



La MAH en ingénierie tissulaire : application à la régénération du tissu osseux

Mathilde Fénelon

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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE
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ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ
SPÉCIALITÉ BIOLOGIE CELLULAIRE ET PHYSIOPATHOLOGIE

Par Mathilde FÉNELON

**LA MAH EN INGENIERIE TISSULAIRE : APPLICATION A LA
RÉGÉNÉRATION DU TISSU OSSEUX**

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Soutenue le 22 Novembre 2019

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La MAH en ingénierie tissulaire : application à la régénération du tissu osseux

La régénération osseuse guidée (ROG) est une technique couramment utilisée pour la régénération de perte de substance osseuse. Elle repose sur l'utilisation d'une membrane jouant un rôle de « barrière » en isolant le défaut osseux. Afin de pallier les limites des membranes actuellement utilisées, des recherches récentes tentent de développer de nouvelles membranes dites « bio-actives ». Du fait de ses propriétés biologiques, la membrane amniotique humaine (MAH) pourrait être une alternative aux membranes conventionnellement utilisées pour la ROG. L'objectif principal de ce travail était de déterminer les meilleures conditions d'utilisation de la MAH pour la régénération de pertes de substances osseuses. Dans une première partie expérimentale, l'influence des faces de la MAH appliquées au contact du défaut ainsi que l'effet de la cryopréservation ont été étudiés. Dans une seconde partie expérimentale, une nouvelle méthode de décellularisation de la MAH, simple et reproductible a été développée. Dans une troisième partie expérimentale, la réparation osseuse de défauts de taille non-critiques et critiques a été évaluée en présence de la MAH préservée selon différentes méthodes. Les résultats ont montré que ni les cellules souches contenues dans la MAH, ni la face appliquée au contact du défaut n'avaient d'influence sur la régénération osseuse. La MAH décellularisée/lyophilisée semblait être la méthode de préservation la plus prometteuse en vue de son utilisation en régénération osseuse.

Mots clés : Membrane amniotique ; Os ; Préservation ; Membrane induite

Amniotic membrane for tissue engineering: applied to the bone regeneration field

Guided bone regeneration (GBR) is commonly used to repair damaged bone. GBR is based on the application of a membrane which will act as a physical barrier to isolate the intended bone-healing space. The development of bioactive membranes has been suggested to overcome some limitations of the currently used membrane. Due to its biological properties, the human amniotic membrane (HAM) is a new biological membrane option for GBR. This study aimed at investigating the most suitable conditions to use HAM for GBR. First, the influence of both HAM sides and the impact of cryopreservation were studied. Then, a new decellularization process of HAM, that is simple and reproducible, has been developed. In a third part, bone regeneration of non-critical and critical sized defects depending on the preservation method of HAM was assessed in rodents. Results showed that neither stem cells found in HAM, nor the HAM layer used to cover the defect had an influence on its potential for bone regeneration. The most promising results were achieved with the decellularized/lyophilized HAM for the field of bone regeneration.

Key words : Amniotic membrane; Bone; Preservation method; Induced membrane

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LISTE DES ABREVIATIONS

ADDM : matrice dentinaire autogène déminéralisée

ALP : Phosphatase alkaline

BMP-2 : Bone morphogenic protein-2

CPC : Calcium phosphate cement

DNA: Acide DésoxyriboNucléique

HA: Hydroxyapatite

hAM: human amniotic membrane

hBMSCs : Cellules souches issues de la moelle osseuse humaine

IM : Induced membrane

MA : Membrane amniotique

MAH : Membrane amniotique humaine

MI : Membrane induite

PMMA : Polyméthyl méthacrylate

PLA : Acide polylactique

PLGA : acide poly(-lactique- polyglycolique)

PRF : Plasma riche en fibrine

PRP : Plasma riche en plaquette

PTFE : Poly-tétra-fluoro-éthylène

ROG : Régénération osseuse guidée

RTG : Régénération tissulaire guidée

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INTRODUCTION GENERALE

INTRODUCTION GÉNÉRALE

Les pertes de substances osseuses constituent l'une des problématiques fréquemment rencontrée par le chirurgien oral et maxillo-facial. Ces pertes de substances peuvent être d'origine carcinologique, traumatique (balistique, accident de la voie publique ...), inflammatoire ou infectieuse (maladies parodontales), iatrogène (ostéoradionécrose, ostéochimionécrose), être une séquelle d'anomalie congénitale (fente faciale) ou bien résulter d'un mécanisme physiologique, telle que la résorption osseuse post-extractionnelle [1]. Ces pertes de substances peuvent avoir des conséquences fonctionnelles, morphologiques et/ou sociales avec un retentissement significatif sur la vie du patient [1].

En fonction de la taille et de la localisation du défaut une régénération osseuse spontanée peut parfois être observée. [2–4]. Lorsqu' une reconstitution *ad integrum* du défaut osseux ne peut pas être obtenue de façon physiologique, le chirurgien pourra avoir recours à différente technique de reconstruction osseuse, dont la régénération osseuse guidée (ROG) [1,5]. La ROG est une technique couramment utilisée pour la réparation de perte de substance osseuse. Elle repose sur l'utilisation d'une membrane qui peut être associée ou non à un biomatériau de substitution osseuse.

Il existe de nombreuses membranes utilisées couramment en pratique clinique pour la reconstruction de perte de substance à l'aide de la technique de la régénération osseuse guidée.

Néanmoins, dans le but de surmonter les limites des membranes actuellement utilisées, des recherches récentes ont porté sur le développement de nouvelles membranes dites « bio-actives » [6]. Du fait de ses propriétés biologiques, la membrane amniotique humaine (MAH) pourrait être une alternative aux membranes conventionnellement utilisées.

L'hypothèse de ce travail était que les conditions d'utilisation et de préservation de la MAH influencent la régénération osseuse dans des défauts osseux critiques et non critiques.

Ce manuscrit s'articule en quatre parties :

- La **première partie** consiste en une revue de la bibliographie portant sur la technique de la ROG et les membranes couramment utilisées, avant de décrire la membrane amniotique ainsi que son intérêt pour l'ingénierie tissulaire, et plus particulièrement pour la régénération osseuse.
- La **deuxième partie** du manuscrit expose l'objectif général de cette thèse.
- La **troisième partie** présente les résultats obtenus dans le cadre de ces travaux de recherche organisés sous forme de publications scientifiques : deux articles publiés et deux projets d'article. Le premier article (publié) évalue l'influence des faces épithéliales versus mesenchymateuses des MAH fraîches et cryopreservedes pour la ROG. Le second article (publié) s'intéresse au développement de nouvelles méthodes de préservation de la MAH et à leurs impacts sur ses propriétés biologiques et mécaniques. Le troisième article (article soumis) compare le potentiel ostéogénique de quatre types de MAH pour la ROG de défauts osseux non critiques. Le dernier article (article soumis) compare l'effet des MAH sélectionnées dans les études précédentes à la membrane induite pour la réparation de perte de substance osseuse de taille critique.
- La **quatrième partie** de ce travail correspond à une conclusion générale sur les travaux menés et résultats obtenus. Elle détaille également les nouvelles perspectives de travail envisagées.

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REVUE BIBLIOGRAPHIQUE

REVUE BIBLIOGRAPHIQUE

1. LA REGENERATION OSSEUSE GUIDÉE

1.1. Historique

L'apparition de la ROG découle directement de la technique de la régénération tissulaire guidée (RTG) et repose sur le concept de compétition cellulaire. Il s'agit d'induire le repeuplement cellulaire sélectif d'une zone lésée en excluant, à l'aide d'une barrière membranaire, certains types cellulaires indésirables tout en favorisant la prolifération de cellules tissulaires bien définies au niveau du site de cicatrisation [7]. Cela permet d'obtenir la colonisation et la cicatrisation du site lésé par le tissu souhaité. L'idée de promouvoir la cicatrisation d'un site anatomique en l'isolant a été initialement décrite dans les années 1940 dans le domaine de la régénération nerveuse. Il avait alors été observé que la régénération d'un nerf sectionné était altérée par l'invagination de tissu fibreux et la formation de cicatrices [8]. Mais, quand le principe de compartimentation était appliqué, en utilisant de l'aorte allogénique comme conduit protecteur sur les extrémités coupées d'un nerf, la régénération du nerf était alors observée. Le concept de régénération tissulaire guidée s'est ensuite développé pour être appliqué au traitement des maladies parodontales [9,10]. L'objectif était d'isoler la surface radiculaire de la dent de l'épithélium gingival (Figure 1), afin de favoriser la colonisation et la cicatrisation des tissus durs parodontaux (cément, ligament alvéolo-dentaire et os), dont la vitesse de cicatrisation est plus lente [11].

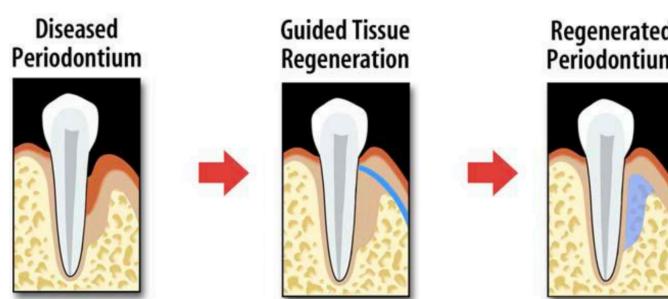


Figure 1. Schémas représentant le principe de la régénération tissulaire guidée (RTG). D'après [12].

Plus récemment, le concept de ROG a vu le jour en s'appuyant sur le principe que la prolifération des cellules épithélio-conjonctives est plus rapide que la cicatrisation osseuse.

L'objectif de la ROG repose sur l'utilisation d'une membrane barrière pour recouvrir et isoler un défaut osseux des cellules épithélio-conjonctives environnantes. Cette membrane va jouer le double rôle de barrière biologique et physique, afin de permettre la régénération osseuse du défaut [13]. C'est Murray et coll. 1957, qui ont mis en évidence pour la première fois le principe de régénération osseuse guidée, en isolant mécaniquement, à l'aide d'une cage en plastique un défaut osseux créé dans un os iliaque de chien [14]. De l'os néoformé était retrouvé à l'intérieur de la cage après cicatrisation. Des expériences similaires ont été menées en 1962 par Melcher et Dreyer qui ont réalisés des défauts diaphysaires de 2mm au niveau de fémurs de rats. Ces défauts étaient ensuite recouverts par une membrane en polytetrafluoroéthylène [15]. En s'appuyant sur ces résultats, Dahlin *et al.* ont également mené une étude chez le rat en 1988 reposant sur l'utilisation d'une membrane en polytetrafluoroéthylène expansé pour prévenir l'invasion d'un défaut osseux par les cellules des tissus mous adjacents [16]. Des défauts osseux bicorticaux au niveau des angles mandibulaires ont été réalisés de façon bilatérale chez le rat. Soit chaque extrémité du défaut était recouverte par la membrane, soit le défaut cicatrisait sans membrane. Dès la sixième semaine, une réparation osseuse complète était observée dans les défauts recouverts de membrane alors qu'aucune cicatrisation osseuse n'était observée dans les défauts témoins et ce, jusqu'à 22 semaines. Les défauts témoins étaient majoritairement comblés par du tissu conjonctif fibreux. Basés sur ces données pré-cliniques précoces, des études cliniques ont ensuite été menées confirmant l'intérêt de la ROG pour la réparation de perte de substance osseuse chez l'homme [17–19]. Ces études reposaient sur l'utilisation de membranes non-résorbables.

1.2. Principe

1.2.1. Physiologie de la cicatrisation osseuse

En cas de lésion tissulaire, le déroulement de la cicatrisation osseuse physiologique peut être divisé en trois étapes : une phase inflammatoire, une phase de réparation et le remodelage osseux final (Figure 2) [20,21] . Il s'agit d'un processus régénératif complexe qui implique un nombre crucial de cellules progénitrices ainsi que des cellules inflammatoires, endothéliales et hématopoïétiques [22]. Les événements cellulaires et moléculaires sont strictement régulés pendant la cascade de cicatrisation.

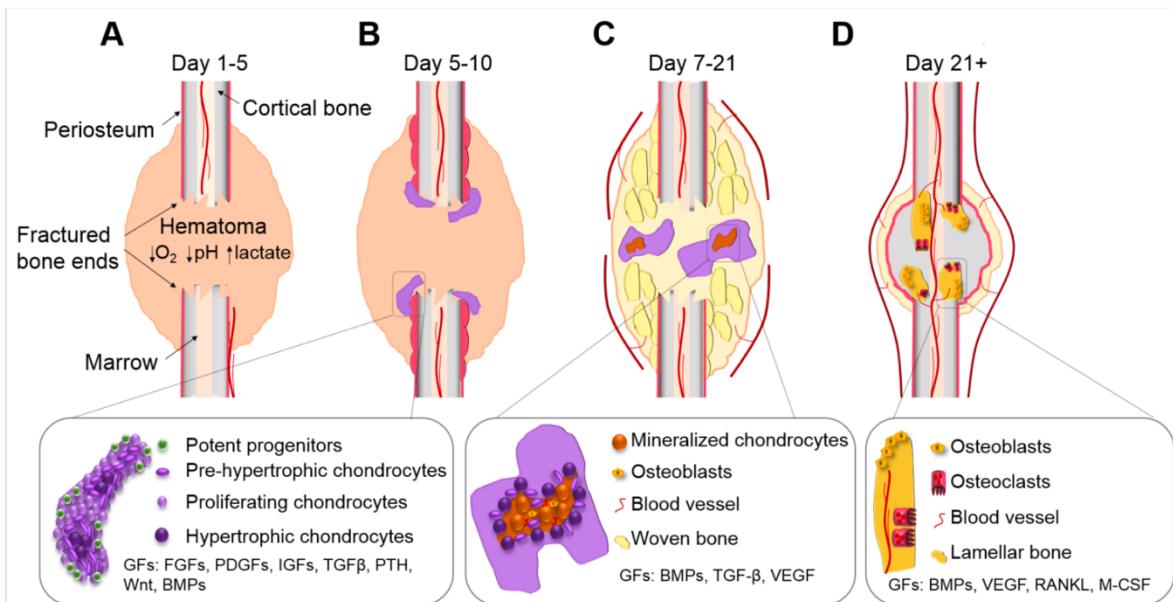


Figure 2. Chronologie de la cicatrisation osseuse. D'après [22].

La première étape de la cicatrisation osseuse est la phase inflammatoire qui est elle-même divisée en trois temps. Celle-ci consiste initialement en une phase d'hémostase aboutissant à la formation d'un caillot sanguin. Ce caillot fibrino-plaquettaire constitue une source de molécules de signalisation (IGF-1, PDGF...) capables d'initier des cascades d'événements cellulaires nécessaires à la cicatrisation osseuse. La libération de facteurs de croissance et de cytokines permet le recrutement des macrophages ainsi que d'autres cellules de l'immunité telles que des monocytes et lymphocytes. Ces cellules immunitaires recrutées vont à leur tour sécréter différentes molécules (FGF, TNFa, VEGF, TGF-b, IL-1, IL-6) qui stimulent, entre autres, la synthèse de matrice extracellulaire et l'angiogenèse (temps inflammatoire). Ces cytokines pro-inflammatoire présentent également un effet chimiotactique sur les cellules de l'immunité et les cellules mésenchymateuses circulantes. Par la suite, l'hématome et la réaction inflammatoire aiguë sont éliminés après une semaine et le caillot est progressivement remplacé par un tissu de granulation (troisième temps) qui va permettre la détersion du site sous l'action des macrophages. Ce tissu de granulation assure également la prolifération et la différentiation de cellules progénitrices et une néovascularisation de la matrice extracellulaire encore non organisée [20,22].

Le tissu de granulation, composé de vaisseaux, de cellules inflammatoires et d'érythrocytes, est ensuite remplacé par une matrice fibroconjonctive (phase de réparation) [23].

La phase de réparation, qui débute quelques jours après la phase inflammatoire, permet l'évolution du tissu conjonctivo-vasculaire en une structure plus organisée aussi appelée « cal ». Cette phase correspond ainsi au processus d'ostéogenèse, avec deux types d'ossification possibles. L'ossification membraneuse correspond à la synthèse directe d'os compact et trabéculaire, sans phase intermédiaire de cal cartilagineux. L'ossification endochondrale qui est un procédé en deux temps. Elle se caractérise par la formation d'un cal cartilagineux déposé par les chondroblastes, qui se calcifie et se vascularise dans un second temps, pour être progressivement remplacé par le nouveau tissu osseux.

Durant la phase de remodelage le tissu osseux ostéoïde nouvellement formé (immature) est ensuite remodelé pour former un tissu osseux mature minéralisé (tissu osseux lamellaire et moelle osseuse). Cette étape se réalise sous l'action de l'unité multi-cellulaire basique (BMU) avec une alternance de phases de résorption par les ostéoclastes et de formation par les ostéoblastes [20]. Elle permet également de restaurer un apport vasculaire normal. Alors que le modelage osseux initial intervient rapidement, le remodelage osseux est un processus long qui se poursuit plusieurs semaines, voire plusieurs mois [21].

1.2.2. Principe de la ROG

Le principe de la ROG repose sur le concept de « compétition cellulaire » : selon les tissus, les cellules ont des vitesses de prolifération différente. Dans le cas d'une lésion osseuse, les cellules épithéliales et conjonctives avoisinantes possèdent une croissance plus rapide que les cellules ostéoblastiques. Elles vont donc plus rapidement envahir et coloniser la cavité osseuse que les cellules à potentiel ostéogénique. La ROG consiste à créer ou à maintenir une cavité osseuse et à l'isoler à l'aide d'une membrane. La ROG s'appuie donc sur l'utilisation d'une membrane dite « barrière » pour recouvrir un défaut osseux et ainsi isoler le caillot sanguin des cellules épithéliales et conjonctives sous-jacentes (Figure 3). Cette technique chirurgicale a pour objectif d'induire un repeuplement cellulaire sélectif en assurant la colonisation du caillot par des cellules à fort potentiel ostéogénique. La membrane va jouer le double rôle de barrière biologique et physique, afin de permettre la régénération osseuse du défaut [13]. La membrane peut également assurer un rôle mécanique de maintien de l'espace au niveau du défaut à reconstruire. C'est dans cet espace délimité entre le défaut osseux et la membrane

que des cellules provenant exclusivement de l'os avoisinant pourront migrer et se multiplier [7].

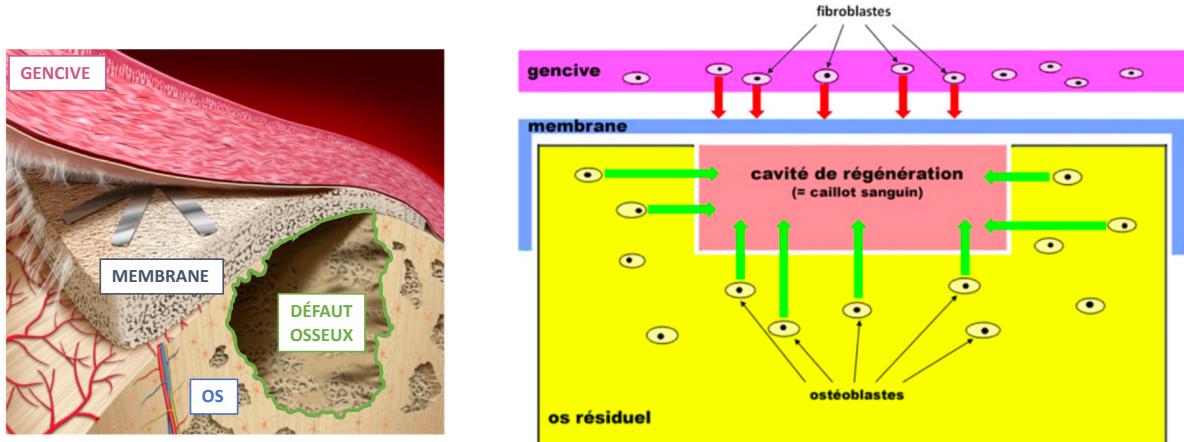


Figure 3. Schémas représentant le principe de la ROG. La membrane assure à la fois le maintien de l'espace [24] au niveau du défaut osseux à régénérer et elle joue le rôle de barrière cellulaire en empêchant l'invasion du site par des cellules non-ostéogéniques [5].

1.3. Cahier des charges des membranes utilisées en ROG

Quel que soit le matériau utilisé, un cahier des charges des membranes utilisées en ROG a très rapidement été défini [11,25,26]. Cinq critères principaux ont été identifiés :

- **Biocompatibilité.** les interactions entre la membrane et les tissus environnants ne doivent pas interférer avec la cicatrisation osseuse. La membrane ne doit pas provoquer une réponse immunitaire, une sensibilisation ou une inflammation chronique qui pourraient avoir des effets néfastes sur les tissus avoisinants, nuire à la guérison ou présenter un danger pour le patient [27].
- **Maintien de l'espace.** La membrane doit jouer le rôle d'un mainteneur d'espace qui va assurer la protection et la stabilisation du caillot sanguin, support naturel de l'ossification. La membrane doit avoir la force nécessaire pour résister aux contraintes exercées par les muscles et tissus mous avoisinants [26]. Elle ne doit pas s'affaisser au sein du défaut afin de ne pas compromettre le volume désiré de l'augmentation osseuse (Figure 4).

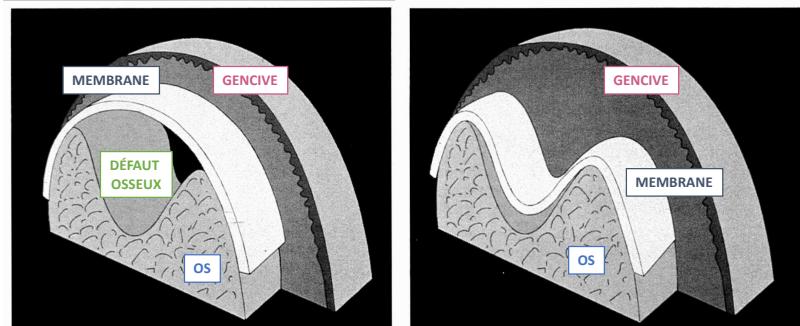


Figure 4. Le maintien de l'espace par la membrane de ROG permet de conserver le volume à régénérer. En cas d'affaissement de la membrane au sein du défaut on observe une diminution du volume disponible pour la ROG. D'après [11].

- **Repeuplement cellulaire sélectif** [13]. Empêcher l'invasion du défaut osseux par les cellules épithéliales ou conjonctives issues des tissus mous avoisinants afin de permettre sa colonisation par des cellules à potentiel ostéogénique. Comme les cellules épithéliales et conjonctives possèdent une croissance plus rapide que les cellules ostéoblastiques, la membrane permet d'éviter une compétition tissulaire défavorable à la néoformation osseuse. Cela empêche l'invasion du tissu conjonctif fibreux au sein du défaut.
- **Intégration tissulaire.** Une bonne intégration tissulaire de la membrane est indispensable au processus de cicatrisation et permet d'obtenir une bonne adhésion entre l'os et le matériau [28]. Cela nécessite une microstructure organisée des membranes, afin de favoriser l'intégration tissulaire, limitant ainsi la migration épithéliale, tout en créant des tissus stables pour la cicatrisation de la plaie [11].
- **Maniabilité.** Les membranes doivent être facile à manipuler [6]. Elles doivent pouvoir être facilement découpées pour être conformées à la taille du site [11]. Il est également important qu'elles puissent maintenir leur forme lors de leur mise en place. Une bonne adaptation au site opératoire limite les risques d'exposition [6]. Elles doivent également pouvoir être aisément retirées [11].

Avec le temps, d'autres critères secondaires ont été rajoutés :

- **Résorption.** Bien que des résultats similaires soient obtenus avec des membranes résorbables et non résorbables, la membrane doit conserver son intégrité et ainsi sa fonction le temps nécessaire pour obtenir un résultat régénératif prédictible. Bien que le temps idéal de résorption membranaire n'ait pas encore été établi [29,30], il semble que plus la membrane

conserve sa fonction longtemps, plus la maturité de l'os régénéré est grande. De plus, la réponse inflammatoire provoquée par la résorption de la membrane ne devrait pas entraver le processus de régénération [6,29].

- **Tolérance à l'exposition** [29]. Que la membrane soit résorbable ou non, son exposition et l'éventuelle contamination bactérienne qui en découle peuvent entraver les résultats de la ROG. En cas d'exposition, et en l'absence de signes d'infection, la membrane exposée doit pouvoir être maintenue *in situ* et continuer à fonctionner pendant le processus de régénération [29].

En fonction du défaut osseux (volume et localisation), la membrane pourra être utilisée seule ou associée à un matériaux de substitution osseuse [31]. Elle participera alors à empêcher l'encapsulation du matériaux de substitution par du tissu fibreux.

2. LES MEMBRANES EN REGENERATION OSSEUSE GUIDÉE

2.1. Les membranes d'origine non humaines

2.1.1. Les membranes non résorbables

Les membranes non résorbables correspondent à la première génération de membrane utilisée en ROG. Développées dans les années 60-70, ces membranes avaient pour objectif de se rapprocher des propriétés physiques du tissu remplacé tout en induisant le moins de réponse toxique chez l'hôte. Il s'agit de membranes biocompatibles et dont l'intégrité structurale n'est pas altérée au cours du temps [32].

a. Les membranes en polytétrafluoroéthylène et dérivés

Les membranes non résorbables utilisées pour la ROG sont en téflon : il s'agit de membrane en PolyTétraFluoroEthylène expansé (e-PTFE) ou de membrane PTFE à haute densité appelée d-PTFE. Ces deux membranes peuvent éventuellement présenter un renforcement en titane. Elles possèdent la même composition chimique, et proviennent d'un même polymère synthétique biologiquement inerte et qui ne se délite pas après implantation [29,33]. Ce matériau est poreux pour permettre la colonisation tissulaire superficielle et donc une relative stabilité après implantation sous le tissu conjonctif gingival. Les porosités de ces deux type de membrane en PTFE sont différentes et peuvent également varier en fonction du domaine

d'application. En effet, ces deux membranes diffèrent dans leur procédé de préparation, ce qui affecte la densité et la taille des pores. La membrane e-PTFE est un matériau fritté avec des pores de grande taille, et la membrane d-PTFE est un matériau non fritté avec une taille de pore beaucoup plus petite.

Les membranes en e-PTFE, largement connues sous le nom de membrane Gore-tex, présentent deux type de microstructure : une partie coronaire dont la taille des pores est comprise entre 5 et 30 microns, et une partie apicale, dite occlusive dont la porosité est de 8 microns. Cette partie apicale permet l'apport d'éléments nutritifs tout en empêchant l'infiltration d'autres types cellulaires. Néanmoins, en cas d'exposition dans la cavité buccale, une infiltration bactérienne de la membrane peut être observée risquant de compromettre la régénération de la perte de substance osseuse. En effet, plusieurs études ont rapporté une diminution du gain osseux en cas d'exposition de la membrane e-PTFE [34,35], justifiant leur ablation de façon systématique en cas d'exposition [6]. Une exposition prématuée de ces membranes étaient rapportées dans 30 à 40 % des cas [26]. Actuellement, les membranes en e-PTFE ont ainsi été abandonnées en chirurgie orale [28]. Le praticien a recours à d'autres types de membrane non résorbables, telles que les membranes d-PTFE.

Du fait de leur porosité inférieure, les membranes d-PTFE sont largement utilisées. Elles présentent des pores d'environ 0,2 microns, ce qui les rendraient résistantes à la pénétration bactérienne [26,28]. Le risque de contamination bactérienne et d'infection en cas d'exposition est ainsi diminué par rapport aux membranes e-PTFE [6]. Certains auteurs rapportent même qu'il ne serait pas nécessaire de réaliser de fermeture étanche du site en vue d'une cicatrisation tissulaire de première intention, permettant ainsi de laisser la membrane exposée [36] (Figure 5).

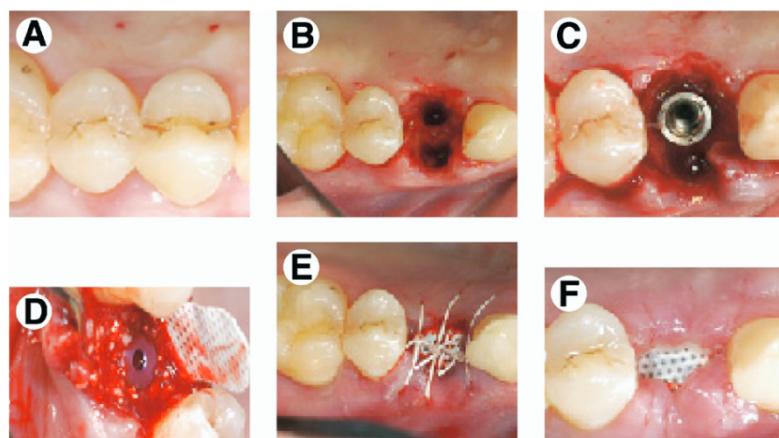


Figure 5. Utilisation d'une membrane en d-PTFE pour une ROG péri-implantaire. La membrane a été laissée exposée. D'après [36].

Par ailleurs, du fait de leur faible porosité, les membranes d-PTFE présentent une invagination tissulaire plus limitée, réduisant le traumatisme des tissus mous avoisinants lors de leurs retraits [6].

Ces deux membranes peuvent parfois être renforcées par du titane afin d'augmenter leur rigidité [26].

b. Les membranes titane

Du fait de ses propriétés biologiques (biocompatibilité) et mécaniques (résistance et rigidité, faible densité, résistance à la corrosion), le titane est couramment utilisé en chirurgie orale, maxillo-faciale et orthopédique [33]. Ainsi, les membranes titane sont une autre famille de membranes non résorbables utilisées pour la reconstruction de perte de substance osseuse [28]. Les membranes à mailles de titane ont une résistance suffisante pour assurer le maintien de l'espace et prévenir un affaissement dû à la compression des tissus mous environnants du fait de leur élasticité [26,28]. Elles sont rigides et non poreuses. Du fait de leur surface lisse, ces membranes semblent moins susceptibles aux contaminations bactériennes [28]. Cependant, la rigidité de ces membranes est susceptible de causer une irritation mécanique des tissus mous adjacents, associée à un risque d'exposition de la membrane [37]. De plus, la présence d'arêtes vives, causées par la coupe et la flexion des mailles en titane, pourrait être responsables de l'exposition de la membrane [26,28].

- Avantages et limites des membranes non résorbables :

- Avantages : Les membranes non résorbables ont l'avantage d'être bio-inertes. Elles possèdent également de bonnes propriétés mécaniques évitant tout risque d'affaissement de la membrane au sein de la cavité osseuse, maintenant ainsi le volume osseux à reconstituer [28].

- Limites : Le risque fréquent d'exposition, pouvant nécessiter leur retrait anticipé, constitue l'une de leurs limites. La présence de macro-porosité augmente le risque de contamination bactérienne en cas d'exposition endo-buccale [5]. De plus, ces membranes doivent être retirées ce qui nécessite une ré-intervention chirurgicale [32].

2.1.2. Les membranes résorbables

Les membranes résorbables sont la 2^{ème} génération de membrane développées pour la ROG. Elles ont été conçues dans l'objectif de s'affranchir du 2^{ème} temps chirurgical destiné au retrait des membranes non résorbables [27,32].

Les membranes résorbables sont d'origine naturelle ou synthétique. Les membranes naturelles non humaines sont principalement des membranes de collagène, mais on retrouve également des membranes à base de chitosane [27]. Les membranes synthétiques disponibles dans le commerce sont constituées de polymères aliphatiques organiques tels que l'acide polyglycolique ou l'acide polylactique, et leurs modifications [26]. Les membranes résorbables doivent avoir une durée de résorption compatible avec le délai de formation osseuse.

a. Les membranes naturelles de collagène

Les membranes de collagène sont les membranes résorbables les plus fréquemment utilisées en pratique clinique. Les membranes de collagène sont majoritairement composées de collagène de type I [30]. Elles sont le plus souvent d'origine bovine, porcine ou équine [30]. Leur vitesse de résorption peut varier de 4 semaines à 6 mois en fonction des études [6,30]. La structure fibrillaire des membranes de collagène est assimilable à une micro-porosité qui varie grandement en fonction du degrés de réticulation des fibres et des traitements chimiques liés à leur fabrication [5]. Les premières membranes de collagène utilisées étaient constituées de deux couches. La face interne poreuse placée au contact du défaut osseux était

constituée de fibres de collagène disposées de manière lâche permettant aux ostéoblastes de coloniser le site. La surface externe, faisant face aux tissus mous, était dense, agissant comme une barrière pour empêcher la prolifération des fibroblastes dans le défaut osseux [6]. Les membranes résorbables de collagène possèdent des propriétés mécaniques faibles. Lors de leur mise en place au niveau du défaut osseux, ces dernières vont très rapidement être au contact de liquides biologiques tels que le sang ou la salive. Leurs propriétés mécaniques vont le plus souvent se détériorer une fois humidifiée puis au cours de leur résorption [38]. Afin de compenser ce manque de rigidité et pour leur apporter un soutien mécanique, elles seront très souvent associées à des biomatériaux de comblement. Selon la membrane utilisée et les conditions locales, la durée de résorption de ces membranes est variable et difficilement prévisible ni contrôlable [32]. Des techniques de réticulation ont été développées afin d'augmenter la durée de résorption des membranes de collagène. Lorsqu'elles sont exposées, on peut observer une ré-épithérialisation au niveau des membranes de collagène dense, contrairement aux membranes e-PTFE non résorbables qui ont tendance à s'infecter [39].



Figure 6. Utilisation d'une membrane de collagène pour une ROG péri-implantaire.

- Avantages et limites des membranes de collagène :

Avantages : Les membranes résorbables ne présentent pas d'éléments radio-opaques ce qui permet d'effectuer un contrôle radiologique sans risque d'artefacts. Elles sont facilement manipulables et elles ne nécessitent pas de 2^{ème} temps chirurgical pour les retirer.

Limites : Les membranes résorbables de collagène possèdent des propriétés mécaniques faibles [38]. En outre, les risques de transmission de maladies dus à l'utilisation de collagène d'origine humaine ou animale peuvent entraîner des restrictions réglementaires ou soulever d'autres types de problématiques, notamment en lien avec des croyances religieuses [6,40]. Manque de prédictibilité de la vitesse de résorption. Bien que probablement très faible, il existe un risque de transmission à l'homme d'agents infectieux provenant du fait que ces membranes soient d'origine animale [27]

b. Les membranes naturelles de chitosane

Le chitosane est un polysaccharide et un polymère naturel obtenu principalement par hydrolyse de la chitine issue de carapace de crabe. Il possède une structure proche de celle de l'acide hyaluronique. Le chitosane est biocompatible et résorbable [29,41,42]. De plus, il possède des propriétés bactériostatiques [29,33]. Le taux de dégradation des membranes de chitosane varie en fonction de leurs poids moléculaire et des méthodes de préparation [33]. Il possède des propriétés hémostatiques [43]. Bien que plusieurs études pré-cliniques rapportent l'utilisation de membranes en chitosane pour la ROG de perte de substance osseuse, il n'existe pas à ce jour d'études cliniques dans ce domaine [5,29,33].

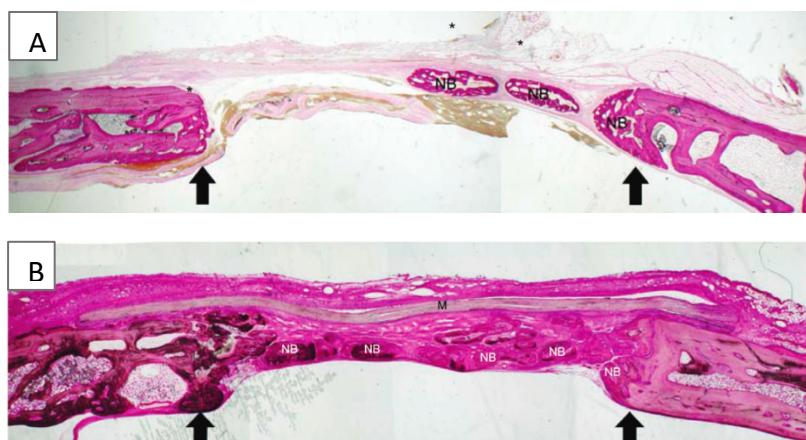


Figure 7. Réparation osseuse de défauts de calvaria chez le lapin à 4 semaines. Le défaut était soit laissé vide (A) soit recouvert d'une membrane de chitosan (B). NB : os néoformé.
D'après [41].

- Avantages et limites des membranes de chitosane :

Avantages : Le chitosane présente de nombreuses propriétés biologiques (biocompatibilité, effet bactériostatique et hémostatique). Il s'agit d'une membrane résorbable ne nécessitant pas de deuxième temps opératoire.

Limites : Jusqu'à présent son utilisation reste expérimentale.

c. Les membranes synthétiques

Les membranes synthétiques résorbables sont généralement à base d'acide polyglycolide (PGA), d'acide polylactide (PLA) ou de copolymères de PLA/PGA. Il s'agit de matériaux biocompatibles mais ils ne sont pas biologiquement inertes puisque leur dégradation est susceptible d'induire une réaction tissulaire [27]. Elles ont l'avantage d'être facilement manipulables [29]. Il est possible de jouer sur leur composition et le procédé de fabrication, afin d'ajuster le temps de résorption, la porosité ou les propriétés mécaniques des membranes [32] (Figure 8).

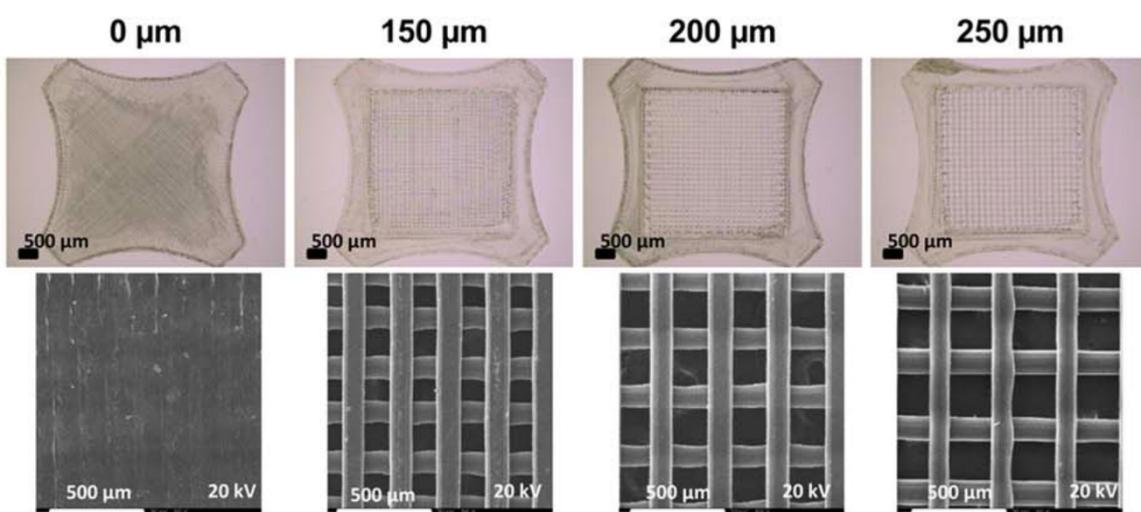


Figure 8. Impression par la technique FDM (dépot de fil fondu) de matrice en PLA de différentes porosités pour l'ITO. D'après [44]

Tout comme pour les membranes de collagène, les membranes PLA/PGA présentent un manque de rigidité qui, en l'absence de soutien mécanique, provoque l'affaissement des membranes dans la lésion et, par conséquent, la diminution du volume du tissu régénéré.

Contrairement aux membranes faites de polymères naturels, elles ne possèdent pas de propriétés biologiques telle que la présence de facteurs de croissance [45].

- Avantages et limites des membranes résorbables synthétiques :

Avantages : Possibilité d'ajuster le temps de résorption, les propriétés de manipulation et les propriétés mécaniques en modifiant la composition et le procédé de fabrication.

Limites : Une toxicité liée à leurs produits de dégradation leur est souvent reprochée [5,32].

2.2. Les membranes d'origine humaine

2.2.1. *Le concept membranaire PRF*

Le PRF (plasma riche en fibrine) a été développé en France en 2001 par Choukroun *et al.* pour une application en chirurgie orale et maxillo-faciale [46]. Le PRF se définit comme un caillot de fibrine riche en plaquettes obtenu à partir du sang total du patient [47]. Les membranes de PRF se préparent de façon extemporané durant l'intervention à partir du sang du patient qui va être centrifugé (Figure 9).

Cela permet d'obtenir une membrane de fibrine autologue. Elle concentre les leucocytes, des plaquettes et des molécules de cicatrisation et de l'immunité : ces membranes contiendraient l'ensemble des cytokines (ou facteurs de croissances) plaquettaires telles que le TGFb-1, le PDGF, VEGF l'IGF-1 et le PD-ECGF [5,48,49]. La membrane de PRF assure un relargage régulier de ces différents facteurs de croissance et cytokines durant 10 jours [49]

Le délai de résorption du PRF est très court. Il est équivalent au délai de résorption physiologique de la fibrine (8 à 10 jours).

Bien qu'il ait été démontré que les membranes de PRF amélioraient la régénération des tissus mous parodontaux et limitaient la perte osseuse post-extractionnelle, le niveau de preuve des études portant sur son utilisation pour la ROG est encore insuffisant [49,50].

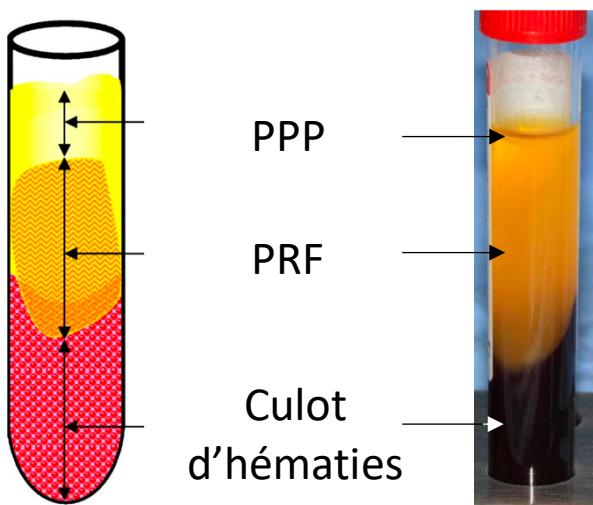


Figure 9. Trois fractions sont identifiables après centrifugation. Le plasma pauvre en plaquette (PPP), le caillot de fibrine (PRF) et le culot d'hématies.

D'après [48].

- Avantages et limites des membranes de PRF:

Avantages : C'est une membrane à faible coût et qui assure une sécurité d'utilisation puisqu'il s'agit d'un produit autologue [27]. La membrane de PRF possède des propriétés biologiques dues à la libération de nombreux facteurs de croissance [5].

Limites : Ces membranes possèdent des propriétés mécaniques très insuffisantes si elles sont utilisées seules et une vitesse de résorption très rapide [5]. Faible niveau de preuve concernant leur utilisation en ROG.

2.2.2. Le derme allogénique

Le derme allogénique constitue une autre source de membrane résorbable. Il s'agit de derme prélevé sur la peau de cadavre fourni par des banques de tissus humains. Le derme est traité et décellularisé afin d'éliminer un risque de rejet immunologique chez le patient receveur [51]. Initialement décrite pour le traitement des brûlures cutanées sévères, ces membranes sont de plus en plus utilisées en chirurgie orale. Bien que de nombreuses études rapportent son intérêt pour les pertes de substances des tissus muco-gingivaux, son utilisation en ROG reste encore peu décrite [52]. D'autres études sont nécessaires pour conclure sur son intérêt dans la régénération de perte de substance osseuse.

2.2.3 Le périoste

Le périoste autologue est la seule membrane dite ostéogénique. Le périoste recouvre les surfaces externes de la plupart des os, et représente une zone de transition entre l'os cortical et les tissus mous ou la musculature qui le recouvrent [53]. La structure du périoste repose sur deux couches : i) une couche externe, dite fibreuse, composée de fibroblastes, de collagène et d'élastine ainsi que d'un réseau nerveux et micro-vasculaire, et ii) une couche interne, en contact direct avec la corticale osseuse, présentant une densité cellulaire importante [53,54]. Cette dernière contient des cellules souches mésenchymateuses, des cellules progénitrices ostéogéniques différencierées et des ostéoblastes (Figure 10).

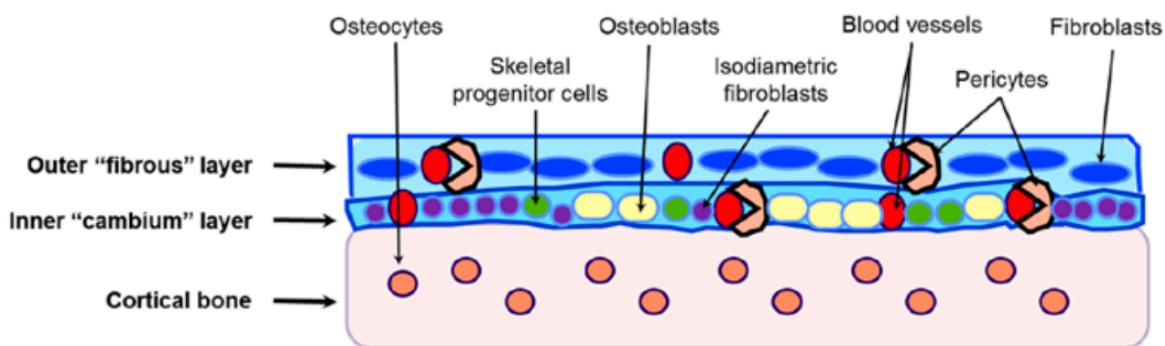


Figure 10. Structure du périoste. D'après [54].

Peltola *et al.* ont comparé la cicatrisation de défauts osseux chez le lapin, comblés par deux matériaux de substitution osseuse différents, puis recouverts soit par un lambeau de périoste pédiculé soit par une greffe de périoste libre [55]. Quel que soit le matériaux de comblement utilisé, la réparation osseuse était plus importante lorsque le défaut était recouvert par le lambeau de périoste plutôt que par la greffe de périoste libre. Ainsi, il semblerait que le maintien de la vascularisation du périoste soit nécessaire dans des situations pathologiques ou lors de la manipulation du périoste à des fins d'ostéogénèse [56]. Une autre étude pré-clinique a alors comparé l'utilisation d'un lambeau de périoste à une membrane en PTFE pour la reconstruction de défauts osseux bilatéraux de 5 mm réalisés au niveau de l'os frontal de lapins [57] (Figure 11). Aucune différence significative n'a été observée concernant le taux de réparation osseuse à 4 et 12 semaines. Ces résultats suggèrent que le périoste pourrait jouer un rôle de barrière mécanique similaire à celui des membranes non résorbables couramment

utilisées pour la ROG [57]. Néanmoins, l'os néoformé était plus fin lorsque le défaut était recouvert par le périoste. Cela pourrait s'expliquer par les faibles propriétés mécaniques du périoste qui maintiendrait moins bien le volume du site à régénérer par rapport à la membrane en PTFE qui est plus rigide.

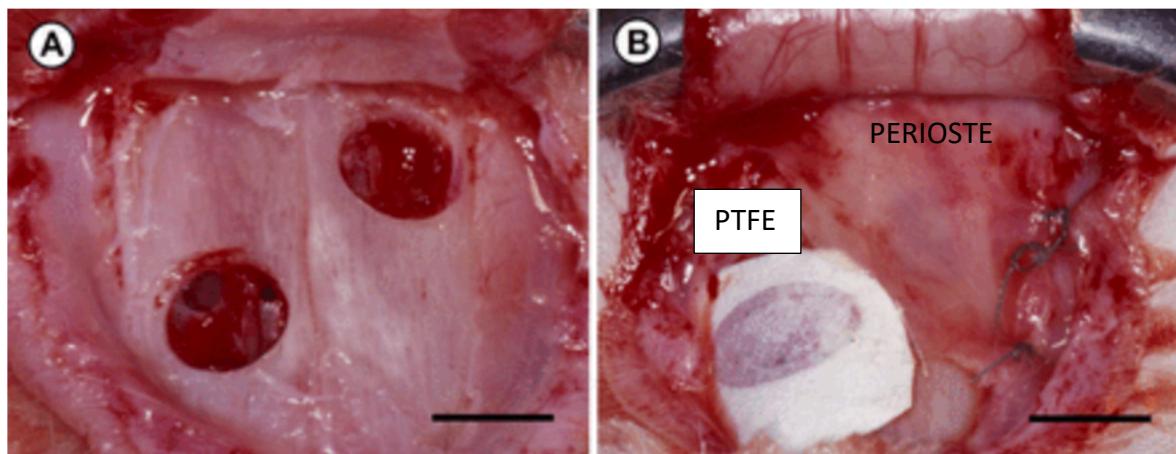


Figure 11. Comparaison de la réparation osseuse d'un défaut de calvaria en présence d'un lambeau de périoste ou d'une membrane PTFE. D'après [57].

En plus de son rôle de barrière mécanique, le périoste joue un triple rôle biologique dans l'ostéogénèse. Tout d'abord, il assure un contingent de cellules ostéoprogénitrices de par les précurseurs présents au sein de ses couches internes et externes. Son décollement lors de sa manipulation va également déclencher l'émission locale de facteurs de croissance permettant le recrutement d'autres précurseurs ostéochondrogéniques présents dans l'environnement vasculaire, médullaire ou musculaire. Enfin, il joue un rôle métabolique en participant à la néo-angiogénèse du site [56].

Des études cliniques ont montré que l'intégrité du périoste est un élément clé pour la régénération osseuse spontanée de perte de substance osseuse [54,58–60]. Le périoste agit comme une source de cellules progénitrices ostéogéniques, fournit un apport vasculaire pour l'os néoformé, et son intégrité lui permet de jouer un rôle de barrière contre l'infiltration de tissu de granulation, créant ainsi un environnement propice à la régénération osseuse [58,60]. Récemment, différents lambeaux de périoste ont été proposés comme alternative aux membranes conventionnellement utilisées en chirurgie orale pour la préservation alvéolaire post-extractionnelle, le traitement parodontal d'atteintes de furcation dentaire ou encore dans un contexte de ROG [54,60] (Figure 12).

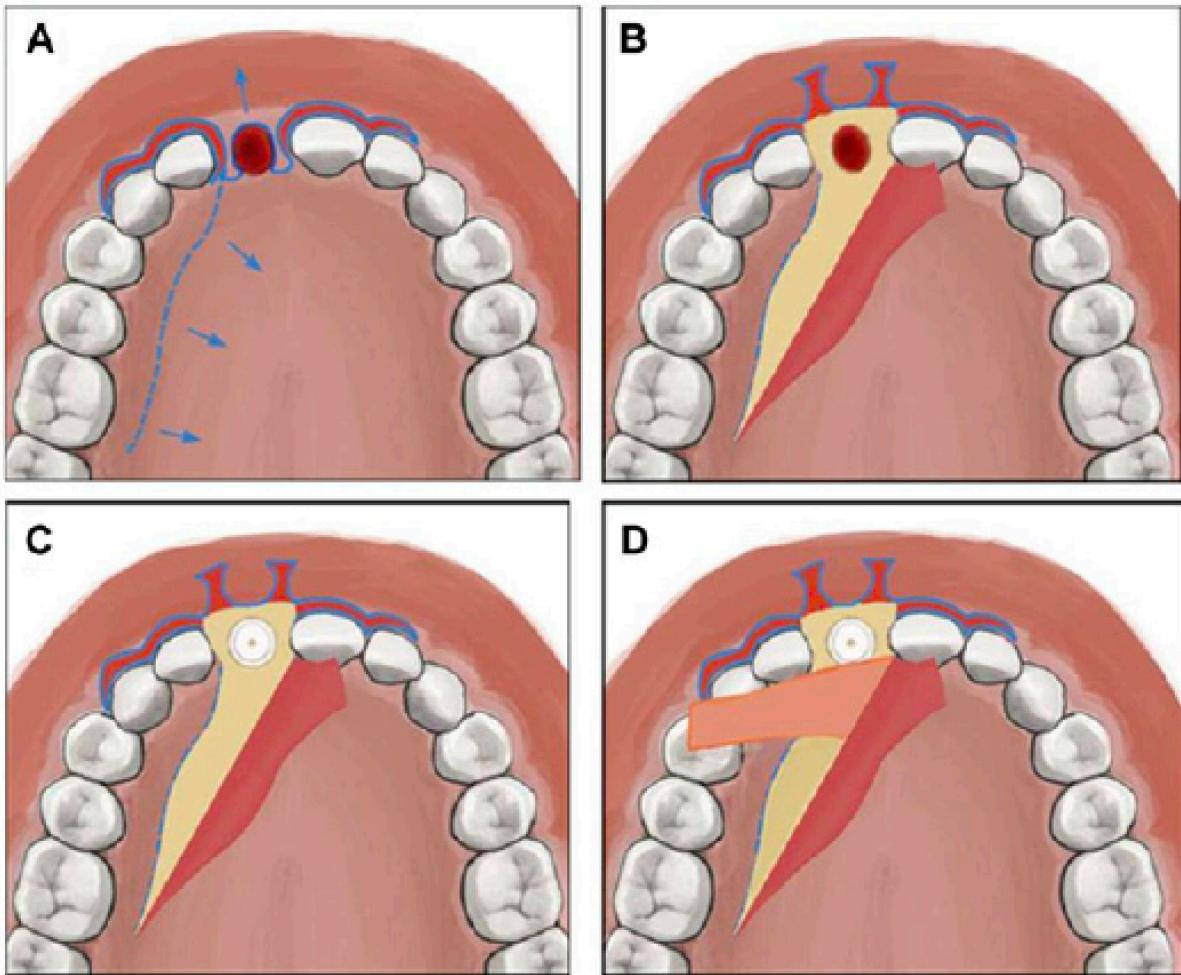


Figure 12. Lambeau de périoste palatin pédiculé. D'après [54].

- Avantages et limites du périoste:

Avantages : Membrane autologue riche en cellules souches.

Limites : Le périoste ne peut pas être préservé dans certaines situations cliniques. Ses propriétés mécaniques sont faibles. Il est disponible en quantité limité. Allongement de la durée de la chirurgie lors de la réalisation de lambeau de périoste.

2.2.4. La membrane de Masquelet ou membrane induite

a. Principe de la technique

Quelle que soit leur étiologie, la reconstruction de segments osseux diaphysaire des os longs constitue un défi majeur pour la conservation des membres. Il a été démontré qu' au-delà de 6 cm, les greffes osseuses autologues subissent un phénomène de résorption même dans

un environnement musculaire bien vascularisé [61,62]. Plusieurs auteurs ont cherché à limiter la résorption de la greffe par une membrane d'interposition, d'abord non résorbable [63] puis résorbable [64], mais ces expérimentations n'ont pas dépassées le stade préclinique (colonisation du site par du tissu fibreux non vascularisé [63], nécrose osseuse en l'absence de pré-perforation de la membrane [64]).

Développée en France à partir de 1986 par le docteur Masquelet, la technique de la membrane induite a été décrite dans les années 2000 pour la reconstruction de perte de substance osseuse segmentaire des os longs [65]. Cette technique permet de limiter les mécanismes de résorption des greffes osseuses [66]. Il s'agit d'une technique chirurgicale en deux temps opératoire reposant sur une greffe d'os spongieux autologue au sein d'une membrane préalablement induite par un conformateur (Figure 13).

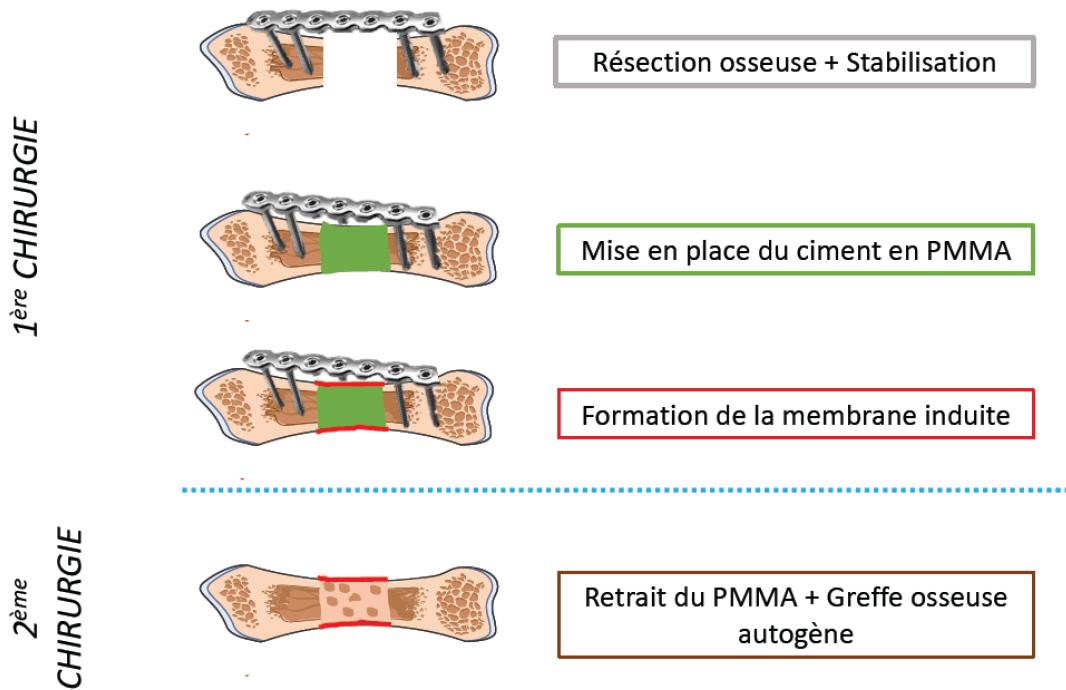


Figure 13. Technique de Masquelet : principe de la membrane induite.

Le premier temps chirurgical consiste à effectuer un débridement des tissus mous et osseux dévitalisés et infectés. Une stabilisation instrumentale des deux fragments osseux est réalisée le plus souvent au moyen d'une plaque d'ostéosynthèse vissées, d'un clou centro-médullaire ou d'un fixateur externe. Un ciment chirurgical (polyméthyl méthacrylate, PMMA) est ensuite inséré au niveau de la lésion osseuse entre le fragment proximal et distal. Cette entretoise en

ciment doit être de taille supérieure au diamètre de l'os à reconstruire afin d'augmenter la quantité d'os autologue secondairement greffé. Le rôle de cette entretorse en ciment est double : elle empêche l'invasion du tissu fibreux dans la zone lésée, tout en induisant le développement d'une membrane environnante pseudo-synoviale, à la suite d'une réaction à corps étranger.

Le deuxième temps chirurgical est réalisé 6 à 8 semaines plus tard [67] une fois la cicatrisation des parties molles acquise et lorsqu'une membrane dite « induite » (MI) s'est formée autour du conformateur en ciment utilisé. Lors de ce deuxième temps opératoire, la membrane induite est incisée longitudinalement de façon à retirer délicatement l'entretorse tout en laissant en place la membrane induite [68]. La cavité est alors comblée par des fragments d'os spongieux autologue prélevés à partir des crêtes iliaques [65,69]. Lorsque la quantité de greffe autologue n'est pas suffisante, un substitut osseux peut y être ajouté [68].

b. Structure et rôle de la membrane induite

La MI est une membrane constituée de deux couches qui mesurent 1 à 2 mm d'épaisseur. La partie interne (au contact du ciment) est un épithélium synovial et la partie externe est constituée de fibroblastes, de myofibroblastes et de collagène [69,70] (Figure 14).

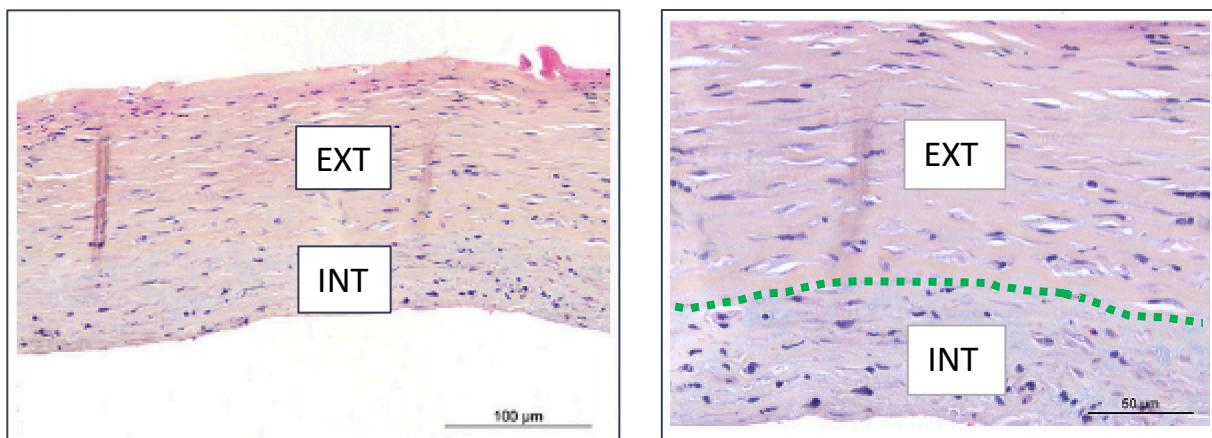


Figure 14. Analyse histologique de la MI en coloration HES. EXT: couche externe; INT: couche interne. D'après [71].

Bien que plusieurs auteurs aient rapporté que la MI possédait des propriétés ostéo-inductrices et angiogéniques, ses propriétés ostéo-inductrices restent encore discutées [69–72].

Pelissier *et al.* ont réalisé une étude histologique et immunohistochimique d'une membrane induite au contact de ciment PMMA implanté en sous cutané chez le lapin [70]. Les auteurs ont conclu qu'il s'agissait d'une membrane richement vascularisée, responsable de la sécrétion de facteurs de croissance comme le VEGF (vascular endothelial growth factor), le TGF (transforming growth factor) et la BMP-2 (bone morphogenic protein-2). Une quantité importante de VEGF a également été mise en évidence suite à la transposition de la technique de la membrane induite pour la reconstruction de perte de substance osseuse segmentaire mandibulaire [73]. Des résultats contradictoires ont été obtenus lors de l'analyse des propriétés ostéoinductrices des membranes induites sur l'ostéogenèse hétérotopique au sein d'une céramique phosphocalcique biphasique composée à 75% d'hydroxylapatite (HA) et à 25% de phosphate tricalcique β (β -TCP), associée ou non à une greffe osseuse autologue [72]. En l'absence d'os autologue, aucune formation osseuse n'était observée. Les auteurs ont conclu sur l'absence d'effet ostéo-inducteur de la MI sur la céramique phosphocalcique biphasique en site hétérotopique. Néanmoins, une quantité plus importante de VEGF a également été mise en évidence dans cette étude [72]. Dans leur étude, Henrich *et al.* ont montré que la composition cellulaire et le contenu en facteurs de croissance de la MI variaient selon l'endroit où la membrane avait été induite (défaut fémoral *versus* implantation sous-cutané) [74]. Dès la deuxième semaine, des cellules souches mésenchymateuses étaient retrouvées au niveau de la MI en site osseux, alors que ces dernières n'étaient pas présentes au niveau de la MI en site sous-cutané. De même, l'expression des facteurs de croissance tels que la BMP-2, le VEGF et le TGF- β était significativement plus élevée en site osseux par rapport à la MI en site sous cutané (Figure 15) [74]. Par ailleurs il semblerait que la concentration en BMP-2 soit la plus élevée à 4 semaines [69–71].

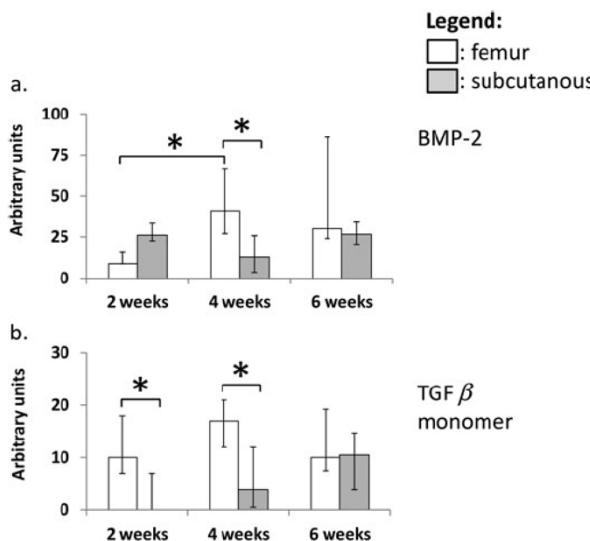


Figure 15. Expression des facteurs de croissances de la MI en fonction du site d'implantation du PMMA. D'après [74].

c. Applications cliniques

L'expérience clinique initiale de la technique de la membrane induite concernait des pseudarthroses septiques post-traumatiques de jambe. Progressivement les indications ont été élargies à d'autres étiologies et l'efficacité de la membrane induite a été rapportée dans des séries de cas portant sur le traitement de pertes de substance osseuse post-traumatiques, dans les cas de retard de consolidation osseuse ou pseudarthrose septique et aseptique, les résections tumorales et dans le cas de pseudarthrose post-fracturaire en territoire irradié [67]. Cette technique, initialement mise au point en chirurgie orthopédique au niveau des os longs des membres inférieurs et supérieurs, s'est ensuite développées pour le traitement de pertes de substances au niveau du poignet et de la main ainsi qu'en chirurgie maxillo-faciale [73,75].

En 2000, Masquelet *et al.* rapportaient une série de 35 cas, traités entre 1986 et 1999, et portant sur la reconstruction par la technique de la MI de perte de substance osseuse diaphysaire des membres supérieurs et inférieurs de 4 à 25 cm [65]. Une consolidation osseuse était radiologiquement observée dès le 4^{ème} mois. Lorsque la cicatrisation a été conduite à son terme (31 cas sur 35), la consolidation complète a été acquise dans un délai moyen de 8,5 mois (6-17 mois). Les échecs étaient tous inhérents à des troubles trophiques et vasculaires compromettant la vascularisation du segment de membre correspondant.

En 2012, une série de 84 patients, traités entre 1988 et 2009, portait sur le traitement de perte de substance osseuse diaphysaire supérieure à 5 cm dans plus de la moitié des cas par la technique de la membrane induite [76]. Ils rapportaient un taux de consolidation de 90 % avec un délai moyen de consolidation de 14 mois (4-18 mois). Les auteurs rapportaient l'absence de corrélation entre la taille du défaut osseux et le délai de consolidation. Cependant, la technique différait ici de celle initialement décrite par Masquelet, puisque la greffe osseuse autologue était accompagnée de l'injection de BMP-2 (25 patients) ou associé à un lambeau osseux revascularisé (7 patients).

Peu d'étude rapporte l'association de facteur de croissance ostéoinducteur avec la technique de la MI [69,76]. Parmi ces études, on retrouve une série de 11 patients traités par Masquelet *et al.* par la technique de la MI entre 2000 et 2004 pour des pertes de substance osseuse diaphysaire des membres supérieurs et inférieurs de 5 à 18 cm [69]. Dans cette étude, lors du deuxième temps opératoire les patients bénéficiaient d'une greffe osseuse autologue associée à une injection locale de facteur de croissance BMP-7. La durée moyenne de consolidation a été de 11,5 mois (6-18 mois) chez 10 sujets, mais trois patients ont présenté une déformation progressive du segment reconstruit quelques mois après. De plus, une modification de l'aspect radiologique du segment reconstruit a été observé chez tous les sujets avec dans un premier temps une densification de l'os, suivie par l'apparition de zones de raréfaction osseuse évoquant un processus de résorption. Les auteurs concluaient que l'adjonction de facteur de croissance tel que la BMP-7 n'améliorait pas les résultats de la technique de la MI.

Avec le temps, plusieurs variantes chirurgicales de la technique de la MI ont ainsi été proposées : adjonction de facteurs de croissance, application de ciments préalablement imprégnés par des antibiotiques, utilisation de substituts osseux [67]... Une récente revue systématique de la littérature a montré que ces modifications ne semblaient pas avoir d'impact sur l'efficacité de la technique de la MI et une consolidation osseuse était observée dans 90% des cas [67]. Une corrélation positive a seulement été retrouvée entre la nécessité de réinterventions et des taux d'union osseuse plus faibles ($p = 0,005$) ainsi que la survenue de complications ($p < 0,001$). De même il a été montré que les patients bénéficiant d'une reconstruction osseuse par la technique de la MI en raison d'infections osseuses présentaient un risque plus élevé de complication chirurgicale [67].

- Avantages et limites de la membrane induite:

Avantages : La MI est une membrane autologue, richement vascularisée, jouant un rôle de barrière physique limitant la résorption osseuse au niveau du site greffé. La MI possède également des propriétés biologiques intéressantes telles qu'une action anti-fibrotique, antalgique ainsi que la libération de facteurs de croissance [77]. En cas d'échec, elle peut être réalisée à nouveau en respectant un temps de latence.

Limites : Il s'agit d'une technique nécessitant deux temps opératoire. De plus, son association avec de l'os autologue reste la technique de référence ce qui augmente la morbidité pour le patient du fait du deuxième site opératoire nécessaire pour le prélèvement osseux [68].

2.3. Évolution et perspectives de la ROG : ingénierie tissulaire

La très grande majorité des membranes actuellement utilisées pour la ROG ne revendique seulement qu'un rôle passif de barrière cellulaire et de mainteneur d'espace [5]. Avec le développement de l'ingénierie tissulaire, le concept de membranes de « troisième génération » a évolué, suggérant le développement de nouvelles membranes possédant des propriétés biologiques. Ainsi, de nombreuses équipes s'attachent à mettre au point des membranes dites « bio-actives » [5]. L'objectif est de créer des membranes qui agissent non seulement comme des barrières mais aussi comme des dispositifs de libération d'agents spécifiques (tels que des antibiotiques, des facteurs de croissance ou des facteurs d'adhésion) sur le site lésé [27]. Cette libération d'agents, à un moment précis ou en fonction du besoin, pourrait permettre de mieux organiser et diriger la guérison naturelle du site. D'autres alternatives ont été proposées pour potentialiser l'effet des membranes, telles que l'optimisation de leurs propriétés mécaniques et physico-chimiques, ou encore de les combiner avec des matériaux ostéo-conducteurs ou ostéo-inducteurs [24]. Cela permettrait également d'obtenir une cicatrisation osseuse satisfaisante dans des cas cliniques complexes ou bien chez des patients atteints d'affections osseuses compromettantes [24]. Ces différentes stratégies d'optimisation des membranes de ROG sont détaillées ci-dessous et résumés de façon schématique dans la figure 16.

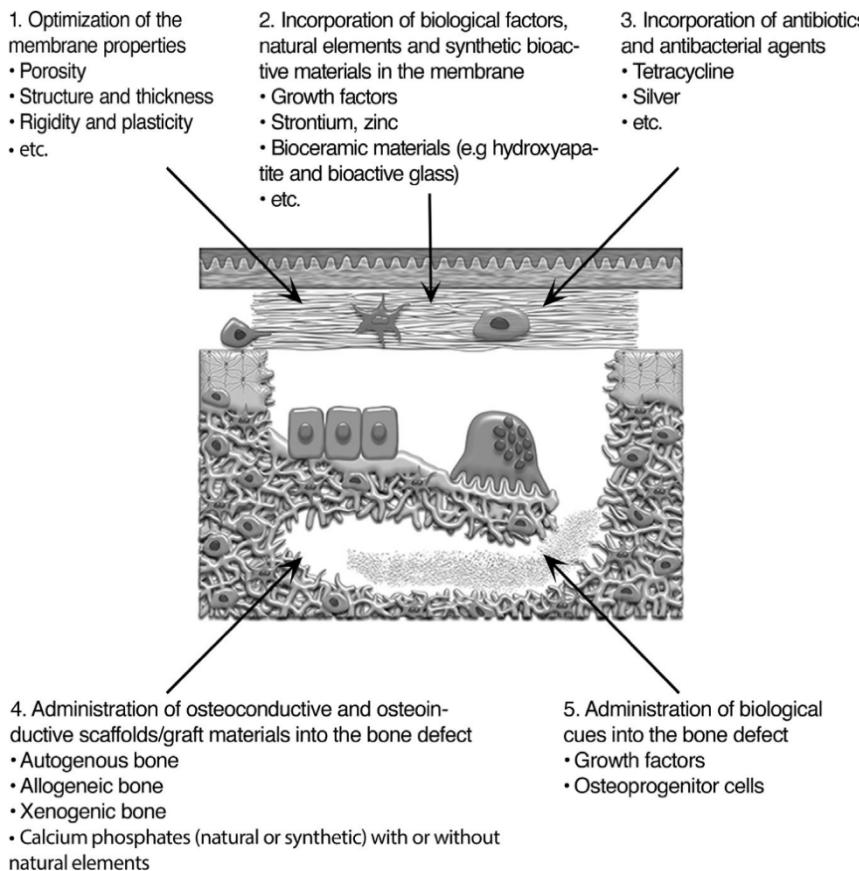


Figure 16. Représentation schématique des stratégies d'amélioration des membranes en ROG. D'après [24].

- Activité anti-microbienne : La contamination bactérienne, le plus souvent inhérente à l'exposition endo-buccale des membranes, représente un des facteurs de risque les plus compromettants lors d'une ROG. Afin de diminuer ce risque, plusieurs études ont suggéré l'incorporation d'antibiotique aux membranes, tels que l'amoxicilline ou la tétracycline [78,79]. Dans leur étude, Bottino *et al.* ont montré que l'ajonction de metronidazole à une membrane de PLA/gélatine permettait de diminuer la charge bactérienne et la formation d'un biofilm à son contact [40].

- Adjonction de cellules ou de facteurs de croissance [29,32]. L'administration de facteurs de croissance ou de cellules associée à la mise en place d'une membrane de ROG repose sur l'hypothèse que la présence de ces éléments biologiques peut déclencher le développement d'un microenvironnement osseux favorable au sein du défaut et ainsi favoriser la régénération

osseuse. On obtiendrait alors une membrane dite ostéoinductrice, favorisant le recrutement et la différentiation de cellules ostéoformatrices au sein de la cavité osseuse. Cela reposerait sur l'incorporation aux membranes de facteurs de croissances et de différentiations cellulaires tels que : FGF, BMP-2, BMP-7, TGFb, VEGF, PDGF [5,24,27,29,80]. Les facteurs de croissance n'ont pas seulement été incorporés dans les membranes pour améliorer la bio activité de celles-ci, mais ont également été délivrés localement au sein du défaut par injection ou en combinaison avec un matériau support biocompatible [24]. De nombreuses études précliniques ont comparé l'apport de membranes natives à ces même membranes couplés à des facteurs de croissances (tels que BMP-2, BMP-7, bFGF/FGF-2, PDGF) pour la régénération osseuse (Figure 17). La plus part de ces études ont rapporté une régénération osseuse significativement plus élevée en présence de facteur de croissance [24].

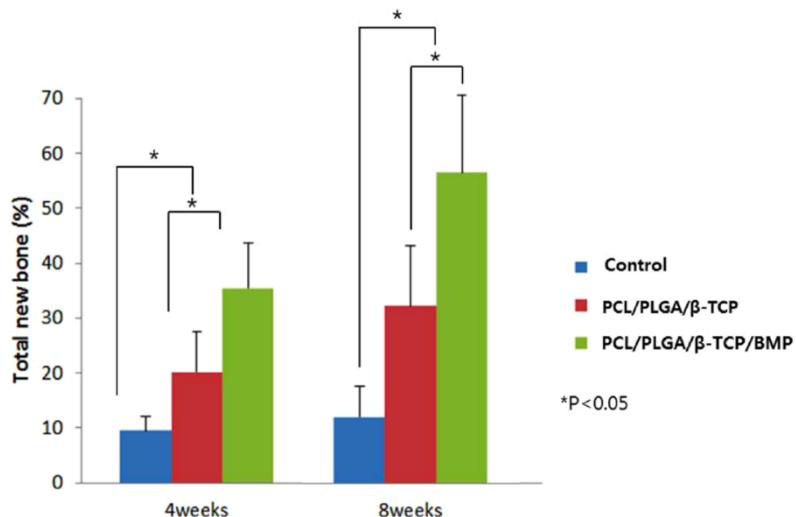


Figure 17. Réparation osseuse de défauts de calvaria chez le rat en présence d'une membrane de PLA/PLGA/bTCP préalablement chargée ou non en BMP-2. D'après [80].

Des résultats encourageants ont également été obtenus lorsque la membrane induite était comblée par un matériaux de substitution osseuse (HA-β-TCP) préalablement chargé en BMP-7 en site osseux fémoral chez le rat [81]. Par ailleurs, plusieurs études pré-cliniques suggèrent l'adjonction de cellules souches, pré-ostéodifférencierées ou non, pour potentialiser l'effet des membranes. Il s'agit majoritairement de cellules souches provenant de la moelle osseuse ou du tissu adipeux [24,29]. Kaigler *et al.* ont effectué le premier essai clinique pour évaluer l'apport de la thérapie cellulaire à la ROG de défauts post-extractionnel avant la pose d'implants [82]. Après l'extraction, les alvéoles dentaires étaient comblées par des éponges de collagène avec ou sans cellules progénitrices autologues issues de la moelle osseuse des

patients. Le site était recouvert par une membrane de collagène dans les deux groupes. Six à douze semaines après la chirurgie, une biopsie osseuse était réalisée et les implants étaient placés. Les analyses cliniques et histomorphométriques effectuées 6 et 12 semaines après le traitement ont montré que la thérapie cellulaire réduisait le risque d'exposition osseuse lors de la mise en place de l'implant et accélérait la régénération osseuse.

- Incorporation de substances minérales au sein des membranes [5,27,40]. Il a été démontré *in vitro* que l'adjonction de nano-particules d'HA favorisait la prolifération et la différentiation cellulaire précoce [83,84].

- Fonctionnalisation des membranes : La fonctionnalisation de la surface et de la structure des membranes a également été proposée pour améliorer les propriétés des membranes en ROG [5,27]. Cette fonctionnalisation repose sur l'utilisation de membranes multicouches [83,85] avec différents gradients de composition et/ou de structure qui répondent aux exigences fonctionnelles locales en améliorant la croissance osseuse tout en empêchant l'envahissement par les tissus gingivaux [27]. L'electrospinning ou electrofilage des membranes permet de produire des polymères naturels ou synthétiques biocompatibles et dégradables dont la disposition ressemble normalement à de la matrice extracellulaire native [40]. L'une des possibilités est également de jouer sur la porosité de la membrane en fonction des couches : une faible porosité en surface afin de jouer le rôle de barrière et une porosité plus importante en profondeur pour améliorer l'adhésion et la prolifération des ostéoblastes [5]. D'autres études suggèrent d'optimiser la composition structurale de chaque couche pour une fonction particulière [83,85]: en utilisant par exemple un film en PLGA non poreux pour la face superficielle, et une face profonde poreuse (au contact du défaut osseux) composée d'hydroxyapatite, de collagène et de PLGA [83]. Une autre possibilité consiste à réaliser une membrane par impression 3D à partir d'une pâte contenant déjà les différents composants souhaités. Dans leur étude, Shim *et al.*, ont réalisé une pâte contenant un mélange de PLA/PLAGA/ β -TCP qui est ensuite placée dans une seringue à extrusion en vue de l'impression 3D d'une membrane pour la ROG [80].

3. LA MEMBRANE AMNIOTIQUE

Depuis peu, l'utilisation de membrane résorbable d'amnion ou d'amnion/chorion a été suggérée comme une alternative à l'utilisation des membranes traditionnelles [6,26,32,77]. La membrane amniotique contient des cellules souches et des facteurs de croissance lui conférant de nombreuses propriétés biologiques. Elle pourrait alors être considérée comme une membrane « bio-active » pour la ROG.

Dans cette partie nous décrirons dans un premier temps la structure et les propriétés de la membrane amniotique, puis nous aborderons son utilisation en ingénierie tissulaire avant de conclure sur ses applications pour la régénération du tissu osseux.

La partie concernant la description et l'apport de la membrane amniotique en ingénierie tissulaire est présentée sous la forme d'un chapitre d'ouvrage international qui sera prochainement publié dans « Micro- and Nanotechnologies in Biomedical Engineering ; Volume 1 : Tissue engineering » (Elsevier).

3.1. Anatomy and physiology of amniotic membrane

The human placenta starts growing a few days after fertilization. This complex organ plays a key role for the development and survival of the fetus during the foetal development, nutrition and tolerance, acting as a physical and biological protection [86].

The placenta is composed of two fetal membranes and separates the fetus from the endometrium. The two fetal membranes consist of an outer chorionic membrane and the amniotic membrane or amnion. Human amniotic membrane (hAM) is the innermost layer, lining the amniotic cavity and is in contact with the amniotic fluid [87], in which the fetus is suspended during pregnancy. hAM contains three main layers: an epithelial monolayer that is separated from the stroma layer by a basement membrane (Figure 18).

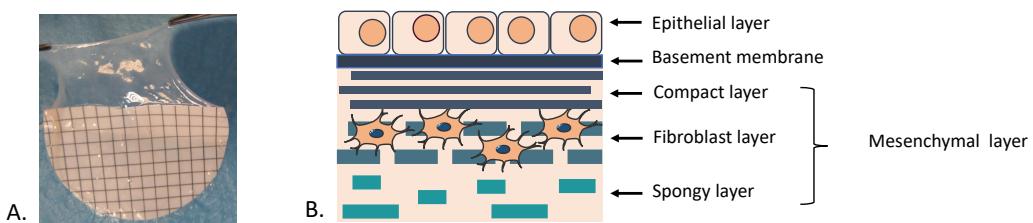


Figure 18. (A) Macroscopical appearance of amniotic membrane. (B) Structure of amniotic membrane.

The amniotic epithelium is characterized by a single layer of human amniotic epithelial cells (hAECs), which have usually a columnar or cuboidal shape [88]. It has been reported that hAECs express stem cell markers and have the ability to differentiate toward all three germ layers [41–45,51,52][89]. hAECs are densely adherent to the basement membrane, which lies at their outer edge. These cells secrete collagen type III and IV as well as noncollagenous glycoproteins (laminins, nidogen, and fibronectin) that form the basement membrane of the hAM [90]. This basement membrane is one of the thickest found in humans and it provides a support to the fetus throughout gestation. The third layer, called the stroma layer, is a collagen-rich mesenchymal layer and contains three components: (i) a compact layer, that is a dense and almost acellular layer mainly composed of collagen type I and III and fibronectin; (ii) a fibroblastic layer, where dispersed fibroblast-like mesenchymal cells and rare macrophages with a loose fibroblast network can be observed ; and (iii) the outer layer, called spongy layer, because of its high quantity of proteoglycans and glycoproteins leading to a spongy appearance on histological sections [87,88,91,92]. This spongy layer is made of loosely arranged collagen fibers and separates the amniotic and chorionic mesoderm. Collagens types I, III, V and VI are major proteins of the extracellular matrix in the stroma layer [93], and they are secreted by mesenchymal cells situated in the fibroblast layer. Two cell types compose the amniotic membrane: hAECs and human amniotic mesenchymal stromal cells (hAMSCs). They are responsible for the production of extra-cellular matrix, different cytokines and growth factors [94]. This membrane is a translucent biological structure that is neither vascularized nor innervated. Nutriments and oxygen are provided by the surrounding chorionic fluid, amniotic fluid and the fetal surface vessels, through diffusion mechanisms [95].

3.2. Amniotic membrane collection and preservation methods

Amniotic membrane is easy to obtain and readily available because the human placenta is considered as a surgical waste after delivery. hAM is generally obtained from healthy pregnant patients undergoing elective caesarian surgery, after proper informed consent. A rigorous serological screening must be performed on pregnant donors, for human immunodeficiency virus-1/2, Hepatitis B, Hepatitis C, human T-cell lymphotrophic virus, syphilis, cytomegalovirus, and tuberculosis [96]. Placentas obtained from caesarians is the preferred source because placentas from vaginal deliveries can be contaminated and therefore unsuitable for transplantation [94]. After the delivery, the collected placenta is placed in a sterile container containing a sterile transport medium to avoid drying [10–12]. Then the placenta is processed under aseptic conditions to obtain hAM. After repeated rinses of the placenta, the hAM is easily separated from the underlying chorion along their natural cleavage plane, since hAM spongy layer is loosely connected to the chorion. The placenta is routinely washed using a saline sterile solution containing antibiotics such as streptomycin, or penicillin, and amphotericin prior to storage [97].

Long-time storage before use is recommended by regulatory agencies of many countries to avoid the possibility that the donor is in the “window period” of infection. Thus, several preserving methods such as cryopreservation, freeze-drying or air-drying, have been developed. Moreover, preserved hAM can be used intact, denuded (without epithelium) or decellularized (without epithelial and mesenchymal cells). Whatever the method used, the processing and preservation of hAM will affect the properties of the biological material [98].

Cryopreservation in glycerol, acting as a cryoprotectant, is the most commonly used preservation method. Several studies reported the use of DMSO as an alternative solution to cryopreserve hAM [13–15][99]. The use of cryopreserved hAM is safe and effected as reported by many experimental and clinical studies [100,101]. Cryopreservation allowed a better preservation of proteins and growth factor compared to lyophilization, which is especially important when the tissue has low protein levels [102]. However, cryopreservation has some limitations: the viability of amniotic cells is low after cryopreservation process [103,104] and it requires expensive and cumbersome equipment to freeze a high quantity of amniotic tissue

to -80°. Besides, storage can not exceed several months. Another difficulty is the necessary respect of the cold chain, making transportation difficult [96,97].

Lyophilization or freeze-drying is a preservation technique that consists in removing water from a tissue by the process of sublimation. This process will induce some alterations concerning structure, biological and physical properties [102]. However, it results in a decrease of destructive chemical reactions, avoiding tissue deterioration, and the samples can be stored safely for several years at room temperature [97,102,105]. Transportation is simple, in contrary to cryopreserved human amniotic membrane [105]. A pre-treatment with trehalose prior to lyophilization was proposed to improve its quality. Because the water loss caused by lyophilization may affect the physical and biological structure of hAM, trehalose can replace some water content in the cells and it might have a positive effect in terms of stabilization of proteins and other components [97,106]. Air-drying is another proposed preservation technique that is low cost and the final product is easy to store at room temperature [98,107]. hAM is kept at room temperature under a hood and exposed to air for different time period. Lyophilization and air-drying are usually followed by a sterilization of the amniotic tissue by gamma-radiation [92]. Sterilization with paracetic acid (PAA) has also been proposed as an alternative to gamma-radiation [108].

3.3. Biological properties of amniotic membrane

Due to its biological and mechanical properties, hAM is highly attractive for tissue engineering applications. Here, we will describe its properties regardless of the preservation method used (Figure 19). Besides, all these properties have to be modulated by the variability of hAM due to inter- and intra-donor variations [109].

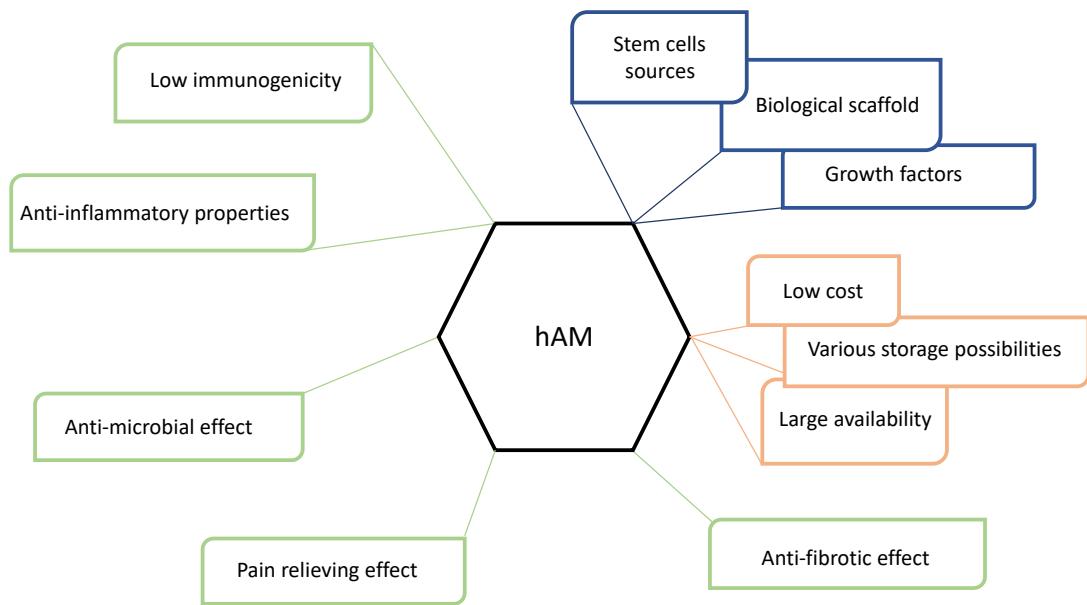


Figure 19. Amniotic membrane properties.

hAM is known to possess a low immunogenicity and it has the ability to exert an anti-inflammatory, antifibrotic, antimicrobial effect and to favor wound healing. Amniotic membrane seems to be an immune-privileged tissue and to contain some immunoregulatory factors, including HLA-G (an immunosuppressive factor) and Fas ligand [110]. This effect is also supported by the low/absent level of expression of HLA class I molecules and the absence of HLA class II molecules [111], avoiding allograft rejection of hAM. Several growth factors are produced by amniotic membrane and its cells such as : epidermal growth factor (EGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) basic fibroblast growth factor (bFGF) and macrophage colony-stimulating factors (M-CSF) [112,113]. Besides, hAM has an anti-inflammatory effect driven by both hAECs and hAMSCs, which express various anti-angiogenic and anti-inflammatory proteins such as interleukin (IL)-1 receptor antagonist; tissue inhibitors of metalloproteinase (TIMPs)-1, -2, -3, -4; and IL-10 [114]. hAM has both angiogenic and anti-angiogenic properties [115]. hAM is also known to induce an anti-adhesive and anti-scarring effect. It reduces proteases activity via the secretion of tissue inhibitors of TIMP's, and down-regulates the expression of transforming growth factor beta (TGF- β), which is responsible for the activation of fibroblasts, thereby inducing an anti-fibrotic effect [116,117]. hAM is also known to exert an anti-microbial effect and therefore protects the wound from infection [86]. hAM expresses natural antimicrobial molecules such as β -

defensins and elafin [118] and has an inhibitory effect against several bacteria (streptococcus group A or S. aureus...) [119], resulting in an anti-bacterial effect. It can also be explained by its close adherence to the wound surface avoiding contaminations [120]. This close adherence is also known to maintain a moist environment, which contributes to the pain-relieving effect of hAM. Indeed, hAM can be used to reduce the pain of burn wounds or surgical wounds, acting as a biological dressing that protects the exposed nerve [109,121,122]. Finally, few studies suggested that amniotic cells may exert an anticancer effect [123,124] mainly explained by the anti-angiogenic, pro-apoptotic and immunoregulatory activity of hAM.

3.4. Mechanical properties of amniotic membrane

The physical properties of hAM, such as elasticity, stiffness and mechanical strength, are another key element of its attractivity for tissue engineering [97]. hAM is one of the human thickest membranes, which adheres firmly to an exposed surface [125]. Fresh hAM is a translucent tissue and its thickness ranges from 0.02 to 0.5 mm. Collagens, elastin and other ECM components play an important role in hAM biomechanical properties [93,126]. Indeed, it has been suggested that collagen proteins play a key-role in the stress tolerance of fetal membranes because it was observed that the collagen content was reduced in pathological fetal membranes that ruptured early [93]. Besides, it seems that collagen types I and III predominate and form parallel bundles, providing the mechanical integrity of AM. Collagen type V and VI form filamentous connections between interstitial collagens and the epithelial basement membrane [94]. To enhance mechanical properties of hAM or to overcome the lack of space-maintenance capabilities, it has been suggested to use multi-layered hAM [127,128], or to reinforce it with a stronger biomaterial such as electrospun nanofibers of polymers [129,130]. Physical and mechanical properties are affected by preservation methods and sterilization of hAM. Depending on the preservation process, hAM thickness can change: cryopreservation often increases hAM thickness, whereas lyophilized hAM is thinner [131]. One study also suggested that the sterilization process of hAM by gamma-radiation had a weakening effect on its mechanical properties [132]. However, the clinical impact of such changes has not be evaluated yet [98].

3.5. Human amniotic stromal cells as a source of stromal cells for tissue engineering

One of the objectives of tissue engineering is to provide complex living constructs for regenerative medicine applications to replace or repair damaged tissues and organs. Stromal cells are one of the most important components to fabricate these constructs, mainly by seeding these cells on an appropriate scaffold matrix, on which they will grow and differentiate. The ability of stromal cells to differentiate towards multiple cell lineages offers promising possibilities for various targeted tissues.

Using routine protocols, two types of cells can be isolated from amniotic membrane: hAECs and hAMSCs [89,133]. After isolation, hAECs display a cobblestone morphology, while hAMCs have a fibroblast-like morphology [134]. Due to their specificities, these amniotic stromal cells are considered as an attractive source of stem cells for TE [95]. First, they are easily available and they constitute a favorable source of stromal cells for allogenic transplantation since they display immunomodulatory properties [89]. The pluripotent potential of hAM-derived cells has been suggested, meaning that hAM-derived cells are capable of self-renewing, and to differentiate into the 3 germ layers of the developing embryo (ectoderm, mesoderm, and endoderm) [135], and hAMSCs and hAECs have been shown to express pluripotent markers [129].

3.5.1. *Amniotic epithelial cells for tissue engineering*

hAECs derived from embryonic ectoderm, which belongs to the continuous monolayer in contact with the amniotic fluid [136]. 50 to 70 millions of hAECs can be obtained from one placenta [111]. Compared to human embryonic stromal cells, hAECs are a noncontroversial source of cells for tissue engineering because their harvest and use do not raise ethical concerns and they are easy to isolate. For the isolation of epithelial cells, the amniotic membrane is stripped from the underlying chorion and digested with trypsin or other digestive enzymes [137]. Normally, 2–6 passages are possible before proliferation ceases [89].

Several authors suggested that hAECs were pluripotent cells. It was first stated because of the early origin of fetal membranes, which begin to develop before gastrulation. Then, the fact that amniotic ectoderm derives itself from the epiblast, suggested that the amniotic epithelium might retain a reservoir of stromal cells all throughout pregnancy [111]. Epiblast is

also the source of the three germ layers of the embryo through the process of gastrulation. These statements were corroborated by several *in vitro* and *in vivo* studies that showed that hAECs display the potential to differentiate into all three germ layers: endoderm (liver, pancreas), mesoderm (cardiomyocytes), and ectoderm (neural cells) [111,138,139].

In the field of tissue engineering, several studies suggested to seed hAECs on a scaffold prior its graft. For example, hAECs were seeded onto rabbit corneal stroma lamellar and transplanted on a corneal stromal cell deficiency model in rabbits to promote ocular reconstruction [140]. Concerning skin regeneration, Jiang *et al.* seeded hAECs and fibroblasts onto an acellular dermal matrix prepared from allogenic skin to form an organotypic skin. Histological and immunohistochemical analysis showed that hAECs successfully developed a stratified epithelium on the allogenic dermis and exhibited the main ultrastructural features of normal human skin [141]. Another method to create an artificial tissue-engineered skin substitute is to co-culture hAECs and hAMCs. In that way, hAECs were cultured in a fibrin scaffold, in order to promote hAECs differentiation towards keratinocyte cells [142]. Another study suggested to seed hAECs onto gel made of type I collagen and hAMCs for skin tissue engineering [143].

The potential of AECs associated to a scaffold, namely a bone substitute, to promote bone regeneration has also been assessed. Ovine AEC were seeded on a biomaterial composed of hydroxyapatite and beta-tricalcium phosphate (beta-TCP) scaffold before grafting the loaded scaffold into the maxillary sinus in sheep. Bone regeneration observed with the AECs seeded on the bone substitute was significantly higher compared to the bone substitute without cells [144]. Another study failed to induce ectopic mineralized bone formation when hAECs were seeded on a beta-TCP scaffold and then implanted subcutaneously [145].

3.5.2. Amniotic mesenchymal stromal cells for tissue engineering

hAMSCs derived from embryonic mesoderm are sparsely distributed in the stroma layer (fibroblast layer). hAMSCs can be obtained from the amniotic membrane after removal of hAECs or after scraping the mesenchymal layer from the amniotic membrane without affecting the epithelial layer [146,147]. Whatever the process used, the remaining hAMSCs are then isolated by digestion with collagenase or collagenase plus DNase [89]. Normally, 5–

10 passages are possible before proliferation ceases [89], and the yield of mesenchymal cells was approximately 1 million placenta[95].

Several studies reported the ability of hAMSCs to differentiate towards the three lineage. Studies have shown that hAMSCs could differentiate into multilineages such as endothelial cells [148], neuronal cells [149], hepatocytes [150]. hAMSCs has a great mesodermal differentiation capability, as assessed by their capability to exhibit cardiogenic, adipogenic, osteogenic and chondrogenic differentiation potential [136,145,151–153]. Besides, hAMSCs produced significantly greater quantities of mineralized and cartilaginous matrix at earlier time points compared with human adipose derived stromal cells (hADSCs) [152].

Despite this great differentiation potential, there are very few studies that described the use of hAMSCs associated with a scaffold for tissue engineering. As mentioned above, the use of hAMSCs associated with hAECs has been assessed to create tissue-engineered skin substitute. In addition, the potential of hAMSCs used alone without hAECs has also been suggested. One study reported the transplantation of hAMSCs through two different carrier on a mice wound model. Whatever the carrier used, hAMSCs enhanced the neovascularization of the wound area [154].

3.6. Human amniotic membrane as a biological scaffold for tissue engineering

The scaffold is an essential component for tissue engineering. It acts as a supporting matrix upon which cells can growth and differentiate. Several prerequisite are needed during the selection of a suitable scaffold, such as biocompatibility or biodegradability. Cell adhesion properties of tissue engineering scaffolds are also critical [155].

hAM is considered as an attractive biological scaffold for tissue engineering since it is an easy to obtain and cost-effective natural tissue [92]. The first use of hAM in medicine was reported in the early 1900s by Davis for skin transplantation. Since then, hAM has been routinely used in Ophtalmology. Thanks to its satisfying outcomes in this field and its biological properties, hAM has been widely studied in various areas of tissue engineering. hAM has the ability to promote cells adhesion and proliferation thanks to its extracellular matrix structural components (collagen, laminin and fibronectin). Fresh or preserved hAM, as well as, intact or

denuded (with or without epithelium) and decellularized hAM have been used as a biological substrate for cell growth, as various cell types can be seeded on it [94,131] (Tableau 1). It was also used as a delivery system [156,157]. Owing to its unique structure and biological properties, hAM can be used alone as a scaffold already containing stromal cells and growth factors. Depending on the targeted application in tissue engineering, hAM can also be processed with other materials or other source of stromal cells to improve mechanical properties or different elements needed to repair the damaged tissue [120,158]. Moreover, hAM can be prepared as a single or multi-layered structure to enhance its mechanical properties, or it can be rolled to realize more complex three-dimensional scaffolds. hAM can also be used as a barrier to separate newly-forming tissues from surrounding tissues.

Tableau 1. Amniotic membrane for tissue engineering.

Authors	Tissue engineering applications	Cells seeded on amniotic membrane	Preservation methods of amniotic membrane	Sides of amniotic membrane upon which cells were seeded
Shortt <i>et al.</i> [108]	Ocular tissue engineering	Human limbal epithelial stem cells	Decellularized +Cryopreserved or Cryopreserved	NS
Niknejad <i>et al.</i> [131]	Ocular tissue engineering	Rat vascular endothelial cells	Fresh Cryopreserved Lyophilized	Epithelial side
Zhang <i>et al.</i> [159]	Ocular tissue engineering	Human limbal epithelial cells	De-epithelialized	Basement membrane side
Yang <i>et al.</i> [160]	Skin tissue engineering	Human keratinocytes	De-epithelialized	Basement membrane side
Redondo <i>et al.</i> [161]	Skin tissue engineering	Human melanocytes	De-epithelialized	Basement membrane side
Tsai <i>et al.</i> [162]	Vascular tissue engineering	Porcine vascular endothelial cells	De-epithelialized	Basement membrane side
Lee <i>et al.</i> [163]	Vascular tissue engineering	Porcine vascular endothelial cells	De-epithelialized + Glutaraldehyde	NS
Amensag <i>et al.</i> [164]	Vascular tissue engineering	Human umbilical vein endothelial cells Human vascular smooth muscle cells	Decellularized	Stromal side

Authors	Tissue engineering applications	Cells seeded on amniotic membrane	Preservation methods of amniotic membrane	Sides of amniotic membrane upon which cells were seeded
Swim <i>et al.</i> [165]	Vascular tissue engineering	Human thymus derived mesenchymal stem cells Human umbilical cord blood stem cells Human umbilical vein endothelial cells Cardiac Myocytes Arterial Smooth Muscle Cells	Decellularized + Lyophilized	NS
Díaz-Prado <i>et al.</i> [125]	Cartilage tissue engineering	Human chondrocytes	Cryopreserved Decellularized +Cryopreserved	Stromal side Epithelial side Basement membrane side
Tan <i>et al.</i> [156]	Cartilage tissue engineering	Rabbit bone marrow mesenchymal stem cells	Dried Lyophilized	NS
Jin <i>et al.</i> [166]	Cartilage tissue engineering	Rabbit chondrocytes	Cryopreserved De-epithelialized	Epithelial side Stromal side Basement membrane side
Wu <i>et al.</i> [167]	Periodontal tissue engineering	Human adipose derived stem cells	De-epithelialized	Basement membrane side
Amemiya <i>et al.</i> [168]	Periodontal tissue engineering	Dog periodontal ligament cells	De-epithelialized	NS
Amemiya <i>et al.</i> [169]	Periodontal tissue engineering	Periosteum derived stem cells	De-epithelialized	NS
Iwasaki et al. [170]	Periodontal tissue engineering	Human periodontal ligament stem cells	Decellularized +Cryopreserved	NS

Authors	Tissue engineering applications	Cells seeded on amniotic membrane	Preservation methods of amniotic membrane	Sides of amniotic membrane upon which cells were seeded
Tsugawa et al. [171]	Bone tissue engineering	Mouse bone marrow-derived osteoblast cells	De-epithelialized	Stromal side
Semyari et al. [172]	Bone tissue engineering	Rabbit adipose-derived mesenchymal stem cells	Decellularized	NS
Tang et al. [173]	Bone tissue engineering	Human umbilical vein endothelial cells Rat bone marrow mesenchymal stem cells	De-epithelialized	NS
Amemiya et al. [174]	Oral mucosa tissue engineering	Human oral mucosal epithelial cells	De-epithelialized	Basement membrane side

NS : Not Specified

3.6.1. Amniotic membrane for ocular surface reconstruction

The transplantation of hAM was introduced in ophthalmology in 1940 by De Rotth who used fresh fetal membranes in the treatment of conjunctival reconstructions. Since then, amniotic membrane is increasingly used for ocular surface reconstruction and it has been successfully applied in a wide variety of ocular pathologies [99], including corneal disorders associated with limbal stem cell failure, conjunctival applications or glaucoma surgery [159]. For its clinical applications in ophthalmology, hAM is used either as a biological dressing to favor healing, or as a scaffold for corneal, conjunctival and limbal epithelial cell growth. Depending on the desired therapeutic purpose, there are three main surgical techniques, by which hAM can be applied on ocular surfaces [99,109]: it can be placed as a graft (onlay) or as a patch (overlay). Finally, multi-layered hAM can be used to fill the defect. When hAM is intended to be used as a substrate for epithelial regeneration, it is grafted as a scaffold for epithelial cells and it will become incorporated into the host tissue. The patch or overlay technique aims to graft a patch of hAM larger than the underlying defect to promote host epithelium growth below the membrane. In this technique, hAM protects the underlying healing epithelium but does not become incorporated into the host tissue since it will be removed few days later [99]. The last way to use hAM for ocular surface reconstruction, called “layered” or “fill in” technique, combines the two previous techniques and is used to repair deep corneal ulcer. In this application, multilayered hAM is grafted to fill the entire depth of a corneal ulcer or crater and the outer layer aims to promote epithelium growth [159].

As mentioned above, another technique to promote ocular surface reconstruction consists in using hAM as a scaffold previously seeded with cells before its transplantation. Indeed, the use of hAM as a substrate for expanding limbal epithelial stromal cells (LEC) for subsequent transplantation in limbal stromal cell deficiency (LSCD) has been widely described [108]. Corneal epithelial stromal cells were harvested from limbal biopsies for *ex vivo* expansion on AM, which can then be transplanted onto the eye to treat LSCD [159]. Whereas cryopreservation is commonly used for hAM graft as a dressing material, Niknejad *et al.* suggested that lyophilized hAM is more suitable than the fresh and cryopreserved hAM to culture endothelial cells [131]. Several process to de-epithelialize hAM have also been evaluated to expose the basal membrane, thus promoting cell growth onto the hAM [160].

3.6.2. Amniotic membrane for skin reconstruction

hAM has been used for centuries as a biological dressing to treat acute and chronic wounds injury and burns, acting as a physical and biological barrier [161]. More than 200 clinical trials have reported its efficacy for wound healing treatment [162]. Several studies highlighted its benefits, such as prevention of infection or its ability to enhance re-epithelialization or to reduce pain after wound covering [96,163]. An analgesic effect was observed in patients treated for the regeneration of non-healing wounds and it could be explained by limited contact of the wound bed and environment through the covering of the nerve terminals [164]. The use of hAM as a biological dressing also provides a reduction of water loss through evaporation, by acting as water barrier, and thus favor a moist environment for cell survival and growth, therefore enhancing the healing process. Furthermore, as mentioned before, hAM has the ability to exert an anti-fibrotic effect, in particular through down-regulation of TGF- β and its receptor expression, which is sought to reduce scar formation.

Moreover, there is a similarity between normal human skin layers and amniotic membrane layers, thereby suggesting a great potency of hAM to be used as a skin substitute [164]. That's why, in addition to wound dressing, Yang *et al.* also suggested to use hAM as a scaffold to create a skin substitute for wound closure. Amnion scaffolds seeded with human keratinocytes have generated living skin equivalents and have been successfully transplanted into an animal model [165]. Redondo *et al.* suggested to use hAM as a new strategy for inducing re-pigmentation in patients with vitiligo disease. They cultured autologous melanocytes on a denuded hAM, which were implanted onto lesions of four patients with vitiligo : a repigmentation of 90–95% was obtained, showing promising results [166]. Finally, a new interesting and promising approach has been developed by Murphy *et al.* concerning the use of a novel amnion membrane-derived product for skin tissue engineering [162]. After grinding lyophilized hAM, they combined this solubilized amniotic membrane with hyaluronic acid and they made a composite hydrogel delivery system. This process aimed to result in a cell-free solution, while maintaining high concentrations of cell-derived cytokines and growth factors. This new amnion membrane-derived showed encouraging results to promote wound healing and reduce scar contraction in a full-thickness murine wound model [162].

3.6.3. Amniotic membrane for vascular tissue engineering

Several studies stated that amniotic membrane could be used as a scaffold for the fabrication of tissue engineered blood vessels. In this objective, the cell culture of porcine arterial endothelial cells on amniotic membrane has been proposed [167,168]. They first demonstrated that porcine endothelial cells can successfully be seeded on sows amniotic membrane with an increase of the expressions of junctional proteins while the expression of the adhesive inflammatory molecules were decreased. Then, they realized a tissue engineered blood vessels made with rolled hAM, thus creating a tube of hAM which was endothelialized with porcine vascular endothelial cells [168].

An *in vitro* study reported the use of decellularized hAM seeded with human umbilical vein endothelial cells and human vascular smooth muscle cells, prior to be rolled into a dense construct, as an alternative strategy to develop cell-dense vascular bioscaffolds. It resulted in a mechanically stable, multi-layered tissue-engineered blood vessel conduit, that can be manufactured into different diameters and shapes to suit targeted applications [169]. Swim *et al.* combined a decellularization and freeze-drying approach to produce a mono-layer or a multi-layer amnion-based scaffold suitable for tissue engineering constructs destined to reconstructive heart surgery application [170]. Whereas both preservation procedure enhanced cells viability and growth of various cells types seeded on hAM *in vitro*, the multi-layered construct had enhanced biomechanical properties. The multi-layered construct was implanted in a piglet model of left pulmonary artery grafting, and showed its *in vivo* suitability and biocompatibility for vascular repair, as demonstrated by the development of newly-formed endothelium in the intima, a smooth muscle cell-rich medial layer and an adventia containing new vasa vasorum, endothelial cell layer in the inner side of the graft and a smooth muscle layer in the outer side. Finally, they proposed to seed this amniotic scaffold with the patient's own MSCs to produce an autologous vascular graft [170].

3.6.4. Amniotic membrane and nerve regeneration

Several studies reported the use of fresh or preserved hAM as a scaffold for nerve regeneration, highlighting a pro-regenerative effect on injured peripheral nerves. Fresh hAM was implanted in a rat model of sciatic nerve scarring to treat recurring perineural adhesions, and associated nerve scarring. An accelerated recovery of sciatic nerve function was observed

when the epithelial side of hAM was applied toward the nerve [171]. Another study reported the use of cryopreserved hAM to treat nerve injury. For this purpose, cryopreserved hAM was wrapped around the damaged nerve, and scar formation and functional recovery were assessed. Although both functional and morphological parameters were not significantly improved, nerves wrapped with hAM had significantly fewer adhesions and less scar formation than controls [172]. Dried acellular hAM sheets were rolled around Teflon stints, resulting in tubes of various diameters. After resection of a sciatic nerve segment, the tube of hAM was inserted around the defect, so that the gap and the edges of the nerve were covered by the tube. Morphological aspect as well as motor and sensory reinnervation of the sciatic nerve were assessed. It was concluded that 1–2-mm diameter tubes made of hAM can serve as an effective conduit for nerve regeneration, including functional recovery [173]. All these studies stated that hAM has a pro-regenerative effect on injured peripheral nerves, and it seems to be mainly provided by its anti-fibrotic and anti-scarring effect.

3.6.5. Amniotic membrane for cartilage regeneration

The biological properties of the extracellular matrix of hAM have also been studied in the field of cartilage regeneration since hAM is rich in hyaluronan acid, proteoglycans and collagen which is found in native cartilage tissue [156]. *In vitro* studies performed on fresh and cryopreserved hAM stated that the chorionic side of hAM is a more suitable scaffold than its epithelial side to promote chondrocytes proliferation and to maintain their phenotype [125,174]. *In vitro*, hAM was also associated with fibrin to develop a new 3D scaffold. They observed that bovine chondrocytes were able to proliferate when seeded on this new scaffold [175]. hAM has also been combined with a synthetic scaffold in PLGA as a cell-free material for cartilage repair. Once implanted in osteochondral defects, this combined scaffold promoted regeneration of hyaline-like cartilage [176]. Another study investigated the potential of intact and denuded hAM to act as a chondrocyte delivery matrix for cartilage regeneration. *In vitro*, denuded hAM appeared to be more suitable for chondrocytes proliferation, compared to the epithelial side of intact hAM. *In vivo*, denuded hAM was compared to denuded hAM seeded with chondrocytes to repair a rabbit osteochondral defect. The rate of regenerated cartilage was significantly higher when chondrocytes seeded on hAM were facing the defect, suggesting that denuded hAM can act as a cell carrier matrix for

cartilage regeneration [174]. The potential of dried and lyophilized hAM to act as a MSC cell carrier matrix and to promote their chondrogenic differentiation was also reported [156].

3.6.6. Amniotic membrane for ligament and tendon healing

The interest of hAM for ligament and tendon healing has also been explored [177,178]. hAM has the ability to prevent tendons adhesions around after injury and reconstruction [179]. One study investigated the effects of fresh denuded amniotic membrane and hyaluronic acid, alone and in combination, on adhesions and healing following chicken flexor tendon repair. The prevention of adhesion formation was superior when amniotic membrane was wrapped around the repaired tendon [180]. Another study reported the effectiveness of decellularized amniotic membrane to promote endogenous healing and to prevent tendon adhesion in the same model [181]. This method of tendon-wrapping, in which hAM is laid over the damaged tendon has also been successfully reported in human using cryopreserved hAM [182,183].

Periodontal disease affects the supportive tissue of the teeth that includes the periodontal ligament. Several preclinical studies aimed to investigate the efficacy of hAM to treat periodontal disease. hAM was seeded with adipose-derived stromal cells (ADSCs) [184], and by periodontal ligament stem cells or periosteum derived cells [185–187]. All of these studies concluded that hAM could be a useful scaffold for periodontal regeneration, by avoiding the proliferation of connective tissue on the denuded root surface in periodontal defect. Another *in vitro* study suggested the potential of dental pulp-derived cell sheet cultured on amniotic membrane substrate for periodontal tissue engineering [188]. Another potential application is to use hAM as interpositional material to prevent temporomandibular joint re-ankylosis [189,190]. One study stated that hAM could be used as an interpositional arthroplasty material to prevent temporomandibular joint ankylosis, preventing fibrous adhesion in a rabbit model [189]. Similar results were observed when cryopreserved hAM was compared to fresh hAM [190].

3.6.7. Amniotic membrane for mucosal tissue reconstruction

Since its first use in 1985 by Lawson *et al.* for the treatment of oral mucosa defects [191], hAM has been widely studied in the field of oral and maxillofacial surgery, and, promising results

are achieved by hAM for oral soft tissue regeneration [120]. Multilayered hAM showed its ability to close oronasal fistula in minipigs [192]. Similar results were observed when hAM was used to close oronasal fistula in four patients [128]. Several studies reported the ability of hAM to stimulate healing and, especially, to enhance epithelial regeneration of the human oral mucosa defect after excision of benign and precancerous lesions [193,194]. In this context, hAM has been used alone or seeded with oral mucosal epithelial cell sheets and transferred to the mucosal defect [195,196]. The use of hAM to treat root exposure caused by gingival recession was successfully reported in several clinical studies. When the hAM graft was associated with a gingival flap, the root coverage and the gingival thickness and biotype were improved [197–199].

Finally, and in a very different field, several authors reported the use of hAM for vaginoplasty in patients suffering from congenital absence of the vagina or for gender reassignment surgery. The creation of a neovagina is thus associated with amnion graft. Both fresh and preserved hAM were assessed for this purpose and resulted in satisfying anatomic and functional outcome [200,201]. Satisfactory outcomes were also obtained when autologous fibroblasts were seeded onto hAM prior its graft to cover the neovagina. The two layers of amnion and fibroblasts appeared to be more resistant to trauma and laceration than amnion without seeded cells [202].

3.7. Applications de la MAH pour la réparation osseuse

3.7.1. *In vitro*

Plusieurs études se sont attachées à isoler les cellules souches de la MAH et à étudier leur potentiel d'ostéo-différentiation. Il a ainsi été montré que les cellules amniotiques épithéliales et mésenchymateuses avaient un fort potentiel ostéogénique *in vitro* lorsqu'elles étaient cultivées dans un milieu approprié [136,203,204]. Plus récemment, Wang *et al.* se sont intéressées à l'influence des hAMSCs sur la prolifération et l'ostéodifférentiation de cellules souches de moelle osseuse humaine (hBMSCs). Ils ont utilisé un système d'insert (tranwell) pour mettre en co-culture indirecte des hBMSCs avec des hAMSCs [205,206]. La prolifération des hBMSCs était significativement plus rapide lorsque celles-ci étaient co-cultivées avec les hAMSCs. Les hAMSCs stimulaient l'augmentation de l'activité de la phosphatase alcaline (ALP),

ainsi que l'expression d'ARNm des gènes marqueurs de l'otéodifférentiation, et le dépôt de matrice minéralisée [205,206]. De plus, l'effet des hAMSCs a été significativement inhibé par l'U0126 qui est un inhibiteur hautement sélectif de la signalisation extracellulaire de la kinase 1/2 (ERK1/2), démontrant ainsi que les hAMSC favorisent la différenciation ostéogénique des hBMSC en influençant la voie de signalisation ERK1/2 [205].

Lindenmair *et al.* se sont intéressés au potentiel d'ostéodifférentiation de la MAH dans son intégralité, sans chercher à isoler les cellules de la matrice [207]. Pour cela ils ont cultivé durant 28 jours des patchs de MAH fraîches dans un milieu basal et dans deux types de milieu ostéoinducteur. Les résultats de cette étude montrent une minéralisation de la MAH en milieu ostéogénique (Figure 20) ainsi que l'expression de marqueurs précoces et tardif de l'ostéodifférentiation.

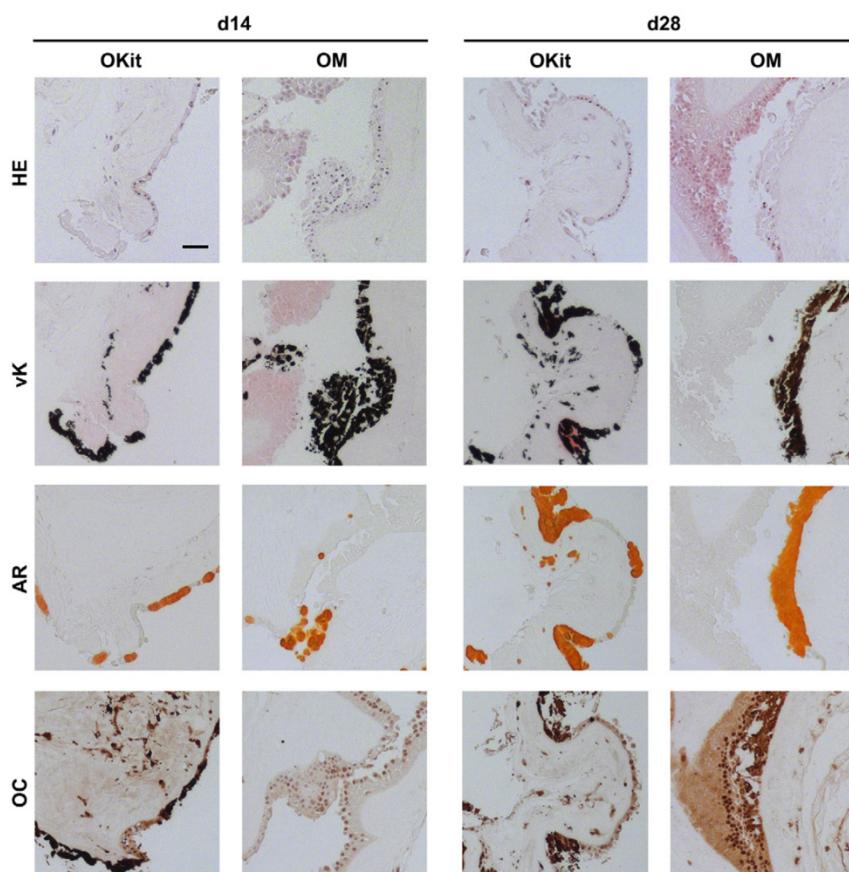


Figure 20. Analyse histologique et immunohistochimique montrant le potentiel ostéogénique de la MAH cultivée en milieu ostéoinducteur. HE: Hematoxylin Eosin; vK: Von Kossa ; AR: Rouge Alizarine; OC: Ostéocalcine. D'après [207].

Des résultats similaires ont été obtenus par Gualdi *et al.* qui se sont également intéressés au potentiel osteogénique de la MAH [208]. La MAH était soit fraîche, soit cultivée en milieu basal, ou alors cultivée dans un milieu ostéoinducteur. Cependant, une altération structurale et fonctionnelle de la MAH a été mise en évidence lorsque celle-ci était cultivée en milieu ostéo-inducteur, suggérant le recours à de la MAH fraîche plutôt qu'à de la MAH pré-ostéodifférenciée en ITO.

3.7.2. Études pré-clinique *in vivo*

De façon similaire aux études menées *in vitro*, des études pré-cliniques ont été menées pour évaluer soit l'apport des cellules amniotiques isolées de la matrice, soit le potentiel de la membrane amniotique utilisée comme matrice pour la réparation osseuse.

a. Études pré-cliniques portant sur l'apport des cellules souches amniotiques pour la réparation osseuse

Plusieurs études ont isolé puis implanté chez l'animal des cellules souches amniotiques afin d'étudier leur potentiel ostéogénique *in vivo*. Mattioli *et al.* se sont intéressés au potentiel ostéogénique de cellules amniotiques épithéliales de mouton pour la réparation de défauts osseux réalisés au niveau des tibias de trois mouton [209]. Des défauts osseux de 3 mm de diamètre étaient réalisés bilatéralement. Ils étaient soit comblés par de la colle biologique (fibrinogène + thrombine) soit par des cellules amniotiques épithéliales de mouton recouvertes par la colle biologique. Les cellules amniotiques épithéliales avaient été préalablement tagguées. Après 45 jours d'implantation, la survie des cellules amniotiques épithéliales était observée en microscopie à fluorescence et une analyse histologique qualitative a été réalisée pour observer la néoformation osseuse. Lorsque les défauts avaient été comblés par les cellules amniotiques épithéliales, une survie de ces cellules était observée ainsi qu'une formation osseuse, tandis que les défauts contrôle semblaient comblés par du tissu fibreux (Figure 21).

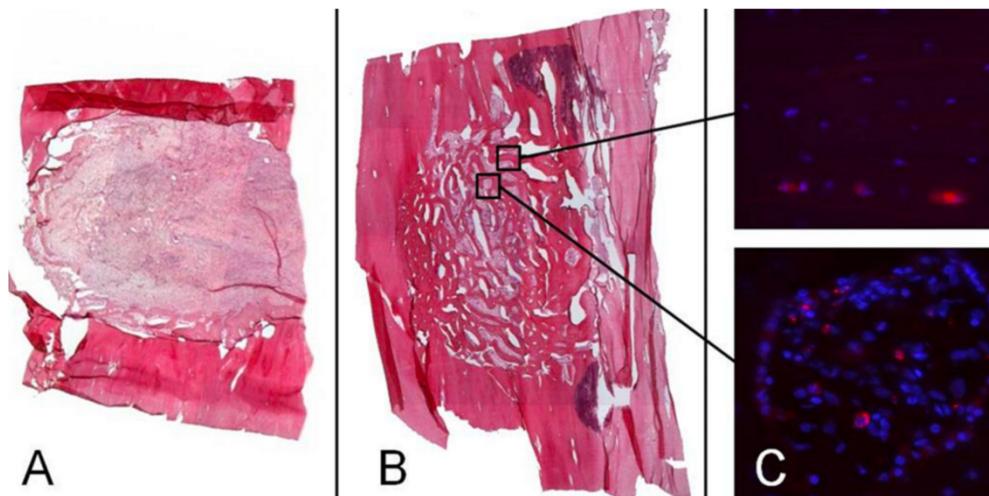


Figure 21. Réparation osseuse de défauts de tibia de mouton 45 jours après l'implantation de (A) colle biologique ou de (B) cellules amniotiques épithéliales recouvertes de colle biologique. (C) Observation des cellules amniotiques épithéliales taguées. D'après [209].

D'autres études se sont intéressées au potentiel ostéogénique des cellules souches amniotiques épithéliales lorsqu'elles étaient préalablement ensemencées sur un « scaffold » notamment sur des matériaux de substitution osseuse. Des cellules amniotiques épithéliales de mouton préalablement ensemencées sur des matériaux composés d'hydroxyapatite et de phosphate tri-calcique (β -TCP) ont été utilisées en complément sous-sinusien dans un modèle de sinus lift chez la brebis [144]. Des défauts bilatéraux ont été réalisés chez 6 brebis et ils étaient soit comblés par le matériau préalablement ensemencé soit comblé par le matériau sans cellules. Une analyse histologique et radiologique ont été réalisées. La néoformation osseuse était significativement plus rapide et plus importante en présence de cellules amniotiques épithéliales. Une autre étude s'est intéressé à la réparation osseuse d'alvéoles maxillaires comblées par un matériau en β -TCP ou par ce même matériau préalablement ensemencé par des hAECs [210]. Les rats ont été sacrifiés après 4 et 8 semaines d'implantation et des analyses histologiques et radiologiques ont été réalisées. La régénération osseuse avait lieu de façon plus précoce et était significativement plus importante en présence d'hAECs. Des résultats divergents ont été obtenus lorsque les implantations avaient lieu en site ectopique. En effet, l'implantation sous cutanée d'un matériau en β -TCP préalablement ensemencé d'hAECs n'induisait pas la formation d'os minéralisé chez la souris [145].

Le potentiel ostéogénique des hAMSCs a également été étudié *in vivo*. Tsuno *et al.* ont ensemencé des matériaux en β -TCP avec une sous population de hAMSCs qui ont ensuite été implantés dans des défauts de calvaria de rats de 5 mm de diamètre [211]. Le groupe contrôle correspondait à l'implantation du matériaux sans cellules. Après 6 semaines d'implantation les cellules amniotiques étaient toujours vivantes et sécrétaient de la matrice extracellulaire. Après 12 semaines, une quantité plus importante d'os mature était observée quand le matériaux avait été préalablement ensemencé par des cellules amniotiques.

b. Études pré-cliniques portant sur l'apport de la membrane amniotique pour la réparation osseuse

Plusieurs études précliniques portant sur le potentiel ostéogénique de la membrane amniotique ont été menées. Selon les études, la membrane amniotique était utilisée : seule, en association avec un matériaux de substitution osseuse, associée à des polymères ou encore préalablement ensemencée par des cellules souches ont été menées. De plus, en fonction du modèle pré-clinique utilisé, la membrane amniotique était soit appliquée comme **membrane barrière** de ROG pour recouvrir un défaut osseux, soit elle était utilisée comme **matériaux de comblement** osseux (Tableau 2).

→ En site ectopique

Une étude s'est intéressée au potentiel ostéogénique de la MAH fraîche ou ostéodifférenciée en site sous-cutané chez la souris [212]. Les MAH fraîche ou ostéodifférenciée étaient implantées seules ou enroulées autour d'un matériau de substitution osseuse. L'analyse histologique a mis en évidence l'absence de formation osseuse quelle que soit la condition étudiée.

→ En site orthotopique

Six études précliniques ont rapporté l'utilisation de la MAH comme membrane pour la ROG [213]. Deux études se sont intéressées à l'apport de la MAH utilisée seule comme membrane pour la ROG [214,215]. Tang *et al.* ont réalisé des défauts de 2.2 mm x 2.5 mm dans des fémurs de rats. Les défauts étaient soit laissés vide, soit recouverts par de la MAH désépithélialisée. L'analyse histomorphométrique montrait une réparation osseuse significativement plus

importante en présence de la MAH [214]. Koushaei *et al.* ont comparé l'apport de la MAH à une membrane de collagène résorbable et commercialisée pour la ROG de défauts de tibia chez le chien [215]. Les défauts de 16 mm de diamètre étaient soit laissés vide (control), soit recouverts par de la MAH ou alors par la membrane de collagène. La réparation osseuse était significativement plus importante lorsque le défaut était recouvert par de la MAH par rapport au groupe contrôle. Cependant, aucune différence significative n'était observée entre la membrane de collagène et la MAH concernant la réparation osseuse [215]. Gomes *et al.*, ont comparé l'apport de la MAH seule ou associée à un matériau de comblement (matrice dentinaire autogène déminéralisée ou ADDM) pour la ROG de défauts de calvaria chez le lapin [213]. Un phénomène de réparation osseuse pouvait être observé dès le 30^{ème} jour après implantation dans les deux conditions, et une réparation osseuse complète du défaut était observée au 120^{ème} jour. Cependant, celle-ci était plus rapide et plus importante en présence de ADDM. Enfin, Li *et al.* ont utilisé un modèle de défaut osseux péri-implantaire, réalisé au niveau de tibia de rats, pour comparer l'apport de la MAH placée en multi couches et associée à un matériau de comblement à une membrane de collagène. Cinq conditions étaient étudiées: 1) Pas de défaut péri-implantaire, 2) défaut laissé vide, 3) comblement du défaut par des particules de Bio-oss®, 4) comblement du défaut par des particules de Bio-oss® recouvertes par une membrane de collagène, 5) comblement du défaut par des particules de Bio-oss® recouverte par de la MAH. Il a été montré que la MAH pouvait jouer le rôle de barrière biologique en empêchant l'envahissement du site osseux par du tissu fibreux et de barrière mécanique en stabilisant le biomatériau en place. Ainsi, l'os néoformé était plus mature et en quantité plus importante avec une meilleure connexion implant-os néoformé lorsque le matériau de comblement était recouvert par de la MAH par rapport à la membrane de collagène [127] (Figure 22).

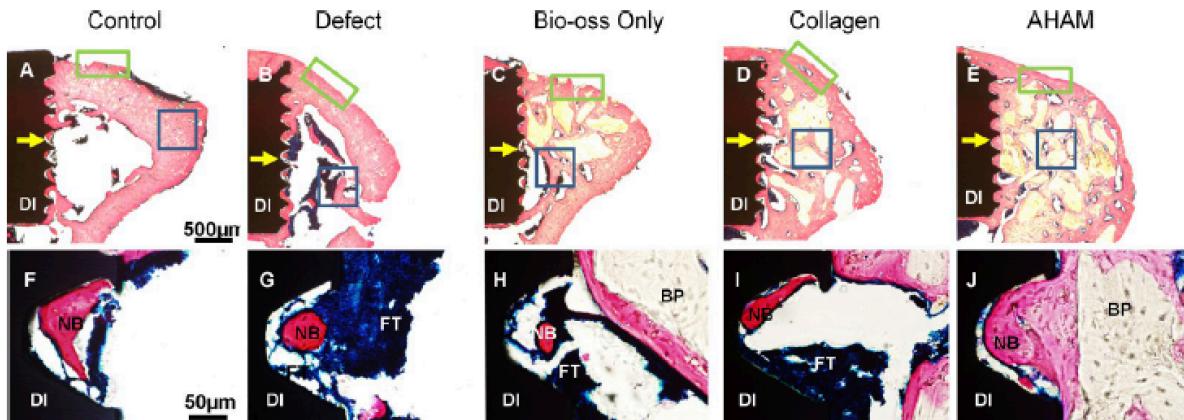


Figure 22. Analyse histologique des tissus au contact de l'implant (flèche jaune) à deux grossissements différents. Une meilleure connexion os-implant était observée en présence de MAH. NB : os néoformé ; BP : particules de bio-oss. [127].

Tsugawa *et al.* ont réalisé des défauts de calvaria chez la souris et ont comparé la réparation osseuse obtenue soit en présence de MAH seule, soit en présence de cellules ostéoblastiques murines implantées dans le défaut puis recouverts par de la MAH ou par de la MAH dont la face mésenchymateuse était précédemment ensemencée de cellules ostéoblastiques murines [216]. Une réparation osseuse complète du défaut a été observée dans la condition où la MAH était ensemencée par des cellules ostéoblastiques, alors que celle-ci n'était que de 50% lorsque les cellules ostéoblastiques étaient implantées puis recouvertes par de la MAH. Enfin, la MAH seule n'a pas permis dans cette expérimentation une réparation osseuse [216]. Semyari *et al.* ont également étudié l'apport de la MA pour la régénération osseuse avec un modèle de défaut de calvaria chez le lapin [217]. L'objectif était d'étudier l'apport de trois types de matrice (la MA et deux matrices polymériques) ensemencées par des ADSC (cellules souches issues du tissu adipeux) autologues *versus* ces trois mêmes matrices décellularisées. Il n'y avait pas de différence significative concernant la réparation osseuse entre les groupes MA seule et MA ensemencée [217].

L'apport MAH a également été évaluée dans un modèle de fente palatine. Li *et al.* ont assemblé une double couche de MAH décellularisée à un polymère synthétique [218]. Ils ont comparé l'apport de la MAH décellularisée seule à de la MAH décellularisée associée au polymère synthétique dans un modèle de fente palatine chez le rat. La MAH a été appliquée de façon à recouvrir un défaut palatin ostéo-muqueux de 1.3 x 7 mm. Une néoformation osseuse

plus importante était observée lorsque le défaut était recouvert par la MAH associée au polymère (Figure 23). Trois études pré-cliniques s'étaient déjà intéressés à l'apport de la MAH pour la fermeture de fente palatine. Des résultats satisfaisants avaient été obtenus mais ces études portaient uniquement sur la fermeture muqueuse [128,192,219].

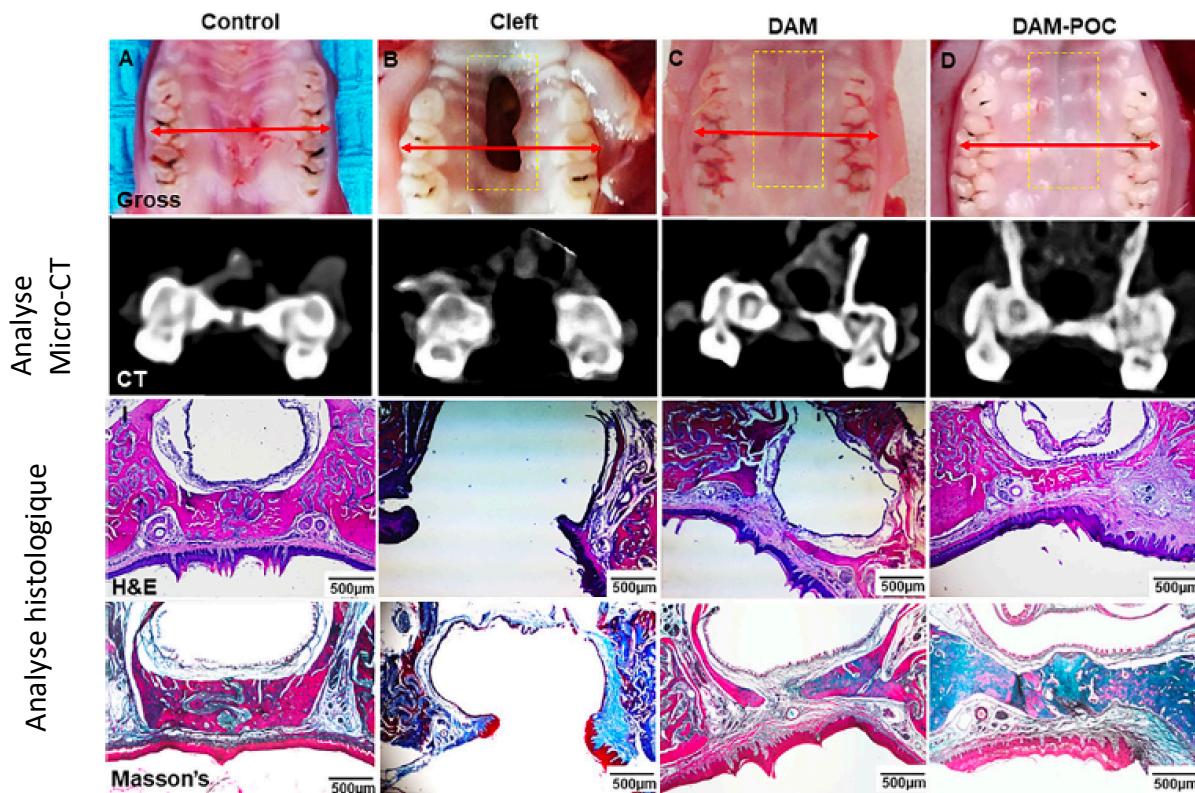


Figure 23. La membrane amniotique associée à un polymère permet la réparation osseuse dans un modèle de fente palatine chez le rat huit semaines après implantation.

Par ailleurs, quatre études pré-cliniques se sont intéressées à l'utilisation de la MAH comme matériaux de comblement pour la régénération de défauts osseux. Celle-ci pouvait être utilisée seule ou en association avec des cellules souches mésenchymateuses. Wu *et al.* ont étudié l'apport de la MAH cryopréservée comme matrice ensemencée en cellules souches humaines issues du tissu adipeux (hADSC) pour la régénération osseuse post-extractionnelle chez le rat [184]. Un défaut osseux était créé trois semaines après l'extraction d'une molaire maxillaire et l'étude comportait quatre conditions : un groupe contrôle, un groupe où le défaut était comblé par des hADSC, un groupe où le défaut était comblé par de la MAH et le dernier groupe où le comblement était réalisé par la MAH ensemencée en hADSC. La

régénération osseuse était significativement plus importante dans les deux groupes MAH seule et MAH utilisée comme matrice ensemencée en hADSC. Les auteurs ont également souligné l'apport de la MAH comme matrice d'échafaudage pour le comblement de défauts osseux. Khalil *et al.* se sont intéressés au potentiel ostéogénique de la MAH utilisée seule comme matériau de comblement. Ils ont réalisé des défauts de 4 x 5 mm dans des fémurs de lapin qui étaient soit laissés vide, soit comblés par de la MAH lyophilisée. L'analyse histomorphométrique montrait une régénération osseuse significativement supérieure en l'absence de MAH [220]. Parmi ces quatre études, deux études ont comparé le potentiel de la MAH utilisée en matériau de comblement osseux *versus* la MAH comme membrane. Ghanmi *et al.* ont comparé l'apport de la MAH fraîche utilisée comme matériau de comblement à la MAH employée comme membrane barrière pour la régénération osseuse de défauts segmentaires chez le lapin. La régénération osseuse était significativement plus importante lorsque la MAH était utilisée comme membrane barrière [221]. Moosavi *et al.* ont également réalisé des défauts osseux segmentaires chez le lapin pour comparer l'apport de la MAH en comblement et comme membrane barrière [222]. Le défaut était soit laissé vide, soit recouvert par de la MAH (mimant un tube), soit comblé par de la MAH, soit comblé par un matériau de substitution osseuse puis recouvert par de la MAH. La réparation osseuse était plus importante lorsque la MAH était enroulée autour du défaut comme membrane barrière par rapport à son utilisation en comblement.

Enfin, deux études ont porté sur l'utilisation de produits dérivés de la MAH et commercialisés comme matériau de comblement pour la régénération osseuse. Konofaos *et al.* ont utilisé de l'AmnioMTM® réalisé à partir de morceaux de membrane cryopréservée pour le comblement osseux de défaut de calvaria [223]. Le défaut était soit comblé par un matériaux de substitution osseuse, soit par ce même matériaux préalablement mixé à l' AmnioMTM®. Aucune différence significative de formation osseuse n'a été mise en évidence entre les deux groupes. Dans leurs travaux, Starecki *et al.* se sont intéressés à l'apport d'une suspension active de MAH commercialisée (Nucel®) utilisée en association avec un comblement osseux [224]. Des défauts fémoraux de 8 mm ont été réalisés chez le rat. Ils étaient soit laissés vides, soit comblés par un substitut osseux ou alors ils étaient comblés par le substitut osseux mélangé au produit Nucel®. De meilleurs résultats étaient obtenus lorsque le produit dérivé de la MAH était associé au comblement.

Tableau 2. Apport de la membrane amniotique pour la régénération osseuse: études pré-cliniques.

Article	Modèle	Indication	Utilisation et préservation de la MAH	Conditions	Résultats
Tang 2017 [173]	- Rats (n =30) - Défaut fémoral épiphysaire - Taille : 2.2 x 2.5 mm	- MAH utilisée seule comme membrane pour la ROG	Désépithélialisée + Lyophilisée	1) Vide 2) MAH	Condition 2 > 1
Koushaei 2018 [217]	- Chien (n = 4) - Défauts de tibia - Diamètre : 16 mm	- MAH utilisée seule comme membrane pour la ROG	Cryopréservée	1) Vide 2) MAH 3) Membrane de collagène	Condition 2>1 Pas de différence significative entre 1 et 3 Pas de différence significative entre 2 et 3
Gomes 2001 [216]	- Lapins (n =36) - Défaut de calvaria - Taille : 10 x 5 mm	- MAH utilisée comme membrane seule ou associée à un substitut osseux pour la ROG	Lyophilisée	1) MAH 2) MAH + ADDM	Condition 1 et 2 : Réparation osseuse totale à J120, mais plus rapide et plus importante dans condition 2
Li 2015 [127]	- Rats (n=30) - Défaut osseux péri implantaire au niveau du tibia - Taille : 2 x 2 x 2.5mm	- MAH utilisée comme membrane associée à un substitut osseux pour la ROG péri implantaire	Décellularisée + Lyophilisée	1) Pas de défaut péri implantaire 2) Défaut vide 3) Bio-oss 4) Bio-oss + Membrane de collagène 5) Bio-oss + MAH	Condition 1 > à 3 et 4 Pas de différence significative entre 1 et 5 Condition 5 : Meilleure connexion implant-os néoformé

Article	Modèle	Indication	Utilisation et préservation de la MAH	Conditions	Résultats
Tsugawa 2011 [171]	- Souris - Défaut de calvaria - Diamètre : 4.6 mm	- MAH utilisée comme membrane , seule ou associée à des cellules, pour la ROG	Cryopréservée	1) Contrôle 2) MAH 3) MAH ensemencée en OB 4) OB recouverts par MAH	- Condition 3 > 4 > 1
Semyari 2015 [172]	- Lapins (n=12) - Défaut de calvaria - Diamètre : 8mm	- MAH utilisée comme membrane , seule ou associée à des cellules pour la ROG	Décellularisée	Ensemencement de 3 matrices par des ADSC dont la MA versus les 3 matrices sans ADSC	- Réparation osseuse similaire avec MA ou MA + ADSC
Li 2019 [218]	- Rat (n= 20) - Défaut palatin ostéomuqueux - Taille : 1.3 x 7 mm	- MAH utilisée seule ou associée à un polymère synthétique (PS) dans un modèle de fente palatine	Décellularisée	1) Pas de défaut 2) Contrôle 3) MAH 4) MAH + POC	- Conditions 4 > 3 > 2
Wu 2015 [167]	- Rats (n=20) - Défaut osseux post extractionnel	- MAH utilisée seule ou associée à des cellules souches comme matériau de comblement pour la RO	Cryopréservée	1) Contrôle 2) hADSC 3) MAH 4) MAH + hADSC	- Conditions 3 et 4 > à 1 et 2

Article	Modèle	Indication	Utilisation et préservation de la MAH	Conditions	Résultats
Khalil 2018 [220]	- Lapins (n= 18) - Défaut fémoral - Taille : 4 x 5 mm	- MAH utilisée seule en matériau de comblement pour la RO	Lyophilisée	1) Vide 2) MAH	- Condition 1 > 2
Ghanmi 2018 [221]	- Lapins (n= 40) - Défaut segmentaire de tibia - Taille : 20 mm	- MAH utilisée seule comme membrane pour la ROG/ MAH utilisée seule en matériau de comblement pour la RO	Fraîche	1) Vide 2) Vide + périoste préservé 3) MAH (comblement) 4) MAH (membrane)	- Condition 2 > 3 - Conditions 2 et 3 > conditions 1 et 4
Moosavi 2018 [222]	- Lapins (n= 20) - Défaut segmentaire de radius - Taille : 15 mm	- MAH utilisée comme membrane pour la ROG seule ou associée à un biomatériau / MAH utilisée seule en matériau de comblement pour la RO	Fraîche	1) Vide 2) MAH (comblement) 3) MAH (membrane) 4) Os déminéralisé + MAH (membrane)	- Condition 3 > 4 > 2 > 1
Konofaos 2015 [223]	- Rats (n= 10) - Défaut de calvaria - Diamètre : 10mm	- Produit dérivé de la MAH utilisé associé à un biomatériau en matériau de comblement pour la RO	AmnioMTM®	1) Os déminéralisé 2) Os déminéralisé + AmnioMTM	- MicroCT : Pas de différence significative entre conditions 1 et 2 - Histomorphométrie : condition 1 > 2

Article	Modèle	Indication	Utilisation et préservation de la MAH	Conditions	Résultats
Starecki 2014 [224]	- Rats (n = 21) - Défaut segmentaire fémoral - Taille : 8mm	- Produit dérivé de la MAH utilisé associé à un biomatériau en matériau de comblement pour la RO	NuCel®	1) Vide 2) Substitut osseux (NP) 3) Substitut osseux (NP) + NuCel	- Condition 3 > 2 > 1

ADDM : Matrice dentinaire autogène déminéralisée ; ADSC : Cellules souches issues du tissu adipeux ; hADSC : Cellules souches humaines issues du tissu adipeux ; MA : Membrane amniotique ; MAH : Membrane amniotique humaine ; NP : Non précisé ; OB : Ostéoblaste ; POC : Polymère synthétique poly 1,8-octamethylene-citrate ; RO : Régénération osseuse ; ROG : Régénération osseuse guidée

3.7.3. Études cliniques

Plusieurs cliniques ont rapporté l'intérêt de la membrane amniotique pour la réparation de perte de substance osseuse.

Sept études cliniques menées chez des patients atteints de parodontite se sont intéressées à l'utilisation de la MAH pour la régénération du ligament alvéolo-dentaire (RTG) et la réparation osseuse (ROG) [225–231]. Kothiwale *et al.* ont réalisé une étude randomisée chez des patients atteints de parodontite et présentant des atteintes de furcation dentaire de classe II bilatérales. Chaque côté bénéficiait d'un comblement par de l'os allogénique ou de l'os xénogénique recouvert par de la MAH lyophilisée. A neuf mois on observait une amélioration des paramètres cliniques, associée à une augmentation du niveau osseux dans les 2 conditions [230]. Kumar *et al.* ont étudié l'apport de la MAH lyophilisée pour la RTG chez des patients atteints de parodontite. Les poches parodontales étaient soit comblées par de l'hydroxyapatite (HA) seule ou par de l'HA associée à de la MAH. L'évaluation de l'apport de la MAH reposait sur la mesure de paramètres cliniques (profondeur de poche et niveau d'attache), radiologiques, et sur la quantification de marqueurs de l'inflammation. Les auteurs ont observé une amélioration significativement plus importante des paramètres cliniques ainsi que du comblement osseux dans le groupe HA + MAH. Par ailleurs, un effet anti-inflammatoire de la MAH a été observé [229]. Une étude similaire a été menée par Pajnigara *et al.* chez 20 patients présentant des atteintes de furcation dentaire de classe II bilatérales [225]. Les défauts étaient soit comblés par un substitut osseux soit par le substitut puis recouvert par la MAH. Les paramètres cliniques et radiologiques osseux étaient significativement améliorés en présence de la MAH. Cependant, Sali *et al.* n'ont pas trouvé de différence significative concernant le gain osseux de défauts parodontaux comblés par un substitut osseux recouvert ou non par de la MAH [227]. Une étude randomisée a comparé l'apport de la MAH lyophilisée utilisée en bi-couche à une membrane de collagène pour le traitement de défauts osseux parodontaux. Le défaut osseux était comblé par de l'os bovin avant d'être recouvert par l'une des deux membranes. Une amélioration des paramètres cliniques a été observée dans les 2 conditions [228]. Kaur *et al.* ont évalué l'apport de la membrane de PRF à l'association d'une membrane de PRF avec de la MAH pour le traitement d'atteintes de furcation dentaire de classe II chez 15 patients [226]. Le gain osseux était

significativement plus élevé lorsque la MAH était présente. Kalra *et al.* ont suggéré l'utilisation d'un gel à base d'acide hyaluronique associé à de la MAH pour le comblement d'une atteinte de furcation de Classe II chez un patient. L'évaluation radiologique montrait une reminéralisation verticale quasi complète au niveau de la lésion de furcation traitée [231]. La MAH était utilisée en mono couche dans toutes les études sauf une [228], où la MAH était appliquée en double couche.

Bien que la majorité des études portant sur l'utilisation de la MAH pour la régénération osseuse aient été menées dans un contexte de maladies parodontales, d'autres applications ont été recherchées en chirurgie orale. Elles portent essentiellement sur l'apport de la MAH pour les pertes de substances muqueuses [120]. Néanmoins, une étude prospective portant notamment sur la reconstruction de large perte de substance osseuse maxillaire ou mandibulaire a utilisé de la MAH décellularisée comme membrane servant à recouvrir une greffe osseuse (greffon illiaque + particules d'os d'origine bovine) [232]. Le greffon osseux était soit recouvert par de la MAH ($n= 5$ patients), soit par de la MAHensemencée par des cellules souches autologues provenant du corps adipeux de la joue (boule de Bichat) ($n= 4$ patients) (Figure 24). Un gain osseux dans le sens vertical et horizontal a été observé quelle que soit la condition. Le volume osseux néoformé dans le sens horizontal était significativement plus important en présence de cellules souches adipeuses autologues. Enfin, une série de deux cas a rapporté l'utilisation de la MAH comme membrane pour la ROG de lésions osseuses péri-apicales [233]. Les défauts osseux étaient comblés par des particules de xénogreffe mélangées à une membrane de PRF avant d'être recouverts par de la MAH.

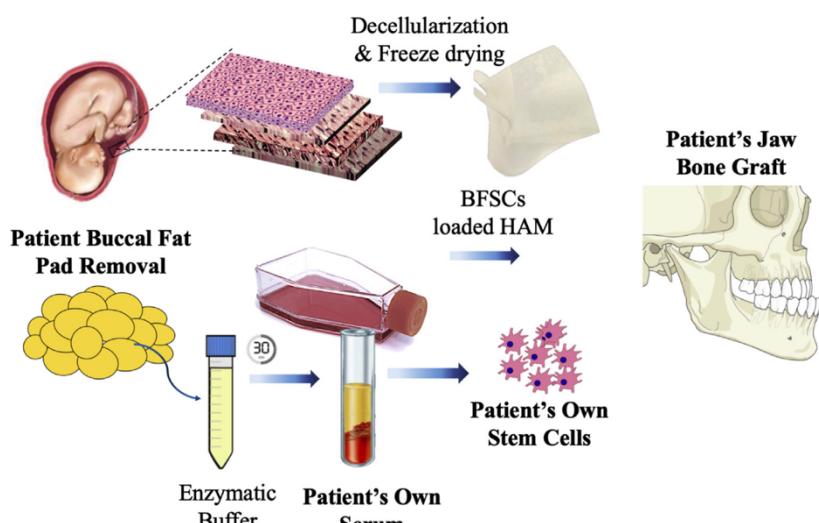


Figure 24. Représentation schématique de l'étude de Akhlaghi *et al.* [232]

Dans toutes les études cliniques précédemment décrites, nous pouvons observer que la MAH était utilisée comme membrane barrière et non comme matériaux de comblement.

Enfin, en chirurgie orthopédique, une étude rétrospective s'est intéressée à l'utilisation d'une suspension active de MAH commercialisée (Nucel®) associée à une allogreffe osseuse pour la réalisation d'arthrodèse chez 72 patients [234]. Un essai clinique prospectif multi-centrique portant sur 200 patients est en cours afin d'évaluer l'apport du produit Nucel® pour la réalisation d'arthrodèse.

- Avantages et limites de la membrane amniotique:

- Avantages : La MAH est un déchet opératoire dont l'obtention en grande quantité est simple et à faible coût. Elle possède de nombreuses propriétés biologiques telles qu'un effet anti-inflammatoire pro-angiogénique et anti-adhésif, elle accélère également la cicatrisation épithéliale...

- Limites : La MAH ne peut pas être utilisée fraîche et nécessite d'être préservée. Or il n'existe pas de consensus sur la méthode de préservation à utiliser en ingénierie tissulaire osseuse.

OBJECTIFS

OBJECTIFS

La revue bibliographique a mis en évidence certaines limites présentées par les membranes actuellement utilisées en ROG. Ces limites justifient la recherche d'alternatives telle que le développement de membrane de troisième génération dite « bio-active ». Du fait de ses nombreuses propriétés biologiques, la MAH pourrait avantageusement se substituer aux membranes existantes.

Nous avons également souligné l'utilisation de différentes méthodes de préservation de la MAH en vue de son application en régénération osseuse. Quelques études ont également montré que son potentiel était plus important lorsque la MAH était utilisée comme membrane barrière plutôt que lorsqu'elle comblait le défaut osseux.

L'objectif principal de ce travail était de déterminer les meilleures méthodes d'utilisation de la MAH pour la régénération de perte de substance osseuse.

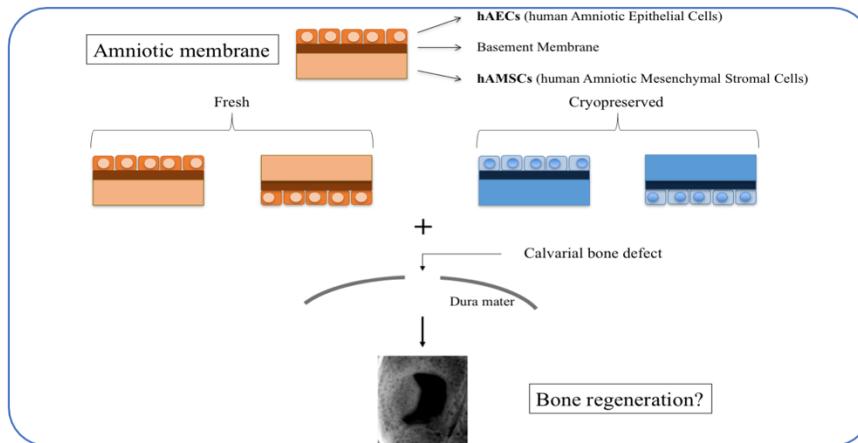
La première partie de ce travail s'est intéressée à l'influence des faces épithéliales et mésenchymateuses sur la réparation osseuse, et, a également permis de comparer le potentiel des MAH fraîche et cryopréservée pour la ROG.

La deuxième partie de ce travail avait pour objectif de développer de nouvelles méthodes de préservation de la MAH et d'étudier leur impact sur les propriétés biologiques et mécaniques de ces 4 MAH (fraîche, cryopréservée, lyophilisée et décellularisée/lyophilisée).

La troisième partie de ce travail avait pour but de comparer le potentiel de ces 4 types de MAH (fraîche, cryopréservée, lyophilisée et décellularisée/lyophilisée) pour la ROG d'un défaut osseux non critique afin de sélectionner les meilleures méthodes de préservation pour la régénération osseuse.

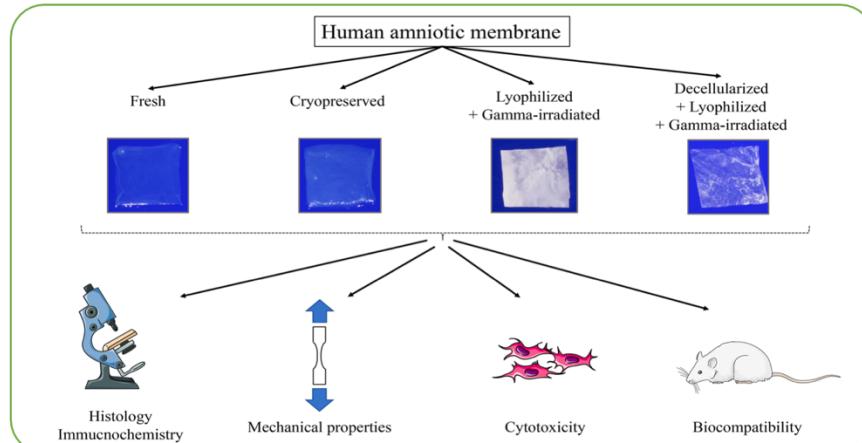
Enfin, la dernière partie de cette thèse visait à comparer les MAH sélectionnées dans la partie 3 (MAH lyophilisée et décellularisée/lyophilisée) à la technique de la membrane induite dans un défaut osseux segmentaire critique.

ARTICLE 1



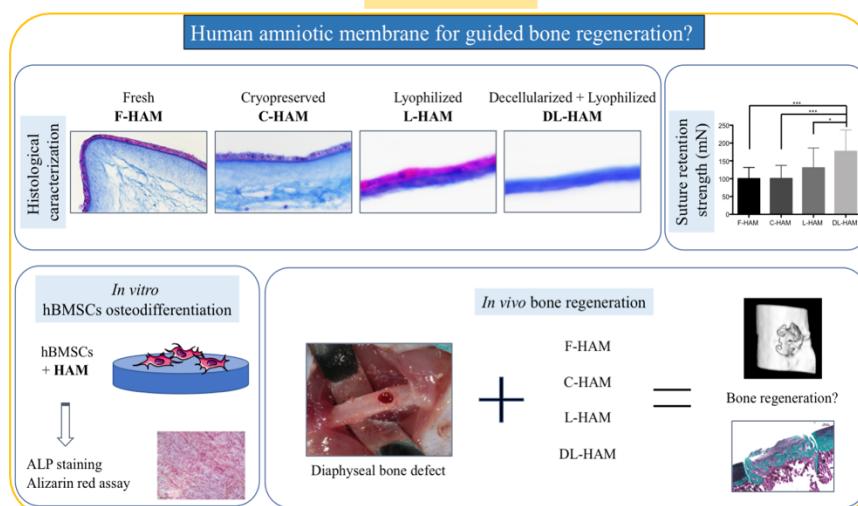
Influence des faces épithéiale *versus* mésenchymateuse et de la cryopréservation pour la ROG d'un défaut de calvaria chez la souris

ARTICLE 2



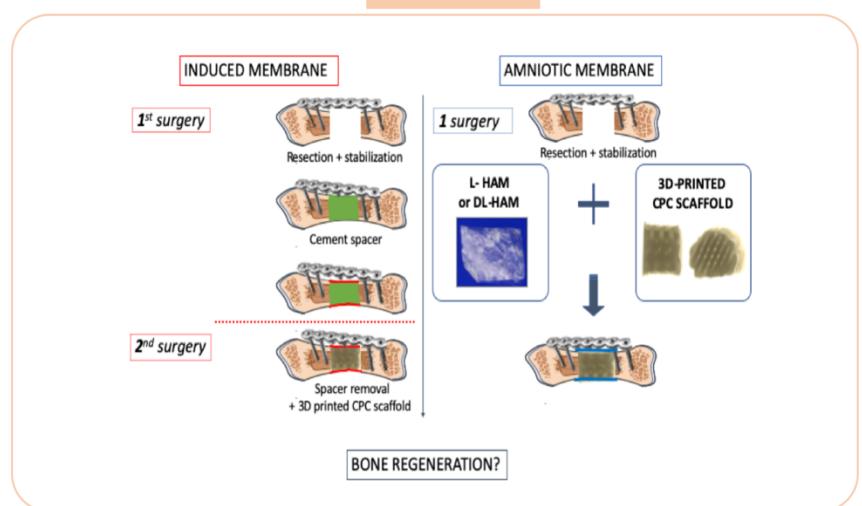
Développement de nouvelles méthodes de préservation de la MAH
Impact sur les propriétés biologiques et mécaniques de la MAH

ARTICLE 3



Impact des méthodes de préservation de la MAH sur son potentiel en ITO.
Sélection des meilleures méthodes de préservation pour la ROG

ARTICLE 4



Comparaison de la membrane induite à la MAH pour la réparation osseuse de défaut segmentaire chez le rat

RÉSULTATS

RESULTATS

1. COMPARAISON DE L'APPORT DES FACES EPITHELIALE VERSUS MESENCHYMATEUSE AINSI QUE DE L'IMPACT DE LA CRYOPRESERVATION SUR LA ROG DANS UN DEFAUT DE CALVARIA CHEZ LA SOURIS.

1.1. Introduction

La revue de la littérature a mis en évidence le potentiel d'ostéodifférentiation des cellules souches amniotiques : hAECs et hAMSCs *in vitro* et *in vivo* [144,145,209–211]. Il a également été démontré qu'il était possible d'ostéodifférencier la MAH fraîche dans son intégralité (face épithéliale et mésenchymateuse) sans avoir isolé les cellules au préalable [207]. Enfin, une étude pré-clinique a mis en évidence « l'effet périoste » de la MAH fraîche lorsqu'elle recouvrait des défauts osseux segmentaires chez le lapin [221].

Cependant, afin d'éviter le risque de transmission de maladies infectieuses, la MAH ne peut pas être utilisée fraîche et doit être préservée. La cryopréservation dans une solution de RPMI/Glycerol est la méthode de préservation de référence de la MAH utilisée par l'Etablissement Français du Sang en France. Néanmoins, il n'existe pas de données concernant l'influence de la cryopréservation sur les propriétés ostéogéniques de la MAH.

Les objectifs de ce travail étaient : i) de préciser l'influence respective des faces épithéliales et mésenchymateuses sur les propriétés ostéogéniques de la membrane amniotique et ii) de déterminer l'influence de la cryopréservation sur les propriétés ostéogéniques de la membrane amniotique.

Pour cela, nous avons d'abord évalué *in vitro* la viabilité cellulaire des hAECs et hAMSCs dans la MAH fraîche et cryopréservée. *In vivo*, nous avons évalué le potentiel de régénération osseuse guidée des MAH fraîche et cryopréservée dans des défauts de calvaria de souris en appliquant soit la face épithéliale, soit la face mésenchymateuse au contact du défaut. Enfin, nous avons comparé l'apport de la MAH cryopréservée à une membrane de collagène

commercialisée pour la ROG de défauts de calvaria préalablement comblés par un substitut osseux associé à un facteur de croissance.

Ce travail a été mené en collaboration avec l’Établissement Français du Sang (EFS). Les placentas étaient récupérés par l’EFS qui nous fournissait ensuite des patchs de MAH conditionnés sur des filtres de nitro cellulose et placés dans du milieu de conservation. Une fois au laboratoire les patchs de MAH étaient soit conservés dans du milieu de culture et placés en étuve pour être utilisés frais, soit cryopréservé selon le même protocole que celui réalisé à l’EFS.

1.2. Article n°1

HUMAN AMNIOTIC MEMBRANE FOR GUIDED BONE REGENERATION OF CALVARIAL DEFECTS IN MICE

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Original Research

Human amniotic membrane for guided bone regeneration of calvarial defects in mice

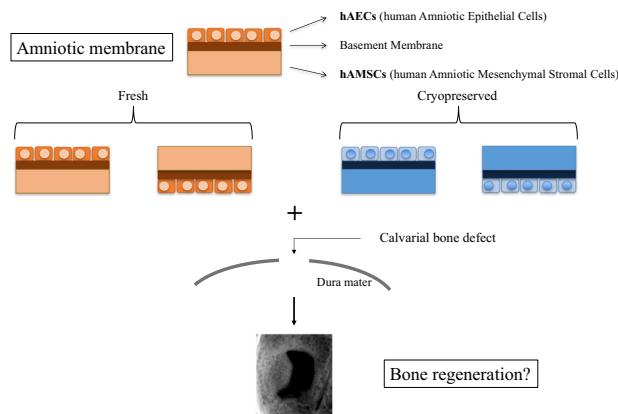
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Abstract

Due to its biological properties, human amniotic membrane (hAM) is widely studied in the field of tissue engineering and regenerative medicine. hAM is already very attractive for wound healing and it may be helpful as a support for bone regeneration. However, few studies assessed its potential for guided bone regeneration (GBR). The purpose of the present study was to assess the potential of the hAM as a membrane for GBR. *In vitro*, cell viability in fresh and cryopreserved hAM was assessed. *In vivo*, we evaluated the impact of fresh versus cryopreserved hAM, using both the epithelial or the mesenchymal layer facing the defect, on bone regeneration in a critical calvarial bone defect in mice. Then, the efficacy of cryopreserved hAM associated with a bone substitute was compared to a collagen membrane currently used for GBR. *In vitro*, no statistical difference was observed between the conditions concerning cell viability. Without graft material, cryopreserved hAM induced more bone formation when the mesenchymal layer covered the defect compared to the defect left empty. When associated with a bone substitute, such improved bone repair was not observed. These preliminary results suggest that cryopreserved hAM has a limited potential for GBR.

Graphical Abstract



Jean-Christophe Fricain and Claudine Boiziau are co-directed.

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List of abbreviations

BMP	Bone morphogenetic protein
GBR	Guided bone regeneration
HA	Hydroxyapatite
hAM	human amniotic membrane
hAECs	human amniotic epithelial cells
hAMSCs	human amniotic mesenchymal stem cells
f-hAM	fresh hAM
Cryo-	Cryopreserved hAM
hAM	

1 Introduction

Oral surgeons aim to restore the health and function of soft and hard tissue from the oral cavity. Guided tissue regeneration (GTR) and guided bone regeneration (GBR), associated or not with grafting materials, are widely used to repair damaged tissue in oral and maxillofacial surgery. These therapies were initially used to regenerate periodontal tissue [1]. Owing to the rise in dental implant surgery, GBR was used for the development of alveolar bone sites before or during implant placement [2]. Nowadays, GTR and GBR are new tools to treat implants affected by peri-implantitis [1, 3]. GTR and GBR use membranes which should act as a physical and biological barrier to isolate the damaged tissues and promote their regeneration. Both resorbable and non-resorbable membranes can be used [4]. One of the disadvantages of non-resorbable membranes is the high frequency of premature exposure and the need to perform additional surgery to remove them after tissue healing. Resorbable membranes have low mechanical strength and a strong inflammatory response during the postoperative healing phase may occur [5]. Therefore, new approaches are still needed to improve the outcome of the current techniques.

Human amniotic membrane (hAM) is the innermost layer of the fetal membrane, lining the amniotic cavity. Due to its biological and mechanical properties, hAM is widely studied in regenerative medicine [6–9]. Indeed, hAM has the ability to exert an anti-inflammatory [10], anti-fibrotic [9] and an anti-tumorigenic effect, and possesses low immunogenicity [11, 12]. Moreover, it is a source of stem cells [13] and growth factors [14, 15]. The clinical use of HAM in medicine was first reported by Davis in 1910 for skin replacement. Due to its biological properties, and large availability, hAM is already widely used in the field of ophthalmology and dermatology [8, 16, 17], and several studies reported the use of hAM for tissue engineering.

Human amniotic membrane (hAM) is a highly abundant and readily available tissue that may be helpful as a support

for bone regeneration in the field of oral and maxillofacial surgery [18]. At the structural level, hAM contains two different cell types: human amnion epithelial cells (hAECs) and human amnion mesenchymal stem cells (hAMSCs) which have a strong osteoinductive potential [19–21]. The clinical use of hAM in oral surgery was first reported by Lawson *et al.* in 1985 [22]. Since then, five preclinical studies reported the use of amniotic membrane for guided bone regeneration [23–27] and two preclinical studies concluded that hAM enhanced periodontal tissue regeneration [28, 29]. Several studies conducted on human beings reported the use of hAM as a membrane to regenerate periodontal tissue and to favor the healing of exposed bone in the oral cavity [30–32].

The objective of this study was to assess the efficacy of hAM to act as a membrane in guiding bone growth. First, we investigated the role of epithelial and mesenchymal layers of fresh and cryopreserved hAM on bone regeneration efficiency in a model of calvarial bone critical size defect. Then its efficacy was compared to that of a collagen membrane currently used for GBR, associated with grafting material and the growth factor BMP2, in the same calvarial defect.

2 Materials and Methods

2.1 Preparation of hAM

hAM was collected by a local tissue bank (French Blood Establishment (Etablissement Français du Sang, EFS), CHU de Bordeaux, France) according to the procedure used for corneal graft with hAM. Human placentas were recovered after elective cesarean surgery from consenting healthy mothers (tested seronegative for HIV, cytomegalovirus, Toxoplasma gondii, Hepatitis B and C virus, and syphilis). hAM was peeled from the chorion, washed with phosphate-buffered saline solution (PBS 1×) and put on discs of nitrocellulose (d = 50 mm). hAM was cut into pieces of 6 mm × 6 mm and transferred into 24-round-bottom-well plates with a well-defined orientation: either the epithelial or the mesenchymal layer was in contact with the nitrocellulose filter to allow *in vivo* implantation of a defined layer in contact with the bone. Pieces of fresh hAM (f-hAM) were cultured in plates containing 250 µl of α-minimum essential medium (MEM alpha, GIBCO®), 10% fetal bovine serum (Eurobio®) and 1% antibiotics (amoxicillin/ streptomycin Invitrogen®) in incubator (37°C, 5% CO₂, 100% humidity). For cryopreservation, pieces of hAM were put in 24-well plates containing 250 µl of RPMI/Glycerol 1:1, stored 1 h at -20°C, then kept frozen at -80°C. After thawing, hAM patches (cryo-hAM) were washed twice with PBS 1×. Three human placentas were used for this study.

2.2 Preparation of collagen membrane

A resorbable collagen membrane (Geistlich Bio-Gide®) was cut into 6 mm × 6 mm pieces and stored at room temperature in a sterile petri dish.

2.3 Preparation of hydroxyapatite particles and BMP2

Hydroxyapatite (HA) synthesis: HA was prepared by wet chemical precipitation as previously described [33, 34]. Briefly, a 0.5 M solution of calcium hydroxide (Alfa Aesar, Germany) was stirred vigorously, degassed and heated at 40°C for 1 h. Then, 100 ml of a 0.3 M solution of phosphoric acid (Rectapur, Prolabo®, Paris, France) were added drop-by-drop to 100 ml of the 0.5 M solution of calcium hydroxide (Alfa Aesar, Germany), under vigorous stirring at 40°C. At the end of the reaction, the pH was adjusted to 9 with ammonium hydroxide 30%, then stirring was continued for 12 h. The solution was decanted overnight and lyophilized. Then, the powder was sterilized by gamma radiation at 25KGy (Gamacell® 3000 Elan, NORION MDS, Ottawa, Canada).

A commercially available kit (InductOs®, Medtronic, Biopharma B.V., Heerlen, Netherlands) was used to prepare the solution of rhBMP-2 following the manufacturer's protocol (solution concentration: 125 µg/mL).

2.4 Cultivation of fresh and cryopreserved hAM and viability assay

Viability assays of both f-hAM and cryo-hAM were performed 1, 3 and 7 days after culture in plates containing 250 µl of α-minimum essential medium (MEM alpha, GIBCO®), 10% fetal bovine serum (Eurobio®) and 1% antibiotics (amoxicillin/streptomycin Invitrogen®) in an incubator (37°C, 5% CO₂, 100% humidity) (n = 3 per condition). A live/dead viability assay (Thermo Fisher Scientific, Invitrogen) was performed according to the manufacturer's instructions. Pieces of hAM were then observed with a confocal microscope (Leica TCS SPE Model DMI 4000B). Living cells emitted green fluorescence in the cytoplasm (exc: 494 nm, em: 517 nm), whereas dead cells emitted red fluorescence in the nucleus (exc: 527 nm, em: 617 nm). Once the live-dead images were obtained by confocal microscopy, we calculated areas covered by live cells using ImageJ® software. Briefly, we selected three images of the epithelial and mesenchymal layer for each condition (f-hAM and cryo-hAM) and for each time point (day 1, 3 and 7). Color channels (green and red) were split for each of these 36 images and percentage of covered areas were calculated for each color [35].

2.5 Animal model and implantation procedure

The present study was approved by the French Ethics Committee (agreement APAFIS n°2685-20l5111012075358 v4). Female C57Bl/6 mice (10 weeks old, 18–23 g) were used (8 mice per condition and one defect per mouse were used). Short-term anesthesia was induced by inhalation of 4% isoflurane (Air:1 L/min) and maintained using 2% isoflurane. The surgical site was aseptically prepared and an incision was made on the skull skin. A unilateral critical bone defect of 3.3 mm diameter was made with a trephine bur on the right calvarial bone in all mice.

In the first *in vivo* experimentation, standardized circular calvarial defects were left empty (control group) or they were covered by cryopreserved hAM, either with the mesenchymal side (cryo-hAM hAMSCs), or the epithelial side (cryo-hAM hAECs) in contact with the defect, or the defect was covered by fresh hAM, either with the mesenchymal side (f-hAM hAMSCs) or with the epithelial side (f-hAM hAECs) in contact with the defect (n = 8 per condition).

In the second *in vivo* experimentation, the same standardized circular calvarial defects were performed (8 mice per condition and one defect per mouse calvaria). Two groups were considered as control, in which either HA particles alone (0.5 mg) or HA particles followed by deposition of 4 µl (=0.5 µg) of BMP-2 (HA-BMP2) were inserted into each defect. Two other groups of bone defects were filled either by HA or HA-BMP2 and then covered by cryo-hAM hAMSCs (with the mesenchymal side in contact with biomaterials). Calvarial defects of the last two groups were filled either by HA or HA-BMP2 and then covered by the collagen membrane (Geistlich Bio-Gide®).

In all cases, the edges of the two membranes were at least 1 mm beyond the borders of the surgical site. Skin was sutured with synthetic absorbable suture (4-0 Vicryl; Ethicon, Inc). Analgesia was performed by intraperitoneal injection of 0.1 mg/kg buprenorphine (Buprecare®, 0.3 mg/ml) one hour before surgery and the day after.

Sacrifice was performed by cervical dislocation 8 weeks and 6 weeks after implantation for the first and the second experiment respectively. The calvaria were dissected, rinsed in PBS 1×, fixed in 4% paraformaldehyde overnight at 4°C, and then processed for X-ray analysis and histology.

2.6 X-Ray analysis of bone regeneration

Radiographic analysis was performed on each calvarial specimen using a Faxitron X-Ray MX20-DC2 digital imaging instrument (Faxitron Bi optics, Arizona, USA). The region of interest (ROI) was defined as a circle with a diameter of 3.3 mm corresponding to the initial surgical site, as defined by the X-ray image of a mouse sacrificed

immediately after defect creation. Using ImageJ® software, a binary thresholding was performed inside each ROI (white corresponding to new bone and black corresponding to the lack of bone). The percentage of bone healing was assessed by the new bone area compared to the surface of the ROI.

2.7 Histology

Each sample was decalcified in a commercially available decalcification solution (DC3, Labonord, France), and was cut in the middle of each defect. Then, samples were dehydrated and processed for conventional embedding in paraffin. Seven- μm -thick serial sections were prepared through the middle of the defect and stained with Masson's trichrome staining. Images were obtained with an Eclipse 80i light microscope (Nikon, Japan) and captured with a DXM 1200C CCD camera (Nikon, Japan).

2.8 Histomorphometry

Using the photo of calvaria sections stained with the Masson's trichrome techniques, the residual hAM was delimited inside each section of sample using Gimp® software. This area was then measured using ImageJ software. The surface of residual hAM was expressed as a percentage of ROI.

2.9 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software (La Jolla/CA, USA). The first normality test was performed using a D'Agostino and Pearson omnibus normality test. Statistical significance for independent samples was evaluated with the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test (for more than two groups) or with the Mann-Whitney test (for two compared groups). Differences were considered statistically significant if $p < 0.05$. Statistical significances are marked by stars with * indicating a two-tailed $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3 Results

3.1 Viability assay of fresh and cryopreserved hAM

As hAM is a biological membrane that may contain living cells able to participate in bone repair through cytokine and growth factor secretion, we analyzed the survival capacity of hAM cells cultivated in a cell culture medium. Therefore, hAM fragments were cultured for a few days (1, 3 and 7), either immediately after recovery as a fresh tissue ("f-hAM") or after cryo-preservation ("cryo-hAM"). Live/dead assays were performed on f-hAM and cryo-hAM to assess

the cell viability of their epithelial (hAESC) and mesenchymal layers (hAMSCs) (Fig. 1a). Live/dead staining showed significant heterogeneity regarding the viability of cells within f-hAM and cryo-hAM, and even in the same piece of membrane (Fig. 1a).

Since the acetoxymethyl ester of calcein colors the cytoplasm of live cells (green fluorescence), whereas ethidium homodimer-1 colors the nucleus of dead cells (red fluorescence), it is not possible to compare the surfaces covered by live cells to the surfaces covered by dead cells. Therefore, we compared the surface covered by live cells according to the method of preservation of hAM (fresh or cryopreserved) (Fig. 1b, c). Quantitative assessment was easier for the epithelial side that consists in a single layer of cuboidal cells, whereas the mesenchymal side is composed of three different layers. Because of the high variability inside each condition, no statistical difference was observed between the conditions (f-hAM or cryo-hAM) or between the different times of culture in a same condition.

At day 1, f-hAM showed a high cell survival rate inside the two layers (epithelial and mesenchymal layers). The living epithelial cells were grouped in large clusters. From the third day of culture, a large number of dead cells was observed inside the epithelial layer of f-hAM. The majority of MSCs were alive at days 1 and 3 (green staining), but very few living mesenchymal cells were observed in f-hAM at day 7.

At day 1 and 3, cryo-hAM showed a high percentage of dead cells inside the epithelial layer: less than 10% of the area was covered by cells that emitted a green fluorescence. Living epithelial cells were grouped into small clusters. At day 7, the highest number of dead cells was observed inside the epithelial layer of cryo-hAM.

Concerning the mesenchymal layer of the cryo-hAM, very few hAMSCs were alive at day 1. At day 3, the typical morphology of mesenchymal stem cells of hAMSCs could be observed and dividing cells were detected. Quantitative analysis also showed a higher density of live cells inside the mesenchymal layer of cryo-hAM at day 3 compared to day 1, so the surviving cells present in the mesenchymal layer were able to proliferate when cultured in appropriate conditions. At day 7, the amount of living cells seemed to be similar between f-hAM and cryo-hAM (Fig. 1b, c).

3.2 Bone regeneration in the presence of fresh or cryopreserved hAM

The objective of this study was to compare the properties of the human amniotic membrane in the context of bone regeneration of a critical size bone defect. As we observed some survival differences between, on the one hand, the fresh hAM and its cryo-preserved counterpart and, on the other hand, between the two cell layers (see above), we

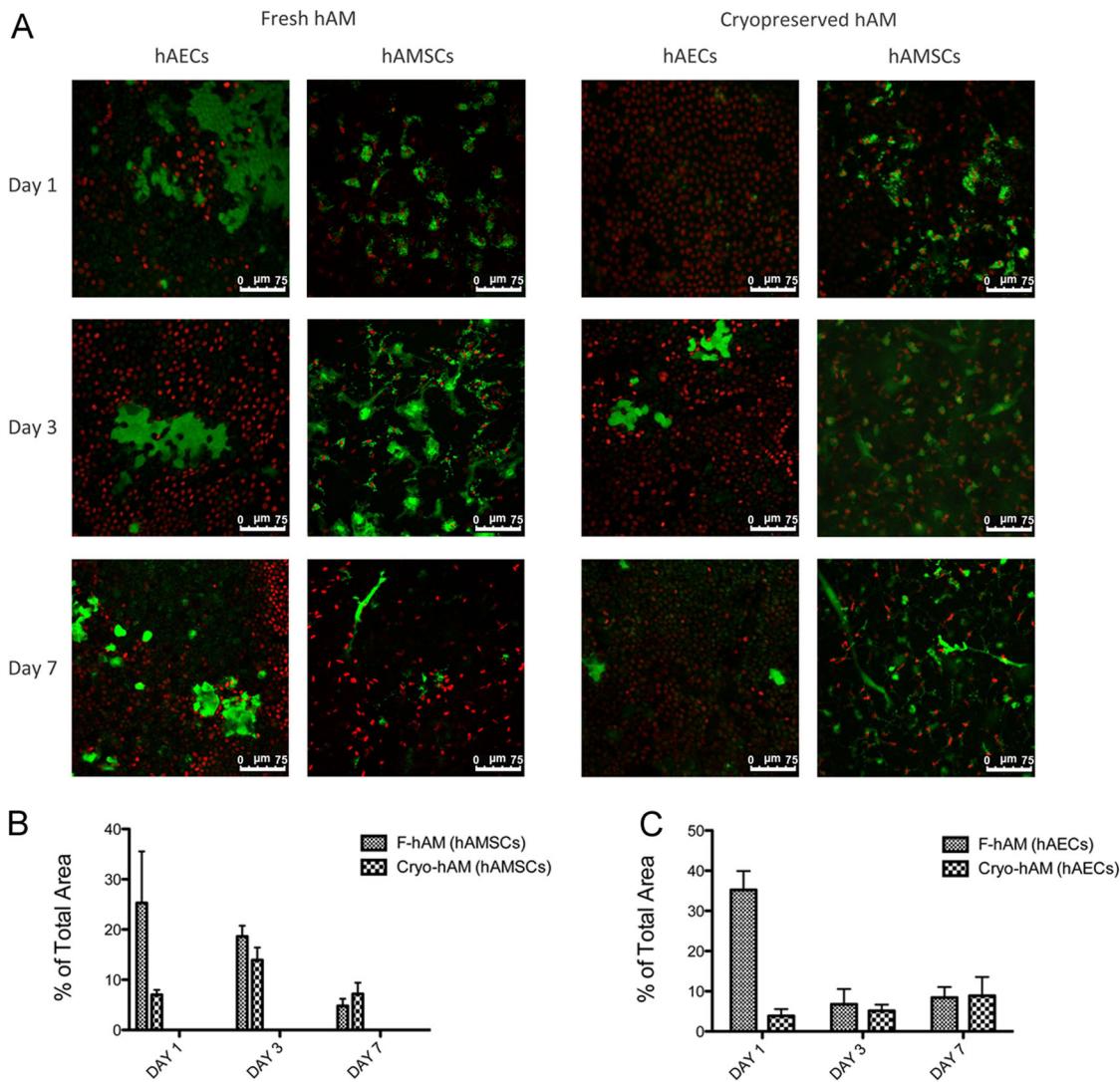


Fig. 1 In vitro cell viability assays. **a** Fresh and cryopreserved hAM pieces were cultivated in α -MEM at 37 °C for one, three and 7 days. The viability of human amniotic epithelial cells (hAECs) and human amniotic mesenchymal stem cells (hAMSCs) was analyzed by LIVE/DEAD™ assay: green fluorescence showed living cells whereas dead cell nuclei emitted red fluorescence. The epithelial and mesenchymal layers were imaged separately by confocal microscopy. Significant

heterogeneity was observed regarding the viability of cells within the same piece of membrane. The scale bar represents 75 μ m. **b** Quantitative analysis of the % of total area covered by live hAMSCs calculated from three samples for each condition. **c** Quantitative analysis of the % of total area covered by live hAECs calculated from three samples for each condition. The percentage of total area covered by live cells is expressed as mean +/- standard deviation

hypothesized that various bone regenerative properties might be obtained depending on the membrane used (fresh “f-hAM” vs cryo-preserved “cryo-hAM”) and on the layer (epithelial or mesenchymal layer) in contact with the bone to be regenerated. Therefore, to investigate the role of epithelial and mesenchymal layers of fresh *versus* cryopreserved hAM on bone regeneration efficiency, we performed a critical calvarial defect on 40 mice. There were five treatment modalities: 1) covered by fresh hAM with the mesenchymal side in contact with the defect (f-hAM hAMSCs); 2) covered by fresh hAM with the epithelial side in contact with the defect (f-hAM hAECs); 3) covered either by cryopreserved hAM with the mesenchymal side in

contact with the defect (cryo-hAM hAMSCs); 4) or covered by cryopreserved hAM with the epithelial side in contact with the defect (cryo-hAM hAECs); 5) left empty ($n = 8$ per condition). After 8 weeks, the mice were sacrificed and we evaluated bone repair using 2-D X-ray analysis and histology (Fig. 2). The D’Agostino and Pearson omnibus normality test was performed. Statistical significance for these samples was evaluated with the Kruskal-Wallis test followed by Dunn’s multiple comparison test (for more than two groups) or with the Mann-Whitney test (for two compared groups).

Representative X-ray images of each group are shown in Fig. 2a. As illustrated in this figure, none of the five

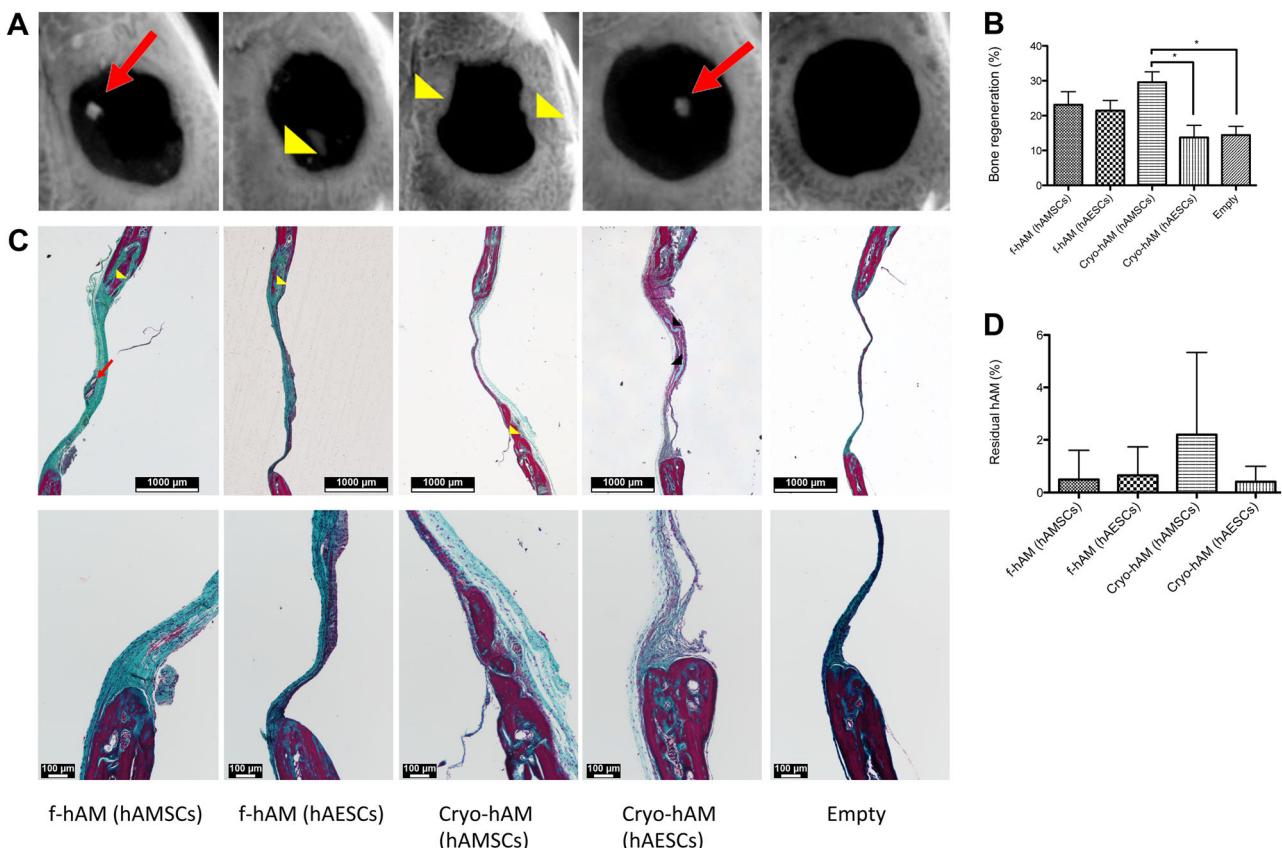


Fig. 2 Bone regeneration using fresh or cryopreserved hAM. **a** Planar X-rays showing the remaining defects 8 weeks after surgery (representative images of each group; $n = 8$ in each group). **b** Quantification and statistical analysis of bone formation. Mean \pm standard deviation. * $p < 0.05$; ** $p < 0.01$. **c** Histological examination of decalcified

calvarial defects stained with Masson's trichrome staining and observed. Magnification: $\times 2$ (C) and $\times 10$ (C insert). **d** Histomorphometric analysis of residual hAM. Yellow arrowhead: bone formation at periphery of defect; Red arrow: isolated bone island formation; Black arrow: residual hAM

modalities tested could promote *ad integrum* bone regeneration eight weeks after implantation. Most of the healing occurred at the periphery of the defect (Fig. 2a, yellow arrowheads). When the defects were covered by hAM, small islands of new bone formation were occasionally observed inside the defect. In contrast, islands of new bone were not observed when the defects were left empty.

Quantitative analysis of bone regeneration showed that cryo-hAM, with the mesenchymal side in contact with the defect, induced significantly more bone formation (bone regeneration: $29.58 \pm 8.386\%$) compared to the empty defect ($14.41 \pm 7.17\%$, $p = 0.0012$). Furthermore, bone regeneration was significantly greater when the defect was covered with the mesenchymal side of cryo-hAM compared to its epithelial side ($13.75 \pm 9.841\%$, $p = 0.0059$). No statistical difference in the repaired surface could be evidenced with the fresh hAM in both orientations (f-hAM (hAMSCs): $23.10 \pm 11.25\%$; f-hAM (hAECs): $21.46 \pm 9.09\%$) (Fig. 2b).

To further assess healing, the demineralized calvaria were paraffin-embedded and sections were stained by Masson's trichrome (Fig. 2c). This histological analysis

confirmed the previous results, i.e. bone formation mainly at the periphery of the defect and very few bone nodules inside it (Fig. 2c, yellow arrowheads and red arrows, respectively). Most of the defect was filled by connective tissue. Histomorphological analysis also showed that fresh or cryopreserved hAM were almost entirely resorbed 8 weeks after implantation with only a little of the initially implanted hAM remaining detectable (Fig. 2c, black arrows and Fig. 2d).

3.3 Addition of hydroxyapatite and BMP-2 to stimulate bone regeneration

In the previous animal experimentation, the highest rate of bone formation was observed when the mesenchymal side of cryo-hAM (cryo-hAM (hAMSCs)) was applied to the defect. Therefore, we decided to compare its bone regeneration potential to a resorbable collagen membrane currently used in oral surgery. To promote bone repair, we inserted hydroxyapatite (HA) particles or HA particles associated with the growth factor BMP2 (HA-BMP2). After 6 weeks, the mice were sacrificed and bone repair was

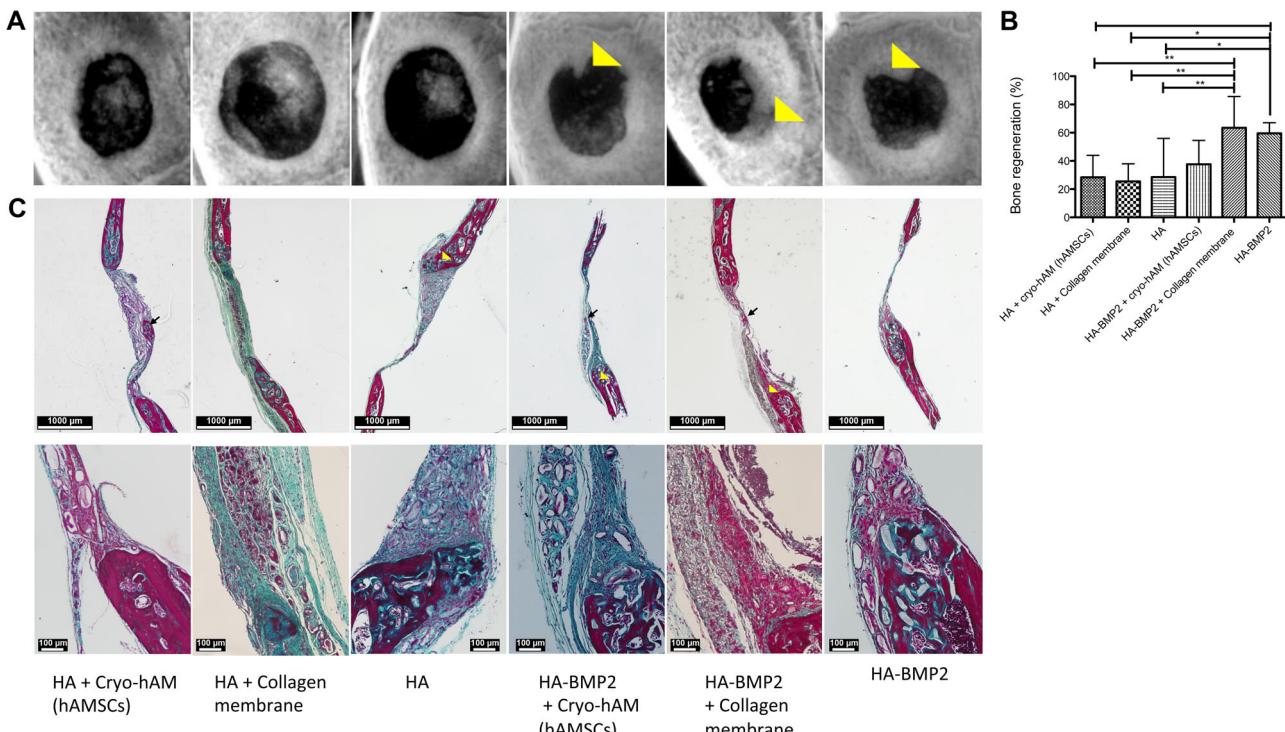


Fig. 3 Stimulation of bone regeneration by addition of hydroxyapatite and BMP2. **a** Planar X-rays of calvaria after 6 weeks healing (representative images of each group; n = 8 in each group). **b** Quantification and statistical analysis of bone formation. Mean +/- standard deviation. *p < 0.05; **p < 0.01; ***p < 0.001. **c** Masson's trichrome staining of decalcified calvarial defect observed. Magnification: x2 (C) and x10 (C insert). Yellow arrowhead: bone formation at periphery of defect; Black arrow: isolated bone island formation

deviation. *p < 0.05; **p < 0.01; ***p < 0.001. **c** Masson's trichrome staining of decalcified calvarial defect observed. Magnification: x2 (C) and x10 (C insert). Yellow arrowhead: bone formation at periphery of defect; Black arrow: isolated bone island formation

evaluated by 2-D X-ray analysis and histology (Fig. 3). Statistical significance for these samples was evaluated with the Kruskal-Wallis test followed by a Dunn's multiple comparison test or with the Mann-Whitney test (for two compared groups).

Representative images of X-rays taken after 6 weeks healing are shown in Fig. 3a: they show enhanced but incomplete bone healing. When the defects were filled by HA alone or filled by HA and then covered by a membrane, a radiopaque material was observed. When the defects were filled by HA-BMP2 or by HA-BMP2 then covered by a membrane, newly mineralized tissue was mostly observed at the edges of the defects. Some isolated islands of new bone were also observed in the center of the defects.

Bone regeneration was significantly greater when HA particles associated with BMP2 were inserted into the defects ($59.58 \pm 7.456\%$) compared to the three modalities without BMP2 (Bone regeneration: HA = $35.88 \pm 32.81\%$, HA + cryo-hAM = $30.60 \pm 15.75\%$, HA + collagen membrane = $25.96 \pm 11.62\%$; p = 0.0205, p = 0.0003 and p = 0.0003 respectively). In addition, bone regeneration was significantly greater when HA particles associated with BMP2 were inserted into the defects, then covered by the collagen membrane (Bone regeneration = $63.46 \pm 22.18\%$) compared to the three modalities

without BMP2 (p < 0.01), whereas no statistical differences were observed between HA particles covered by cryo-hAM and HA-BMP2 covered by cryo-hAM (Bone regeneration = $30.60 \pm 15.75\%$, $37.66 \pm 16.91\%$ respectively).

However, no significant differences were observed between HA, HA covered by the collagen membrane and HA covered by cryo-hAM (hAMSCs). Similarly, no significant differences were observed between HA-BMP2, HA-BMP2 covered by the collagen membrane and HA-BMP2 covered by cryo-hAM (hAMSCs) (Fig. 3b).

Histological analysis evidenced new bone formation at the edges of the defects (Fig. 3c, yellow arrowheads). HA particles were still visible 6 weeks after surgery and were surrounded with newly formed bone (Fig. 3c, green arrowheads). The rest of the defect was filled by connective tissue (Fig. 3c).

4 Discussion

We assessed the use of hAM for GBR using the calvarial defect model in mice. hAM is an abundant tissue that is easy to obtain since it is usually discarded after parturition. Already widely used in the field of ophthalmology [16], the procedures for preparing and conditioning hAM were

optimized several years ago to prevent infectious disease transmission and to minimize host inflammatory reactions. Several conditioning and preserving methods have emerged to avoid the use of fresh hAM [36–39], one of which is cryopreservation. Cryopreservation reduces the risk of infectious disease transmission by exceeding the period of viral incubation and makes it possible to obtain a large quantity of immediately available material [40]. To reduce the risk of a potential host reaction to chemical products used in the preservation procedure, the EFS, which is in charge of hAM preparation in France, recommends using glycerol for hAM cryo-preservation instead of dimethyl sulfoxide (DMSO), which is still commonly used in research laboratories to cryopreserve cells but is not advisable for tissue contact (skin, eye,...). For this reason, the hAM fragments used in this study were cryopreserved as recommended by the EFS. However, whatever technique is used to cryopreserve hAM, a low viability is always reported (<20%), even in 10% DMSO [39].

Our first *in vivo* experiment analyzed the osteoconductive properties of hAM *per se* and aimed at defining the best preparation process (fresh vs cryo-preserved) and the cell layer with the best regeneration efficiency. In the second experiment, a bone substitute (hydroxyapatite particles) and BMP2 were added and covered by hAM to test its GBR properties.

hAM *per se* led to a small bone repair increase in the presence of cryo-hAM(MSCs). The mechanism of action might be due to different factors: i) the already described anti-fibrotic property of hAM cells; ii) the secretion by hAM cells of growth factors in the few days following surgery, before they die; or iii) the release of growth factors presents in hAM. Besides, recent observations suggested that human amniotic fluid derived mesenchymal stem cells possess higher osteogenic capacity than hAECs [41]. In addition, the issue of cell survival during surgery remains to be explored.

As shown here and by others [18, 39], a very poor cell survival is observed after cryopreservation, although some surviving cells – both cryo-hAMSCs and cryo-hAECs – are able to proliferate, at least when incubated in optimal cell culture conditions, which is not necessarily the case during *in vivo* implantation. As the greatest bone repair was observed with cryo-preserved hAM patches, we hypothesize that the percentage of cells surviving after surgery is not a major issue. Indeed, most hAMSCs were alive in our preparation of fresh hAM but contact of this layer with the bone defect did not improve the repair rate.

Based on a previous report [42, 43] and considering the results obtained with different configurations of the hAM alone, a second implantation procedure using the mesenchymal side of cryo-hAM was performed and we compared its bone regeneration potential to a resorbable

collagen membrane currently used in oral and dental implant surgery [44]. To enhance its osteogenic potential, we inserted hydroxyapatite (HA) particles or HA associated with BMP2 into the calvarial bone defects. Indeed, the chemical composition of HA is very similar to the inorganic components of bone matrix. Therefore, HA is widely used in the field of bone regeneration thanks to its excellent biocompatibility and its osteoconductive properties [45]. BMP2 is a naturally potent growth factor that induces osteogenic differentiation from mesenchymal stem cells [46].

The X-ray analysis showed enhanced but incomplete bone healing 6 weeks after implantation in all groups, including the three groups that received BMP2. The relatively poor regeneration observed in the presence of BMP-2 could be due to the administration of a low dose of BMP2. We administered 0.5 µg of BMP2 which is much lower compared to other studies performed on calvarial defects in mice that used between 1 and 4 µg of BMP2 [47, 48]. We observed that HA associated with BMP2 increased bone formation significantly compared to HA. Similarly, HA-BMP2 covered by the collagen membrane increased bone formation significantly compared to HA covered by the collagen membrane. Other authors found that HA associated with BMP2 induced more bone formation compared to HA alone in a critical calvarial defect in rats [49]. However, in our study, this enhancement was not observed when HA-BMP2 was covered by cryopreserved hAM. In contrast with our first implantation performed in the absence of any HA and BMP2 that suggested that cryo-hAM(MSC) had some osteoconductive potential, this second implantation demonstrated that cryo-hAM associated with HA and BMP2 does not significantly enhance bone regeneration compared to cryo-hAM associated with HA. Additional experiments will be required to understand what led to the failure of hAM in this context.

We chose to study bone repair potential of hAM using calvarial defect because it is one of the most commonly used model to assess biomaterials and surgical strategies for craniofacial applications [50]. Whereas this is a thin bone, this model is currently used to study GBR [24, 51]. This can be explained by the fact that it allows to realize a reproducible and standardized defect and it is easy to analyze by radiographic and histological assessment [51]. Besides, this model allows to quantify bone regeneration using 2D-X-Rays analysis [52].

This preclinical study confirms clinical reports in which collagen membrane was used as a resorbable membrane for GBR and GTR [44, 53]. However, these membranes have some limitations and alternatives are still required [4, 5]. hAM was already compared to a collagen membrane currently used in oral surgery in two studies. One preclinical study assessed the efficacy of bone xenograft associated

with hAM *versus* a collagen membrane for GBR around titanium implants in rats. X-ray analysis showed extensive ossification throughout the defect site in both groups. A better bone–implant connection was evidenced by histological analysis in the group treated by hAM [27]. In another study, a randomized controlled blinded clinical trial was done to compare the efficacy of xenogenic bone graft associated with hAM *versus* a collagen membrane in GTR for the treatment of intrabony periodontal defects. Periodontal clinical parameters were significantly improved in both groups [31].

5 Conclusion

This study aimed to assess the osteogenic potential of fresh and cryopreserved hAM in a critical size bone defect. Cryopreserved hAM seemed to induce greater bone formation when the mesenchymal side covered the defect. When a bone substitute was inserted into the defect, bone repair was not improved by addition of cryopreserved hAM. This showed that, in this model of bone defect, the properties of hAM for bone regeneration are actually limited.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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1.3. Conclusion

Les expérimentations menés dans cet article tendaient à montrer que la face de la MAH appliquée au contact du défaut a un faible impact sur la régénération osseuse et que le potentiel ostéogénique de la MAH ne dépend pas ou peu des cellules souches contenues dans celle-ci.

Dans les conditions actuelles de récupération et de préservation de la MAH selon le protocole de l'EFS , nous avons observé *in vitro* une mort cellulaire importante au sein des MAH fraîches et cryopréservées. *In vivo*, la réparation osseuse était légèrement augmentée lorsque le défaut était recouvert par la face mésenchymateuse de la MAH cryopréservée. Ce phénomène n'était pas observé avec la face mésenchymateuse de la MAH fraîche bien que la viabilité de la plus part des cellules souches mésenchymateuses semblaient autant ou plus élevée que pour la MAH cryopréservée. Nous émettons ainsi l'hypothèse que la viabilité des cellules souches contenues dans la MAH ne constitue pas un critère nécessaire à la ROG.

Enfin, nous avons comparé *in vivo* l'apport de la MAH à une membrane de collagène couramment utilisée en pratique clinique. Pour cela les défauts osseux étaient soit comblés par de l'HA ou par de l'HA associé à un facteur de croissance (BMP-2), soit comblés par ces deux même conditions puis recouverts par la MAH cryopréservée ou par la membrane de collagène. Aucun effet membrane n'a été observé dans ce modèle: il n'y avait pas de différence significative entre les défauts comblés par de l'HA *versus* ceux comblés par de l'HA puis recouverts par l'une des deux membranes. Les mêmes résultats étaient obtenus en présence de BMP-2 : la réparation osseuse n'était pas augmentée lorsque les défauts étaient comblés par HA+ BMP-2 puis recouverts par l'une des deux membranes par rapport aux défauts comblés par HA+ BMP-2 sans membrane. Néanmoins, l'effet ostéoinducteur de la BMP-2 était retrouvé dans la condition sans membrane et lorsque le comblement était recouvert par la membrane de collagène. Cet effet n'a pas été retrouvé en présence de la MAH cryopréservée (face mésenchymateuse au contact du défaut). Par ailleurs aucune différence n'avait été retrouvé dans cette deuxième expérimentation *in vivo* entre la face mésenchymateuse et la face épithéliale de la MAH cryopréservée (résultats non publiés).

2. DEVELOPPEMENT D'UNE NOUVELLE METHODE DE DECELLULARISATION DE LA MAH ET COMPARAISON DE L'IMPACT DES METHODES DE PRESERVATION SUR LES PROPRIETES BIOLOGIQUES ET MECANIQUES DE LA MAH.

2.1. Introduction

L'étude précédente n'a pas mis en évidence d'intérêt de la MAH fraîche par rapport à la MAH cryopréservée sur la régénération osseuse de défauts de calvaria de souris. La présence de cellules vivantes au sein de la MAH ne semblait pas avoir d'influence sur son potentiel ostéogénique. Basés sur ces résultats, et les limites inhérentes à la cryopréservation (durée limitée de stockage, implantation d'un matériau dont on ne maîtrise pas la viabilité cellulaire, nécessité de disposer de matériel encombrant et couteux pour préserver le tissu, décongélation et rinçage avant l'usage...), nous avons cherché à développer de nouvelles méthodes de préservation de la MAH au sein du laboratoire.

En dehors de la cryopréservation, la lyophilisation ou bien la décellularisation suivie de la lyophilisation font parties des méthodes de préservation de la MAH le plus souvent rapportées [99,105,120,235–237]. Les méthodes de décellularisation de la MAH décrites dans la littérature sont souvent longues (plusieurs jours) et complexes à mettre en œuvre, ou alors elles nécessitent une étape de décellularisation physique par raclage de la MAH afin de détacher et d'éliminer les cellules résiduelles. Ce procédé de « scrapping » (raclage mécanique) peut présenter une certaine variabilité car il est opérateur dépendant et peut également causer des dommages tissulaires de la matrice. De plus, lorsque nous avons essayé de reproduire certains des protocoles déjà décrits (avec ou sans raclage mécanique) lors d'expérimentations préliminaires, nous observions la persistance de résidus cellulaires.

Les méthodes de préservation peuvent modifier les propriétés biologiques, physiques et mécaniques de la MAH [131,160,238]. Pourtant, bien que l'ensemble de ces méthodes de préservation de la MAH soient souvent utilisées en ingénierie tissulaire, il n'existe pas d'étude comparant leurs propriétés et aucun consensus sur le choix d'une méthode de préservation.

L'objectif de ce travail était i) de mettre au point de nouvelles méthodes de préservation de la MAH et ii) de comparer l'impact des différentes méthodes de préservation sur les propriétés biologiques et mécaniques de la MAH.

Dans un premier temps, nous avons mis au point la technique de lyophilisation de la MAH au sein du laboratoire. Puis nous avons cherché à développer un nouveau protocole de décellularisation qui ne soit pas chronophage et qui ne nécessite pas d'étape de « scrapping » afin de s'affranchir de toute variabilité liée à ce processus de détachement mécanique des cellules.

Dans un second temps, nous nous sommes intéressés à l'impact des méthodes de préservation de la MAH sur son aspect morphologique, histologique, sur la persistance de certaines protéines de sa matrice extracellulaire. Pour cela, nous avons comparé 4 types de MAH : la MAH fraîche, la MAH cryopréservée, la MAH lyophilisée et la MAH décellularisée/lyophylisée. Nous avons également comparé les propriétés mécaniques en traction uniaxiale de ces 4 types de MAH.

Enfin, nous avons comparé la cytotoxicité *in vitro* et la biocompatibilité *in vivo* de ces quatre types de MAH. *In vitro*, nous avons d'abord comparé la cytotoxicité indirecte d'extraits de 4 types de MAH sur des cellules souches humaines de moelle osseuse (hBMSCs). Puis nous avons évalué *in vitro* le potentiel de ces MAH pour servir de matrice support à la prolifération de hBMSCs. *In vivo*, des patchs des quatre types de MAH ont été implantés en sous-cutané chez le rat afin de comparer leur biocompatibilité et de quantifier leur résorption.

À noter qu'à partir de cette deuxième étude, les conditions de prélèvement des placentas ont été modifiées. Une convention a été établie avec la Maternité Aliénor d'Aquitaine, ce qui nous a autorisé à récupérer directement les placentas lors des accouchements par césarienne au CHU et de ne plus être dépendant de l'EFS. Cela nous a permis de récupérer de la membrane amniotique fraîche en plus grande quantité et de pouvoir la traiter au sein du laboratoire dans l'heure suivant le prélèvement du placenta.

2.2. Article n°2

**COMPARISON OF THE IMPACT OF PRESERVATION METHODS ON
AMNIOTIC MEMBRANE PROPERTIES
FOR TISSUE ENGINEERING APPLICATIONS**

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Comparison of the impact of preservation methods on amniotic membrane properties for tissue engineering applications



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ABSTRACT

Human amniotic membrane (hAM) is considered as an attractive biological scaffold for tissue engineering. For this application, hAM has been mainly processed using cryopreservation, lyophilization and/or decellularization. However, no study has formally compared the influence of these treatments on hAM properties. The aim of this study was to develop a new decellularization-preservation process of hAM, and to compare it with other conventional treatments (fresh, cryopreserved and lyophilized).

The hAM was decellularized (D-hAM) using an enzymatic method followed by a detergent decellularization method, and was then lyophilized and gamma-sterilized. Decellularization was assessed using DNA staining and quantification. D-hAM was compared to fresh (F-hAM), cryopreserved (C-hAM) and lyophilized/gamma-sterilized (L-hAM) hAM. Their cytotoxicity on human bone marrow mesenchymal stem cells (hBMSCs) and their biocompatibility in a rat subcutaneous model were also evaluated.

The protocol was effective as judged by the absence of nuclei staining and the residual DNA lower than 50 ng/mg. Histological staining showed a disruption of the D-hAM architecture, and its thickness was 84% lower than fresh hAM ($p < 0.001$). Despite this, the labeling of type IV and type V collagen, elastin and laminin were preserved on D-hAM. Maximal force before rupture of D-hAM was 92% higher than C-hAM and L-hAM ($p < 0.01$), and D-hAM was 37% more stretchable than F-hAM ($p < 0.05$). None of the four hAM were cytotoxic, and D-hAM was the most suitable scaffold for hBMSCs proliferation. Finally, D-hAM was well integrated *in vivo*.

In conclusion, this new hAM decellularization process appears promising for tissue engineering applications.

1. Introduction

The human amniotic membrane (hAM) is the innermost layer of the fetal membrane and is in contact with the amniotic fluid. It contains three layers: an epithelial layer, a mesenchymal layer also known as the stromal layer, and a basement membrane, which separates them. Its thickness ranges from 20 to 500 μm [1]. The epithelial layer contains a single layer of human amniotic epithelial cells (hAECs) with a columnar or cuboidal shape [2]. The epithelium lies on a basement membrane containing mostly type IV collagen and laminin. The latter play a key

role in the attachment of epithelial cells and in the cellular proliferation, migration and differentiation of hAECs [3]. Fibronectin, another component of the basal membrane, is also found in the stroma layer [3]. The amniotic stroma is comprised of three layers from inside to outside: an inner compact layer, a fibroblast layer and a spongy layer [1,2]. The stromal extracellular matrix mainly contains collagen type I, III, V, VI, laminin and fibronectin [4]. This membrane is neither vascularized nor innervated so it is a translucent biological structure.

Because it is considered as surgical waste after delivery, hAM is easy to procure and is widely available. Since its first clinical use for skin

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replacement in 1910 by Davis, hAM is routinely used in ophthalmology and dermatology and remains the gold standard substrate for the *ex vivo* expansion of human limbal stem cells to treat corneal blindness [5–8]. hAM contains immunoregulatory factors such as HLA-G and Fas ligand, which have been linked to its low immunogenicity [9–11]. hAM is also known to display an anti-inflammatory and antifibrotic effect and to enhance wound healing [12–16].

Thanks to these biological properties, the low cost of harvesting and good clinical outcomes, hAM has become a highly attractive and promising scaffold for tissue engineering. Several studies have used hAM as a biological scaffold upon which different cell types can grow and differentiate [17–19]. To allow prolonged storage, several preservation methods of hAM have been developed in order to prepare hAM prior to cell seeding for tissue engineering [6,20–22]. Cryopreservation and lyophilization are the techniques which are the most commonly used [23,24]. However, cryopreservation leads to very poor cell viability in hAM [25,26]. Therefore, to avoid implanting a tissue whose cell viability is not controlled, the decellularization of hAM emerged. Preserved hAM can thus be used directly or decellularized, *i.e.* without HAECs and human amniotic mesenchymal stromal cells (hAMSCs).

Various attempts have been made to decellularize hAM and they usually require two steps. First, hAM is exposed to chemical or enzymatic agents, then mechanical scraping is performed to remove the loosened cells [21,27–31]. This adjunctive scraping step requires the removal of residual cells under light microscopy, so the procedure is operator-dependent. Furthermore, mechanical scraping may cause severe damage to the basement membrane integrity [32,33]. Only a few studies have suggested new decellularization methods that do not require additional mechanical scraping [16,34–38]. Despite favorable reports, the techniques proposed are very time-consuming since they last several days. Whatever the treatment used (cryopreservation, lyophilization, decellularization or gamma-sterilization), they all have some limitations because the processing and preservation of hAM affect its properties [6,39]. The composition or the distribution of the extracellular matrix and basement membrane components of hAM is often affected by preservation [23,30,40]. It has also been shown that the preservation of hAM decreases the amount of growth factors [40,41], and leads to changes in its physical and mechanical properties [23,39,42]. However, few studies have compared the properties of these different hAM with fresh hAM for tissue engineering applications, and there is still no consensus about the optimal method for preserving hAM prior to its use as a scaffold for tissue engineering.

The objective of this study was first to develop a simple and reproducible method for the effective decellularization and preservation of hAM. We also aimed to establish the most suitable preservation method based on morphological, biomechanical, histological, *in vitro* cytocompatibility and *in vivo* biocompatibility parameters.

2. Materials and methods

2.1. Harvest and preservation methods of hAM

2.1.1. Tissue collection

Eleven human placentas were collected after elective cesarean surgery from consenting healthy mothers (tested seronegative for HIV, cytomegalovirus, Toxoplasma gondii, Hepatitis B and C virus, and syphilis). Patients provided written informed consent as requested by the institutional review board and their placentas were anonymized. The placentas were kept in a sterile solution containing PBS 1× (Gibco®) supplemented with 1% antibiotics (penicillin/ streptomycin, Invitrogen®) to be transferred to the laboratory. Then, they were rinsed with sterile distilled water and residual blood clots were removed. The amniotic membrane was peeled from the chorion and rinsed with sterile distilled water again before storing. All these steps were performed under sterile conditions.

2.1.2. Preparation and storage of hAMs

Four treatments of hAM were performed in this study: fresh (F-hAM), cryopreserved (C-hAM), lyophilized (L-hAM) or decellularized then lyophilized hAM (D-hAM). All steps were done under aseptic condition. Fresh hAM (F-hAM) was kept in plates containing α-minimum essential medium (MEM alpha, GIBCO®), 10% fetal bovine serum (FBS, Eurobio®) and 1% antibiotics (amoxicillin/ streptomycin Invitrogen®). It was stored in this medium in an incubator (37 °C, 5% CO₂, 100% humidity) for a maximum of two days before use. For C-hAM, pieces of hAM were put in a solution of RPMI/glycerol 1:1 and kept frozen at –80 °C. When needed, C-hAM was thawed and washed twice with sterile PBS 1× before further analysis. To prepare L-hAM, patches were frozen at –80 °C, then dried under vacuum in a freeze dryer. For D-hAM, hAM was first treated with trypsin and ethylene-diaminetetraacetic acid (T/EDTA, 0.125%) for two minutes at 37 °C. It was washed with sterile PBS for 15 min, transferred to a decellularization solution composed of 8 mM CHAPS, 25 mM EDTA, 0.12 M NaOH and 1 M NaCl in PBS and then incubated under gentle agitation for 7 h at room temperature. Following this treatment, hAM was washed thoroughly overnight in three changes of sterile distilled water with vigorous shaking. Finally, D-hAM was frozen at –80 °C, before being dried in the freeze dryer. L-hAM and D-hAM were put in sterilization pouches before being sterilized by gamma radiation at 25 kGy (Gammacell® 3000 Elan, NORION MDS, Ottawa, Canada). They were stored in their sterilization pouches at room temperature and kept in the dark until analysis.

2.1.3. Validation of decellularization method

To ensure the effectiveness of the decellularization process, we used previously established guidelines for decellularization [32]. D-hAM was compared to non-treated amnion using qualitative and quantitative criteria (*n* = 3 for each experiment). First, DAPI staining was conducted to visualize the presence of any residual nuclei on D-hAM and non-decellularized hAM using confocal microscopy (Leica TCS SPE Model DMI 4000B). In addition, samples of hAM from the four groups were fixed in 4% paraformaldehyde (Antigenfix, Microm Microtech, France), dehydrated by baths of increasing ethanol concentrations and paraffin-embedded. Samples of fresh and preserved hAM were then sectioned with a microtome (7 μm) and stained with DAPI. Cross-sections were observed with the same confocal microscopy. In the second experiment, after freeze-drying and grinding hAM, residual DNA was extracted from D-hAM and non-treated hAM in order to be quantified with a DNA extraction kit (QIAamp® DNA Mini Kit, Qiagen, USA) according to the manufacturer's instructions. DNA was then quantified with a spectrophotometer by determining its absorbance at 260/280 nm wavelength (Implen NanoPhotometer® P-Class P330). The value obtained (ng/μL) was plotted against the weight of the dry samples (ng/μg). Finally, to determine the size of the remaining DNA, equal concentrations of extracted DNA from non-treated and decellularized hAM were separated by agarose gel electrophoresis 1.5% and visualized with ultraviolet transillumination using a ladder (FastRuler low range DNA Ladder, ThermoFisher®).

2.2. Characterization of fresh and preserved hAM

2.2.1. Histological assessment

For histological analysis, samples of hAM from the four groups were fixed in 4% paraformaldehyde (Antigenfix, Microm Microtech, France), dehydrated by baths of increasing ethanol concentrations and paraffin-embedded. Samples of fresh and preserved hAM were then sectioned with a microtome (7 μm) and stained with hematoxylin and eosin safron (HES). Images were obtained with an Eclipse 80i light microscope (Nikon, Japan) and captured with a DXM 1200C CCD camera (Nikon, Japan).

2.2.2. Immunohistological study

To assess the effect of the preservation procedure, the distribution of 7 proteins of hAM extracellular matrix was assessed by immunohistochemistry. Sections of the paraffin-embedded samples were obtained with a microtome (5 µm), then glued with an albumin-glycerol mixture on treated slides. After dewaxing and rehydration of sections, a hyaluronidase pretreatment was performed. After washing in PBS, samples were incubated with the primary antibody overnight at 4 °C to detect the presence of type I, III, IV, V collagen, elastin, laminin and fibronectin (See Supplementary Table S1). Having blocked endogenous peroxidase activity with 0.5% hydrogen peroxide, samples were incubated with the secondary antibody for 45 min at room temperature. Antigen-antibody complexes were revealed by tetrahydrochloride diaminobenzidine (DAB, Dako, K3468) and cells were slightly counterstained with Mayer's hematoxylin. Sections were mounted with aqueous medium for microscope observation.

2.2.3. Biomechanical behavior

The physical and biomechanical properties of F-hAM, C-hAM, L-hAM and D-hAM were investigated. Briefly, the average thickness of fresh and preserved hAM was assessed with a laser sensor (Aeroel®, XLS 13XY XACTUM™). Uniaxial tensile tests were performed by using an Autograph tensile tester AGS-X (Shimadzu®). hAM samples were designed by using a dog-bone shaped punch similar to the ASTM D-638 type V (width: max = 7.5 mm, min = 2.5 mm; linear length = 6 mm; overall length = 38.63 mm). They were pre-tested at 20 mm/min until 0.1 N and stretched at a speed of 1% of loaded initial length (L_0) per second (typically around 0.24 mm/s). The samples remained wet during the mechanical testing. If failure did not occur in the center of the sample, the sample was discarded. Maximal force before rupture (Fmax) and strain at failure (Smax) were recorded using Trapezium X® software.

2.3. In vitro cytocompatibility studies

Human bone marrow mesenchymal stem cells (hBMSCs) were utilized for the cytocompatibility studies. The hBMSCs were isolated from consenting patients who had undergone hip surgery (experimental agreement with *CHU de Bordeaux* and *Etablissement Français du Sang*, agreement CPIS 14.14), and expanded according to well-established protocols [43]. Cells were used at passage 1 for this study.

2.3.1. Extract cytotoxicity assay

First, we wanted to assess the cytotoxicity of the preservation method. For this purpose, the cytotoxicity of soluble extracts obtained from the four membranes (F-hAM, C-hAM, L-hAM and D-hAM) was evaluated according to the NF-EN-ISO 10993-5 standards by measuring the cell viability and the metabolic activity of hBMSC using a neutral red assay and a 3-(4-5 dimethylthiasol-2-yl) diphenyl tetrazolium (MTT) assay, respectively. For both studies, cell culture medium extracts were prepared according to the EN 30993-5 European standard. Pieces of the four hAMs ($n = 12$ for each preservation method) were put in 24-well plates containing 400 µL of α-minimum essential medium (MEM alpha, GIBCO®), 1% antibiotics (Invitrogen®), then incubated at 37 °C in a humidified atmosphere containing 5% CO₂ in air. The medium extracts were collected after 24 h (E1) and replaced by the same volumes of medium. Medium extracts were stored at -20 °C. The procedure was repeated every day for three days (E2 and E3). For both Neutral Red and MTT assays, hBMSCs were plated at 1.5×10^4 cells/cm² in 96-well plates and cultured for 72 h to reach cell confluence. After removal of culture media, soluble extracts (E1, E2 and E3) of the four hAMs supplemented with 10% FBS were added and incubated for 24 h. Triton 100× at 0.1% was used as a negative control and α-MEM culture medium alone was used as a positive control.

To assess the effect of hAM soluble extracts on the cell viability of hBMSCs, the culture medium was removed after 24 h of contact with

soluble extracts, and a solution of 100 µL of Neutral Red (Sigma-Aldrich Co), diluted in 1.25% IMDM supplemented with 10% FBS, was added to each well and cultured at 37 °C, 5% CO₂ for 3 h. Then, the supernatant was removed and 100 µL of a solution made of 1% acetic acid in 50% ethanol were added to lyse the cells. Staining intensity was quantified by measuring the absorbances at 540 nm with a spectrophotometer (Perkin Elmer®, 2030 Multilabel Reader Victor™X3).

To assess the metabolic activity, the culture medium was removed after 24 h of contact and replaced with 125 µL solution of MTT (Sigma-Aldrich Co, 5 mg/mL in 0.1 M PBS, pH = 7.4), which was diluted (20% in IMDM without phenol red (Gibco®) and cultured for 3 h at 37 °C, 5% CO₂ to form blue formazan crystals. Subsequently, the supernatant was removed and 100 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich Co) were added to the plates to dissolve the formazan crystals. Staining intensity was quantified by measuring the absorbance at 570 nm with a spectrophotometer (Perkin Elmer®, 2030 Multilabel Reader Victor™X3). For both assays, the results of each condition were normalized to positive controls (cells cultured on plain plastic surfaces in basal medium) for each incubation time (as 100% cell viability and metabolic activity).

2.3.2. Contact cytotoxic assay

We aimed to compare the suitability of fresh and preserved hAM to be used as a scaffold upon which hBMSCs can adhere and grow. Thus, the metabolic activity of hBMSCs cultured over fresh and preserved hAM was evaluated at 1, 3 and 10 days post-seeding by using a resazurin-based assay [44,45]. Metabolic activity of hBMSCs cultured in 2D conditions on tissue culture polystyrene (TCPS) plates served as a positive control. Briefly, samples of hAM were cut into circles, put in 24-well plates and maintained in place with home-made rings ($n = 9$ for each condition). hBMSCs were seeded on each sample at a density of 2.5×10^4 cells per well and cultured for 10 days. An alamar blue assay was performed on day 1, 3, 7 and 10. Briefly, a solution of resazurin (0.1 mg/mL in PBS) was added to each well to a final 10% (v/v) concentration. After 3 h incubation at 37 °C, 200 µL of the medium were transferred to a 96-well plate and measured by fluorescence (exc. = 530 nm, em. = 590 nm, Victor X3, Perkin Elmer). Results were expressed as percentage of metabolic activity of cells relative to 2D conditions at day 1.

2.4. In vivo biocompatibility

2.4.1. Subcutaneous implantation

The present study was approved by the French Ethics Committee (agreement APAFIS n°4375-2016030408537165v8). Thirty 10-week-old female Wistar rats were used. The biocompatibility of the four hAM was assessed using a rat subcutaneous implant model. The aim was to implant patches of F-hAM, C-hAM, L-hAM and D-hAM (10 × 10 mm) in the dorsal subcutaneous tissue of adult rats and to compare their biocompatibility. As hAM is a resorbable material, we used blue non-absorbable sutures (Prolene™ Visi-Black™ 6-0, Ethicon®) to fix it at each of the four corners of the samples and to act as a marker for identifying the implantation sites. Surgery was carried out under aseptic conditions. Short-term anesthesia was induced by inhalation of 4% isoflurane (Air:1.5 L/min) and maintained using isoflurane 2% (Air: 0.4 L/min). Analgesia was performed by intraperitoneal injection of 0.1 mg/kg buprenorphine (Buprecare®, 0.3 mg/ml). The back was shaved, the surgical site was aseptically prepared, and a midsagittal incision was made in the back area. Three conditions were implanted on either side of the mid-dorsal line ($n = 6$ conditions per rat). In addition to the four implanted hAM, two negative controls were performed for this study: a sham-operated control with no biomaterial implantation (sham) and a negative control for which only non-absorbable sutures were made (suture). Implants were spaced at least 10 mm apart, and each implant base was > 10 mm from the line of incision. After surgery, food and water were supplied *ad libitum*. Euthanasia was performed one week, one month and two months after implantation using CO₂ inhalation

($n = 30$ rats; 10 rats per time point). After shaving, the samples were carefully harvested and rinsed with PBS 1×, then placed in 4% paraformaldehyde (Antigenfix, Microm Microtech, France) overnight. Then, the 180 explanted samples were rinsed in PBS 1× and processed for histology and immunofluorescence.

2.4.2. Histological and immunolabeling analysis of implants

Samples were dehydrated and processed for conventional embedding in paraffin. Seven-μm-thick serial sections were made. First, immunolabeling of type I collagen was performed in order to reveal the presence of residual hAM (Abcam, ab34710). Briefly, deparaffinized sections were pretreated with citrate (pH 6.0) for 20 min at 95 °C and were then washed with PBS. To block non-specific binding sites, 5% BSA (bovine serum albumin) in PBS was used for 30 min at room temperature. Anti-collagen I antibody diluted in 5% BSA in PBS (1:200) was applied on sections overnight at 4 °C. Secondary antibody Alexa Fluor 568-conjugated goat anti-rabbit (Invitrogen, A11036) was diluted (1:300) and applied for 1 h30 at room temperature in the dark. Cross-sections of the suture control samples were used as control.

Then, the 180 samples were stained with HES and images were acquired with a slide scanner (Hamamatsu Nanozoomer 2.0HT). The resorption of hAM patches over time and for each condition was measured on HES staining using NDPview software (one section per sample) by two investigators. The dimension of residual hAM calculated on the stained section was plotted against the initial dimension of the implants. Finally, according to the NF-EN-ISO 10993-6 standard, a blinded independent trained investigator scored the inflammatory reaction around the implants semi-quantitatively by HES staining. The following biological response parameters were assessed and recorded: cellular infiltration and inflammatory cell type (polymorphonuclear, lymphocytes, macrophages, plasma cells and giant cells), vascularization, fatty infiltration and extent of fibrosis. The scoring system was as follows: the test sample was considered as non-irritant (0.0 up to 2.9), slightly irritant (3.0 up to 8.9), moderately irritant (9.0 up to 15.0) or severely irritant (> 15) to the tissue as compared to the sham-operated control sample.

2.5. Statistical analysis

Results are expressed as mean ± standard deviation, with n indicating the number of hAM samples tested. Statistical analysis was performed using GraphPad Prism Software (La Jolla/CA, USA). First, a normality test was performed using a D'Agostino and Pearson omnibus normality test. If data assumed Gaussian distribution, differences were assessed by one-way analysis of variance (ANOVA) with the Bonferroni post-test, whereas statistical significance for independent samples was evaluated with the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. In both cases, statistical significances are marked by stars with * indicating a two-tailed $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Results

3.1. Decellularization: DAPI staining DNA quantification and agarose gel electrophoresis assessment

No positive staining was observed after the decellularization process, demonstrating the absence of residual nuclei (Fig. 1A and B). This result was confirmed by DNA quantification. The amount of residual DNA in D-hAM was lower than 50 ng/mg of dry tissue (remaining DNA of non-treated hAM and D-hAM: 5408 ± 3341 and 42 ± 12 ng/mg respectively, $p < 0.05$) (Fig. 1C). Furthermore, no residual DNA was visible in the decellularized tissue gel electrophoresis after the decellularization process (Fig. 1D).

3.2. Morphological aspect, ECM composition and mechanical properties of fresh and preserved hAM

The morphology of fresh and preserved hAM was assessed by HES staining (Fig. 2A). The organization of the epithelium was maintained as a single layer of columnar or cuboidal cells in F-hAM, C-hAM and L-hAM, whereas the complete lack of residual cells was obvious after decellularization. This result supported the validation of the decellularization process of D-hAM. The epithelial cells were slightly damaged by cryopreservation and lyophilization and vacuolar degeneration was observed in C-hAM and L-hAM. The trilaminar architecture of hAM (epithelium, basement membrane and mesenchymal layer) was preserved in F-hAM. The epithelial and the mesenchymal layers could be identified in C-hAM and L-hAM, whereas the architecture was no longer observable in D-hAM. The stroma layer remained unchanged after cryopreservation, although it was much thinner and denser in L-hAM and D-hAM.

Immunohistochemistry was also performed to investigate whether the extracellular matrix and basement membrane proteins remained unchanged after the treatments (Fig. 2B). Before treatment, F-hAM was distinctly labeled for type I, III, IV and V collagen, elastin, fibronectin and laminin in the basement membrane and stromal layer. After cryopreservation, the architecture of the amnion seemed unchanged. However, type IV collagen and laminin labeling were slightly decreased by the treatment. After lyophilization, we observed a labeling of type I, IV and V collagen, elastin, fibronectin and laminin, whereas the labeling of type III collagen was reduced or absent. The other labeling persisted in D-hAM except for type I, type III collagen and fibronectin labeling, which were absent or greatly reduced.

Finally, to compare the physical and mechanical properties of hAM, the thickness, maximal force (FMax) and strain at failure (SMax) of fresh and preserved hAM were assessed. L-hAM and D-hAM were significantly thinner than F-hAM. C-hAM appeared thicker than F-hAM, but no statistical difference was observed (Fig. 3A). D-hAM was significantly stronger than C-hAM and L-hAM ($p < 0.001$). Furthermore, no statistical difference in FMax was observed between F-hAM and D-hAM, suggesting that this treatment did not compromise the mechanical properties of hAM (Fig. 3B). D-hAM was also significantly more stretchable than F-hAM (Fig. 3C).

3.3. In vitro cellular response

Cell viability was evaluated with red neutral assay. There was a significant reduction in the viability of hBMSCs when they were cultured with the first soluble tissue extract of C-hAM (Fig. 4A). No decrease in cell viability was observed with the second and third extracts. The metabolic activity of hBMSCs was evaluated by the MTT test. The first extracts from C-hAM also significantly reduced the metabolic activity of hBMSCs and this reduced activity was also observed with the third extract (Fig. 4B). However, since the cell viability and metabolic activity of hBMSCs were always higher than 70%, the four hAM may be considered non-cytotoxic according to NF-EN-ISO 10993-5 standards.

We also assessed the capacity of hBMSCs to attach and proliferate over fresh and preserved hAM. At day 1, the metabolic activity of hBMSCs seeded on any hAM was significantly higher than that of hBMSCs cultured on plastic. At subsequent time points, metabolic activity was significantly enhanced compared to control (hBMSCs cultured on plastic) only when cells were seeded over D-hAM (Fig. 4C).

3.4. In vivo biocompatibility

All implants could be sutured to the subcutaneous tissue of rats during surgery (Fig. 5). However, L-hAM and D-hAM were easier to handle and to suture than F-hAM and C-hAM. They were stiffer, more resistant to tearing and did not fold. As non-absorbable sutures were used to mark the implantation site, all the 174 samples could be easily

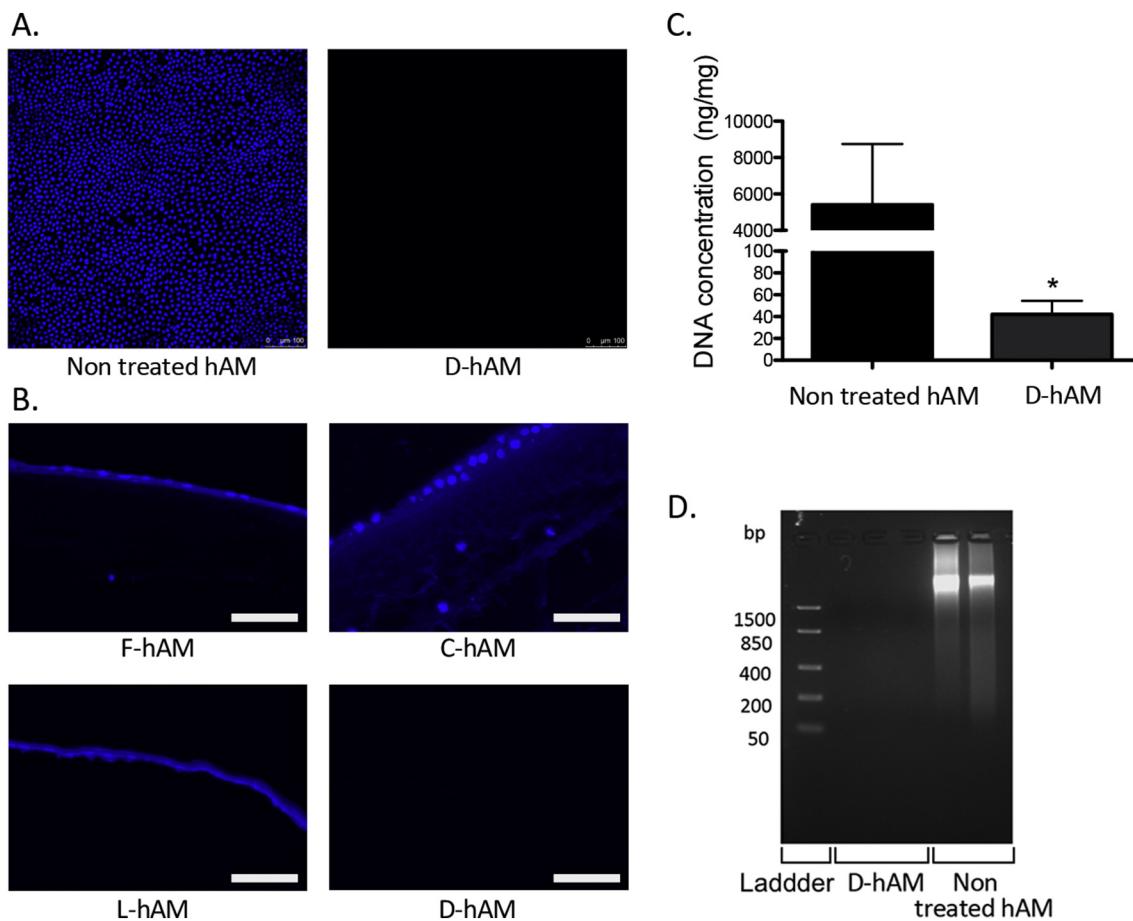


Fig. 1. Validation of decellularization process.

The effectiveness of the decellularization method was assessed according to three previously established criteria [32]. (A) DAPI staining of nuclear material was strong on the non-treated hAM, whereas no fluorescent labeling was observed on D-hAM. This lack of staining showed that the decellularization process completely removed the nuclear material. (B) DAPI staining of histological sections of the four hAMs. The epithelial cells were clearly visible in F-hAM, C-hAM and L-hAM, and few hAMSCs were observed in F-hAM and C-hAM. Scale bar: 50 μ m. (C) After extraction, the DNA content of D-hAM was lower than 50 ng/mg of dry tissue. (D) In the decellularized sample, no residual DNA was observed on agarose gel electrophoresis, whereas in non-treated hAM, the residual DNA ranged from 100 bp to > 1500 bp and likely included intact full-length DNA. Data are presented as means + /– standard deviation. The symbol * indicates a statistically significant difference compared to the other group with $p < 0.05$.

located one week, one month or two months after surgery ($n = 10$ per time point for each condition, except at one week since one rat died during surgery, so $n = 9$).

3.4.1. Resorption of hAM

First, the immunofluorescence staining of type I collagen showed the presence of F-hAM and C-hAM one week after surgery, whereas no staining was evidenced thereafter in these membranes (Fig. 6A). Staining of L-hAM was no longer visible two months after surgery and D-hAM was the only condition in which type I collagen staining was still present two months after surgery (Fig. 6A).

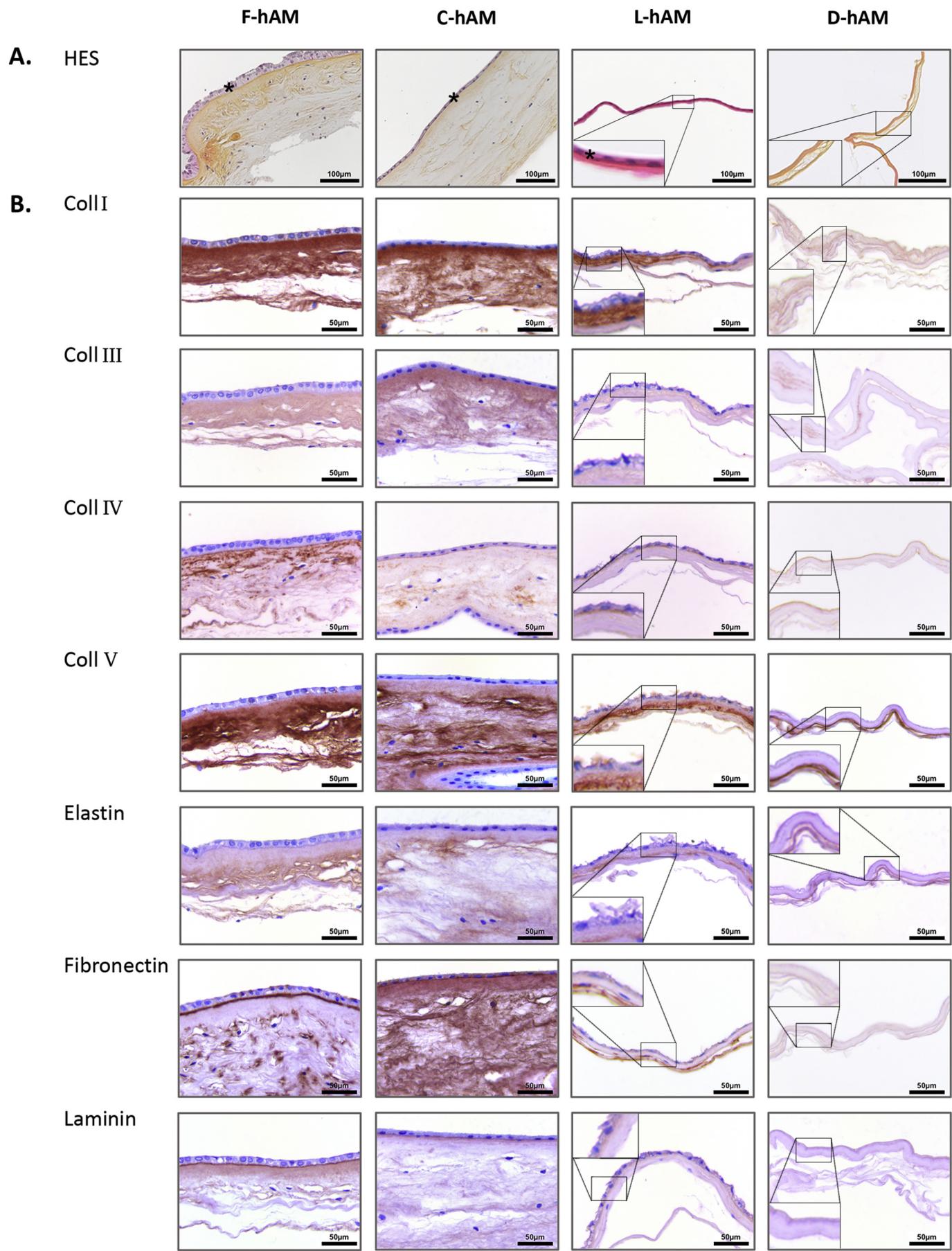
Then, these data were corroborated by measuring the resorption rate of fresh and preserved hAM by histological analysis (Fig. 6A and B). One week after surgery, the degradation of fresh and preserved hAM had already started. D-hAM had the lowest resorption rate but there was no significant difference between the four membranes at one week. One month after surgery, F-hAM and C-hAM were almost fully resorbed. L-hAM seemed to have degraded faster than D-hAM after one week and one month, but the difference became statistically significant only after two months. Thus, D-hAM had the slowest rate of resorption since it was the only membrane that was still present two months after implantation.

3.4.2. Inflammatory reaction

To evaluate the host response, a blinded independent trained investigator scored the inflammatory reaction around the implants semi-quantitatively by HES staining (Fig. 7A and B). Values were expressed as the difference between the test sample (hAM and non-absorbable suture control) and the control sample (sham-operated control).

One week after surgery, a slight inflammatory reaction was observed around fresh and preserved hAM compared to the sham-operated control. The acute inflammatory reaction was clearly visible around F-hAM and C-hAM implants, with cells penetrating the implant (Fig. 7A). L-hAM and D-hAM caused less inflammation and no host cell infiltration was observed in the implant (Fig. 7A). One month after surgery, L-hAM and D-hAM had induced a higher cellular response than F-hAM and C-hAM, which were completely degraded by then. Two months after surgery, the inflammatory reaction had abated in all the conditions. However, only D-hAM maintained a slight active inflammatory reaction, where host cell infiltration associated with delamination of D-hAM was observed (Fig. 7A).

Based on ISO 10993-6:2007 scoring, fresh (F-hAM), preserved hAM (C-hAM, L-hAM and D-hAM) and suture controls were considered slightly-to-moderately irritant to the tissue as compared to the sham-operated control (Fig. 7B). Similar results were obtained with the suture control samples, which induced a slight inflammatory reaction compared to the sham samples too.



(caption on next page)

Fig. 2. Influence of preservation methods on morphological aspect and components of hAM.

(A) Light microscopy of amniotic membrane stained using HES. C-hAM closely resembles F-hAM, whereas lyophilization caused compaction of L-hAM and D-hAM. Black asterisks show epithelial layer of hAM. Scale bar: 100 µm. (B) Representative immunohistochemical staining of extracellular matrix and basement membrane components of amniotic membranes. Abbreviations: Coll I, type I collagen; Coll III, type III collagen; Coll IV, type IV collagen; Coll V, type V collagen. Scale bar: 50 µm.

4. Discussion

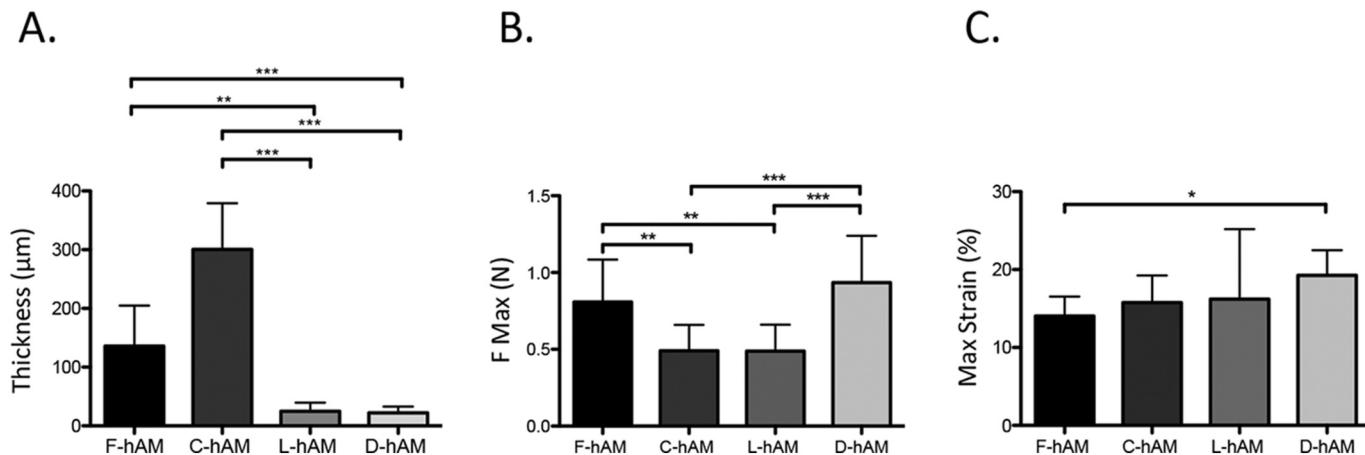
We developed and characterized a new acellular amnion-based scaffold suitable for tissue engineering, and compared it with all conventional methods to preserve hAM. Some hAM decellularization processes have already been used to obtain an acellular amniotic membrane. Although they were successful in removing cells, they either required mechanical scraping [27–29], which induces variability, or involved multi-day treatments with enzymatic and/or harsh chemical agents [34–36,38,46]. Our new method is effective, not time-consuming and does not require mechanical scraping. First, to avoid exposing tissues to cell removal agents for long incubation times, we added a short incubation time with T/EDTA as a first step. For dense tissue, exposure to trypsin may be needed to improve the penetration of the decellularization agent and obtain a completely acellular scaffold [47]. Then, we used a decellularization method that has proved successful for decellularizing tissue-engineered vascular grafts [48]. There were no residual nuclei with DAPI staining and residual DNA was < 50 ng/mg, which is considered as an acceptable threshold to avoid an adverse host response [32,49].

Cryopreservation and lyophilization are the most commonly used hAM preservation procedures [23]. Once the hAM was decellularized, we performed lyophilization and gamma sterilization prior to its use to allow long-term storage. Freeze-drying (*i.e.* lyophilization) allows the safe storage of samples for several years at room temperature [22,25,50], whereas cryopreservation requires expensive equipment that may be unavailable in some institutions, and the storage time cannot exceed 12 to 24 months [6,51]. Furthermore, the cold chain involves complex transportation procedures and the samples need to be thawed before use [50,51]. Lyophilized hAM appears easier to store, and it is usually followed by sterilization of the amniotic tissue by gamma radiation [52]. Gamma radiation is used worldwide for sterilizing medical products, and it is considered the most reliable and effective method to sterilize tissue allografts [25].

Treatment of hAM raises issues regarding its biological and mechanical properties. Several studies have already reported some damage in the expression or the distribution of the extracellular matrix and basement membrane components of hAM after preservation [23,30,40].

In this study, the morphology of the hAM and the protein distribution were altered by the three preservation methods tested. Delamination of the stroma was also observed in D-hAM. This disruption is commonly induced by decellularization processes [30,40,53]. In our study, except for fibronectin, labeling of the other proteins was still observed after the decellularization of hAM, although it was sometimes slightly or highly reduced. In addition, our process did not damage the integrity of the basement membrane components. Indeed, type IV collagen and laminin, which are abundant in the basement membrane, were still expressed. This could be due to the zwitterionic detergent used in this study (*i.e.* CHAPS), which usually preserves the ultrastructure better than ionic detergents [32]. Moreover, not having used mechanical scraping may have contributed to preserving the basement membrane integrity.

The preservation and sterilization of hAM may also affect its biophysical properties as allogenic grafts [39]. However, to our knowledge, no study has simultaneously compared the mechanical properties of fresh, cryopreserved, lyophilized/gamma-sterilized and decellularized/lyophilized/gamma-sterilized hAM. Preservation caused significant changes in the thickness of hAM: it was significantly reduced in L-hAM and D-hAM, whereas C-hAM seemed thicker than F-hAM. These results are consistent with previous studies in which cryopreservation led to the uptake of hydrophilic glycerol and water, thus resulting in the swelling of C-hAM, whereas freeze-drying resulted in the loss of liquid [23,39,54]. Because the thickness of hAM varies significantly between donors [55] and also depends on the preservation procedures used, we decided to assess the mechanical characteristics of hAM using thickness-independent parameters, as done previously [23]. The tensile Fmax of F-hAM was significantly higher than that of cryopreserved and lyophilized hAM (65% higher, $p < 0.01$), and the Fmax of D-hAM was also 92% higher than that of C-hAM and L-hAM. Similar results were obtained by Niknejad et al., who found that both cryopreservation and lyophilization induced lower maximal loads to failure than with fresh hAM [23]. This could be due to the extracellular matrix alterations that they induce. Other authors compared the mechanical properties of hAM after lyophilization and after decellularization and lyophilization [29]. They found similar results between both membranes, whereas in our study D-hAM was stronger than L-hAM. This could be because, unlike

**Fig. 3.** Comparison of physical and mechanical properties of fresh and preserved hAM.

(A) Preservation significantly changed the thickness of hAM ($n = 16$). (B) Fresh and decellularized hAM were significantly stronger than C-hAM and L-hAM ($n = 15$ per condition). Fmax: Maximal force before rupture. (C) Decellularization made hAM significantly more stretchable than F-hAM ($n = 15$ per condition). Max Strain: strain at break. (ANOVA; Mean + / - standard deviation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

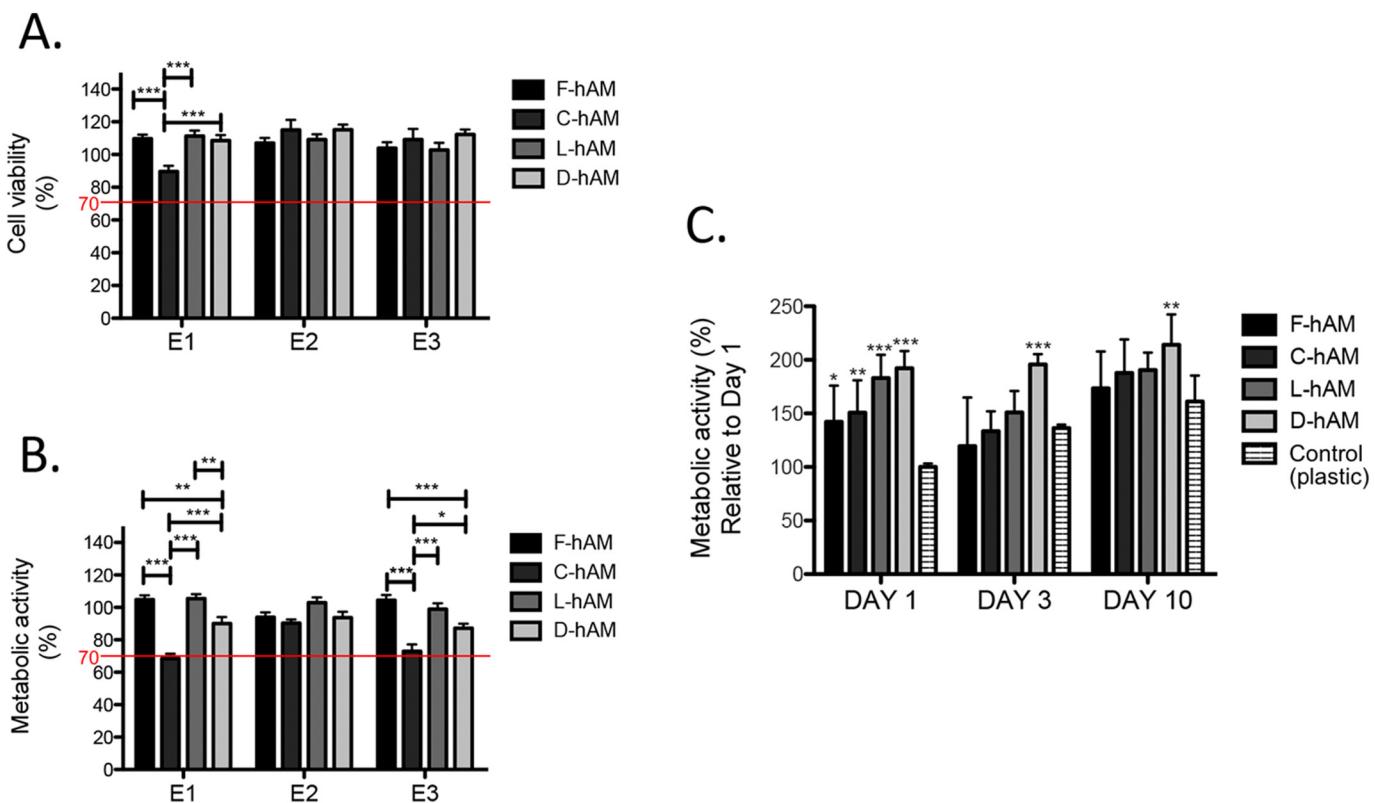


Fig. 4. Extract and contact *in vitro* cytotoxicity of fresh and preserved hAM on human bone marrow stem cells (hBMSCs).

(A) Neutral red assay. Relative cell viability expressed as a percentage of untreated blank. Soluble extracts of cryopreserved hAM (C-hAM) significantly reduced hBMSCs cell viability after one day (E1) compared to F-hAM, L-hAM and D-hAM. No statistical difference in hBMSCs cell viability was observed with soluble extracts collected at day 2 and 3 (E2-E3) ($n = 12$ per condition; ANOVA, *** $p < 0.001$). (B) MTT assay. Relative metabolic activity expressed as percentage of untreated blank. The decrease in number of living cells due to soluble extract of C-hAM at day 1 (E1) resulted in a significant decrease in metabolic activity. However, the relative cell viability and metabolic activity of hBMSCs were higher than 70% of the control group, demonstrating the non-cytotoxic effect of soluble extract of fresh and preserved hAM. ($n = 12$; ANOVA, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (C) Relative metabolic activity of hBMSCs seeded on fresh and preserved hAM. Data were normalized to positive control that represented 100% metabolic activity at day 1. Their metabolic activity was significantly enhanced when hBMSCs were seeded on decellularized hAM (D-hAM) compared to control. ($n = 9$ per condition; ANOVA, * Indicates a statistically significant difference compared to control, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Results are expressed as: mean + / - standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

them, we did not use scraping, thus avoiding mechanically damaging the membrane. Another study comparing the mechanical properties of fresh and decellularized hAM showed no statistical difference between them [37]. The membrane was decellularized without scraping, then sterilized by using paracetic acid [37]. We thus hypothesized that the decellularization process play a protective role that preserved the

matrix from a decrease of its mechanical properties following cryopreservation/lyophilization. We hypothesize that the cell removal caused by the decellularization created space inside the matrix to allow ice crystals to form without damaging the collagen network. This would result in better preservation of mechanical properties. Another explanation could be that the decellularization process somehow induces

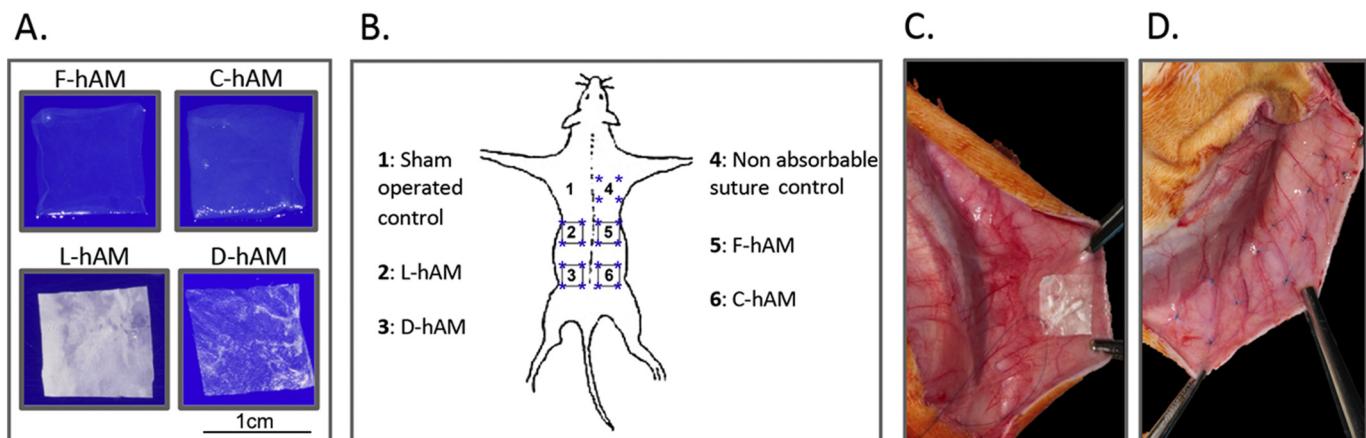
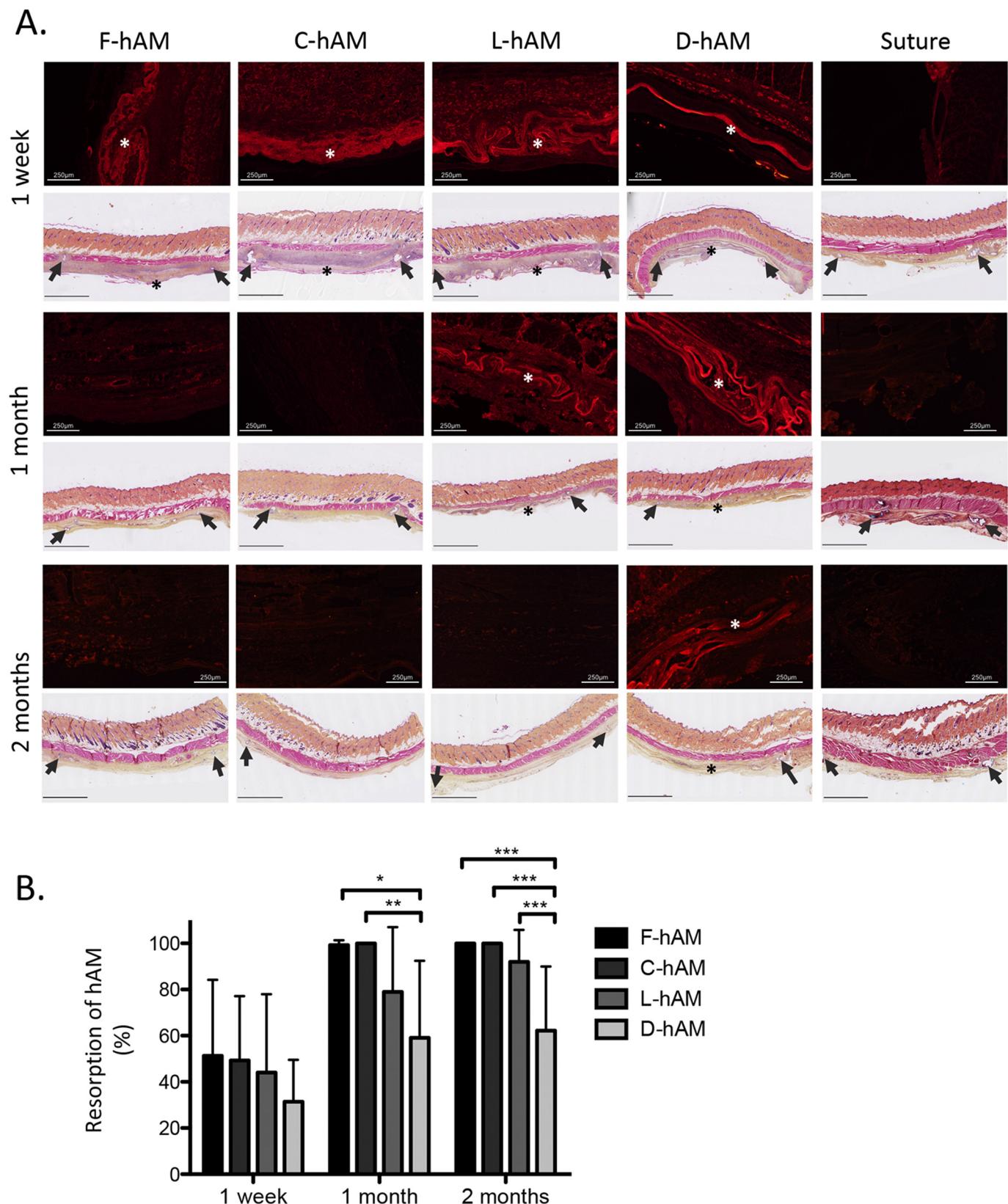


Fig. 5. Implantation of hAM patches in rat subcutaneous model

(A) Macroscopic appearance of fresh and preserved hAM implants. (B) Implantation procedure. (C) and (D) Surgery and suture of hAM patches.

**Fig. 6.** Resorption of hAM after subcutaneous implantation in rats

(A) Representative histological and immunofluorescence (IF) staining of explanted fresh and preserved hAM and suture control. Black and white asterisks show residual hAM and black arrows indicate non-absorbable suture. Scale bar histological analysis: 2.5 mm; Scale bar IF: 250 µm. (B) Resorption rate of fresh and preserved hAM was measured using HES staining. D-hAM had the slowest rate of resorption. ($n = 9$ samples per condition at one week, $n = 10$ samples at one and two months; ANOVA; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

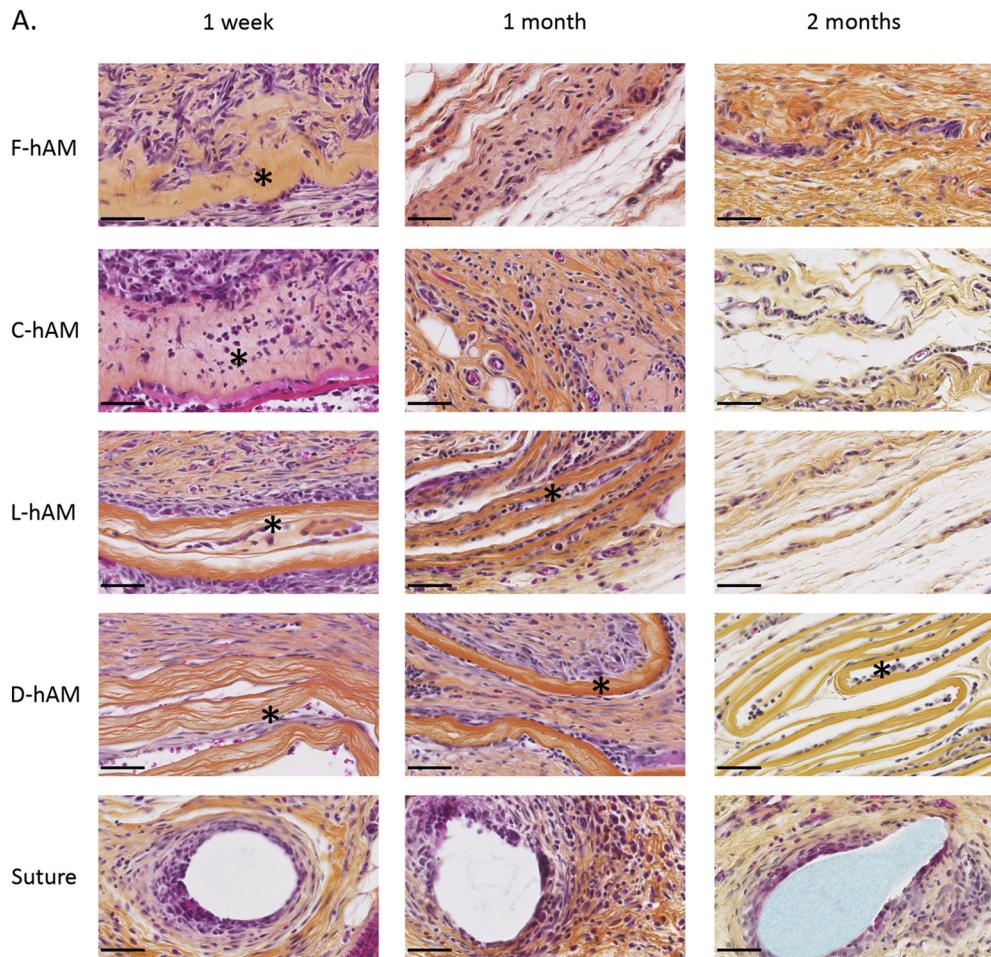
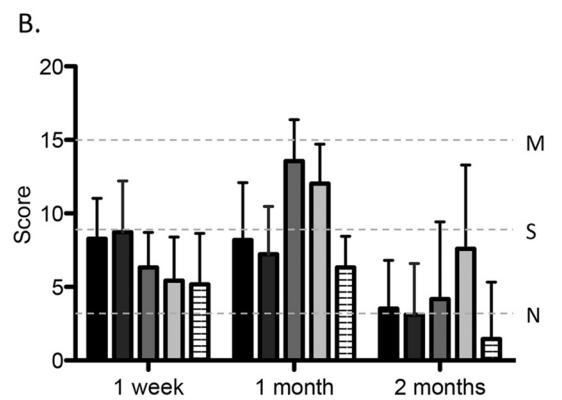


Fig. 7. Biocompatibility of fresh and preserved hAM

(A) Representative histological sections stained with HES after subcutaneous implantation. At one week, F-hAM and C-hAM exhibited higher host-cell infiltration. Black asterisks show residual hAM (Scale bar: 50 μ m). (B) Histological sections were evaluated and scored according to NF-EN-ISO 10993-6 standard. The test samples were considered as non-irritant (N: up to 2.9), slightly (S: 3.0 up to 8.9) to moderately irritant (M: 9.0 up to 15.0) and severely irritant (> 15) to tissue as compared to sham-operated control sample ($n = 9$ samples per condition at one week, $n = 10$ samples at one and two months).



the formation of smaller ice crystal. A 3D-analysis would be necessary to be able to link the collagen network structure of hAM to its mechanical properties. Interestingly, we also found no statistical difference in Fmax between D-hAM and F-hAM, and D-hAM appeared to be 37% more stretchable than F-hAM ($p < 0.05$). Mechanical results showed that decellularization did not decrease the overall strength of the tissue. In addition, it made hAM more rigid than F-hAM and C-hAM, resulting in a membrane easier to handle and to suture without tearing during *in vivo* experiments. Figueiredo et al. also reported that decellularization followed by gamma irradiation stiffened hAM tissues [38]. However, depending on the targeted application, it could be interesting to further enhance the thickness and mechanical properties of D-hAM, perhaps by designing a multi-layered D-hAM scaffold [36,56].

Several differentiated cells such as human keratinocytes [17], human oral mucosal epithelial cells, human chondrocytes [18] and

smooth muscle cells [35,57] have been successfully seeded on acellular amnion scaffolds. However, fewer studies have investigated its potential as a matrix for growing stem cells, a cell source that could be more promising for tissue engineering [36] and especially for bone regeneration [56]. Here we assessed the cytotoxicity of fresh and preserved hAM and compared their ability to support the proliferation of hBMSCs. As shown here and by other groups [23,29], we observed that fresh and preserved hAM were non-cytotoxic. However, cryopreservation caused a significant reduction in cell viability and metabolic activity. These results are consistent with previous studies, likely for two reasons [19,58]. First, they may be due to the use of glycerol as a cryoprotectant. Shortt et al. found that glycerol impaired the ability of hAM to act as a substrate for cell seeding compared to hAM cryopreserved in Hank's Balanced Salt Solution without glycerol [19]. Glycerol is known to display a growth-inhibitory effect that is dose-dependent on

various cell types [59,60]. It may be induced by osmotic pressure change leading to a stress to cells, because water moves faster across the cellular membrane than glycerol [61]. Another possible explanation for this significant reduction is that the presence of autolytic enzymes released by the cells dying in the amniotic membrane could decrease cell viability after the extract cytotoxicity assay [37]. This is supported by the very poor survival of amnion-derived cells previously observed after cryopreservation [26]. Finally, in the context of tissue-engineering applications, we assessed the capacity of hBMSCs to attach and proliferate over fresh and preserved hAM. Whatever the preservation method used, the metabolic activity of hBMSCs was significantly greater once seeded on hAM than cultured on plastic at day 1. Over time, D-hAM was the most suitable scaffold for hBMSCs proliferation, as demonstrated by a significant enhancement of their metabolic activity. These results are consistent with previous studies that reported the successful proliferation of human and rat BMSCs seeded on an acellular hAM [62] [29,56]. In our study, the best results were achieved with D-hAM. This could be due to the fact that the decellularization process led to exposure of the basement membrane of hAM, thereby promoting its ability to support cell adhesion and proliferation. Our decellularization process did not damage the integrity of the basement membrane components, which is essential to promote cell seeding.

Finally, we compared the biocompatibility of fresh and preserved hAM using subcutaneous implants in rats. The host response to fresh or preserved hAM implantation has already been studied in immunocompetent rodents [29,34,63]. Indeed, hAM is an immune-privileged tissue that contains some immunoregulatory factors such as HLA-G and Fas ligand, and has a low-to-absent level of expression of HLA class I and II molecules [9,64]. These characteristics should thus avoid the rejection of hAM by an allograft or xenograft. hAM is a resorbable biological scaffold, so unlike other authors [29,34], we decided to stabilize it with non-absorbable sutures so as to easily identify the implant site after sacrifice. As described earlier and by other groups, we observed an early degradation of fresh and cryopreserved hAM once implanted [15,63]. Lyophilization and gamma sterilization especially after decellularization significantly prolonged graft survival. In some applications such as bone regeneration, which requires around three months, the longevity of hAM grafts might be a critical parameter for proper healing. In such cases, D-hAM appears to be the most suitable scaffold since it displays the slowest rate of resorption. Another way to enhance their longevity would be to stack hAM to obtain multi-layered scaffolds [63]. We observed that fresh and preserved hAM are a biocompatible matrix, inducing a slight-to-moderate reaction as compared to the sham-operated control samples. However, the inflammatory reaction score for D-hAM was higher two months after surgery, probably because it was the only membrane still present two months after implantation. Furthermore, host cell infiltration of D-hAM occurred later, suggesting a protection effect against cellular infiltration like that offered by the barrier membrane used in clinical practice.

A limitation is that we did not assess the growth factor level of hAM as a function of the preservation method. Contradictory findings have been reported regarding the effect of preservation on the level of growth factors contained in hAM [22,25,41]. First, it could be because, depending on the targeted application, different growth factors were investigated, making the comparison between studies difficult. Second, preservation methods affect each growth factor differently. Third, a variation in growth factor content in amniotic membrane samples has been shown between donors, but it also depends on the region of the membrane and the delivery method [65,66]. In addition, several studies have reported successful scaffold function with preserved hAM despite their low concentration in growth factors [22,40].

5. Conclusion

We have developed a novel and rapid method to decellularize hAM that does not require an operator-dependent mechanical scraping step.

This study is the first to compare decellularized hAM with fresh and conventionally preserved hAM. Disruption of the architecture of D-hAM was observed but the integrity of the basement components was preserved. Whatever the treatment used, hAM had no cytotoxic effect, and D-hAM significantly enhanced the metabolic activity of hBMSCs once seeded on D-hAM compared to other treatments. Our method also enhanced the mechanical properties of hAM and prolonged its longevity after implantation, making it an attractive matrix for tissue engineering.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.msec.2019.109903>.

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2.3. Conclusion

Cette étude a permis de développer et de valider une nouvelle méthode de décellularisation de la MAH qui est reproductible et moins chronophage. Ces travaux ont également permis de comparer la MAH décellularisée/lyophilisée aux méthodes de préservation conventionnelles de la MAH.

Ces résultats ont montré une modification de la morphologie ainsi que la composition de la MAH quelle que soit la méthode de préservation utilisée. La membrane basale a un rôle déterminant dans l'attachement et la prolifération cellulaire. Et, bien que celle-ci se retrouve exposée suite au protocole de décellularisation, nous avons mis en évidence qu'il n'y avait pas d'altération du marquage des composants de la lame basale (collagène IV et laminine) au niveau de la MAH décellularisée/lyophilisée.

Cette étude a également montré l'impact de ces méthodes de préservation sur les propriétés physiques et mécaniques de la MAH. Alors que la cryopréservation augmente son épaisseur, la lyophilisation entraîne une diminution significative de l'épaisseur de la MAH. Basés sur ces résultats, et du fait de la variabilité inter et intra-donneur observée dans d'autres études, nous avons choisi de réaliser des tests mécaniques qui s'affranchissaient de l'épaisseur de la MAH. Nous avons observé une diminution significative des propriétés mécaniques des MAH cryoprérvée et lyophilisée alors que la MAH décellularisée/lyophilisée ne présentait pas de différence par rapport à la MAH fraîche. Nous pouvons en conclure que le protocole de décellularisation développé dans cette étude préserve la MAH de la détérioration des propriétés mécaniques observée avec les autres traitements.

Nous avons ensuite évalué *in vitro* la cytotoxicité directe et indirecte de la MAH sur des hBMSCs. Les hBMSCs ont un fort potentiel d'ostéodifférentiation. Nous avons observé que les quatre type de MAH étaient cytocompatibles. De plus, la MAH décellularisée/lyophilisée semble être la meilleure matrice support pour la prolifération des hBMSCs. Cette matrice offre des perspectives intéressantes dans le domaine de l'ingénierie tissulaire osseuse.

Enfin nous nous sommes intéressés à la biocompatibilité et à la résorption des quatre types de MAH. Les quatre types de MAH implantés en sous-cutanés chez le rat étaient biocompatibles. La MAH fraîche et cryopréservée présentaient une résorption significativement plus rapide que les MAH lyophilisée et décellularisée/lyophilisée. La MAH décellularisée/lyophilisée avait le taux de résorption le plus lent, suggérant son intérêt pour la ROG. La régénération osseuse étant un processus qui peut durer plusieurs mois, il est nécessaire que la membrane utilisée ne se dégrade pas trop rapidement.

Cette étude ne nous a pas permis de préciser l'impact de ces méthodes de préservation sur le potentiel de régénération osseuse de la MAH. Ainsi, une nouvelle étude portant sur la comparaison de ces membranes dans un contexte plus spécifique d'ingénierie tissulaire osseuse a été mise en place. Ces expérimentations ont fait l'objet d'un troisième article soumis en septembre 2019.

3. COMPARAISON DE L'IMPACT DES METHODES DE PRESERVATION DE LA MAH SUR LA REGENERATION OSSEUSE GUIDEÉE D'UN DEFAUT OSSEUX FEMORAL NON CRITIQUE.

3.1. Introduction

Les travaux de l'article n°2 ont permis de développer de nouvelles méthodes de préservation de la MAH et de comparer les propriétés biologiques et mécaniques de quatre types de MAH : MAH fraîche, MAH cryopréservée, MAH lyophilisée et MAH décellularisée/lyophilisée. Plusieurs études pré-cliniques et cliniques rapportent l'intérêt de ces 4 types de MAH pour la régénération osseuse. Cependant, leur potentiel pour la régénération osseuse n'a jamais été comparé de façon concomitante dans une même étude.

L'objectif de cette étude était de comparer l'apport des MAH fraîche, cryopréservée, lyophilisée et décellularisée/lyophilisée pour la régénération osseuse guidée.

Dans un premier temps, nous avons comparé la préservation du collagène et des glycosaminoglycans (GAG) au sein des 4 types de MAH. Puis, nous avons évalué l'influence de ces méthodes de préservation sur la suturabilité de la MAH.

Dans un second temps, nous avons étudié *in vitro* le potentiel de ces quatre types de MAH comme matrice support de la prolifération et de l'ostéodifférentiation de hBMSCs.

Enfin, les quatre types de MAH ont été utilisés comme membrane « barrière » pour la ROG de défauts diaphysaires non critiques chez la souris. Les défauts étaient soit laissés vide, soit recouverts par l'une des quatre MAH. La réparation osseuse ainsi que la néovascularisation ont été évaluées à un temps précoce (une semaine) et plus tardif (un mois).

3.2. Projet d'article n°3

ASSESSMENT OF FRESH AND PRESERVED AMNIOTIC MEMBRANE FOR GUIDED BONE REGENERATION IN MICE

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Keywords:	Bone, amniotic membrane, <i>in vivo</i> , tissue engineering, processed amnion

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ABSTRACT

Thanks to its biological properties, the human amniotic membrane (HAM) can be used as a barrier membrane for guided bone regeneration (GBR). However, no study has assessed the influence of the preservation method of HAM in this application. This study aimed to establish the most suitable preservation method of HAM for GBR.

Fresh (F), cryopreserved (C) lyophilized (L) and decellularized and lyophilized (DL) HAM were compared. Impact of the preservation methods on collagen and glycosaminoglycans (GAG) content was evaluated using Masson's trichrome and blue alcian staining. Their suture retention strengths were assessed. *In vitro*, the osteogenic potential of human bone marrow mesenchymal stromal cells (hBMSCs) cultured on the four HAMs was evaluated using alkaline phosphatase staining and alizarin red quantification assay. *In vivo*, the effectiveness of fresh and preserved HAMs for GBR was assessed in a mice diaphyseal defect after one week and one month. Micro-CT and histomorphometric analysis were performed.

The major structural components of HAM (collagen and GAG) were preserved whatever the preservation method used. The tearing strength of DL-HAM was significantly higher. *In vitro*, hBMSCs seeded on DL-HAM displayed a stronger ALP staining and alizarin red staining quantification was significantly higher at day 14. *In vivo*, L-HAM and DL-HAM significantly enhanced early bone regeneration. One month after the surgery, only DL-HAM slightly promoted bone regeneration.

Several preserving methods of HAM have been studied for bone regeneration. Here, we demonstrate that DL-HAM achieved the most promising results for GBR.

Key word : Bone; Amniotic membrane; *In vivo*; Tissue engineering; Processed amnion

1 1. INTRODUCTION

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Guided bone regeneration (GBR) is one of the most commonly used procedure to repair damaged bone in oral and maxillofacial surgery. GBR has been successfully performed to treat periodontal defects (furcation and/or intrabony defects), for ridge preservation procedure, maxillary sinus augmentation, implant site development and to treat peri-implants bone defects [1–3]. This procedure is based on the application of a membrane, which will act as a physical barrier, ensuring selective permeability to prevent the invasion of fibrous tissue and unwanted cells into the expected bone-healing space [1]. Their biocompatibility is the most important requirement to consider when selecting a membrane as it allows integration in the host tissues without triggering inflammatory reaction [4,5]. These membranes should meet other criteria such as space maintaining to ensure the stability of the blood clot and grafted bone substitute [6] as well as easy-handling and shaping of the membrane [1,7]. Depending on the target application, different resorbable or non-resorbable barrier membranes have been developed [4,7,8]. Non-resorbable membranes have the disadvantages of often resulting in premature membrane exposure (causing bacterial contamination) and require an additional surgery to removed them [3,4]. Resorbable membranes can induce a strong inflammatory response during the postoperative healing phase and they have low mechanical strength [1,7,8]. Due to its unique biological properties, such as low immunogenicity as well as anti-inflammatory [9], antibacterial and antiadhesive effects [10,11], the human amniotic membrane (HAM) is a new biological membrane option for GBR.

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HAM is one of the oldest biomaterials used for wound healing in medicine. The first clinical use of fresh HAM was reported by Davis for skin transplantation in 1910. Since then, cryopreserved HAM has been routinely used in ophthalmology [12,13] and fresh and preserved HAM are widely studied in various areas of tissue engineering [14–17]. HAM is the innermost layer of the placenta, lining the amniotic cavity [18]. HAM displays a typical trilaminar architecture characterized by an epithelial monolayer that is separated from the stroma layer by a basement membrane [19]. This thick basement membrane provides a support to the fetus during the gestation. HAM is a source of stromal cells and growth factors [20–22], and it is considered a surgical waste so it is a highly available and cost-effective tissue that might be useful for bone regeneration in the field of oral and maxillofacial surgery [23].

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Several studies have investigated the potential of fresh or various preserved HAM for bone healing in oral surgery, but these HAMs have never been simultaneously compared. Six preclinical studies reported the use of amniotic membrane for guided bone regeneration [23–28]. HAM was used either fresh [23], cryopreserved [23,25,27], lyophilized [24] and/or decellularized [26,28]. Another preclinical study suggested the use of lyophilized HAM for GBR procedure around dental implants [29]. Positive outcomes were also obtained with cryopreserved HAM in a model of extraction socket in rats [30]. Moreover, clinical studies performed on patients with periodontitis have highlighted the potential of HAM to regenerate the ligament and to improve bone repair [31]. Lyophilized HAM has been successfully used to treat periodontal defects such as furcation and/or intrabony defects [32–37]. A case-series also suggested to use of a combination of lyophilized HAM, a bone susbtitute (hydroxyapatite) and platelet-rich fibrin to treat periapical lesions resulting from necrosis of the dental pulp [38].

HAM has thus been used as a fresh, cryopreserved, lyophilized and/or acellular matrix to promote bone healing.

However, there are no studies that have directly compared these four types of HAM when used for bone regeneration. It is thus still unknown if there is an optimal preservation method of HAM to act as a barrier membrane for GBR. This study aimed at investigating the most suitable preservation method of HAM for GBR.

2. **MATERIALS AND METHODS**

Harvest and preparation of HAM

- **Tissue collection :**

Five human placentas were collected after elective cesarean surgery from consenting healthy mothers (tested seronegative for HIV, Hepatitis B and C virus, and syphilis). Patients provided written informed consent as requested by the institutional review board and their placentas were anonymized. After delivery, the placentas were transferred immediately to the laboratory in a sterile solution containing PBS 1x (Gibco®) supplemented with 1% antibiotics (penicillin/streptomycin, Invitrogen®). Then, residual blood clots were removed using sterile distilled water to rinse them. The amniotic membrane was separated from the chorion by blunt dissection and rinsed before storing. All these steps were performed under sterile condition.

- **Preparation and storage of HAMs**

Four treatments of HAM were assessed in this study: fresh (F-HAM), cryopreserved (C-HAM), lyophilized (L-HAM) or decellularized/lyophilized hAM (DL-hAM). The four treatments were performed for the samples from each of the five placentas. They were prepared and stored as previously described [39]. Briefly, fresh HAM (F-HAM) was stored in plates containing α-minimum essential medium (MEM alpha, GIBCO®), 10% fetal bovine serum (FBS, Eurobio®) and 1% antibiotics (amoxicillin/streptomycin Invitrogen®) in incubator (37°C, 5% CO₂, 100% humidity). For cryopreservation (C-HAM), HAM was put in a solution of RPMI/Glycerol 1:1, and kept frozen at -80°C. Before experimentation, C-HAM was thawed and washed with sterile PBS 1x. To realize lyophilized HAM (L-HAM), samples were first frozen at -80°C, then dried in a freeze dryer device. For decellularization (D-HAM), HAM was first treated with trypsin and ethylenediaminetetraacetic acid (T/EDTA, 0.125%) for two minutes at 37°C. After washing HAM with sterile PBS, HAM was transferred to a decellularization solution composed of 8 mM CHAPS, 25 mM EDTA, 0.12 M NaOH and 1 M NaCl in PBS and incubated, for 7h at room temperature under gentle agitation followed by three washes of sterile distilled water. Finally, DL-HAM was frozen, before being freeze-dried in the freeze dryer device. L-HAM and DL-HAM were sterilized by gamma radiation at 25 kGy (Gamacell® 3000 Elan, NORION MDS, Ottawa, Canada) and kept at room temperature until analysis.

Characterization of fresh and preserved HAM

- **Histological analysis**

Samples of the four tested HAMs were fixed in 4% paraformaldehyde (Antigenfix, Microm Microtech, France), dehydrated through graduated baths of increasing ethanol and paraffin embedded. Seven µm sections of fresh and preserved HAM samples were performed with a microtome, and stained with Masson's Trichrome to observe collagen and Alcian Blue to show glycosaminoglycans (GAG). Images were observed using an Eclipse 80i light microscope (Nikon, Japan) and acquired with a DXM 1200C CCD camera (Nikon, Japan).

- Suture retention test

To investigate the influence of preservation methods on HAM mechanical properties, we tested their suture retention strength on four placentas. Four conditions were assessed: F-HAM, C-HAM, L-HAM and DL-hAM and six samples by placenta were tested for each condition of preservation (n=24 per condition). The mechanical resistance against the pull out of a suture was investigated with a specified samples size of HAM (width: 1 cm and length: 2 cm). One edge of each sample was held with a clip in the testing machine, while the other end, was sutured at 2 mm from the rim by a 4–0 absorbable suture (4-0 Vicryl®; Ethicon, Inc). The test was run using a low constant vertical deformation speed of 10 mm/min until failure. During the whole duration of mechanical testing the samples stayed wet with PBS 1x. Uniaxial tensile tests were performed using a 100 N load cell on Autograph tensile tester AGS-X (Shimadzu®). Maximal force before rupture (Fmax) was recorded using the software Trapezium X®.

Osteogenic differentiation of hBMSCs cultured over fresh and preserved HAM

- Culturing hBMSCs and HAMs preparation

We aimed to compare the suitability of fresh and preserved HAM to be used as a scaffold upon which human bone marrow mesenchymal stromal cells (hBMSCs) can differentiate into osteogenic lineage. Thus, the potential of osteogenic differentiation of hBMSCs cultured over fresh and preserved HAM was evaluated using alkaline phosphatase staining and alizarin red quantification assay. First, the hBMSCs were isolated from consenting patients who underwent hip surgery (experimental agreement with *CHU de Bordeaux* and *Etablissement Français du Sang*, agreement CPIS 14.14), and expanded following well-established protocols [40]. Cells were used at passage 1 for this study. HAM scaffolds were trimmed into circles and placed on the bottom of wells of a 24-well plate and maintained in place with home-made rings (n= 3 per condition and per time for each analysis). The hBMSCs were then seeded onto HAMs at an initial cell density of $2,5 \times 10^4$ cells/cm² in 500 µl of α-minimum essential medium (MEM alpha, GIBCO®), 10% fetal bovine serum (FBS, Eurobio®) and 1% antibiotics (amoxicillin/streptomycin Invitrogen®) in incubator (37°C, 5% CO₂, 100% humidity). hBMSCs cultured in 2D conditions on tissue culture polystyrene (TCPS) plates served as a positive control. Then, the standard osteogenic induction medium (StemPro™, GIBCO®) was added in each well and cultured for 14 and 21 days.

- Alkaline phosphatase staining

Qualitative assessment of alkaline phosphatase activity was performed after 7 and 14 days. The hBMSCs were fixed with 4% paraformaldehyde for 15 min. The wells were washed twice with PBS 1x and processed using the Alkaline Phosphatase kit 86 C-1KT (Sigma-Aldrich, USA) according to the manufacturer's instructions. Briefly, cell layers were incubated with Fast Blue RR salt and Naphtol AS-MX Phosphate Alkaline solution 0.25% (Sigma-Aldrich, USA) for 30

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2 min at room temperature in the dark. Images were obtained using a stereo microscope (MZ10F,
3 Leica Microsystems, Germany) coupled to a camera (Leica model DFC 450C).
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- 6 • Mineralization assessment

7 Alizarin red (AR) semi-quantification assay was performed to evaluate calcium-rich deposits
8 by hBMSCs cultured over fresh and preserved HAM after 14 and 21 days according to well-
9 established protocol [41]. The hBMSCs were fixed with 4% paraformaldehyde at room
10 temperature for 15 min. Each well was rinsed with PBS 1x, prior to addition of 0.5 mL of 40
11 mM AR at pH 4.1 (Sigma-Aldrich, USA) per well. The plates were incubated at room
12 temperature for 10 min. The unincorporated dye was removed, then the wells were washed six
13 times. For quantification of staining, AR staining was extracted from the well by incubation in
14 0.5 mL cetylpyridinium chloride buffer for 1 h at room temperature. The dye was then discarded
15 and 100µL aliquots were transferred to a 96-well plate prior to reading at 570 nm using a
16 spectrophotometer (Perkin Elmer®, 2030 Multilabel Reader Victor™X3).
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19 **Implantation of fresh and preserved HAM in a diaphyseal femoral mice defect**

- 20 • Animal model and implantation procedure

21 Experiments were carried out in an accredited animal facility following European
22 recommendations for laboratory animal care (EU Directive 2010/63/EU for animal
23 experiments). The present study was approved by the French Ethics Committee (agreement
24 APAFIS n° 16861-2018092514552144 v4). Thirty adult C57BL/6 male mice were used for the
25 experiment (2 defects per mouse, n=6 defects per condition and per time). Analgesia was
26 performed by subcutaneous injection of 0.1 mg/kg buprenorphine (Buprecare®, 0.3 mg/ ml).
27 Short-term anesthesia was induced by inhalation of 4% isoflurane (Air:1 L/min) and maintained
28 using 2% isoflurane. The surgical site was shaved and aseptically prepared. A straight
29 longitudinal skin incision was made laterally across both legs and dissection of the quadriceps
30 was performed to expose the femoral diaphysis. The periosteum was mechanically removed
31 and unicortical holes were performed in each femoral diaphysis. This circular bone defect was
32 achieved using a 1.3 mm diameter trephine bur. The operating field was frequently irrigated
33 with a sterile saline solution to save the bone from thermal necrosis and to remove bone pieces.
34 The defect was left empty or covered with a 5 mm x 5 mm patch of one of the four tested HAM:
35 fresh, cryopreserved, lyophilized or decellularized/lyophilized HAM. The muscles were
36 subsequently repositioned and sutured with absorbable sutures (5-0 Vicryl®; Ethicon, Inc), and
37 the skin was closed with surgical clips. An antiseptic spray (Aluspray® by Vétoquinol) was
38 applied on the scar.

39 The thirty mice were sacrificed by cervical dislocation one week or one month after the surgery
40 (n=6 femurs per condition and per time). The femurs were dissected, rinsed in PBS 1x and fixed
41 in 4% paraformaldehyde overnight, before being stored into 70% ethanol at 4°C.
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- 44 • Micro-computed tomography

45 The X-ray microtomographic device used in this study was a Quantum FX Caliper (Life
46 Sciences, Perkin Elmer). The X-ray source was set at 90 kV and a current of 160 µA to obtain
47 a 10 µm resolution (field of view : 5 x 5 mm) with an exposure time of 1800 ms. After scanning,
48 cross-sectional slices were reconstructed and three-dimensional analysis was performed using
49 eXplore MicroView® software (General Electric Healthcare, Milwaukee, WI). Reconstruction
50 of the region of interest was performed after correction of the center of rotation and calibration
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of mineral density. A 1.3-mm-diameter, 350- μm thick cylindrical volume of interest corresponding to the initial surgical defect was created. Each scan was reconstructed using the same calibration system to distinguish bone and air. The percentage of bone formation, which corresponded to the volume of newly formed bone inside each cylindrical volume of interest, was measured as follows: % Bone formation = (Bone volume/Total volume) x 100%. Tissue Finally, we assessed the amount of mineral deposited within the defect also called tissue mineral density (TMD). Results were expressed as an average \pm standard deviation.

- Histological preparation

Each sample was decalcified with EDTA-based Microdec® decalcifiant for 6 days under gentle agitation. Then the samples were dehydrated and processed for conventional embedding in paraffin. Seven- μm -thick serial sections were prepared through the middle of the defect and stained with Masson's trichrome and HES staining.

- Histomorphometric analysis

Images of Masson's trichrome staining were observed with an Eclipse 80i light microscope (Nikon, Japan) and captured with a DXM 1200C CCD camera (Nikon, Japan). ImageJ® software was used to perform morphometric analysis for each diaphyseal specimen. Quantification of new regenerated bone was realized as a percentage of bone area from the whole newly regenerated tissue. Finally, images of the 60 samples stained with HES were acquired with a slides scanner (Hamamatsu Nanozoomer 2.0HT) and a quantitative analysis of new blood vessels was performed using NDPview software (one section per sample). The presence of a luminal structure filled with red blood cells were sought to identify blood vessels. Blood vessel density (BVD) was quantified using the number of new blood vessels in the defect area divided by the entire defect area [42–44].

Statistical analysis

GraphPad Prism Software (La Jolla/CA, USA) was used to perform statistical analysis. Results are expressed as mean \pm standard deviation and n indicated the number of HAM sample we tested. We first performed a normality test using a D'Agostino and Pearson omnibus normality test. Statistical significance between several groups was assessed by one-way analysis of variance (ANOVA) followed by Bonferroni post-test for data assuming Gaussian distribution. Differences for independent samples were evaluated with the non-parametric Kruskal-Wallis test and Dunn's multiple comparison test. The non-parametric Mann-Whitney test (two-tailed) was used to compare two groups. In all cases, statistical significances are marked by stars with * indicating a two-tailed $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. RESULTS

3.1 Conservation of extracellular matrix components in fresh and preserved HAM.

Masson's trichrome staining revealed the typical trilaminar architecture of F-HAM: an epithelial and a mesenchymal layer separated by the basement membrane (Fig 1A). These two layers are clearly visible in C-HAM and L-HAM, but no longer in DL-HAM since all epithelial cells had been successfully removed. F-HAM and C-HAM showed a single layer of amniotic epithelial stromal cells with a columnar or cuboidal shape. Their morphology was less easily visible in L-HAM and losses of epithelial cells were seen in some regions. The three

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3 components of the mesenchymal layer were observed in F-HAM: i) a dense fibrillar network
4 of collagen was observed throughout the compact layer, ii) the fibroblastic layer where
5 dispersed fibroblast-like mesenchymal cells were found and iii) the spongy layer. The
6 distribution of collagen seemed unchanged after cryopreservation, whereas a unique and thinner
7 layer with a dense collagen staining could be identified after lyophilization or
8 decellularization/lyophilization.
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11 Blue alcian staining showed an abundant staining of GAG in F-HAM and GAG have been
12 conserved whatever the preservation method used (Fig 1B). Their distribution appeared to
13 remain unchanged after cryopreservation and a strong and homogeneous staining could be
14 identified in L-HAM and DL-HAM.
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17 **3.2 Decellularization of HAM increases its mechanical properties**

18 The suture retention strength tests demonstrated that HAM could be cryopreserved without
19 deteriorating its biomechanical properties, as no statistical difference was observed between the
20 tearing strength of F-HAM and C-HAM (FMax = F-HAM: 100 ± 31 mN; C-HAM: 100 ± 37
21 mN). Interestingly, lyophilization appeared to increase the strength of HAM (L-HAM: $130 \pm$
22 56 mN), but it was not statistically shown to be significant. The decellularization process
23 increased significantly the tearing strength of HAM: DL-HAM mechanical properties were
24 significantly higher than the three other conditions (FMax= 176 ± 61 mN) (Fig 2).
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27 **3.3 DL-HAM enhance osteogenic differentiation of hBMSCs compared to other HAMs**

28 We assessed the suitability of fresh and preserved HAM to be used as a scaffold, upon which
29 hBMSCs can differentiate into osteogenic lineage using early alkaline phosphatase staining
30 (ALP) and late markers (AR semi-quantification) for osteogenesis (Fig 3). At the early time-
31 point (day 7), a slight ALP staining was observed when hBMSCs were seeded on F-HAM and
32 L-HAM, whereas hBMSCs seeded on C-HAM did not show any positive staining. The
33 hBMSCs seeded on DL-HAM showed an early and abundant positive staining for ALP activity.
34 Similarly, hBMSCs stained positively for ALP when seeded on plastic (control group). When
35 examined after 14 days of culture, a slight increase of positive staining for ALP was observed
36 when hBMSCs were seeded on F-HAM, C-HAM and L-HAM, whereas the hBMSCs seeded
37 on DL-HAM displayed a strong ALP staining. An enhancement of positive ALP staining,
38 between day 7 and 14, was also observed in the control group (Fig 3A).
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41 Analysis of absorbance found a significant higher concentration of AR staining when hBMSCs
42 were cultured over a DL-HAM compared to the control group at day 14. After 21 days,
43 quantification of AR staining showed no statistical difference between groups. However, it was
44 significantly enhanced for each condition between day 14 and day 21, which corroborated the
45 osteogenic differentiation of hBMSCs (Fig 3B).
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48 **3.4 The use of L-HAM and DL-HAM as a scaffold for GBR promotes bone regeneration**

49 To determine the most suitable preservation method of HAM for bone regeneration we
50 performed non-critical diaphyseal femoral defects in 30 mice (Fig 4 A and B). There were five
51 treatment modalities: 1) defects covered by F-HAM, 2) covered by C-HAM, 3) covered by L-
52 HAM, 4) covered by DL-HAM, 5) left empty (n= 6 per condition and per time). The aim was
53 to mimic GBR so the edges of the four membranes were at least 1 to 2 mm beyond the borders
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of the surgical site in order to cover the whole defect and not to fill it (Fig 4 C and D). Bone regeneration and TMD were quantified in the five different conditions one week and one month after the surgery using micro-CT analysis (Fig 5A). First, we compared the bone regeneration of the defect covered by the four HAMs to the empty defect. One week after the surgery, L-HAM and DL-HAM significantly enhanced early bone formation compared to the defect left empty (1 week BV/TV (%): L-HAM = 18 ± 8 ; DL-HAM = 23 ± 8 and empty defect = 9 ± 2) (Fig 5B). Covering the defect using F-HAM or C-HAM did not significantly enhance bone formation compared to the empty defect (1 week BV/TV (%): F-HAM = 10 ± 2 ; C-HAM = 12 ± 2). One month after the surgery, the DL-HAM was the only membrane which significantly increased bone formation compared to the defect left empty (1 month BV/TV (%): DL-HAM = 69 ± 7). No significant difference was observed between the three other HAMs and the empty defect (1 month BV/TV (%): F-HAM = 73 ± 20 ; C-HAM = 63 ± 14 and L-HAM = 55 ± 9). However, no significant difference was observed between DL-HAM and other HAMs. Concerning the TMD, no significant difference was observed between conditions one week and one month after the surgery (Fig 5C). However, we observed that TMD was significantly enhanced overtime for each condition.

Micro-CT results were corroborated by the histomorphometrical analysis of Masson's trichrome staining, which stated that L-HAM and DL-HAM significantly enhanced early bone formation compared to the defect left empty at one week (Bone regeneration (%): L-HAM = 26 ± 9 ; DL-HAM = 29 ± 14 and empty defect = 6 ± 4) (Fig 6). After one month, bone formation seemed higher than other conditions with DL-HAM too, without significance difference (Bone regeneration (%): F-HAM = 63 ± 18 ; C-HAM = 51 ± 9 ; L-HAM = 44 ± 18 ; DL-HAM = 72 ± 14 and empty defect = 61 ± 6).

To assess the influence of fresh and preserved HAM on vascularization in bone defects, vessel density was calculated from histological sections stained with HES (Fig. 7). Covering the defect with L-HAM or DL-HAM resulted in a significant increase in blood vessels density compared to the empty defect one week after the surgery. After one month, DL-HAM was the only membrane that generated around 15% more vessels than the defect left empty, without significant difference.

DISCUSSION

It was shown before that fresh amniotic membrane displays a strong osteogenic potential thanks to its periosteum-like effect [45]. However, HAM cannot be used as a fresh tissue for clinical applications because of the risk of infectious disease transmission and cannot be stored in the long-term [46]. To overcome these drawbacks, several preservation methods of HAM have been developed [46]. Cryopreservation, lyophilization and decellularization followed by lyophilization are among the most commonly used preservation methods [12,31,39,46–48]. All of them have already been assessed in a context of bone regeneration. However, depending on the preservation method used, the resulting HAM may have different biological and mechanical properties [49–51], therefore altering its potential for GBR. For the first time, we characterized and directly compared four types of HAM for bone regeneration.

Collagen and GAG are major structural components of HAM that have never been investigated simultaneously in F-HAM, C-HAM, L-HAM and DL-HAM. The content of collagen-rich mesenchymal layer increases the tensile strength of HAM [18] and several authors reported that removal of GAGs from a scaffold can have a negative effect on its viscoelastic behavior [52,53]. They also play a crucial role for cellular proliferation, migration and differentiation [54]. As previously reported, histological analysis showed a dramatic compaction of the mesenchymal layer in L-HAM and DL-HAM [39]. In this study, no apparent loss of collagen and GAG was observed whatever the preservation method of HAM. Our results are consistent with studies that have used alcian blue staining to demonstrate GAG preservation after HAM de-epithelialization or decellularization [55,56]. However, this approach is limited in that it was not possible to assess the structural integrity of these two components using this method.

One of the key factors of GBR is to ensure the stability of the bone-healing space. It, therefore, requires the ability to fix the barrier membrane. Titanium pins are a common way to attach and stabilize resorbable membranes for GBR [57]. To avoid an additional surgical procedure to remove the pins, the use of resorbable sutures for the fixation of the membrane has been suggested [58,59]. We have already reported enhanced mechanical properties of DL-HAM compared to F-HAM, C-HAM and L-HAM using uniaxial tensile test [39]. Here, we investigated the more clinically relevant suture retention strength of HAM depending on the preservation procedure used. We observed that neither cryopreservation nor lyophilization altered the suture retention strength of HAM, as no statistical difference was observed between F-HAM, C-HAM and L-HAM. Interestingly, DL-HAM was significantly stronger than simply lyophilized HAMs. To explain the positive effect of decellularization on lyophilized HAM strength, we hypothesized that the cell removal caused by the decellularization play a key role in the increase of HAM mechanical properties. The decellularization process created porosities inside the matrix, thereby allowing ice crystals formation without causing damages of the collagen network. One study compared the suture retention strength of HAM after lyophilization and after decellularization/lyophilization [60], but the authors did not demonstrate any improvement by adding decellularization. This could be explained by the fact that they used mechanical scraping to decellularize HAM, thus potentially causing mechanical damage of the membrane. However, whatever the preservation method used, HAM strengths reported here are approximately five-fold less than results reported in other studies [60–62]. One possible explanation is the type and size of sutures used. In these studies, the suture retention strength of HAM was mainly investigated for ophthalmological applications where thinner and non-absorbable sutures are indicated (10.0 or 5.0 Nylon). Here, we aimed to reproduce the technique used in oral surgery [58] and, thereby, tested 4.0 non-absorbable suture typically used for GBR. In this context, our results showed that the DL-HAM was the strongest membrane, suggesting its ability to be fixed for GBR applications. However, to further enhance the mechanical properties of HAM, one possibility would be to design a multi-layered DL-HAM scaffold [29,63].

Several differentiated cells have already been successfully seeded on acellular HAM [64–67]. However, few studies have investigated HAM for its potential to promote osteogenic differentiation of stromal cells. Here, we investigated the ability of fresh and preserved HAM

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3 to support early and late osteogenic differentiation of hBMSCs. In our study, the best results
4 were achieved with the DL-HAM, as demonstrated by the early and abundant positive staining
5 of hBMSCs for ALP activity. A higher concentration of AR staining was also observed when
6 hBMSCs were cultured over DL-HAM compared to the control group after 14 days. These data
7 were consistent with our previous study, which investigated the ability of fresh and preserved
8 HAM to support the proliferation of hBMSCs [39]. Indeed, we previously reported that our
9 decellularization process did not damage the integrity of the basement membrane components,
10 which is necessary to promote cell seeding. Furthermore, we had observed that this
11 decellularization process led to exposure of the basement membrane of HAM, thereby
12 increasing its ability to support hBMSCs adhesion and proliferation [39] compared to F-HAM,
13 C-HAM and L-HAM. Two studies have previously investigated the ability of acellular HAM
14 to promote osteogenic differentiation but these were performed with rats BMSCs [28,29]. Tang
15 *et al.* compared the osteogenic potential of rats BMSCs seeded either on an acellular HAM or
16 on a collagen-coated plate in osteogenic medium. Their results showed a stronger positive ALP
17 staining after 7 and 15 days of culture on acellular HAM and the expression of bone specific
18 genes was significantly higher [28]. In a similar study, rats BMSCs displayed early mineral
19 deposition and enhanced osteogenic markers expression on an acellular HAM compared to a
20 cell culture plate. [29] Here, we chose human BMSCs because they are more representative of
21 the clinical situation where they can undergo osteogenic differentiation and contribute to the
22 bone repair process [68,69]. Other sources of stems cells, such as dental apical papilla cells
23 (APCs), have the ability to undergo osteodifferentiation. Indeed, Chen *et al.* have shown that
24 the acellular HAM matrix promoted the osteogenic differentiation of APCs in osteogenic
25 medium and enhanced the expression of osteogenic marker genes [70]. These *in vitro* results
26 suggest that this membrane could promote local stromal cells osteogenic differentiation to
27 support bone regeneration.
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30 Finally, to further assess the osteogenic potential of fresh and preserved HAM, we compared
31 their ability to promote bone regeneration in a mice diaphyseal defect. Depending on how HAM
32 is used, its ability to favor bone regeneration is not established. The potential of HAM to
33 promote bone regeneration have been investigated in various models where it was either applied
34 over the bone defect [23,27,29,32,35,45] or implanted as a filling material inside the bone defect
35 [45,71]. Better results were achieved when HAM was used as a barrier membrane covering the
36 defect [27,29] rather than as a filler [45,71]. We thus decided to investigate the potential of
37 fresh and preserved HAM as a barrier membrane to promote GBR. We performed an uni-
38 cortical, non-critical size bone defect of the mouse femur. We chose this model because it is
39 known to be an easily replicable model and it is simple to quantify [72], and because the healing
40 process is the same to that of the jaws, which occurs through intramembranous ossification
41 [72]. We wanted to assess early and late bone regeneration, we thus performed a wider femur
42 mid-diaphysis defect than previously reported [72–75]: 1.3 mm in the present study *versus* 0.9
43 mm diameter. Since a complete cortical bone healing did not occur one month after the surgery,
44 differences between groups could be clearly observed. Micro-CT and histomorphometric
45 analysis showed that covering the defect with L-HAM or DL-HAM significantly enhanced
46 early bone regeneration compared to the defect left empty. One month after the surgery, only
47 DL-HAM significantly increased bone regeneration compared to the empty defect. We also
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decided to assess the angiogenesis process that occurred into the bone defect. Indeed, the extension of vessel sprouts from the surrounding host bone into the defect is required for osteogenesis [76]. In the present study, a significant increase of new blood vessels density was observed when L-HAM or DL-HAM covered the defect compared to the empty defect one week after the surgery. These results are consistent with the higher rate of early bone formation observed for both groups. After one month, DL-HAM was the only membrane that generated around 15% more vessels than the defect left empty. This data is also positively correlated to the late bone regeneration observed when DL-HAM was applied over the defect. These results are consistent with previous studies which showed that blood vessel density is positively correlated to the quantity of newly formed bone [42,77].

In this study, the best results were achieved with the decellularized HAM. One possible explanation is its superior persistence *in vivo*. Indeed, we previously observed a slower resorption rate of DL-HAM in a subcutaneous model, compared to F-HAM, C-HAM and L-HAM [39]. The DL-HAM was the only HAM still observed one month after its implantation. Our results are consistent with this previous study, as the DL-HAM was the only membrane to promote bone regeneration one month after the surgery. Besides its slower resorption rate, the decellularization may have reduced the inflammatory and/or immune reaction to this xenograft, which may explain the better bone regeneration observed.

CONCLUSION

This study is the first to compare the potential of a decellularized HAM with fresh and conventionally preserved hAM for GBR. Whatever the treatment used, major structural components of HAM were preserved. The decellularization process significantly enhanced mechanical property of HAM and its ability to promote *in vitro* hBMSCs osteodifferentiation. *In vivo*, L-HAM and DL-HAM significantly increased early bone regeneration. One month after the surgery, only DL-HAM seemed to slightly enhance bone formation. In conclusion, our decellularization process enhanced mechanical property and early osteoconductive properties of HAM, making it an attractive resorbable membrane for GBR.

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1
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3 Imaging Center (a service unit of the CNRS-INSERM and Bordeaux University) with the help
4 of Sébastien Marais.
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8 FIGURE LEGENDS 9

10
11 **Figure 1. Histological comparison of extracellular matrix components in fresh and**
12 **preserved HAMs.** (A) Collagen was stained in blue using trichrome Masson's staining.
13 Collagen was abundant in the mesenchymal layer of HAM whatever the preservation method
14 used. (B) Alcian blue staining confirmed the presence of GAG (stained in blue) in fresh and
15 preserved HAMs. Black arrows show epithelial layer of hAM, white and black asterisks show
16 the mesenchymal layer. Scale bar: 100 µm.
17

18 **Figure 2. Comparison of the suture retention strength of fresh and preserved HAMs.**
19 Results showed that cryopreservation did not deteriorate HAM biomechanical properties,
20 whereas the tearing strength of DL-HAM was significantly enhanced compared to F-HAM, C-
21 HAM and L-HAM. (Data are presented as means +/- standard deviation; n=24 per condition;
22 Kruskal-Wallis test followed by Dunn's multiple comparison test; * p<0.05; ***p < 0.001)
23

24 **Figure 3. Osteogenic differentiation potential of hBMSCs cultured on tissue culture plates**
25 **(control), fresh and preserved HAM.** (A) Early osteogenic differentiation was assessed using
26 ALP staining. Scale bar: 1mm. (B) Quantitative assessment of mineralized matrix deposition
27 in hBMSCs using alizarin red semi-quantification assay as a marker of late osteogenic
28 differentiation.
29

30 **Figure 4. Implantation of fresh and preserved HAM over a diaphyseal cortical bone defect**
31 **in mice.** (A) Exposure of the femoral diaphysis after muscle dissection. (B) A circular non-
32 critical defect of 1.3 mm diameter was performed. The defect was left empty or covered by
33 HAM: (C) fresh or (D) preserved HAM. White dotted line represents the edges of the
34 membranes.
35

36 **Figure 5. Analysis of regenerated cortical bone in mice diaphyseal defect.** (A)
37 Representative 3D reconstruction images of X-ray microtomography showing the remaining
38 defects one week and one month after the surgery. (B) Quantitative analysis of bone
39 regeneration (BV/TV (%)) at one week and one month. (C) Analysis of the degree of
40 mineralization of newly formed bone within the cortical gap. Tissue mineral density (mg/cm³)
41 was significantly enhanced for each condition between one week and one month after the
42 surgery. (Data are presented as means +/- standard deviation; n=6 per condition and per time;
43 * p<0.05, **p < 0.01).
44

45 **Figure 6. Histological analysis of bone regeneration in decalcified diaphyseal defects**
46 **stained.** (A) Representative histological sections (Masson's trichrome staining) of explanted
47 defects one week and month after the surgery. Abbreviations and signs used: Black asterisks
48 show native host bone; NB: new bone; BM: bone marrow; White dotted rectangle represents
49 the initial defect in which bone formation was measured. Scale bar: 100 µm. (B)
50 Histomorphometric analysis of bone formation using Masson's trichrome staining. Early bone
51 formation was significantly enhanced when L-HAM or DL-HAM covered the defect. (Data are
52 presented as means +/- standard deviation, n=6 per condition and per time; **p < 0.01)
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3 **Figure 7. Histomorphometric analysis of angiogenesis in decalcified diaphyseal defects.**
4 (A) Representative histological sections (HES staining) of explanted diaphyseal defect one
5 month after the surgery. Scale bar: 250 μm . (B) Higher magnification image of the blue
6 rectangle. Abbreviations and signs used: NB: new bone; BM: bone marrow; Black dotted line
7 represents the initial edge of the defect; Red arrow: new blood vessels; Black arrow: osteocytes.
8 Scale bar: 100 μm . (C) New blood vessels density (vessels/mm²). One week after the surgery,
9 the defects covered by L-HAM or DL-HAM showed significant more new blood vessels. (Data
10 are presented as means + /- standard deviation, n=6 per condition and per time; * p<0.05).
11
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14

15 **Abbreviations:**

16 ALP: Alkaline phosphatase

17 AR: Alizarin red

18 GAG: Glycosaminoglycans

19 GBR: Guided bone regeneration

20 HAM: Human amniotic membrane

21 hBMSCs: human bone marrow mesenchymal stromal cells

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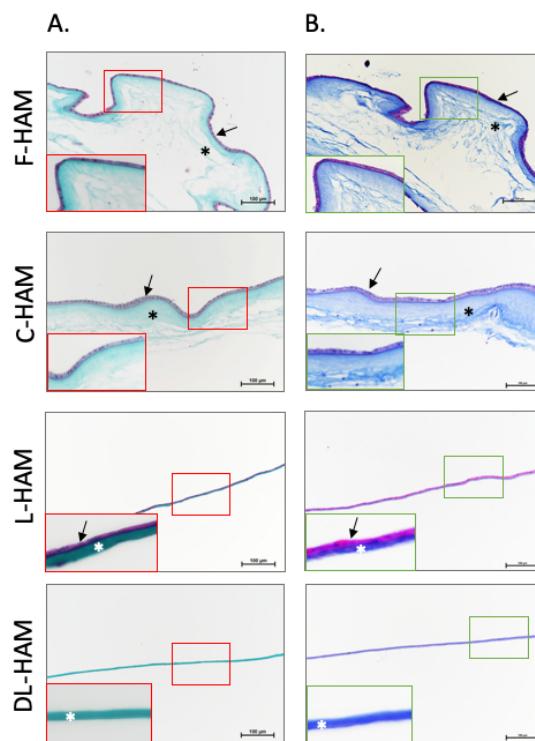


Fig 1

Figure 1

190x275mm (96 x 96 DPI)

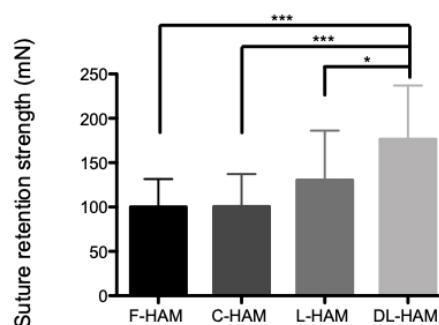


Fig 2

Figure 2

190x275mm (96 x 96 DPI)

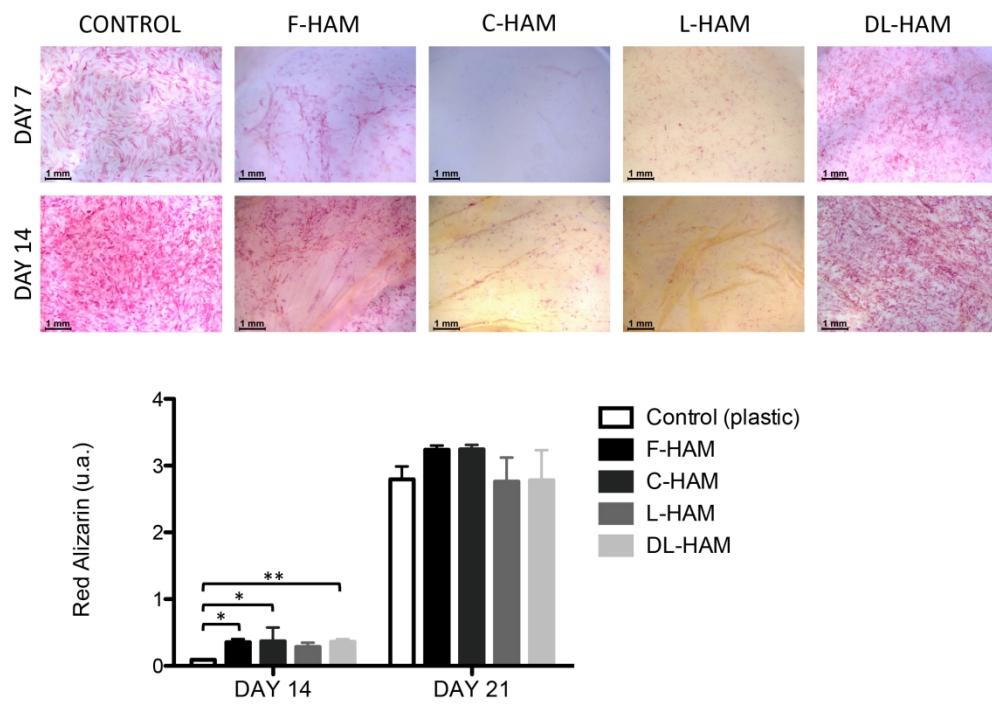


Figure 3

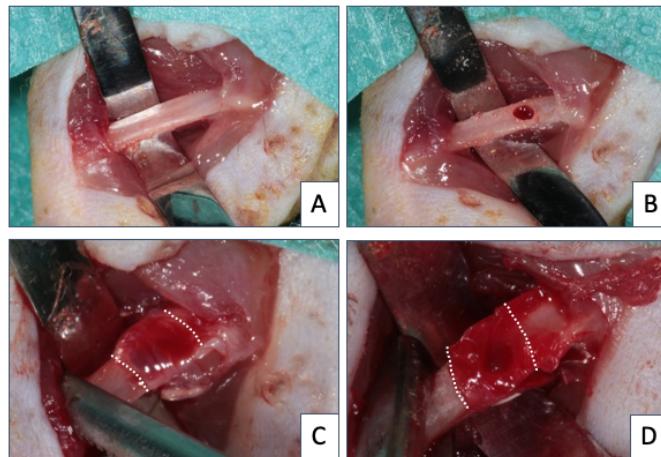


Fig 4

Figure 4

190x275mm (96 x 96 DPI)

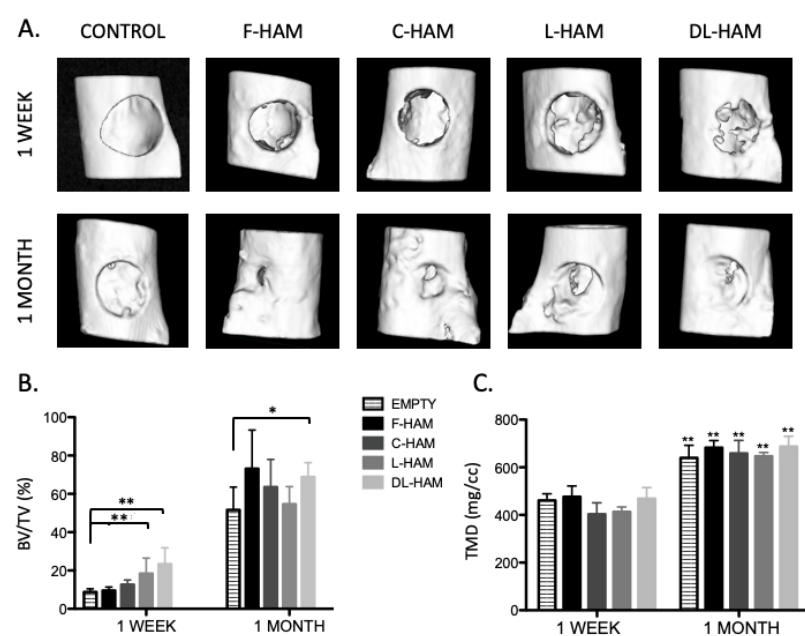
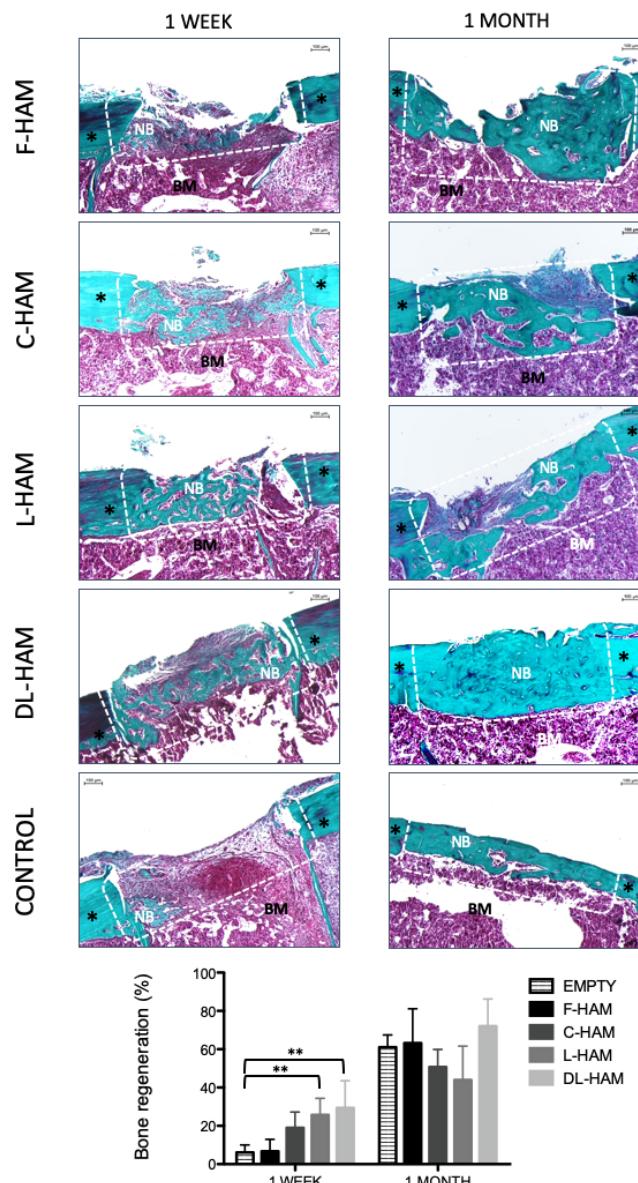


Figure 5

190x275mm (96 x 96 DPI)



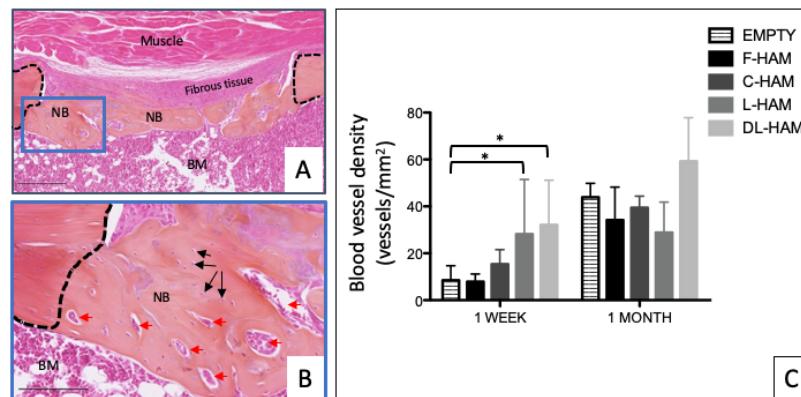


Fig 7

Figure 7

190x275mm (96 x 96 DPI)

3.3. Conclusion

Pour la première fois, cette étude a permis de comparer simultanément le potentiel de quatre types de MAH pour la régénération osseuse : MAH fraîche, MAH cryopréservée, MAH lyophilisée et MAH décellularisée/lyophilisée.

Dans un premier temps, nous avons approfondi la caractérisation des propriétés de la MAH en fonction des méthodes de préservation débutée dans l'article n°2. Bien que leurs répartitions soient modifiées en fonction des méthodes de conditionnement de la MAH, nous avons observé une préservation des GAGs dans les 4 MAH. Les GAGs sont connus pour jouer un rôle crucial dans la prolifération et la différentiation cellulaire. Nous avons également étudié la rétention à la suture de ces quatre membranes. En effet, afin de se rapprocher d'une application clinique, il est nécessaire de pouvoir fixer une membrane pour son utilisation en ROG afin de la stabiliser. Nous n'avons pas observé de différence significative entre la MAH fraîche, cryopréservée et lyophilisée. Par contre, la rétention à la suture de la MAH décellularisée/lyophilisée était significativement supérieure aux trois autres membranes.

Dans l'étude précédente, nous avions observé une prolifération plus importante des hBMSCs lorsqu'elles étaient cultivées sur la MAH décellularisée/lyophilisée par rapport aux autres MAH. Dans l'article n°3, la MAH décellularisée/lyophilisée semblait être la matrice la plus favorable *in vitro* à l'adhésion et à l'ostéo-différentiation de hBMSCs.

La dernière partie de cette étude reposait sur l'apport des quatre types de MAH pour la ROG d'un défaut osseux non critique. La MAH lyophilisée et la MAH décellularisée/lyophilisée augmentaient significativement la réparation osseuse précoce par rapport aux défauts laissés vides. Une néovascularisation significativement plus importante était également observée dans ces deux conditions. Après un mois, seule la MAH décellularisée/lyophilisée augmentait significativement la réparation osseuse par rapport au défaut vide.

L'article n°3 a permis de confirmer l'effet limité des cellules souches de la MAH sur son potentiel de régénération osseuse, comme supposé dans l'article n°1.

Ainsi, sur les quatre types de MAH comparés dans nos travaux, la MAH lyophilisée et la MAH décellularisée/lyophilisée semblaient présenter le meilleur potentiel pour la ROG de défauts osseux non critiques. L'étude de leur potentiel pour la régénération de perte de substance osseuse segmentaire de taille critique a fait l'objet d'un projet d'article présenté ci-après.

4. COMPARAISON DES MEMBRANES AMNIOTIQUES PRESERVEES A LA TECHNIQUE DE LA MEMBRANE INDUISTE POUR LA REGENERATION OSSEUSE GUIDEED'UNE PERTE DE SUBSTANCE OSSEUSE FEMORALE CRITIQUE.

4.1. Introduction

La reconstruction de perte de substance osseuse segmentaire repose en partie sur la technique de la membrane induite ou technique de Masquelet. Bien que couramment utilisée, cette technique présente essentiellement deux limites : elle requiert deux temps chirurgicaux et la nécessité d'une greffe osseuse autologue (morbilité du site donneur).

Plusieurs auteurs ont suggéré l'utilisation de la MAH comme alternative à la technique de la membrane induite sans qu'elles n'aient jamais été comparées dans une même étude. En effet, ces deux membranes présentent de nombreuses similitudes concernant leur structure et leurs propriétés. La substitution de la membrane induite par la MI permettrait de diminuer le nombre d'intervention chirurgicale. De plus, son utilisation en association avec un matériau de substitution osseuse réduirait la morbidité inhérente au prélèvement osseux autologue réalisé pour la technique de la membrane induite.

Les résultats de l'article n°3 ont montré que la ROG de défauts de taille non-critique était significativement supérieure en présence de MAH lyophilisée ou de MAH décellularisée/lyophilisée, ce qui nous a permis de sélectionner ces deux membranes pour la présente expérimentation.

L'objectif de cette étude était de comparer l'apport de la MAH lyophilisée ou décellularisée/lyophilisée à la technique de la membrane induite pour la régénération de perte de substance osseuse segmentaire.

Pour cela, nous avons eu recours dans un premier temps à l'impression 3D afin de modéliser un substitut osseux qui viendrait parfaitement s'intégrer au contact des segments osseux ainsi que de la plaque de reconstruction dans un modèle de défaut segmentaire chez le rat. Sa

capacité à assurer la prolifération de hBMSCs ensemencées sur le matériaux et à jouer un rôle ostéo-conducteur ont été évaluées *in vitro*.

In vivo, le matériau préalablement chargé en BMP-2 a été implanté dans un défaut segmentaire de fémur de rat. Il était soit recouvert par de la membrane induite, soit recouvert par l'une des deux membrane amniotique. Un guide de coupe a été utilisé afin de réaliser des défauts osseux de taille reproductible.

Un suivi radiographique longitudinal en deux dimensions a été réalisé. Une quantification par microscanner ainsi qu'une analyse histomorphométrique ont permis d'évaluer la néoformation osseuse totale et au sein des pores du matériaux. Des scores d'union entre l'os et le biomatériaux ont également été réalisés.

4.2. Projet d' article n°4

COMPARISON OF AMNIOTIC MEMBRANE VERSUS THE INDUCED MEMBRANE FOR BONE REGENERATION IN SEGMENTAL LONG BONE DEFECTS USING CALCIUM PHOSPHATE CEMENT LOADED WITH BMP2

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En préparation pour « Journal of Orthopaedic Research »

Comparison of amniotic membrane *versus* the induced membrane for bone regeneration in long bone segmental defects using calcium phosphate cement loaded with BMP-2

Short title: Amniotic membrane as an alternative to the induced membrane technique

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ABSTRACT

Thanks to its biological properties, the human amniotic membrane (HAM) associated with a bone substitute could be used in a single-step surgery as an alternative to the induced membrane (IM) technique for regeneration of critical bone defects. However, no study has compared the potential of these two membranes simultaneously.

We first designed a 3D printed calcium phosphate cement (CPC) based scaffold and assessed *in vitro* its suitability to act as an osteoconductive scaffold for human bone marrow mesenchymal stromal cells (hBMSCs) and to replace autograft *in vivo*. We then performed a rat femoral critical size defect to compare the barrier membrane effect of IM and HAM using the CPC scaffold loaded with rh-BMP2 (CPC/BMP2) for bone healing. Five conditions were compared. Group 1 was left empty. Group 2 received a single-stage CPC/BMP2 scaffold. Group 3 and 4 received a single-stage CPC/BMP2 scaffold covered with lyophilized or lyophilized/decellularized HAM respectively. Group 5 underwent a two-stage procedure with insertion of a polymethylmethacrylate (PMMA) spacer followed, after 4 weeks, by its replacement with the CPC/BMP2 scaffold wrapped in the IM.

The present study showed that CPC scaffold supported the proliferation and osteodifferentiation of hBMSCs *in vitro*. *In vivo*, the CPC/BMP2 scaffold significantly induced bone formation in a rat femoral segmental defect without autograft. The decellularized/lyophilized HAM could be used as an alternative to the technique of Masquelet allowing a single-step surgical procedure for segmental defects, whereas L-HAM had a negative effect on bone formation.

Key words: Amniotic membrane; Masquelet technique; Bone; 3D-printing; Tissue engineering; Bone morphogenetic protein;

1. INTRODUCTION

One of the most challenging problems in orthopedic surgery remains clinical management of critical sized bone defects of long bones, occurring after tumor resection, trauma or infection [1,2]. Autologous bone graft remains the most commonly used procedure for segmental bone defects up to 5 cm in size [1]. However, when bone defects are larger, reconstruction with autologous bone graft could not provide a complete healing because of graft resorption, even with a good vascularized muscular envelope [3]. For these defects, the Masquelet technique is a recognized and well-established method [4–6].

The Masquelet technique also called the induced membrane (IM) technique is a two-step surgical procedure. First, the segmental defect is radically debrided, the bone is usually stabilized with a plate or other means of fixation and a polymethylmethacrylate (PMMA) cement spacer is inserted into the defect [5,6]. This spacer will induce a foreign body reaction thereby leading to the formation of a biological membrane (i.e. the induced membrane (IM)) [4]. The second stage is performed six to eight weeks later, once the definitive healing of soft tissue is acquired [5,7]. The spacer is carefully removed, ensuring that the formed IM is left intact [6]. Then the cavity is filled up by cancellous bone autograft harvested from the iliac crest [5,8]. If the autologous bone graft is insufficient, it has been suggested to add a bone graft substitute [1,6], without exceeding a 1:3 ratio of bone substitute to autograft [5]. The IM technique has the benefit to create a separate “privileged” compartment, providing an isolated environment for bone regeneration. The IM will prevent bone graft from resorption and invasion of fibrous tissue, and it might produce osteoinductive growth factors and a source of blood vessels [1,6,9].

However, the IM technique has some limitations. The traditional technique involves large quantities of autograft, which can be associated with significant donor site morbidity and additional risks for the patient [10]. The two surgeries required to complete the entire procedure is another important limitation of this technique [7]. To overcome these drawbacks, alternatives to the IM technique for bone defects treatment have been suggested, using bone substitutes (avoiding donor site morbidity) and/or synthetic resorbable membrane (avoiding the first surgical procedure that induces the membrane formation) [1,11–15].

Thanks to their ease of use and customization, without the need for graft harvesting or the risks of disease transmission, synthetic bone substitute are an alternative to autograft and allograft [12]. Calcium phosphate-based materials are one of the most commonly used bone substitute, as they ensure biocompatibility and osteoconductivity, enabling their use as scaffolds for bone regeneration [16–20]. To enhance bone regeneration of bone substitutes in large segmental defect, they usually require growth factors such as bone morphogenetic proteins (BMP). In this study, BMP-2 was chosen as they induce the differentiation of osteoprogenitor cells into osteoblasts and they stimulate the formation of new bone [21].

It has also been suggested that the IM may not have an osteoinductive effect and only acts as a physical barrier, preventing fibrous tissue ingrowth according to the concept of guided bone regeneration (GBR) [22,23]. For this reason, the replacement of the IM by a synthetic membrane has been proposed to ensure a single-stage procedure [12,13,22]. In this context, two

studies reported the use of a synthetic membrane such as polycaprolactone or polytetrafluoroethylene membranes to cover segmental defect. However, these membrane only act as a physical barrier, thereby avoiding fibrous tissue ingrowth without providing any additional biological effects [12,22].

Thanks to its biological properties, human amniotic membrane (HAM) has become a highly attractive and promising biological tissue to act as a bioactive membrane for GBR [24–30]. HAM is a readily available biomaterial, derived from human placenta and lining the amniotic cavity. As the placenta is usually discarded after delivery, the use of HAM does not raise ethical concerns. Furthermore, HAM is a source of stromal cells and growth factors [31–33], and it is also known to possess low immunogenicity [34–36] and an anti-cancer effect [37,38]. Thus, HAM is used for over a century for wound healing in medicine. Few studies have already reported promising results using HAM in the field of orthopedic surgery, and especially, its potential to be used as an alternative to the IM [39–41]. Indeed, similarities between the structure and composition of HAM and IM have been shown: both biological membranes are a highly-organized tissue, sharing similar proteins components and comparable thickness [40]. They both contain growth factors such as VEGF or TGF- β 1 and express anti-inflammatory proteins [9,32,40,42,43]. Finally, several authors reported the osteogenic capacities of HAM [29,44–47]. Taken together, these findings suggest that HAM could replace the IM, using a single surgical procedure.

Although the use of amniotic membrane has already been suggested as an alternative to the IM technique, these two membranes have never been compared during the same experimentation. The objectives of this study were first i) to design and fabricate a 3D custom scaffold made of osteoconductive calcium phosphate, used to replace autologous bone graft in the two-stage IM method, then ii) to compare bone regeneration potential of HAM *versus* the IM when they covered this scaffold loaded with BMP-2.

2. Materials and methods

2.1. Materials

2.1.1. 3D Plotting of the bone substitute and post-processing

The bone scaffolds were fabricated using calcium phosphate cements (CPC) paste (InnoTERE® GmbH, Radebeul, Germany). The powder component consisted of 60 wt % a-TCP, 26 wt % calcium hydrogen phosphate, 10 wt % calcium carbonate and 4 wt % precipitated HA [48]. They were fabricated by pressure-assisted micro-extrusion using a dedicated 3D-printer (3D Discovery®, RegenHU, Switzerland). The following plotting parameters were used: needle diameter = 250 μm ; plotting speed = 4 $\text{mm}\cdot\text{s}^{-1}$; dosing pressure = 0,25 MPa.

Two types of scaffolds were fabricated depending on the experimentations. Circular-shaped scaffolds were used for the *in vitro* characterization experiments. They were plotted in 60° configuration (each layer orientation varied by 60°), provided a triangular inner structure, and a size of 9 mm diameter and 1 mm thick. For the *in vivo* experiments, the scaffold was designed in order to be adjusted into a critical-size rat femoral defect [48]: they were shaped with a

cylindrical outer geometry with a height of 5 mm and a diameter of 4 mm and the same triangular inner structure. One side was flattened to allow fixation onto the osteosynthesis plate [48]. Inside each layer and for both scaffolds, spacing between lines was set at 500 µm. After plotting, the scaffolds were incubated in water-saturated atmosphere at 37°C for 3 days for cement setting, before being sterilized by gamma radiation at 25 kGy (Gamacell® 3000 Elan, NORION MDS, Ottawa, Canada).

2.1.2. Preparation and storage of HAMs

Two treatments of HAM were assessed in this study: 1 / lyophilized (L-HAM) and 2/ decellularized then lyophilized HAM (DL-HAM). They were prepared and stored as previously described [49]. Two human placentas were collected after elective cesarean surgery from consenting healthy mothers (tested seronegative for HIV, Hepatitis B and C virus, and syphilis). Patients provided written informed consent as requested by the institutional review board and their placentas were anonymized. The placentas were kept in a sterile solution containing PBS 1x (Gibco®) supplemented with 1% antibiotics (penicillin/ streptomycin, Invitrogen®) and transferred to the laboratory. They were rinsed with sterile distilled water to remove residual blood clots and the HAM was peeled from the chorion by blunt dissection then rinsed with sterile distilled water. All these steps were performed under sterile conditions. To realize lyophilized HAM (L-HAM), patches were frozen at -80°C, then dried under vacuum in a freeze dryer. The decellularization of HAM was performed according to a previously established protocol [49]. Briefly, HAM was first treated with trypsin and ethylenediaminetetraacetic acid (T/EDTA, 0.125%) for two minutes at 37°C. After washing HAM with sterile PBS, HAM was transferred to a decellularization solution composed of 8 mM CHAPS, 25 mM EDTA, 0.12 M NaOH and 1 M NaCl in PBS and incubated, for 7h at room temperature under gentle agitation followed by three washes of sterile distilled water. Finally, DL-HAM was frozen at -80°C, before being dried in the freeze dryer. L-HAM and DL-HAM were sterilized by gamma radiation at 25 kGy (Gamacell® 3000 Elan, NORION MDS, Ottawa, Canada) and kept at room temperature until analysis.

2.1.3. Preparation of spacers

The bone cement spacers were made of polymethyl methacrylate (PMMA) as previously described [50]. They were 5 mm in length and 5 mm in diameter. PMMA spacers were made of medical grade PMMA bone cement (CMW 3, DePuy International, Blackpool, England) and hardened in silicone moulds (Putty soft, Gumar™, Acteon®). They were sterilized by gamma radiation at 25 kGy (Gamacell® 3000 Elan, NORION MDS, Ottawa, Canada).

2.1.4. BMP-2 solution

A commercially available kit (InductOs®, Medtronic, Biopharma B.V., Heerlen, Netherlands) was used to prepare the solution of rhBMP-2 following the manufacturer's protocol. Prior their implantation *in vivo*, the CPC scaffolds were loaded with a solution containing 10 µg of rhBMP-2 (solution concentration: 0,05 µg/µl): CPC/BMP2

2.2. *In vitro* evaluation of the cytocompatibility of the scaffolds

In vitro viability and osteogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs) cultured over the bone substitute were assessed. First, after getting patient consent, the hBMSCs were isolated from patients who underwent hip surgery (experimental agreement with *CHU de Bordeaux* and *Etablissement Français du Sang*, agreement CPIS 14.14), and expanded following well-established protocols [51]. Cells were used at passage 2 for this study. Then, the circular-shaped scaffolds (9 mm diameter; 1mm height) were put in 48-well plates previously covered by agarose 2 %.

For the viability assay, hBMSCs were seeded onto the cement scaffolds at a density of 10×10^4 cells per scaffold and they were cultured in 1 ml of two different mediums: 1) basal medium (BM= α -minimum essential medium (MEM alpha, GIBCO®), 10% fetal bovine serum (Eurobio®) or 2) osteogenic medium (OM= standard osteogenic induction medium (StemPro™, GIBCO®)). Plates were then incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air. The hBMSCs cultured in 2D conditions on tissue culture polystyrene (TCPS) plates served as a positive control.

A live/dead viability assay (Thermo Fisher Scientific, Invitrogen) was performed according to the manufacturer's instructions 3, 7 and 14 days after culture (n=2 per condition and per time). The staining was visualized with a confocal microscope (Leica TCS SPE Model DMI 4000B). Living cells emitted green fluorescence in the cytoplasm (exc: 494 nm, em: 517 nm), whereas dead cells emitted red fluorescence in the nucleus (exc: 527 nm, em: 617 nm).

Qualitative assessment of hBMSCs osteodifferentiation using ALP activity was performed after 7 and 14 days of culture (n=2 per condition and per time). The samples were fixed with 4% paraformaldehyde for 15 min, then washed twice with PBS 1x and processed using the Alkaline Phosphatase kit 86 C-1KT (Sigma-Aldrich, USA) according to the manufacturer's instructions. Briefly, cell layers were incubated with Fast Blue RR salt and Naphtol AS-MX Phosphate Alkaline solution 0.25% (Sigma-Aldrich, USA) for 30 min at room temperature in the dark before being washed in distilled water. Images were obtained using a stereo microscope (MZ10F, Leica Microsystems, Germany) coupled to a camera (Leica model DFC 450C).

2.3. *In vivo* surgical implantation

2.3.1. Animal model and implantation procedure

The present study was approved by the French Ethics Committee for animal care and experiment (agreement APAFIS n°12543-201712121537848v3). The aim was to compare bone regeneration using L-HAM or DL-HAM (one step surgical procedure), to the induced membrane (IM) technique (two steps surgical procedure) using a rat femoral critical-size defect model. Fifteen 10-weeks-old male Sprague Dawley rats were used (n=2 conditions per rat). They were divided into the following five groups (n=6 per group) and summarized Table 1:

- 1) Empty group: defects were left empty (**EMPTY**)
- 2) Control group: defects were filled by the rh-BMP2 loaded calcium phosphate cements (**CPC/BMP2**)
- 3) The L-HAM group: defects were filled by the rh-BMP2 loaded bone substitute, then covered by the L-HAM (**L-HAM**)

- 4) The DL-HAM group: defects were filled by the rh-BMP2 loaded bone substitute and covered by the DL-HAM (**DL-HAM**)
- 5) The IM group: underwent a two-stage procedure with insertion of a PMMA spacer followed by its replacement with the rh-BMP2 loaded bone substitute wrapped in the IM (**IM**)

Table 1. Description of the five experimental conditions used for the *in vivo* implantation ($n=6$ per group).

Group	Name	Defect Fillings
1	EMPTY	Left empty
2	CCP/BMP2	CPC scaffold + rhBMP-2
3	L-HAM	CPC scaffold + rhBMP-2 covered by L-HAM
4	DL-HAM	CPC scaffold + rhBMP-2 covered by DL-HAM
5	IM	CPC scaffold + rhBMP-2 covered by the induced membrane

A single step surgical procedure was used for groups 1 to 4, whereas a two-steps surgical procedure was used for group 5. Surgery was carried out under aseptic conditions. Short-term anesthesia was induced by inhalation of 4% isoflurane (Air:1.5 L/min) and maintained using isoflurane 2% (Air: 0.4 L/min). Before the surgery, analgesia was performed by intraperitoneal injection of 0.1 mg/kg buprenorphine (Buprecare®, 0.3 mg/ml) and rats were given a subcutaneous injection of cephalosporin (cefazoline 0.06mg/kg). The back and the legs were shaved, the surgical site was aseptically prepared, and a longitudinal 3-cm skin incision was performed laterally across the leg. Dissection of the muscles was performed to expose the femoral shaft. The RatFix™System (RISystem AG, Davos, Switzerland) was mounted to perform standardized 5 mm defect (Fig. 1). Briefly, the osteosynthesis system consisting of a PEEK plate of 23 mm, was mounted in the rat femur by six 0.8×6.5 mm screws after predrilling with a 0.79 mm drill bit, facilitating the placement of the saw guide utilized. Subsequently, two osteotomies were created using a Gigli saw (Fig 1A) and the central mid-diaphyseal bone fragment was removed (Fig 1B). Group 1 defects were left empty for 6 weeks. For groups 2 to 4 the defect was either filled with CCP/BMP-2 alone (Fig 1C), or the CCP/BMP-2 was covered by a HAM (Fig 1D) for 6 weeks. Group 5 received an initial PMMA spacer for 4 weeks (Fig 1E), after which the site was reopened to expose the induced membrane created (Fig 1F). After a slight incision through the IM (Fig 1G), the spacer was removed and replaced by the CPC/BMP2 scaffold (Fig 1H) for 6 weeks. The incision made through the IM was carefully closed with absorbable sutures (vicryl 5.0, Ethicon). Finally, the muscles and the superficial fascia were closed using absorbable sutures (vicryl 4.0, Ethicon, division of Johnson & Johnson, Brussels, Belgium). The skin was closed with Michel staples and then covered with aluminum spray (Aluspray®, Vetoquinol, Lure, France). Injection of buprenorphine and cephalosporin were performed the following day after the surgery. At the final time points, the animals were euthanized using CO₂ inhalation. The femurs were dissected, rinsed in PBS 1x and fixed in 4% paraformaldehyde overnight, before being stored into 70% ethanol at 4°C.

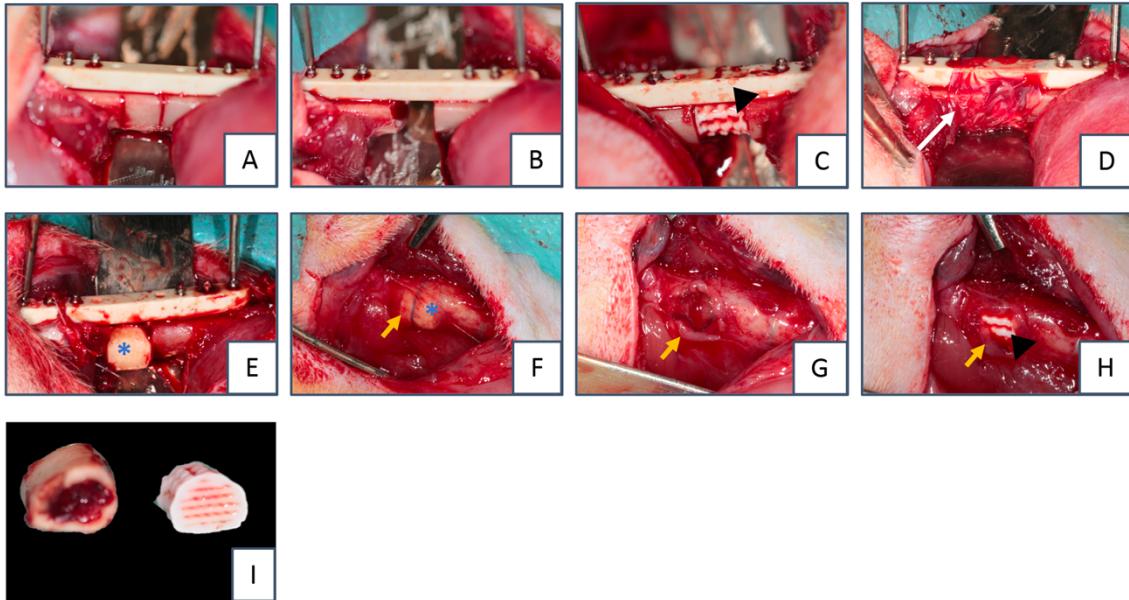


Figure 1. Surgical protocol. (A) Two osteotomies were created using a Gigli saw and the saw guide, (B) the central mid-diaphyseal bone fragment was removed and (C) replaced with the CPC scaffold, (D) before being covered by the HAM. The two steps-protocol consisted of (E) the surgical implantation of the PMMA spacer in the bone defect, followed by the subsequent (F) formation of the induced membrane four weeks later (G) which was incised to remove the spacer and (H) replace it with the CPC scaffold wrapped in the induced membrane. Black arrowhead : CPC scaffold ;White arrow: HAM; Blue asterix: PMMA spacer; Yellow arrow: Induced membrane. (I) Bone fragment and CPC scaffold.

2.3.2. Planar X-Rays

After anesthetizing the animals with isoflurane, X-Ray Radiographs were taken immediately after the surgery (Day 0) and every two weeks using Faxitron X-Ray MX20-DC2 digital imaging instrument (Faxitron Bioptics, Arizona, USA). The percentage of bone formation was scored according to a previously described system [15,52] after two, four and 6 weeks post-implantation. We have used a 4-points system with scores 1 to 4. A score of “1” represented 0 to 25 % bone healing, “2” represented 26 to 50 % healing, ”3” represented 51 to 75 % healing and “4” represented 76 to 100 % bone healing. The degree of union between the bone substitute and each edge of the defect was also assessed every two weeks post-implantation, according to a modified score described previously: 0 = no union, 1 = partial union, 2 = complete radiographical union [53]. Proximal and distal union were evaluated separately and added.

2.3.3. Micro-computed tomography

The X-ray microtomographic device used in this study was a Skyscan 1276 (Bruker, Konitch, Belgium). The X-ray source was set at 100 kV and 150 μ A to obtain a 15 μ m resolution with an exposure time of 450 ms. After scanning, cross-sectional slices were reconstructed using NRecon® reconstruction software (Micro Photonics) and three-dimensional analysis was performed using CTAn® visualization software (Bruker, Konitch, Belgium). A volume of interest of 5 mm length and 333-slice thickness was determined for each femur and applied to all reconstructions for bone analysis. Bone volume fraction (bone volume/total volume [BV/TV]) was assessed. Results were expressed as an average \pm standard deviation.

2.3.4. Histological preparation and histomorphometric analysis

Each sample was decalcified with EDTA-based Microdec® decalcifiant for 3 weeks under gentle agitation. Then the samples were dehydrated and processed for conventional embedding in paraffin. Eight- μm -thick serial sections were prepared through the middle of the defect and stained with Masson's trichrome and HES staining. Images of the 30 samples stained with Masson's trichrome staining (one image per sample) were obtained with an Eclipse 80i light microscope (Nikon, Japan) and captured with a DXM 1200C CCD camera (Nikon, Japan). Morphometric analysis was performed using ImageJ® software for each femoral specimen to quantify the percentage of bone formation inside the pores of each scaffold for group 2 to 5. A previously described histological quantitative scoring system [15] was also used to evaluate the tissue response around the scaffold surface, within the pores, as well as the presence of cartilage surrounding the scaffold (Table 2). Finally, images of the 30 samples stained with HES were acquired with a slide scanner (Hamamatsu Nanozoomer 2.0HT) and a quantitative analysis of new blood vessels within the pores of the CPC scaffold was performed using NDPview software (one section per sample). Blood vessels were identified by their luminal structure and the presence of red blood cells within their boundaries. Blood vessel density (BVD) was determined by the number of new blood vessels in the scaffold divided by the entire scaffold area [54–56].

Table 2. Histological quantitative scoring system.

Hard tissue response at scaffold interface	Score
Direct bone to implant contact without soft interlayer	4
Remodeling lacuna with osteoblasts and/or osteoclasts at surface	3
Majority of implant is surrounded by fibrous tissue capsule	2
Unorganized fibrous tissue (majority of tissue is not arranged as a capsule)	1
Inflammation marked by an abundance of inflammatory cells and poorly organized tissue	0
Hard tissue response within the pores	Score
Tissue in pores is mostly bone	4
Tissue in pores consists of some bone within mature, dense fibrous tissue and/or few inflammatory response elements	3
Tissue in pores is mostly immature fibrous tissue (with or without bone) with blood vessels and young fibroblasts invading the space and few macrophages present	2
Tissue in pores consists mostly of inflammatory cells and connective tissue components in between (with or without bone) or the majority of the pores are empty or filled with fluid	1
Tissue in pores is dense and exclusively of inflammatory type (no bone present)	0
Presence of cartilage tissue formation	Score
Yes	1
No	0

2.4. Statistical analysis

Results were expressed as mean \pm standard deviation, with n indicating the number of HAM sample tested. Statistical analysis was performed using GraphPad Prism® Software (La Jolla/CA, USA). First, normality test was performed using a D'Agostino and Pearson omnibus normality test. Statistical significance for independent samples was evaluated with the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. In all cases, statistical significances are marked by stars with * indicating a two-tailed $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. RESULTS

3.1. *In vitro* viability and osteogenic differentiation of hBMSCs cultured over the bone substitute

We assessed the suitability of the bone substitute to be used as a scaffold, upon which hBMSCs can survive and differentiate into osteogenic lineage. Live/dead staining revealed that hBMSCs attached to and spread over the surface of the plotted CPC scaffolds overtime (Fig 2). At day 14, qualitative observations revealed more cells in the scaffold surface when OM was used. Similar results were observed in control groups.

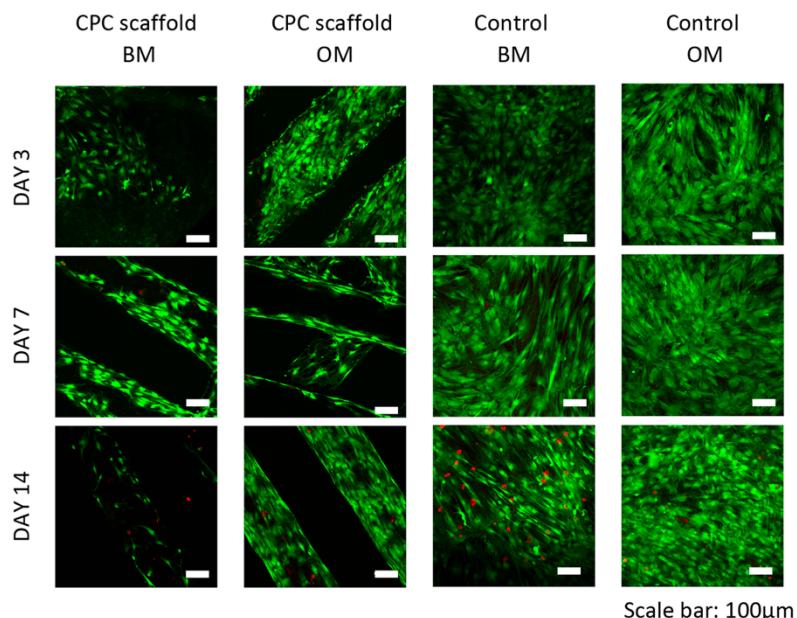


Figure 2. *In vitro* cell viability assays. The 3D-printed CPC based bone substitute was a suitable scaffold for hBMSCs attachment and proliferation. BM: Basal medium; OM: Osteogenic medium. Scale bar: 100 μ m.

At the early time-point (day 3), a slight ALP staining of hBMSCs was observed mainly at the periphery of the scaffold whatever the culture medium used (Fig 3). After seven days, a strong ALP staining of hBMSCs was observed on the scaffold cultured in OM. This intense staining was uniformly distributed over the entire surface of the scaffold. Similarly, an increase of

positive ALP staining was observed when hBMSCs were seeded on plastic in OM compared to BM. This staining appeared less intense than that observed on the scaffold.

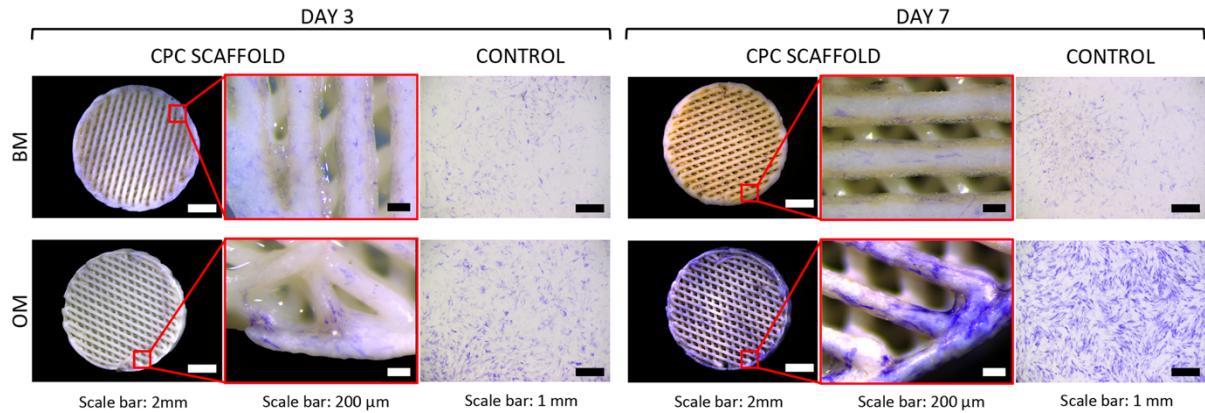


Figure 3. *In vitro* osteodifferentiation. Alcaline phosphatase staining was performed to assess the suitability of hBMSCs to osteodifferentiate when seeded on the CPC scaffold and on tissue culture plates (control).

3.2. *In vivo* study

3.2.1. Planar X-Rays

Figure 4A shows representative x-ray radiographs at day 0 and after 2, 4 and 6 weeks for each group. Whatever the condition studied, bone formation substantially increased between two and four weeks after the surgery. After four weeks, only a slight increase was observed for each condition. Six weeks after the surgery, complete or nearly complete healing (defined as a score greater than 3) was observed in groups 2 to 5 whereas the score was inferior to 3 when the defect was left empty (Fig 4B). The scores resulting from the degree of union showed that bridging between scaffold and bone increased overtime for each condition (Fig 4C). No significant difference was observed between conditions using this score.

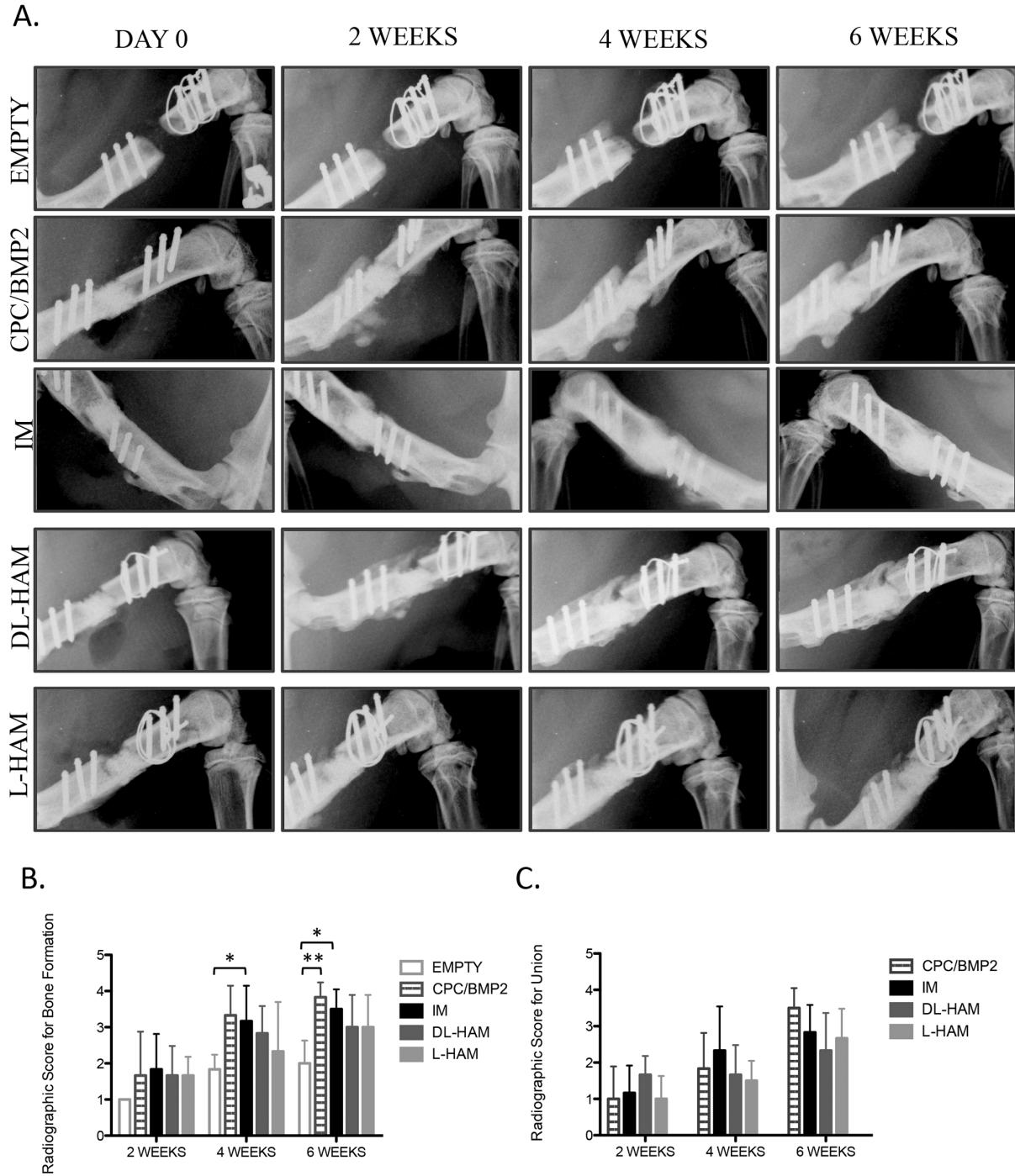


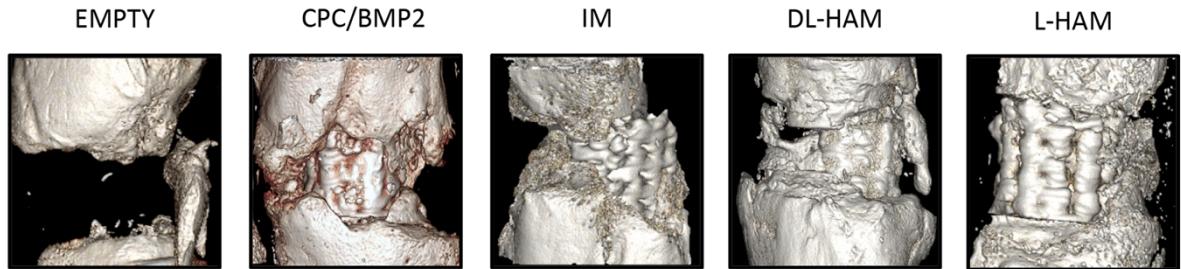
Figure 4. Longitudinal X-Rays follow up. (A) Representative 2D-radiographs showing the segmental defect immediately after the surgery as well as 2, 4 and 6 weeks after the surgery. Radiographic scores were performed to quantify (B) bone formation in the 5 groups and (C) the degree of union between the CPC scaffold and bone edges. Data are presented as means + /- standard deviation; n=6 per condition and per time; * p < 0.05, **p < 0.01.

3.2.2. Micro-CT analysis

Figure 5A shows representative 3D-reconstruction of the region of interest. The amount of bone formed within the defect was evaluated quantitatively at 6 weeks with micro-CT in all experimental groups (Fig 5B). Bone volume fraction (BV/TV) was significantly higher for the following three groups CPC/BMP2, IM and DL-HAM compared to the defect left empty

(BV/TV (%): CPC/BMP2= 58 ± 8 ; IM= 58 ± 9 ; DL-HAM= 61 ± 4 ; Empty= 32 ± 14). Covering the BMP2 loaded bone substitute using L-HAM (BV/TV (%): L-HAM= 42 ± 7), significantly reduced the BV/TV compared to the DL-HAM group.

A.



B.

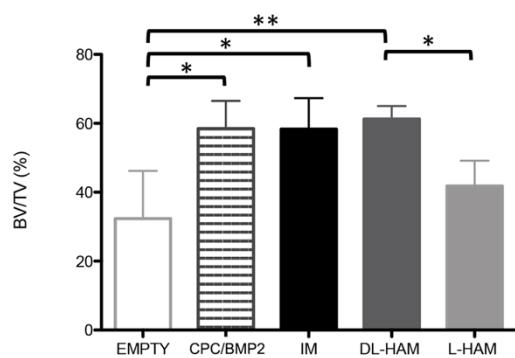


Figure 5. Micro-CT analysis. (A) Representative 3D rendering showing the region of interest. Quantitative analysis was performed for (B) Bone regeneration (BV/TV(%)). Data are presented as means +/- standard deviation; n=6 per condition; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.3. Histomorphological analysis

Representative histological sections for each group stained with Masson's trichrome staining are shown Fig 6A. No fibrous encapsulation was observed in response to either the scaffold or the membranes. Tissues at various stages of bone healing were found in the defect sites, with extensive regeneration of mineralized bone in all groups except for the empty group. Newly formed bone and marrow tissues were found throughout the scaffolds both in direct contact with the scaffold and within the pores.

Histomorphological analysis was performed to quantify bone regeneration inside the pores of the scaffold for groups 2 to 5 (Fig 6B). The nature of the tissue formed inside the pores of the bone substitute was mostly bone, and few dense fibrous as well as marrow tissues were also observed. The highest level of bone formation inside pores was observed when the bone substitute was covered by the IM, without significant difference. The majority of bone ingrowth into the scaffolds occurred from the proximal and distal ends of the defect whatever the condition observed.

To further investigate the tissue response at the interface between the bone and the implanted scaffold and within the pores, an histological tissue response score was realized for groups 2 to 5, as shown in Figure 6 C-E. Fig 6C shows tissue response scores at the rod interface. No

significant difference was observed between conditions. The majority of the samples showed a score greater than or equal to 3 demonstrating a direct bone contact with the scaffold without fibrous tissue encapsulation. The scoring of the hard tissue response within the pores of the bone substitute can be seen Fig 6D. Tissue in pores was mostly bone associated with mature or immature fibrous tissue. Only the L-HAM group showed some samples where inflammatory cells or connective tissue were mostly observed inside pores, as demonstrated by a significantly lower scoring of the hard tissue response within the pores compared to the IM group. No significant difference was found between the other conditions. The presence of cartilage was also investigated at the bone substitute interface. Cartilage was not found except in one of the six samples of the DL-HAM group and of the L-HAM group also. Fig 6E showed the total tissue response score for each condition. The L-HAM group showed the lowest score, without significant difference between conditions.

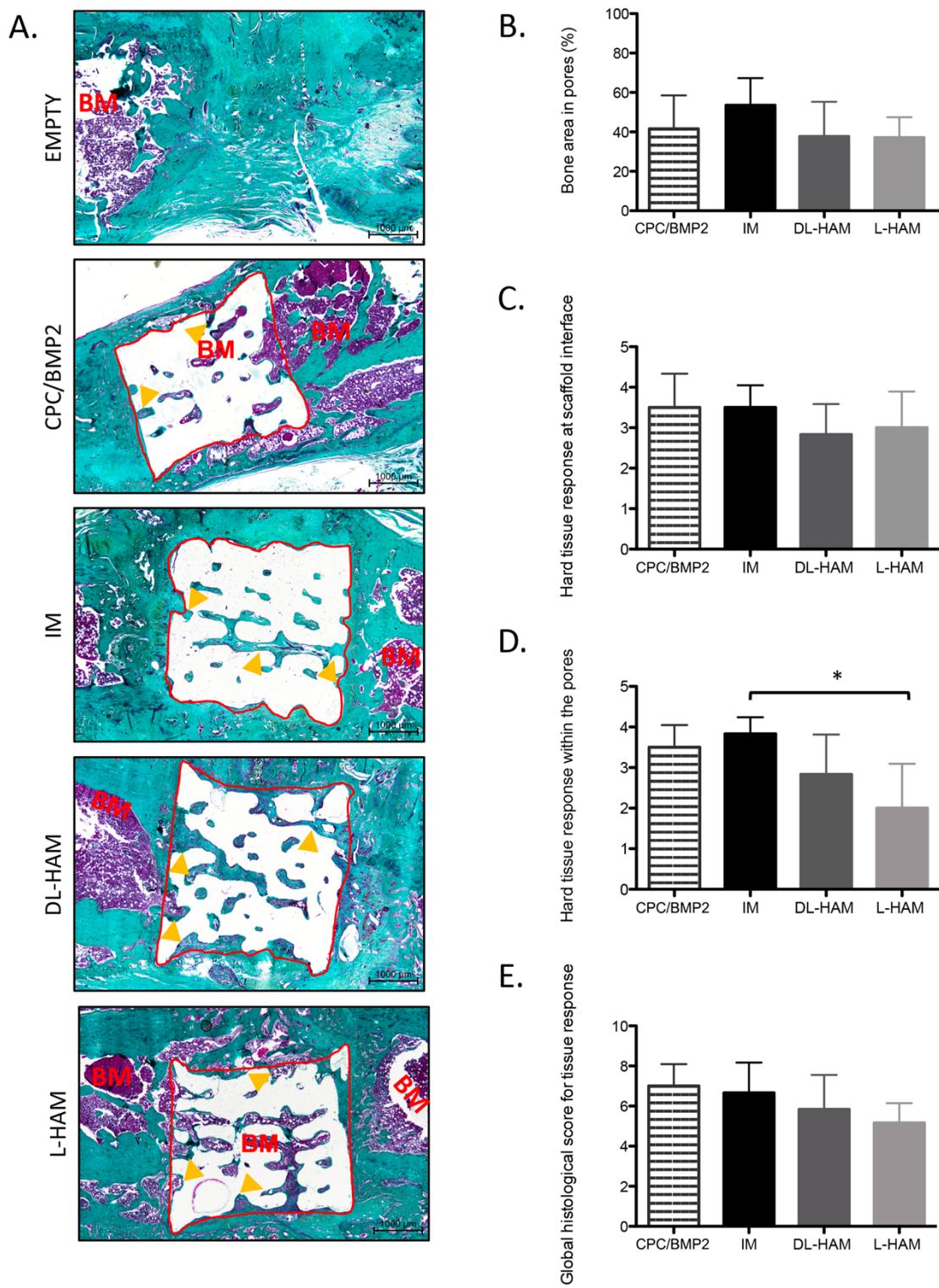


Figure 6. Histomorphological analysis of bone regeneration. (A) Representative histological sections stained with Masson trichrome staining. Yellow arrow: newly formed bone; BM: bone marrow; Red line represents the edges of the CPC scaffold inside which bone formation was quantified (B) Quantitative analysis was performed to assess the percentage of bone formation within pores. (C-E) An histological tissue response score was performed for groups 2 to 5. Data are presented as means + /- standard deviation; n=6 per condition; * p<0.05.

Finally, the vascularization within the pores of the CPC scaffold was assessed and vessel density was calculated from histological sections stained with HES (Fig 7A and B). Newly formed blood vessels were observed within the pores of the scaffold whatever the condition studied without significant difference.

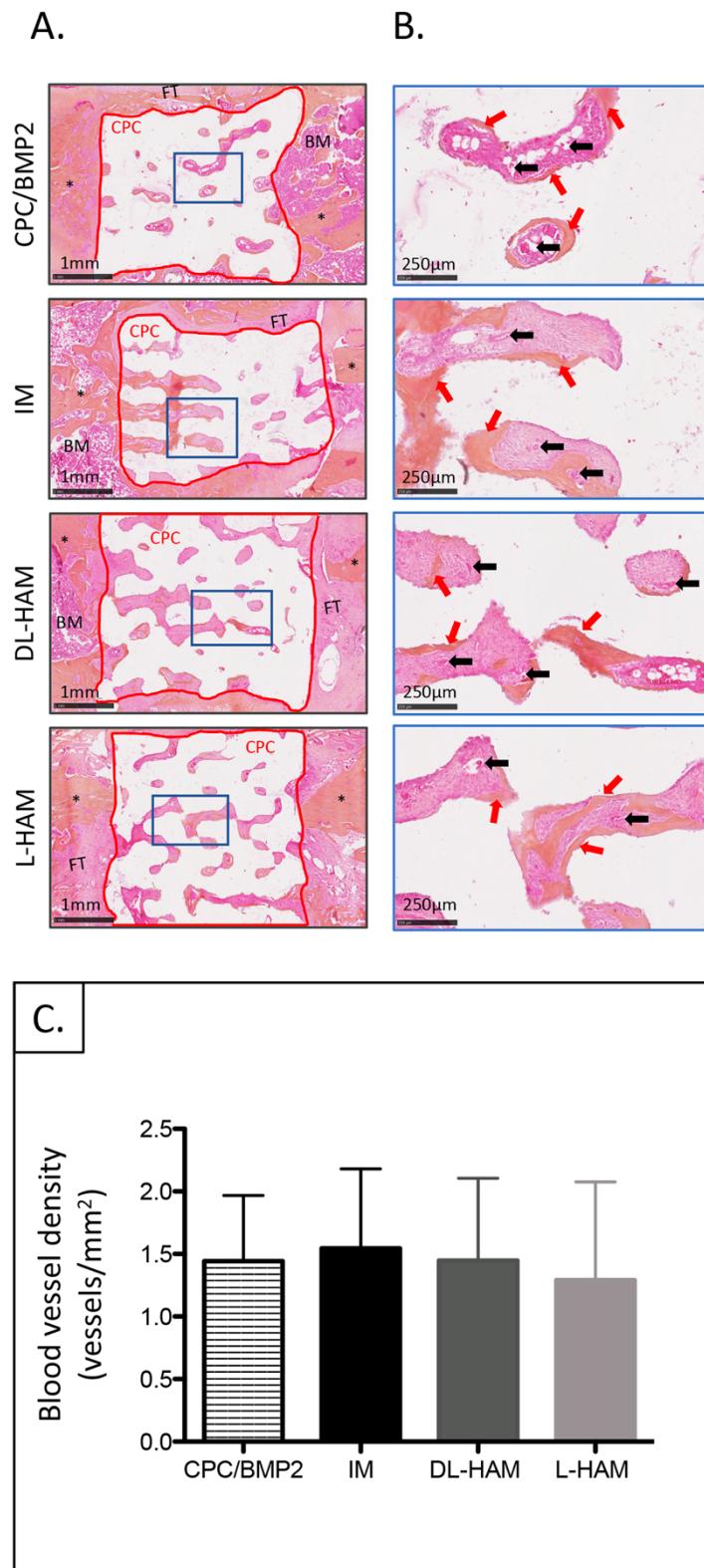


Figure 7. Histomorphometric analysis of angiogenesis in decalcified femoral defects. (A) Representative histological sections (HES staining) of explanted femoral defect six weeks after the surgery. Scale bar: 1 mm. (B) Higher magnification image of

the blue rectangle. Abbreviations and signs used: NB: new bone; BM: bone marrow; Red line represents the edges of the CPC scaffold inside which the vessels were quantified; Black arrow: new blood vessels; Red arrow: newly formed bone. Asterix: surrounding bone of the defect. Scale bar: 250 µm. (C) New blood vessels density (vessels/mm²). Data are presented as means + / - standard deviation, n=6 per condition.

DISCUSSION

This study aimed to compare for the first time the IM with a single step procedure using DL-HAM and L-HAM for bone regeneration. We also aimed to investigate the osteoconductive properties of the 3D printed CPC scaffold and its suitability when loaded with rh-BMP2 to be used to promote bone regeneration without autograft harvest in a Masquelet critical size femoral defect model in rats.

One major limitation of the IM technique is the need for a high quantity of autologous bone graft. However only few studies have already investigated bone substitutes in a Masquelet inspired small animal model using traditional PMMA, without bone graft in the second stage [12]. The biomaterial investigated in this study was a 3D printed CPC scaffold. Fabrication of tissue engineering scaffold with defined architecture using additive manufacturing has gained an increasing interest in regenerative medicine and bone tissue engineering. The layer-by-layer construction of 3D structures allows to design the outershape of the scaffold for a specific defect and to control the inner pore architecture [48]. The porosity of a scaffold play a key role in bone regeneration: complete interconnectivity of the pores as well as pore sizes in an appropriate range are necessary for cell migration, vascularization and new bone ingrowth [57]. 3D printed scaffold also offers greater reproducibility compared to autologous bone harvest. We choose a calcium phosphate based biomaterial because these biomaterials are known to increase osteoprogenitors cells and capillaries ingrowth acting as a biocompatible scaffold for new bone formation in large bone defects [16–20]. We performed an inner-pore architecture using triangular cross-section because it appears to affect positively scaffold vascular colonization and its osteointegration as well as it seems to improve their performances [58,59]. Before its implantation in a segmental defect, we aimed to assess the suitability of the bone substitute to be used as a scaffold upon which hBMSCs can adhere and differentiate into osteogenic lineage. Thus, the viability and the potential of osteogenic differentiation of hBMSCs cultured over the bone substitute were evaluated. Qualitative analysis by means of live/dead staining and ALP staining showed that the inner-pore architecture choose for the CPC scaffold allows hBMSCs proliferation when seeded over the scaffold *in vitro*. Furthermore, homogeneous hBMSCs osteodifferentiation was observed through the entire surface of the scaffold.

The osteoinductive effect of the IM is still discussed [23,60,61]. The CPC scaffold is an osteoconductive biomaterial which lacks growth factors and living cells found in autologous grafts. Furthermore, synthetic bone substitute alone still face challenges in healing large bone defects because of limited re-vascularization [12]. For these reasons, we proposed to load the CPC scaffold with an osteoinductive growth factor to overcome its lack of osteoinductive properties [20]. In this study, biomaterials were loaded with a solution containing 10 µg of rh-BMP-2 before their implantation *in vivo*. This dosage is consistent with previous studies reporting the use of rh-BMP-2 for critical-sized bone defect healing from 3 to 6 mm [62–65].

In our study, complete or nearly complete healing was observed with the CPC scaffold loaded with BMP-2. Besides, as vascularization of a bone substitute in large bone defect is often challenging [66], we investigated the angiogenesis inside the CPC scaffold. Interestingly, results showed blood vessels formation within the CPC pores, which is necessary to allow bone regeneration.

It has been shown that F-HAM displays a strong osteogenic potential [46,47] and has a “periosteum effect” when used to cover a segmental long bone defect in rabbits [45]. However, to avoid the risk of infectious disease transmission and to allow long-term storage, HAM can not be used as a fresh tissue. Cryopreservation, lyophilization and decellularization/lyophilization are among the most commonly used preservation method of HAM [29,67–69]. We previously reported the cytocompatibility and the biocompatibility of cryopreserved HAM, L-HAM and DL-HAM [49]. However, cryopreservation caused a significant reduction in cell viability and metabolic activity of hBMSCs. When implanted subcutaneously in rats, L-HAM degraded faster than DL-HAM, but they both showed a slower resorption rate than fresh and cryopreserved HAM [49]. Moreover, we previously observed that both L- and DL-HAM displayed a higher ability to act as a barrier membrane for GBR in a non-critical size femoral defect in mice than cryopreserved HAM (data not shown).

In the present study, we thus decided to compare the IM to the L-HAM and DL-HAM. We hypothesized that the presence of the membrane limits dispersion of the BMP-2 in the soft tissue and maintains the growth factor inside the bone defect. However, when the IM or the DL-HAM were used to cover the CPC/BMP2 scaffold, no additional effect on bone regeneration was observed. Despite a low dose of rhBMP-2 was used, it seems that the barrier membrane effect was somehow “hidden” by the growth factor. The association of rhBMP-2 and a bone substitute to fill a rat segmental defect using a two-step surgical approach has only been investigated in one study [64]. A silicone spacer was used to induce the membrane formation in one study. Interestingly, they observed a significant higher level of bone formation when they performed a debridement of the IM before filling the defect, therefore suggesting an inhibitory effect between rhBMP-2 and the IM. One preclinical study investigated bone regeneration using a calcium phosphate scaffold loaded with rhBMP-2 covered or not with three different barrier membranes in a rabbit calvarial model. They also observed no additional beneficial effect of barrier membranes on bone regeneration [18]. One possible explanation is an antagonist reaction between the added rhBMP-2 and growth factors secreted by both the IM and the HAM such as TGF- β 1 [9,40]. Indeed, the opposite effects between TGF- β 1 and BMP-2 on osteoblast differentiation and maturation have been previously reported experimentally [70]. This hypothesis would be consistent with our previous study, in which we observed that cryopreserved HAM inhibited the effect of BMP-2 in calvarial defect in mice [24]. Finally, Masquelet *et al.* reported similar results with BMP-7, which is another osteoinductive growth factor, when associated to the IM technique [5]. After removing the spacer during the second surgery, the IM was filled with bone autograft mixed with rhBMP-7 in eleven patients. The authors concluded that the results of this case-series were not improved compared with the conventional technique without growth factor.

To our knowledge, the decellularized HAM had never been evaluated in a segmental bone critical-size defect before. In this study, we observed that the L-HAM interfered with bone

healing (Fig 5 B and fig 6 D), thereby suggesting that the DL-HAM is the most suitable preserved HAM to be compared with the IM. L-HAM significantly reduced bone regeneration when wrapped around the CPC scaffold compared to other membranes. Indeed, radiographical and histomorphological analysis highlighted a negative effect of L-HAM on total bone volume formation and on bone formation within the pores of the CPC scaffold. This could be explained by the higher suitability of DL-HAM to promote hBMSCs proliferation and to support their osteodifferentiation compared to L-HAM (in revision). Moreover, bone healing required several weeks and we previously reported that DL-HAM had a superior persistence *in vivo* [49], thereby preventing the invasion of fibrous tissue over a longer period. Few studies have previously reported promising results achieved with a decellularized HAM for non-critical bone regeneration [29,71]. Positive results were also obtained using a decellularized amnion/chorion membrane to cover bone grafts after segmental mandibulectomy [72].

CONCLUSION

In conclusion the CPC scaffold loaded with BMP-2 showed promising results to ensure effective bone regeneration of large bone defects. Besides, HAM could be used as an alternative to the technique of Masquelet allowing a single-step surgical procedure for segmental defects. Better results were achieved with the DL-HAM compared to the L-HAM, therefore suggesting using of DL-HAM in future studies to further compare both IM and HAM for bone regeneration in a model without BMP2.

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4.3. Conclusion

Grâce à l'utilisation de l'impression 3D par extrusion, cette étude a permis de modéliser un substitut osseux en ciment phosphocalcique qui venait parfaitement s'adapter au défaut osseux réalisé chez le rat. L'adjonction d'un facteur de croissance à ce biomatériaux a permis d'obtenir une réparation osseuse du défaut segmentaire, suggérant leur utilisation combinée comme alternative à l'autogreffe osseuse.

Le modèle animal mis au point dans cette étude semble pouvoir permettre de comparer de façon reproductible la technique de Masquelet avec une membrane ne nécessitant qu'une seule procédure chirurgicale.

Cette étude n'a pas permis de mettre en évidence un effet « membrane ». Aucune augmentation de la réparation osseuse n'a été observée lorsque le biomatériaux chargé en BMP-2 était recouvert par de la MI ou de la MAH décellularisée/lyophilisée, suggérant l'absence d'intérêt de la ROG en présence de BMP2 associé au biomatériaux utilisé dans ce modèle. Néanmoins, cette étude souligne une différence entre les deux types de MAH utilisée puisque la formation osseuse était significativement diminuée en présence de MAH lyophilisée. Ces résultats corroborent l'article n°3 où la MAH lyophilisée n'augmentait pas la quantité d'os néoformé à long terme.

Bien que nous ayons obtenus des résultats prometteurs avec le biomatériaux chargé en BMP-2 en l'absence de membrane dans cette étude pré-clinique, l'utilisation d'une membrane en ROG semble indispensable en pratique clinique. En effet, son rôle de barrière physique permet de maintenir un espace au sein duquel la néoformation osseuse pourra avoir lieu. Dans ce contexte, la MAH décellularisée/lyophilisée semble la méthode de préservation de choix pour de futurs travaux dans le domaine de l'ITO ainsi que pour de prochaines études en rapport avec la technique de la membrane induite. Son intérêt sur la régénération osseuse de défauts critiques en l'absence de BMP2 reste à évaluer.

CONCLUSIONS ET

PERSPECTIVES

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Les propriétés biologiques de la MAH, sa facilité d'obtention et son faible coût en font un tissu prometteur pour son utilisation en ingénierie tissulaire osseuse. Elle peut être utilisée seule ou associée à des matériaux de comblement osseux, des facteurs de croissance, des cellules souches stromales.

Dans le cadre de ce travail, nous avons cherché à déterminer quelle était la meilleure méthode d'utilisation de la MAH, employée comme membrane, pour son application en ingénierie tissulaire osseuse. Dans un premier temps, nous avons établi que l'apport des MAH fraîches et cryopréservée pour la ROG était indépendant de la viabilité cellulaires des cellules souches épithéliales et mésenchymateuses de la MAH. Cette première étude a également permis de décider de s'affranchir de la face de la MAH utilisée (*épithéliale versus mésenchymateuse*) pour la suite de nos travaux. En effet, l'article n°1 a mis en évidence la faible influence de l'une ou l'autre de ces deux faces sur la régénération osseuse. Ces résultats semblent corroborés par le fait que très peu d'études précisent dans leur méthodologie la face de la MAH appliquée au contact du défaut pour la régénération tissulaire de perte de substance. Par exemple, dans notre revue systématique de la littérature incluant 17 études cliniques portant sur l'utilisation de la MAH en chirurgie orale, seulement cinq auteurs précisaien la face de la MAH appliquée au contact du défaut [120].

Dans un second temps nous avons mis au point une nouvelle méthode de décellularisation de la MAH, et, pour la première fois, les quatre méthodes de préservation de la MAH les plus couramment utilisées ont été comparées : MAH fraîche, cryopréservée, lyophilisée et décellularisée/lyophilisée. Nous avons observé que les 4 types de MAH n'avaient pas d'effet cytotoxique et étaient biocompatibles. En revanche, toutes les méthodes de préservation influençaient les propriétés de la MAH. Comparativement aux autres méthodes de préservation, la MAH décellularisée/lyophilisée possédaient de meilleures propriétés mécaniques (traction uniaxiale et rétention à la suture) et une vitesse de résorption plus lente lorsqu'elle était implantée en sous-cutanée. De plus, lorsque des hBMSCs étaient ensemencées sur les différentes membranes, une prolifération et une ostéo-différentiation

plus importantes des hBMSCs étaient observées au contact de la MAH décellularisée/lyophilisée.

Nous avons ensuite évalué l'apport de ces quatre membranes pour la ROG de défauts de taille non critique. La MAH lyophilisée et la MAH décellularisée/lyophilisée augmentaient significativement la réparation osseuse précoce par rapport au défaut laissé vide. Une néovascularisation significativement plus importante était également observée dans ces deux conditions. Après un mois, seule la MAH décellularisée/lyophilisée augmentait significativement la réparation osseuse par rapport au défaut vide. Cette troisième partie de notre travail nous a ainsi permis de sélectionner deux types de MAH : la MAH lyophilisé et la MAH décellularisée/lyophilisée pour la suite de nos expérimentations.

La dernière partie de cette thèse visait ainsi à comparer l'apport de ces deux MAH à la membrane induite dans un défaut segmentaire chez le rat. Dans cette étude, nous avons eu recours à l'impression 3D par extrusion afin de modéliser un substitut osseux sur mesure pour venir combler le défaut segmentaire que nous souhaitions réaliser. Il s'agissait d'un matériau à base de ciment phosphocalcique connu pour ses propriétés ostéconductrices. Ce matériaux préalablement chargé avec un facteur de croissance (BMP-2) a été implanté dans les défauts osseux de taille critique chez le rat puis couverts par la MI ou par l'une des deux MAH. La régénération osseuse en l'absence de membrane était telle qu'aucun effet « membrane » n'a pu être observé. Aucune différence significative n'a ainsi été retrouvée entre le groupe sans membrane, le groupe MI et le groupe MAH décellularisée/lyophilisée. Cependant, nous avons observé un effet délétère de la MAH lyophilisée sur la réparation osseuse, suggérant ainsi que seule la MAH décellularisée/lyophilisée devrait être comparée à la MI dans de futures études.

Plusieurs perspectives de travail peuvent être envisagées à l'issue de cette thèse :

- Apport de la membrane amniotique pour la régénération de défaut osseux segmentaire :

Suite aux travaux menés dans la dernière étude, plusieurs pistes de travail sont envisagées.

- Modification du modèle de défaut segmentaire chez le rat

L'ajout de BMP-2 dans les expérimentations du projet d'article n°4 semblent avoir masqué ou inhibé l'effet « membrane » que nous cherchions à observer. Il serait intéressant de reproduire cette étude sans l'ajout de facteur de croissance et en conservant le ciment de phosphate de calcium imprimé comme matériaux de substitution osseuse. Une autre possibilité serait de refaire cette expérimentation en utilisant seulement de l'os autologue en comblement afin de se rapprocher de la technique de Masquelet conventionnelle.

Par ailleurs, le matériel utilisé dans cette expérimentation avec un guide de coupe commercialisé n'avait jamais été expérimenté auparavant au sein du laboratoire et était très onéreux. Il serait intéressant de mettre au point un modèle reposant sur des plaques orthopédiques classiques associées à cette scie qui permet une plus grande précision lors de la découpe osseuse (par rapport à une fraise montée sur pièce à main). On pourrait également imaginer confectionner et imprimer un guide de coupe au sein du laboratoire.

- Apport de la membrane amniotique pour la réparation osseuse en territoire irradié

Nous souhaiterions également transposer les travaux actuels dans un modèle de territoire irradié. En effet la chirurgie carcinologique cervico-faciale s'accompagne souvent d'un traitement adjuvant par radiothérapie locale. L'objectif serait d'étudier l'impact de la radiothérapie locale sur la MAH et son potentiel de réparation osseuse en conservant le modèle de défaut segmentaire chez le petit animal. Un protocole de radiothérapie avait déjà été mis au point en collaboration avec la compagnie VetoTech à Lille pour ce même modèle.

• Apport de la membrane amniotique pour la régénération de défaut osseux maxillo-faciaux :

Parmi les trois modèles pré-cliniques utilisés dans cette étude pour évaluer la néoformation osseuse en présence de MAH, deux d'entre eux étaient des modèles fémoraux chez le rongeur. La ROG étant principalement utilisée en chirurgie orale et maxillo-facial, il pourrait être intéressant, lors de futurs travaux, d'évaluer l'apport de la MAH décellularisée/lyophilisée pour la ROG en utilisant un modèle de réparation osseuse qui se rapproche de ces deux spécialités [13,239]. Les modèles suivants pourraient ainsi être mis au point au sein de notre laboratoire :

- Préservation alvéolaire

Dans certaines conditions et afin de prévenir la résorption osseuse alvéolaire consécutive à une extraction dentaire, le chirurgien oral peut réaliser une ROG post-extractionnelle [239]. Une étude pré-clinique rapporte l'utilisation de la MAH pour la préservation alvéolaire suite à l'avulsion d'une molaire maxillaire chez le rat [184]. Ainsi une étude pré-clinique pourrait être menée afin d'évaluer la perte osseuse verticale et/ou horizontale consécutive à une avulsion dentaire en présence de la MAH décellularisée/lyophilisée.

- Chirurgie pré-implantaire et péri-implantaire

Une autre perspective serait d'étudier l'apport de la MAH pour la ROG de perte de substance osseuse pré-implantaire. Pour cela, un modèle d'augmentation osseuse verticale pourrait être réalisée chez le rat tel que celui décrit par Donos *et al.* [240,241]. Ils ont réalisé un modèle d'augmentation crestale verticale chez le rat s'étendant de l'incisive centrale à la première molaire maxillaire.

A ce jour, une seule étude pré-clinique s'est intéressé à l'apport de la MAH pour la ROG d'une perte de substance osseuse péri-implantaire. Ce modèle a été réalisé sur un tibia de rat. Des modèles de ROG péri-implantaire localisés à la mandibule ont également été développés chez le gros animal tel que le minipig ou le chien [242,243].

- Chirurgie parodontale

L'apport de la MAH pour la régénération parodontale a été évalué dans deux modèles pré-cliniques. Un défaut parodontal, avec fenestration osseuse et curetage du ligament parodontal et du cément, a été réalisé de façon bilatérale sur des canines de chien [185]. L'autre modèle consistait à créer un défaut de furcation dentaire au niveau de molaires maxillaires chez le rat [187]. De nombreuses études cliniques s'intéressent à l'utilisation de la MAH en chirurgie parodontale et tout particulièrement pour le traitement des poches parodontales et des atteintes de furcations dentaires [120]. Il pourrait donc être intéressant d'évaluer l'apport de la MAH décellularisée/lyophilisée dans un modèle similaire à celui déjà décrit chez le rat.

- Défauts osseux critiques mandibulaires

Ce modèle a particulièrement été mis au point chez le rat. Il consiste à réaliser un défaut osseux cylindrique bi-cortical de 4 à 5 mm de diamètre en regard de l'angle mandibulaire [16,244,245]. Un repère radio-opaque peut être réalisé pour le suivi radiologique à l'aide de gutta-percha [239]. Ce modèle permet de réaliser un défaut de taille critique à la mandibule qui semble facilement reproductible.

- Modèle de fistule bucco-nasale et fente palatine

L'intérêt de ce modèle est qu'il peut permettre à la fois d'étudier la cicatrisation muqueuse et osseuse. Plusieurs études pré-cliniques [128,192,218,219] et une étude clinique [128] ont précédemment rapporté l'utilisation de la MAH dans un modèle de fente palatine ou de communication bucco-nasale. Les études pré-cliniques étaient réalisés chez le petit animal (rat) ou le grand animal (minipig et porcelet). Ils rapportaient l'intérêt de la MAH pour la cicatrisation de défauts muco-periostés ou ostéo-muqueux palatins.

• **Apport de la membrane amniotique pour la régénération des tissus mous:**

Plusieurs études se sont intéressées à l'utilisation de la MAH pour la régénération des pertes de substance muqueuse. Une action anti-fibrotique, l'accélération de la cicatrisation épithéliale ainsi qu'un effet antalgique de la MAH ont fréquemment été rapportés [117,122,246]. En chirurgie orale, la majorité des études cliniques reposant sur l'utilisation de la MAH concernent les tissus mous (récession gingivale, gestion des tissus mous péri-implantaires et cicatrisation muqueuse suite à l'exérèse de lésions de la muqueuse buccale) [120]. Ainsi, il pourrait être intéressant d'évaluer l'apport de la membrane décellularisée/lyophilisée développée dans nos travaux pour la régénération de perte de substance muqueuse.

• **Étude de l'aspect structural et des propriétés de la MAH décellularisée/lyophilisée**

- Analyse structurale

Les résultats de l'article n°2 et 3 mettent en évidence une augmentation des propriétés mécaniques de la MAH décellularisée/lyophilisée. Il serait intéressant d'approfondir l'étude de son aspect structural afin d'expliquer ces résultats. Il pourrait notamment être envisagé de

réaliser une analyse par microscopie électronique à transmission des MAH en fonction des méthodes de préservation afin d'observer l'orientation et la densité des fibres de collagène. Le recours à la microscopie par génération de second harmonique pourrait également compléter ce travail. Enfin, nous pourrions rechercher l'élimination de certaines protéines de la matrice suite à la décellularisation en utilisant la spectrophotométrie de masse.

- Facteurs de croissance

La matrice de la MAH contient de nombreux facteurs de croissance dont certains pourraient être impliqués dans la régénération osseuse. L'une des limites de ces travaux est que nous n'avons pas étudier l'expression de ces facteurs au sein de la matrice après préservation de la MAH. Dans un premier temps, nous souhaiterions étudier la conservation de certains facteurs de croissance, tels que le VEGF, PDGF ou TGF-B, au sein de la matrice de la MAH après sa décellularisation.

- Identifier et quantifier l'expression des marqueurs ostéogéniques.

Il serait également intéressant d'analyser de façon plus précise les mécanismes impliqués dans la réparation osseuse avec la MAH décellularisée/lyophilisée. Nous pourrions notamment recourir à de l'analyse par PCR afin de préciser l'expression de certains marqueurs ostéogéniques par la MAH *in vitro* et *in vivo*.

• Méthodes de préservation de la MAH

Dans cette étude, nous ne nous sommes pas intéressés à la déshydratation et la désépithérialisation de la MAH qui sont d'autres méthodes de préservation souvent retrouvées dans la littérature.

La désépithérialisation (élimination des cellules de la face épithéliale de la MAH) [160,165,216,247], tout comme la décellularisation [169,170,187,214,217,248,249], sont souvent employées pour préparer la MAH avant son utilisation comme « scaffold » en ingénierie tissulaire. Dans le cadre de notre étude, nous avons choisi de réaliser une décellularisation complète de la MAH (cellules épithéliales et mésenchymateuses). En effet, les méthodes de désépithérialisation décrites dans la littérature impliquent une première phase de traitement enzymatique ou chimique suivie d'une étape mécanique pour détacher

les cellules épithéliales restantes. Il n'existe pas de consensus sur la méthode à employer, ces procédés ne semblent pas reproductibles et altèrent le plus souvent l'intégrité de la membrane basale [105,247]. De plus, il n'existe pas de méthode quantitative définie pour s'assurer de l'efficacité de la désépithérialisation. Celle-ci se fait le plus souvent sur la base de paramètres qualitatifs n'évaluant qu'une petite zone de la MAH. La technique de décellularisation que nous avons développée permet de s'affranchir de toute variabilité puisqu'elle permet d'éliminer à la fois les cellules épithéliales et mésenchymateuse.

La déshydratation ou « séchage » de la MAH a été suggérée comme alternative à la lyophilisation. Tout comme la lyophilisation, cette méthode vise à éliminer l'eau contenue dans un produit. Néanmoins, cette méthode de préservation de la MAH est plus rarement rapportée [105,250] et son protocole reste mal défini [99]. Cela nécessite le plus souvent de pouvoir laisser le tissu sécher sous une hotte ou en étude pendant une période allant de 12 à 24 heures [99,236,251]. Cette méthode de préservation altère également les propriétés de la MAH, notamment en diminuant la capacité de la membrane basale à favoriser la prolifération cellulaire à son contact [173].

- **Optimisation de la MAH**

Afin d'améliorer les propriétés biologiques et/ou mécaniques de la MAH :

- **MAH en multicouche**

Plusieurs couches de MAH pourraient être assemblées afin d'augmenter l'épaisseur et la rigidité de la membrane. Seulement quelques études ont déjà rapporté l'utilisation de la MAH en multi-couche. Les différentes couches de MAH étaient maintenues assemblées selon divers protocoles tels qu'à l'aide de suture [127,192] ou encore grâce à l'ajout d'une solution saline stérile entre chaque couche en per-opératoire [246].

- **Association amnion/chorion**

L'utilisation d'une membrane d'amnion/chorion permettrait de faciliter la manipulation de la membrane car le chorion est trois à quatre fois plus épais que l'amnion et cela permettrait aussi possiblement d'améliorer ses propriétés mécaniques. De plus, le

chorion contient également plusieurs facteurs de croissance et on y retrouve notamment le PDGF-a et le VEGF en quantité supérieure par rapport à l'amnion [252]. Le chorion présenterait également des propriétés anti-bactériennes supérieure à la MAH, ce qui réduirait encore le risque d'infection en cas d'exposition de la membrane [253]. Une étude clinique rapporte l'utilisation d'une membrane d'amnion/chorion décellularisée pour recouvrir une greffe osseuse chez quatre patients ayant bénéficié d'une hémi-mandibulectomie interruptrice [254]. Une revue systématique de la littérature portant sur l'utilisation de l'amnion/chorion en chirurgie orale est actuellement en cours dans le cadre de la direction d'une thèse d'exercice en chirurgie dentaire.

- Développement d'une membrane multi-couches/ Fonctionnalisation de la MAH

Il serait intéressant de développer une membrane possédant plusieurs couches permettant d' associer la MAH à un polymère tel que le PL(G)A afin de i) faciliter la manipulation de la MAH, ii) jouer sur la porosité des différentes couches par impression 3D, iii) charger l'une des couches avec des micro-particules d'HA. Après évaluation des facteurs de croissances résiduels au sein de la MAH décellularisée/lyophilisée, il pourrait aussi être envisagé de fonctionnaliser la MAH en y ajoutant d'autres facteurs de croissance ou des cellules pour se rapprocher d'un substitut périosté.

COMMUNICATIONS

SCIENTIFIQUES

COMMUNICATIONS SCIENTIFIQUES

1. Publications

1.1. Articles internationaux

- M Fénelon, S Catros, J.C Fricain. **What is the benefit of using amniotic membrane in oral surgery? A comprehensive review of clinical studies.** Clinical Oral Investigation. 2018 Jun;22(5):1881-1891 DOI 10.1007/s00784-018-2457-3
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2. Communications internationales

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- M Fénelon, D Maurel, R Bareille, R Siadou, A Grémare, S Brun, F Gindraux, N L'Heureux, J.C Fricain. **Development of a new method for decellularization and preservation of human amniotic membrane for bone regeneration: cytocompatibility and biocompatibility assessment**. European Association of Tissue Banks (EATB). Lille. Octobre 2018.
- Grémare, S. Jean-Gilles, P. Musqui, L. Magnan, Y. Torres, M. Fénelon, S. Brun, J.C Fricain, N. L'Heureux. **Development of a new vascular substitute produced by weaving threads of human amniotic membrane: preliminary study**. Termis. Rhodes, Mai 2019.

2.2. Communications affichées

- M. Fénelon, C. Boiziau, O. Chassande, R. Bareille, S. Rey, H. Boeuf, F. Gindraux, Z. Ivanovic, J.C. Fricain. **Human amniotic membrane versus collagen membrane for**

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3. Communications nationales

3.1. Communications orales

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