# ANNEXES

*Evaluation of a formulation of an HA-based gel with fetal progenitor cells for topical treatment of burn wounds.*

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## a) Hydrogels naturels commerciaux utilisés pour les traitements cutanés

*Table 1: Adapté de Murphy et al.(2013) (12)*

| Matériau | Produit commercial | Source du matériau | Application |
|---|---|---|---|
| **Collagène** | Evolence ® Collagen Filler | Tendon de porc | Remplissage dermique |
| | Integra ™ Meshed Bilayer Wound Matrix | Tendon de bovin | Soins de plaies avancés |
| **Acide hyaluronique** | Lifecore Biomedical Corgel Biohydrogel | Biologique : Tissu conjonctif | Chirurgie vasculaire, Ophtalmologie, Orthopédie, Ingénierie tissulaire |
| | Glycosan Biosystems Extracel ™ | Bactérie Bacillus subtilis | Culture 3D de cellules, Ingénierie tissulaire |
| **Chitosan** | CELOX | Carapace de crevette | Pansement hémostatique |
| **Alginate** | 3M™Tegagel™Hydrogel Wound Filler | Alginate de calcium (algue/bactérie) | Gestion plaies exsudatives |
| | Phytacare Alginate Hydrogel wound dressing | | Ulcères cutanées |

## b) Sélection des études relatives aux brûlures

*Table 2: Adapté, Jeffrey Voigt (2012), (17)*

| Interventions | Résultats | Catégorie de la plaie | Réf. |
|---|---|---|---|
| 0,5 mL de **1,5% HA** vs 100% glycérine Recouvrement par du Tegaderm | Les plaies du groupe contrôles sont guéries plus rapidement (9,1 +/- 1,6 jours) par rapport au groupe avec du HA (10,3 +/- 2 jours) Pas de différences au niveau de l'aspect esthétique des plaies après 6 semaines et 3 mois | Brûlures | (63) |
| **Ialugen crème (HA)** vs Placebo, 2x/jour après 1-2 heures de la radiothérapie pendant 6 semaines et ensuite 4 semaines de suivi. | Différence significative en faveur du groupe avec du HA dès la 3ème semaine (la peau apparaissait normale avec un score de 0 sur une échelle de 0-5 où 5 = ulcère) | Brûlures post-radiques | (64) |
| **HA + sulfadiazine d'argent** appliqués topiquement sous forme de crème vs sulfadiazine d'argent seule | Temps de réparation tissulaire statistiquement inférieur dans le groupe avec du HA + sulfadiazine d'argent comparé au groupe avec la sulfadiazine d'argent seule. | Brûlures de 2ème degré superficielles et profondes | (1) |

## c) Tables : Hyaluronic Acid and Wound Healing (48)

**Table 1:** Tissue Regeneration Studies

| Ref | Study type and Model Used | Formulation Containing HA and the HA Level | Efficacy Findings |
|---|---|---|---|
| 2 | Human A431 epidermoid skin cells and mouse fibroblasts exposed to ethanol (50 and 100 mM) | HA (2%, 4% and 8%) | HA reduced ethanol-induced cytotoxicity in a dose-dependent manner between 2% and 4%; 8% HA had no effect; HA decreases production of the pro-inflammatory cytokine TNF-α; HA prevented ethanol-induced apoptosis |
| 3 | Deep dermal wounds produced *in vitro* in human skin | Amniotic fluid rich in nutrients, growth factors and HA | Amniotic fluid (50%) led to similar epithelialization as fetal bovine serum (10%); superior epithelialization vs. cell culture media; HA degradation prevented epithelialization |
| 4 | Rat cardiomyocyte cell line H9C2 treated with hydrogen peroxide as a heart ischemia-reperfusion model | LMW-HA: low molecular weight HA (100 kDa); HMW-HA: high molecular weight HA (1000 kDa) | HMW-HA (0.3%) modulates cell survival and promotes wound healing; HMW-HA (0.3%) significantly facilitates cell migration via cytoskeletal rearrangement (restores activity of migration-associated cytoskeletal proteins); No effect of LMW-HA |
| 5 | Alkali-injured human corneal epithelial cells (HCE-2 cells treated with NaOH) | HMW-HA group: high molecular weight HA (1525 kDa); LMW-HA group: low molecular weight HA (127 kDa) | HMW-HA increased cell viability in dose-dependent manner (24 h incubation after exposure for 1 min to 0.012 N NaOH) |
| 6 | Full-thickness pieces of rabbit skin | HA1 group: porcine acellular dermal matrix + HA (0.3 mL exogenous HA, about 0.17 mg/cm$^2$) + thin skin auto-graft; HA2 group: porcine acellular dermal matrix + HA (0.6 mL exoge-nous HA, about 0.34 mg/cm$^2$) + thin skin autograft; PADM group: porcine acellular dermal matrix + thin skin autograft; TS group: thin skin autograft; NS group: normal skin | Skin grafts grew well in all groups; HA1 and HA2: soft with good elasticity; PADM: quite hard with poor elasticity; TS: thin and susceptible to tearing; NS: best elasticity among all the groups; Collagen I and III content on day 28: higher in HA1 and HA2 than rest; higher in HA1 than HA2; Microvascular density at day 14: higher in HA1 and HA2 than other groups; higher in HA2 than HA1 |
| 8 | Rabbit superficial digital flexor tendon rupture; full thickness tendon transection and surgical repair | NaH group: exogenous sodium hyaluronate injected subcutaneously over the lesion; Control group: saline injection | Time-dependent decrease in the diameter of the injured tendon: superior in NaH vs. control; Treatment was effective in restoring morphological and biomechanical properties of lacerated superficial digital flexor tendon rupture |
| 9 | Rabbit knee articular defect (induced articular cartilage defect) | Treatment: bilateral knee arthrotomies, chondral defects, microfracture + intraarticular HA; Control: saline | Higher potential for healing in the experimental group, with thicker and more organized repair tissue in treatment vs. control |
| 10 | Anterior wall of the maxillary sinus removed in rabbit<br>4 mm circumferential wound on both the nasal and the sinus side | Rapid-gelling HA hydrogel or preformed HA hydrogel filled randomly into the right or left sinus; Blank control or Merogel control into the other sinus | HA preserved neo-ostium opening (prevented ostial stenosis); Degree of lymphocyte or plasmacyte infiltration similar between groups; Preformed HA hydrogel led to lower acute inflammation and heterophile infiltration; Rapid-gelling HA reduced fibrosis and osteogenesis; HA hydrogel promoted wound healing |
| 7 | Full-thickness surgical wound in rats<br>Full-thickness skin defect in diabetic mice | EGF dressing: HMW-HA spongy sheet (upper layer) + arginine, magnesium ascorbyl phosphate and EGF (lower layer); Control: epidermal growth factor-free dressing | Substantially facilitate epithelialization, granulation tissue formation and angiogenesis in rat; EGF dressing superior to EGF-free dressing; Improved wound condition and decreased wound size, and facilitated epithelialization, granulation tissue formation and angiogenesis in mouse; EGF dressing superior to EGF-free dressing |
| 11 | Rat critical size defect model (hole made in parietal bone) | Right hole (treatment): HA, chondro-itin 6 sulphate, dermatan sulphate and 2.5% saline solution; Left hole (control): untreated | Substantial periosteal macroscopic neo-angiogenesis at both sites |

| | | | |
|---|---|---|---|
| 12 | Myocardial infarction induced by cryoinjury in rat | Treatment group: chitosan-HA/silk fibroin cardiac patches implanted in left ventricle; Control group: no patches | Reduced the dilation of the inner diameter of left ventricle in treatment ($p<0.05$); Increased wall thickness of left ventricle in treatment ($p<0.05$); Improved the fractional shortening of left ventricle of hearts ($p<0.05$) |
| 13 | Epithelial defects on the corneas in rat: mechanical scraping and alkali burns | Artificial tears containing HA (0.3% or 0.15%) + high-K ion concentration; PBS | Mechanical scraping model: smaller areas of fluorescein staining in the eyes in HA + high-K artificial tears vs. PBS by 36 h; Alkali burn model: no significant effect of HA + high-K artificial tears vs. PBS by 36 h |
| 14 | RTC (n=21); skin de-epidermised by Er-YAG laser in healthy volunteers | Group A: *Avena rhealba* extract® + HA; Group B: reference product (panthenol and madecassoside); Group C: reference product (resveratrol-copper); Group D untreated control | Laser wound completely healed after 9 days in groups A and B, 12 days in group C, and 16 days in group D; Healing generally slow between days 1 and 6, faster thereafter; Safety profiles of treatments were favorable and comparable |
| 15 | Cohort (n=60); partial thickness burns (average 3% total body surface area); | Zinc-HA gel | On average, wound size reduced by 50% by day 5; Full epithelialization in 93.3% of sample by day 21; Resolution of pain in 91.7% of sample by day 10; No infections |
| 18 | Cohort (n=40); persistent ulcers | Bionect Start® ointment (collagenase product containing HA) | Relevant reduction of the ulcer size after 5 weeks in 80.0% of sample, and mild or no improvement in 20.0% of sample; Decrease in pain in 50.0% of sample, no pain in 40.0% of sample, and no change in pain intensity in 10.0% of sample; Bacterial infection in wound exudates in 23.3% of sample |
| 16 | Cohort (n=30); second-degree skin burns in the phase of re-epithelialization | Topical ozonated oil vs. topical HA gel | Both agents equally effective in reducing symptoms related to skin burns such as erythema, tension, itching and burning sensation; Both agents led to self-reported improved general appearance of skin lesions; Ozonated oil prevented post-lesion hyperpigmentation better than HA |
| 17 | RTC (n=89); venous leg ulcers | Topical HA (gauze pad) vs. neutral vehicle control | Percentage of wound size reduction at day 45: 73 ± 4.6% in HA vs. 46 ± 9.6% in control ($p=0.011$); Number of healed ulcers: 31.1% in HA vs. 9.3% in control at day 45 and 37.8% vs. 16.3% at day 60; Pain intensity based on visual analogue scale lower in HA vs. control; One case of heart attack in HA group and one death not related to therapy |
| 19 | RTC (n=124); pressure ulcers | Control: no treatment; 1 × PRGF: one dose of platelet-rich growth factor (n=34); 2 × PRGF: two doses of platelet-rich growth factor (n=25); 2 × PRGF + HA: two doses of platelet-rich growth factor + HA (n=40) | Reduction in ulcer area in all groups at day 36 (mean reduction 48% vs. baseline); higher in all treatment groups vs. control; greatest in 2 × PRGF + HA (mean 80.4% vs. baseline); Complete wound healing: 32.0% in 2 × PRGF ($p<0.002$) and in 37.5% 2 × PRGF + HA ($p<0.004$); No infections by day 36 |
| 20 | RTC (n=34); chronic periodontitis | Test group: scaling and root planting + HA; Control group: scaling and root planting alone | Probing depth and number of pockets with probing depth ≥5 mm reduced in both groups; superior in test vs. control at months 3 and 6; Reduction in *Treponema denticola* and *Campylobacter rectus* counts; superior in test vs. control |
| 21 | Case series (n=5); wound dehiscence and tendon exposure after Morton's neuroma surgery excision | Platelet rich plasma + 3D polymerised HA-medicated biological dressings containing growth factors | Complete wound healing at day 30; Walking regained after an average 2 weeks; No adverse events |

Ref: reference number; EGF: epidermal growth factor; HA: hyaluronic acid; HMW: high molecular weight; K: potassium; LMW: low molecular weight; NaH: sodium hyaluronate; NaOH: sodium hydroxide; NS: normal skin; PADM: porcine acellular dermal matrix; PBS: phosphate buffered saline; PRGF: platelet-rich growth factor; Ref: reference; RTC: randomized clinical trial; TNF: tumor necrosis factor; TS: thin skin.

**Table 2.** Scaffold for Wound Healing Studies

| Ref. | Model Used | Description of HA Scaffold | Efficacy Findings |
|---|---|---|---|
| 23 | Rat; Full thickness skin wounds | Scaffolds constructed of silk fibroin, chondroitin sulfate and HA | Scaffold containing silk fibroin, chondroitin sulfate and HA led to superior dermis regeneration (smaller wound area), with improved angiogenesis and collagen deposition compared to scaffold lacking collagen; Expression of growth factors was initially superior in the fibroin, chondroitin sulfate and HA group, and decreased with progression of wound healing |
| 24 | Rat; Full thickness skin wounds | Treatment: porous scaffold made up of collagen, HA and gelatin; Control: no treatment | Scaffold ameliorates wound healing, decreases neutrophyils infiltrates and thickens newly generated skin; Small wound area in treatment vs. control, with faster healing |
| 27 | Rat; Midline abdominal incision | Control: no adhesion barrier; Interceed group: Interceed™ absorbable adhesion barrier; HA group: HA alone; mcALG group: mildly cross-linked alginate; HA/mcALG: HA embedded in mildly cross-linked alginate | mcALG and HA/mcALG shielded the injury site, thus preventing peritoneal tissue adhesion and facilitated wound healing |
| 29 | Rat; Critical-size calvaria defects | HA: 1.0% HA; HA + ACS: 1.0% HA + absorbable collagen sponge; ACS: absorbable collagen sponge; Control: no treatment | HA + ACS showed superior bone filling potential vs. control ($p<0.005$) and ACS ($p=0.017$); HA similar to other treatments |
| 25 | Mouse; Full thickness skin wounds | Treatment: human dermal/epidermal cell fractions entrapped directly within a gellan gum/HA spongy-like hydrogel; Control: no treatment | Accelerated wound closure rate, re-epithelialization and neovascularization in treatment vs. control |
| 26 | Rabbit; Full thickness skin wounds | Xenogeneic de-cellularized scaffold from pig peritoneum and HA + basic fibroblast growth factor (1 or 3 μg/mL) | Faster would healing rates and superior dermis regeneration with scaffold, regardless of basic fibroblast growth factor concentration |
| 30 | Rabbit; Critical-size calvaria defects | Treatment: HA hydrogels loaded with growth and differentiation factor 5 (0, 10, 100 or 1000 ng/mL GDF-5);Control: no treatment | More significant new bone formation, larger bone formation area, larger bone volume and bone mineral density in treatment conditions with larger GDF-5 concentration |
| 28 | Rat (*in vitro*); Excised portion of peritoneum: spongy sheet placed on the peritoneal defect in rats | Anti-adhesive spongy sheet composed of HA and collagen + epidermal growth factor<br>Wound surface model (spongy sheet on top of fibroblast-incorporating collagen gel sheet)<br>Inter-tissue model (spongy sheet between two fibroblast-incorporating collagen gel sheets) | Spongy sheet stimulates fibroblasts to release an increased amount of vascular endothelial growth factor and hepatocyte growth factor; Spongy sheet facilitated wound healing and preventing surgically excised tissue from adhering to surrounding tissue |
| 31 | Retrospective study (n=29); Patients with removal of basal cell carcinoma from face | Tissue-engineered dermis composed of autologous cultured dermal fibroblasts seeded on HA sheet | Re-epithelization occurred progressively from the periphery to the center of the wound |
| 32 | Fresh wounds from surgical procedure (n=6); Patients with inveterate disabling scar retraction, with soft tissue defect resulting from surgical scar removal | Hyalomatrix®3D HA scaffolding + autologous skin grafting | Histological observation and immunohistochemical analysis: integration of graft within the surrounding tissues; Regenerated dermis with extracellular matrix rich in type I collagen and elastic fibres, and with reduced type III collagen |

Ref: reference; ACS: absorbable collagen sponge; HA: hyaluronic acid; HMW: high molecular weight; GDF: growth and differentiation factor; LMW: low molecular weight; mcALG: mildly cross-linked alginate

## d) Interaction entre le HA et ses principaux récepteurs (14) (15) (29) (28)

| | CD44 | RHAMM |
|---|---|---|
| Localisation du récepteur | Récepteur de surface de la membrane plasmatique | Récepteur intracellulaire |
| Action dans un état physiologique | Attachement, organisation et turnover de la MEC | • Action intracellulaire : régulation de la mitose<br>• Action extracellulaire (associé au CD44) : activation de cascades intracellulaires<br>• Régulation de la communication intercellulaire *(« Gap junctional intercellular communication (GJIC) ») :* promotion de la fonction de ces canaux par le HA de haut poids moléculaire affectant la différenciation et la prolifération cellulaire.(65) |
| Action spécifique lors de lésion cutanée | • Abrogation de l'inflammation<br>• Stimulation de la production de métalloprotéinases invasion cellulaire à travers la MEC<br>• Adhésion des leucocytes aux cellules endothéliales ainsi que leur extravasation<br>• Stimulation de l'angiogenèse<br>• Induction de la prolifération des cellules endothéliales<br>• Impact sur la ré-épithélialisation (synthèse HA augmentée chez les kératinocytes aux marges de la plaie)<br>• Régulation de la différenciation épidermique et de la synthèse/sécrétion des corps lamellaires lipidiques | • Recrutement des macrophages dans la zone lésée<br>• Remodelage du cytosquelette et migration cellulaire |

## e) Liste des produits et matériel

| Produit et matériel | Code | Fournisseur (Ville, Pays) |
|---|---|---|
| Flasque de 75 $cm^2$ | 90076 | TPP, Trasadingen, Suisse |
| DMEM | 41966-029 | Gibco, Paisley, UK |
| FBS | 10270.106 | Gibco, Paisley, UK |
| L-Glutamine 200nM | 25030-024 | Gibco, Paisley, UK |
| TrypLE express | 12605-010 | Gibco, thermoFisher scientific, CH |
| PBS | 100 0 324 | Bichsel, Interlaken, Suisse |
| Pipette inversée | F148504 | Microman, Gilson, Middleton, WI, USA |
| LIVE/DEAD | L-3224 | Molecular Probes, Life Technologies Ltd., Paisley, UK |
| Microscope à fluorescence IX81 | IX81 | Olympus, Tokyo, Japan |
| Caméra digitale du microscope | iXon | Andor Technology Ltd., Belfast, UK |

## f) Résultats de l'expérience A

Le Synovial 0,8% a montré une survie moyenne des CPF de 55%. À noter que le taux de survie dans le Synovial 1,6% reste plus stable avec une survie moyenne de 50%. L'évolution de la survie cellulaire dans le Viscoseal 0,5% est la plus mauvaise, en montrant une chute dès le premier jour. Il est probable que le gel, étant très liquide, ne fournit pas une matrice de soutien aux cellules et donc celles-ci finissent par se déposer au fond du puit. La meilleure survie à court/moyen terme se constate donc dans le **Synovial 0,8%.**

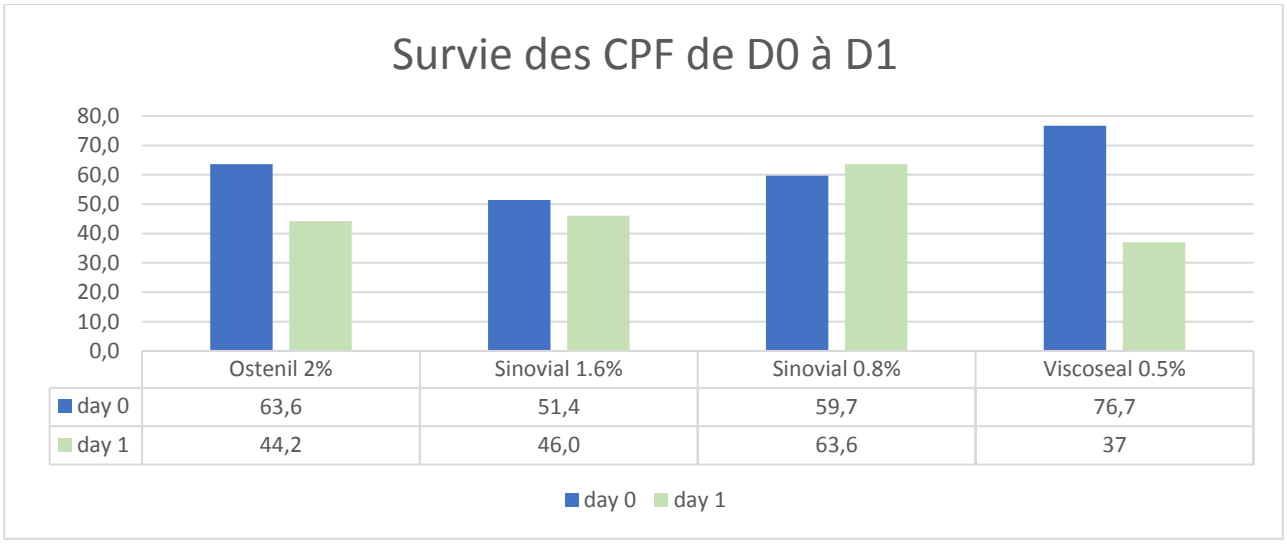



*Figure 1: Survie des CPF de D0 à D1 selon les 4 différentes % de HA (expérience A, EXP 1) ((D0 = jour de l'expérience, D1 = Jour 1)*

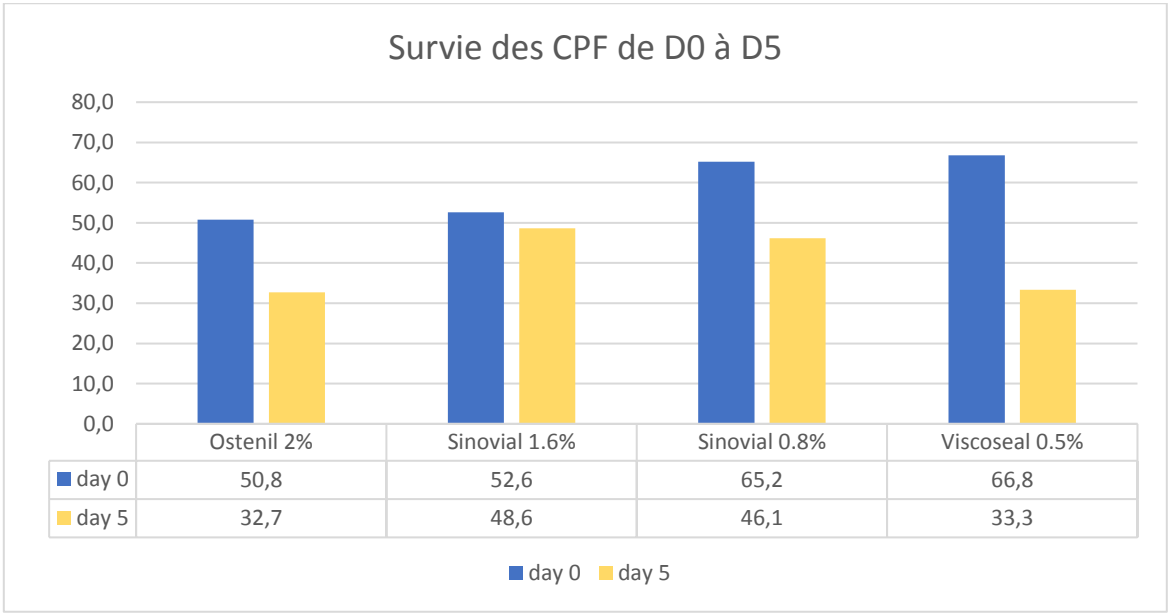
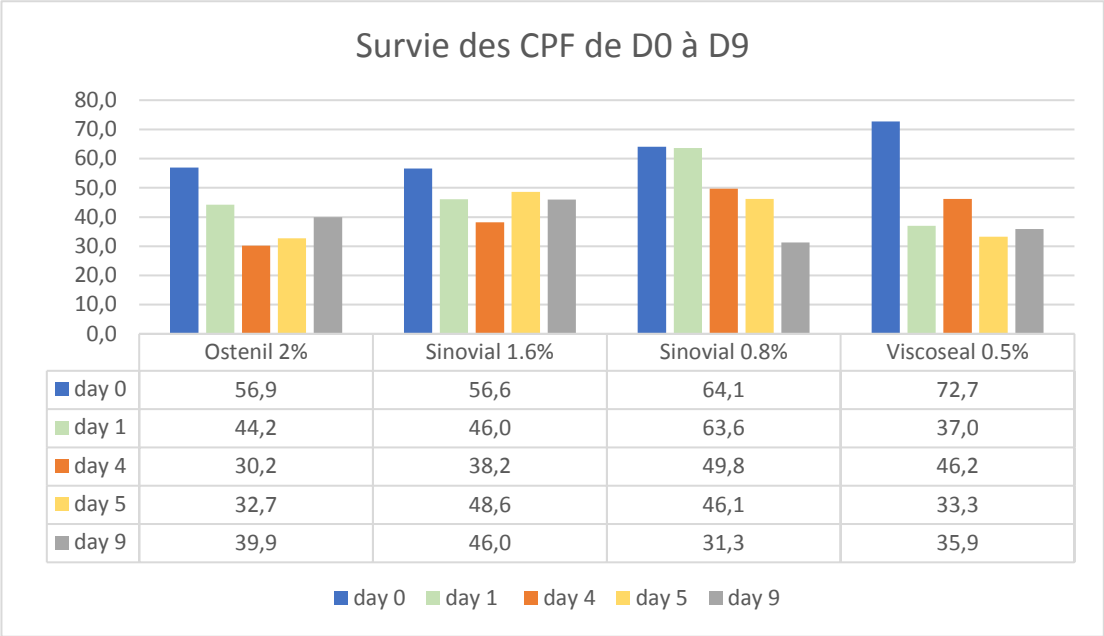



*Figure 2: Survie des CPF de D0 à D5 (Jour de l'expérience (D0) au 5ème jour (D5) selon les 4 différentes % de HA (expérience A, EXP 2)*

Du point de vue de la survie cellulaire à long terme (9 jours), celle-ci semble être plus stable dans le gel Synovial 1,6% (Figure 17).

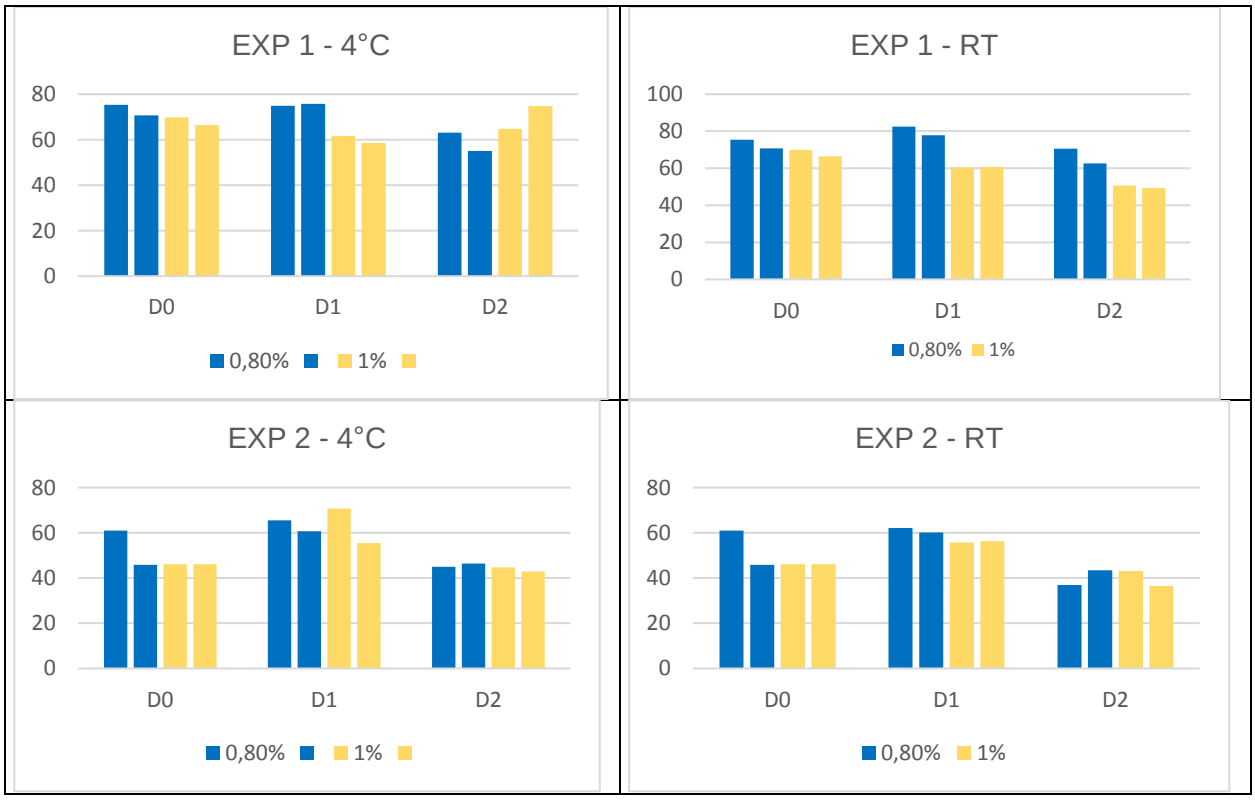


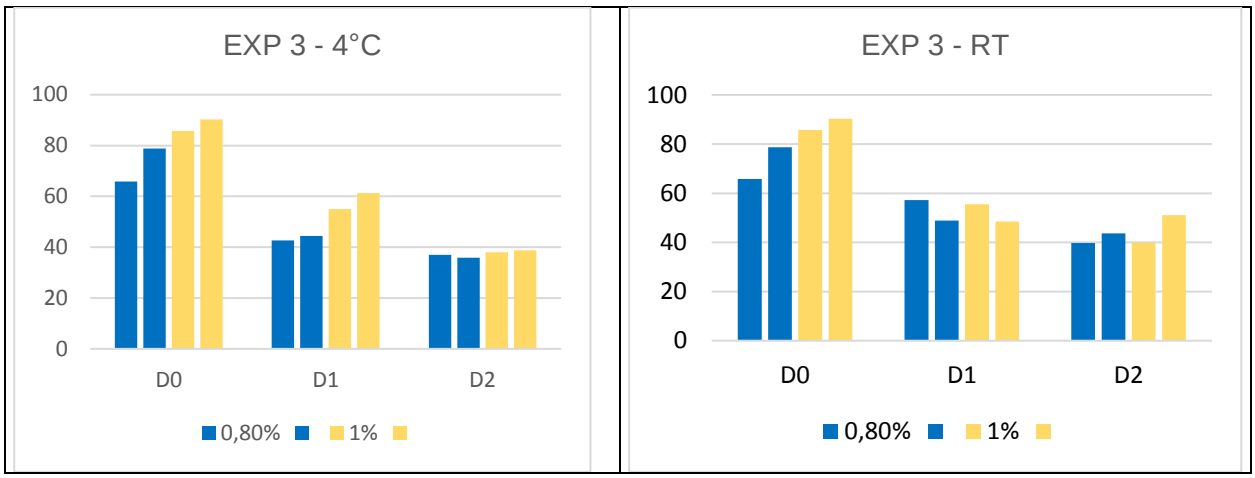

| | Ostenil 2% | Sinovial 1.6% | Sinovial 0.8% | Viscoseal 0.5% |
|---|---|---|---|---|
| day 0 | 56,9 | 56,6 | 64,1 | 72,7 |
| day 1 | 44,2 | 46,0 | 63,6 | 37,0 |
| day 4 | 30,2 | 38,2 | 49,8 | 46,2 |
| day 5 | 32,7 | 48,6 | 46,1 | 33,3 |
| day 9 | 39,9 | 46,0 | 31,3 | 35,9 |

*Figure 3: Moyenne des meilleures survies des CPF de D0 à D9 (Jour de l'expérience (D0) au 9ème jour (D9) selon les 4 différentes % de HA*

## g) Résultats de l'expérience B et analyse statistique

### i) Survie selon la température de conservation et le % de HA du gel






### ii) Analyse statistique

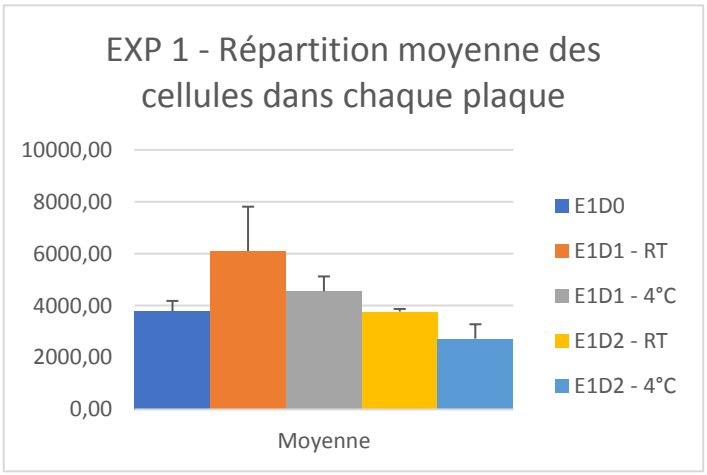
| | 4°C | | | RT | | |
|---|---|---|---|---|---|---|
| | **0.8%** | | | **0.8%** | | |
| % de cellules vivantes | D0 | D1 | D2 | D0 | D1 | D2 |
| | 75,34 | 74,91 | 63,01 | 75,34 | 82,50 | 70,57 |
| | 70,68 | 65,56 | 45,02 | 61,04 | 62,11 | 36,86 |
| | 61,04 | 42,71 | 36,97 | 65,83 | 57,20 | 39,78 |
| | 45,89 | 75,72 | 54,99 | 70,68 | 77,89 | 62,60 |
| | 78,75 | 60,66 | 46,40 | 45,89 | 60,10 | 43,38 |
| | 65,83 | 44,41 | 35,84 | 78,75 | 48,89 | 43,71 |
| | | **t-Test** | | | **t-Test** | |
| | D0 vs D1 | D0 vs D2 | D1 Vs D2 | D0 vs D1 | D0 vs D2 | D1 Vs D2 |
| | 0,50836729 | **0,0289411** | **0,0028868** | 0,82931552 | **0,0279198** | **0,0023946** |
| | **1%** | | | **1%** | | |
| % de cellules vivantes | D0 | D1 | D2 | D0 | D1 | D2 |
| | 69,84 | 61,66 | 64,71 | 69,84 | 60,43 | 50,76 |
| | 46,07 | 70,84 | 44,68 | 46,07 | 55,79 | 43,13 |
| | 85,74 | 55,06 | 38,02 | 85,74 | 55,53 | 39,90 |
| | 66,39 | 58,60 | 74,76 | 66,39 | 60,71 | 49,36 |
| | 46,07 | 55,48 | 42,81 | 46,07 | 56,36 | 36,54 |
| | 90,27 | 61,27 | 38,74 | 90,27 | 48,47 | 51,06 |
| | | **t-Test** | | | **t-Test** | |
| | D0 vs D1 | D0 vs D2 | D1 Vs D2 | D0 vs D1 | D0 vs D2 | D1 Vs D2 |
| | 0,4678835 | 0,17338107 | 0,19854434 | 0,25126848 | **0,0229222** | **0,015936** |

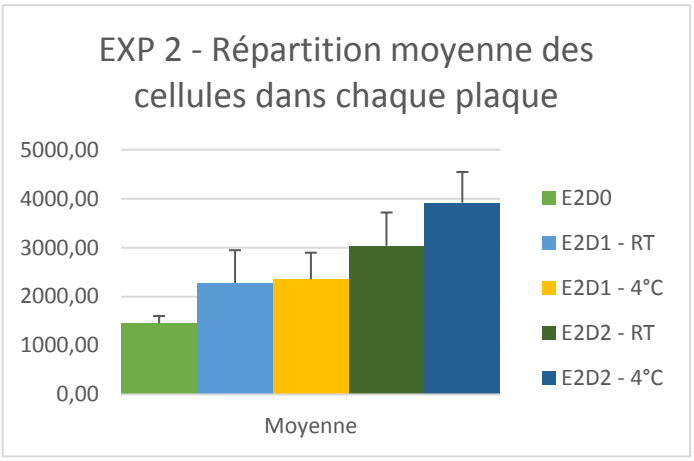
| | **4°C** | | | RT | | |
|---|---|---|---|---|---|---|
| | D0 vs D0 | D1 vs D1 | D2 vs D2 | D0 vs D0 | D1 vs D1 | D2 vs D2 |
| | 0,91768226 | 0,97611092 | 0,33550085 | 0,83285812 | 0,0655616 | 0,37252397 |

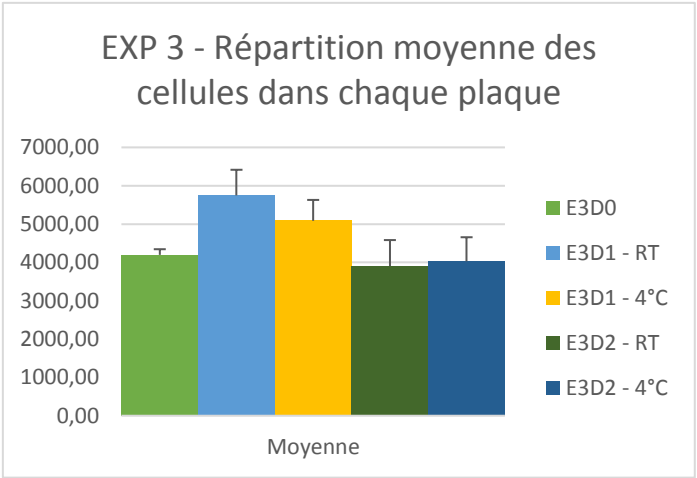
### iii) Répartition cellulaire dans les différentes plaques

La répartition cellulaire dans les différentes plaques a été également examinée afin de percevoir si elle était régulière. En effet, une irrégularité de la quantité des cellules dans chaque plaque est perçue. (Figure 18)

*Figure 4: Répartition moyenne des cellules dans chaque plaque (EXP 1, 2, 3), (RT = température ambiante)*



### iv) Répartition cellulaire dans les différents cadrans des puits

Lors de l'expérience B, les images au microscope ont été acquises selon le schéma illustré dans la Figure 19. Le nombre de cellules retrouvé dans chaque cadran a été respectivement introduit dans un tableau Excel. Dans la Figure 21, nous pouvons constater que la répartition des cellules n'est pas homogène. En particulier, le cadran C (central) résulte être la zone la moins peuplée de cellules au sein du puit. En effet, sur la Figure 20, nous pouvons voir que les cellules s'agglomèrent dans les bords du puit en suivant le gel de HA qui tend également à adhérer aux parois. Ce phénomène entrave la photographie des cellules (qui sont moins visibles) et par conséquent, également le comptage effectif des cellules vivantes/mortes. Ceci s'observe dans les 3 expériences, relativement indépendamment de la concentration du gel et de la température de conservation. Nous pouvons tout de même constater que dans les puits avec le gel HA de 1%, la répartition cellulaire est légèrement plus homogène que dans les puits avec le gel HA 0,8% (Figure 22). Il est probable que le gel HA 1%, étant plus visqueux et moins fluide, permette une répartition moins hétérogène des cellules au sein du puit.

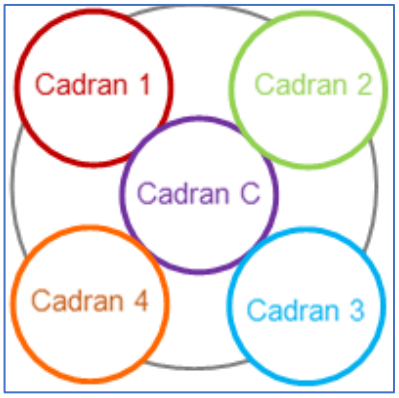



*Figure 5: Schéma illustrant la dénomination des cadrans utilisée pour comptabiliser les cellules au microscope*

*Figure 6: Photo d'un puit au travers de la lentille droite du microscope (EXP3, D2, gel HA 0,8%)*

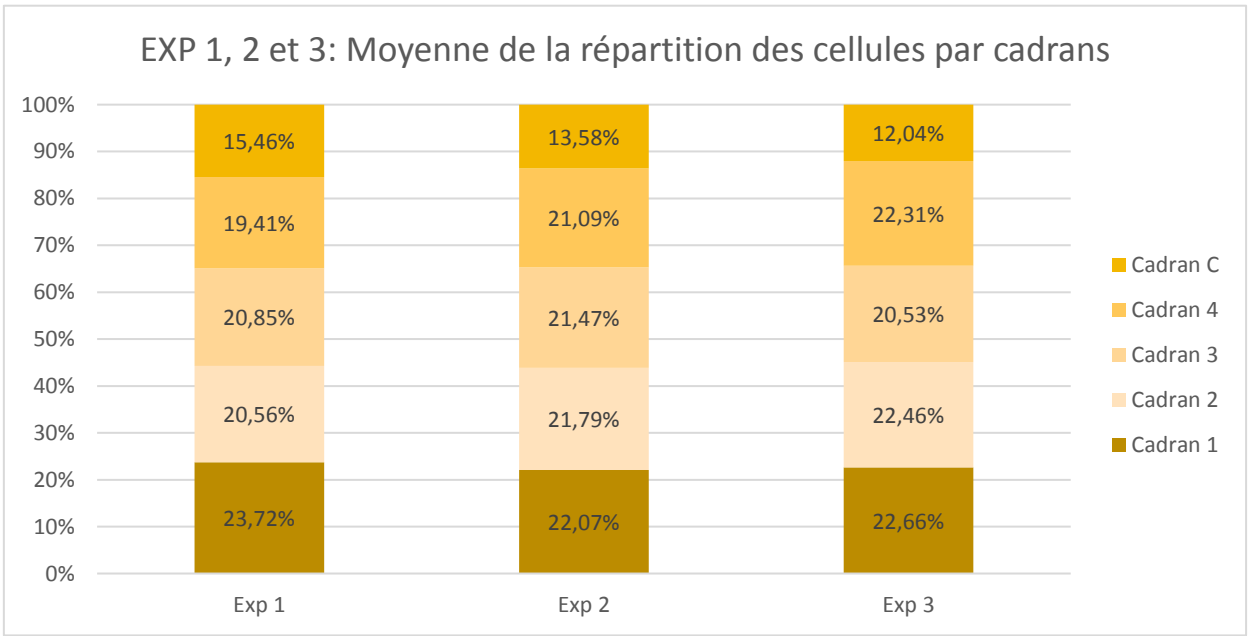



*Figure 7: Moyenne de la répartition des cellules par cadrans*

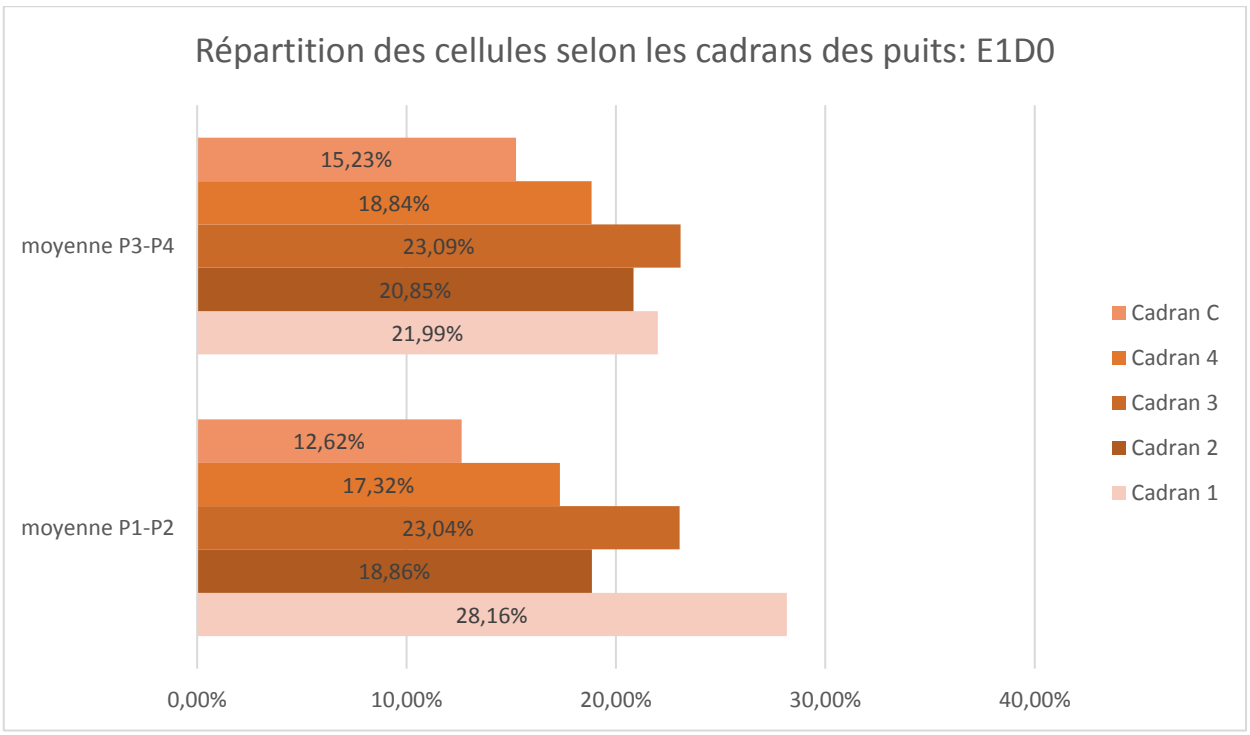



*Figure 8: Répartition des cellules selon les cadrans (EXP 1, Jour de l'expérience (D0)), P1 et P2 sont les puits contenant les gels de HA 0,8% et P3-P4 sont les gels avec du HA 1%.*