UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Ciencias Biológicas y Ambientales

Histological evaluation of closing methods and regenerative agents in wound healing, a preclinical model in mice

.

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RESUMEN

La piel es el órgano más extenso del cuerpo y multifacético del cuerpo. Cuando la piel sufre un daño, se desencadena un proceso de regeneración inmediato. Cualquier alteración en el proceso de curación aumenta el riesgo de infección, desarrollo de ulceras crónicas y el riesgo de amputación. El objetivo de este proyecto es evaluar y comparar el grado histológico de la cicatrización de heridas por incisión utilizando métodos de cierre comunes vs agentes terapéuticos regenerativos. Aprobación del Comité de Bioética de la USFQ numero: 2020-001. Se realizó dos cortes de 1cm en el dorso de cada ratón, uno se cerró usando un punto simple con hilo de sutura Monocryl® y el otro usando el adhesivo tisular Histoacryl®. Para los agentes regenerativos se realizó un corte en el dorso de cada ratón y se aplicó 4 concentraciones de Células Madre mesenquimales (CMMs) (Control, $2.5x10^4$, $5x10^5$, $1x10^6$ células) y 4 concentraciones de un mix de mitocondrias alogénico proveniente de varios donadores (primary allogeneic mitochondrial mix, PAMM) (Control, 25, 50 y 100 ng/uL). A las 48 horas se tomó una biopsia de 1cm2, se preparó la placa histológica con tinción hematoxilina eosina (H&E) y tinción tricrómica de Masson. Se puntuó cada muestra de acuerdo con el grado de regeneración establecido en el índice de regeneración (Wound Healing Index, WHI). Se realizó la prueba de Kruskal-Wallis y la prueba de Mann Whitney. Se encontró que no existe diferencia entre el uso de Monocryl® e Histoacryl® por lo que ambos pueden ser usados en roedores pequeños. Se encontró diferencia significativa entre el punto simple y las concentraciones de $2.5x10^4$ CMMs, $1x10^6$ CMMs, 25 ng/uL y 100 ng /uL de mitocondrias donde los agentes terapéuticos tuvieron una mayor puntuación. Los agentes terapéuticos promueven la cicatrización de heridas dado que pueden interactuar con el medio de la herida y además secretan y promueven la secreción de compuestos que contribuyen a la regeneración. Además, se encontró que las mitocondrias alogénicas mejoran la homeostasis de las células dañadas al participar en sus procesos bioenergéticos y acelerar la regeneración.

Palabras Clave: Regeneración de heridas, sutura tradicional, adhesivo tisular, Células madre mesenquimales (CMMs), Primary allogeneic mitochondrial mix (PAMM), ratones

ABSTRACT

The skin is the body's largest and most multifaceted organ. When the skin is damaged, an immediate regeneration process is triggered. Any alteration in the process prolongs wound healing and increases the risk of infection, development of chronic ulcers and amputations. The objective of this project is to evaluate and compare the histologic extent of incisional wound healing using common closure methods vs. regenerative therapeutic agents. Bioethical Committee of the USFQ approval number: 2020-001. Two 1cm cuts were made on the dorsum of each mouse, one was closed using a single stitch with Monocryl® suture and the other using Histoacryl® tissue adhesive. For the regenerative agents, a single cut was made on the dorsum of each mouse and 4 concentrations of Mesenchymal Stem Cells (MSCs) (Control, $2.5x10^4$, $5x10^5$, $1x10^6$ MSCs). and 4 concentrations of PAMM (Control, 25, 50 and 100 ng/uL) were applied. At 48 hours after the surgical procedure a $1cm^2$ biopsy was taken, and the histological plate was prepared with hematoxylin eosin (H&E) staining and Masson's trichrome staining. Each sample was scored according to the degree of regeneration established in the regeneration index (WHI). The Kruskal-Wallis nonparametric test and the Mann Whitney test were used to analyze results $\alpha = 0.05$. It was found that there is no difference between the use of Monocryl® and Histoacryl®, both can be used for closing wounds in small rodents. Significant difference was found between single point suture and $2.5x10^4$ MSCs, $1x10^6$ MSCs, 25 ng/uL and 100 ng /uL concentrations of mitochondria where the therapeutic agents scored higher. We concluded that therapeutic agents promote wound healing since they can interact with the wound medium and secrete and promote the secretion of compounds that contribute to regeneration. Also, it was found that allogeneic mitochondria improve the homeostasis of damaged cells by participating in its bioenergetic processes and accelerating regeneration.

Key Words: Wound healing, traditional suture, tissue adhesive, MSCs, PAMM, mice

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1. INTRODUCTION

1.1. Wound closure and the skin

Wound closure is a crucial step in the healing of incisional wounds. Healing has direct implications in healthcare and cosmetic research since the main objective is to close the incision in an aesthetic and functional manner. However, there are local and systemic factors that can impede proper healing. For example: age, diseases such as diabetes, obesity or genetic disorders, tobacco, alcohol consumption and more. Additionally, chronic injuries such as advanced grade burns or deep cuts are predisposed to abnormal healing with long-term sequelae (Rodrigues, Kosaric, Bonham, & Gurtner, 2019). In the United States, the treatment of chronic wounds due to healing complications can reach up to $25 billion annually for the healthcare system. A non-healing wound or an infected wound entails negative health consequences for patients. It also leads to economic and social problems since poor healing can trigger loss of mobility and the ability to perform daily activities, implying a loss of participation in the workforce and a challenging quality of life (Ellis et al., 2018). Also, conditions such as advanced grade burns or diabetic foot ulcers mandatorily require therapies that promote healing of injuries since it is very difficult for wounds to heal on their own (Robson, Hill, Woodske, & Steed, 2000). Therefore, much attention is paid to wound healing and new therapies to accelerate the process to obtain functional and aesthetic results. The aim of this project is to analyze the histological level of surgical wound regeneration and the application of innovative therapies to boost this process.

The skin is the largest organ in the body, it participates in various regulatory processes such as hydration and temperature regulation (Tottoli et al, 2020). The skin provides a physical barrier between the internal body and external environment against pathogens, eternal fluids, chemicals and physical trauma (Cañedo-Dorantes & Cañedo-Ayala, 2019). Also, it is involved in endocrine functions such as vitamin D production and exocrine functions by the way of sweat and sebaceous glands (Yousef & Sharma, 2018). The skin is composed of three layers: the epidermis, dermis,

and the hypodermis (Yousef & Sharma, 2018). The epidermis is the external layer of the skin, it is composed of five layers which include: the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. The cells found in the epidermis are keratinocytes, melanocytes, dendritic cells, Merkel's cells, Langerhans cells and other immune and nervous system cells. The dermis is connected to the epidermis, it has two layers: the papillary and the reticular layer which are mostly composed of skin appendages, mast cells, fibroblasts, antigen presenting dermal cells, resident and circulating immune cells. The deepest layer, the hypodermis is composed of adipose lobules with skin appendages such as hair follicles, sensory neurons, and blood vessels (Cañedo-Dorantes & Cañedo-Ayala, 2019; Yousef & Sharma, 2018).

1.2. Wound healing

Due to the complex structure of the skin, wound healing is a sophisticated mechanism that has been studied for a long time in different models. Molecular processes in wound healing are not completely understood as it involves dynamically the interaction of cells, growth factors, cytokines and other complex processes (Zomer & Trentin, 2018). The immune system plays a fundamental role in wound healing, the process of wound healing includes four steps: hemostasis which takes plays immediately after injury, inflammation from 1 to 3 days after, proliferation and repair from 2 to 21 days, and wound recovery from 21-365 days. However, any disturbance in the process could make the healing longer and rises the risk of infection, development of chronic ulcers and in worst cases amputation (Ellis, Lin, & Tartar, 2018). Also, in almost all recovered wounds, skin homeostasis is achieved but a loss of functionality can be found (Zomer & Trentin, 2018).

1.3. Wound healing in mice and humans

Small mammals such as mice, rabbits, guinea pigs and rats are commonly used in wound healing studies because it is inexpensive and easy to manipulate. Even though the first layers of the skin in humans and small mammals have similar cells, anatomical and physiological differences are

found (Sullivan, Eaglstein, Davis, & Mertz, 2001). In mice (*mus musculus*) and humans' differences in the wound healing process are found related to body size, life expectancy, layers thickness, cells type and numb, immune profile, gene expression of cells in the wound, and the presence of congenital or acquired disease diseases determine wound healing process (Zomer & Trentin, 2018). Even if the murine skin is not similar to human skin, ethical animal experimentation allows researchers to understand the process of wound healing and its interaction with regenerative therapies (Grada, Mervis, & Falanga, 2018; Zomer & Trentin, 2018).

1.4. Closing methods and regenerative agents in wound healing

The most common closure methods are traditional sutures or staples. These techniques can induce granulomas, wound dehiscence, hemorrhage, fistulas, loose stitches, ischemia, and necrosis. Furthermore, these traditional wound closing techniques are commonly associated with unaesthetically scarring and bacterial infections (Shekho, Humadi, & Basim, 1994). Nowadays, suturing materials are also under research to achieve an aesthetical scar formation. For example, new biodegradable sutures with drug delivery systems offer great advantages to the healthcare system and patients as they can deliver antibacterial and anti-inflammatory agents (Joseph, George, Gopi, Kalarikkal, & Thomas, 2017).

Other closing methods are tissue adhesives such as cyanoacrylates and hydrogels. Tissue adhesives can bond soft and hard tissues such as mucosa, endothelium, skin, blood and bone (Singh, Degala, Shetty, Rai, & Das, 2019). Cyanoacrylates (Histoacryl®) have shown antibacterial and bacteriostatic properties, good biocompatibility, and fast polymerization in animal models. Unfortunately, its toxicity and carcinogenic potential remains a controversial debate (Borie et al., 2019). Hydrogels on the other side can keep the wound moisturized and its structure similar to the native extracellular matrix (ECM) can provide a barrier against bacteria (Hu & Xu, 2020). Multifunctional hydrogels have a good biocompatibility, good mechanical characteristics, antibacterial, antioxidant and bio adhesive properties (Liang, He, & Guo, 2021).

Also, hydrogels can be composed of a wide range of materials such as polysaccharides and substances that can be suitable to specific wounds (Asadi et al., 2021). Even though both methods have multiple advantages and promising applications, manufacturing costs and industrial production are important constraints (Hu & Xu, 2020).

Regenerative medicine has an important therapeutic potential in wound healing. Therapies such as skin substitutes and scaffolds, advanced dressings, stem cell-derived products and bioactive factors are now catching the researcher's attention. Stem cells, growth factors, autologous cells, gene therapy and tissue engineering can be used to accelerate and promote wound healing (Tottoli et al., 2020). The epithelium of the skin is in constant self-renewal, when the skin is injured, a natural response is displayed to heal the wound. However, under certain conditions such as diabetes or severe burns the natural repair process is not enough (Kanji & Das, 2017).

Stem Cells and Stem Cell-derived products have an advantage over other wound closing methods as stem cells can interact with the wound environment and induce a natural response. Hence, exogenous application of stem cells or its derived products represent a promising alternative to close wounds (Kanji & Das, 2017). Even though advanced therapeutic agents and regenerative medicine have shown optimistic results in clinical and preclinical models, clinical validation is still a limitation. More studies and research in this field is needed to encourage the application of regenerative agents in wound healing. As already mentioned, wound healing can be limited under certain local and systemic conditions. The consequences of poor wound healing or wound infection can be devastating to the patient's quality of life (Rodrigues, Kosaric, Bonham, & Gurtner, 2019). In addition, there are other health conditions that demand the application of therapies to achieve wound regeneration and functional healing.

2. METHODOLOGY

2.1. Ethical Statement

The approval of the project was obtained by The Bioethics Committee for the use of animals in research and teaching of the School of Veterinary Medicine of the Universidad San Francisco de Quito, USFQ. Approval number: 2020-001. The principles of the 3Rs (Replacement, Reduction and Refinement) in animal experimentation were considered to develop the preclinical study design in mice.

2.2. Biological repetitions

The number of biological repetitions was established according to similar studies, considering the reduction criteria for animal experimentation and ethical recommendations of the Bioethics Committee for the use of animals in research and teaching of the School of Veterinary Medicine of the USFQ.

2.3. Histoacryl® and Monocryl® essays

A total of 12 mice were used for the histological analysis of tissue adhesive (Histoacryl®) and traditional suture poliglecaprone (Monocryl®). Four assays were performed, in each assay three mice were used.

2.4. MSCs and PAMM essays

For MSCs three essays were performed to evaluate four conditions (Control, $2.5x10^4$, $5x10^5$, $1x10^6$ MSC). In each essay 12 mice were used.

A preliminary assay was performed with PAMM to evaluate four conditions (Control, 25, 50 and 100ng/ul). The preliminary essay was repeated two times and the optimum concentration of mitochondria was established at 25 ng/ul as higher concentrations showed higher levels of inflammation. One more essay was performed with this condition. A total of 26 mice were used.

2.5. Housing

Mice were placed in their beds in the USFQ biotherium before surgery with food and water. The diet was based on rodent food and water consumption was ad libitum before and after surgery.

2.6. MSC's and mitochondria extraction

MSC were collected from mice skin at a concentration of $2.5x10^4$, $5x10^5$, $1x10^6$ MSCs. For the Primary allogeneic mitochondrial mix, mitochondria of fibroblast and MSCs were extracted and standardized at a concentration of 25, 50 and 100 ng/uL.

2.7. The incision

Before surgery, each mouse was weighed and anesthetized with xylazine 0.5mg/kg + ketamine 80 mg/kg. For evaluation, two cuts of 1cm were performed in the dorsal skin of each mouse. One cut was bonded by Monocryl® and the other cut was bonded with Histoacryl®. For the PAMM application, a cut of 1 cm was performed in the dorsal skin of each mouse. Then, 50uL of the PAMM was injected in three areas at each side of the wound separated by 20mm within them. After surgery, analgesics were provided in the water to decrease the pain.

2.8. Histological Sample Procedure

All mice are euthanized after 48 hours of the surgical procedure and a histological evaluation of the degree of tissue repair was done. For both groups, biopsies of 2 cm2 of each mouse were collected for each cut. The sample processing protocol was established by two pathological anatomy experts. Biopsies were immediately fixed with formaldehyde at 10%. After 24 hours, the macroscopic procedure was performed. First measurements were taken from the samples and cuts were made perpendicular to the major axis with the wound in the center, then the tissue was processed by the tissue processor Leyca TP1020 and Shandon HistocentreTM 3, then cut by a microtome Leyca RM2155 and stained with trichrome, hematoxylin and eosin.

2.9. Histological Analysis

The histological wound evaluation and healing process protocol used in the study was established by two histopathological experts. A histological parameter was established to punctuate the

process of wound healing, with a score of 16 to 19 for a good healing, 12 to 15 for a moderate healing and 8 to 11 for a poor healing. The Wound Healing Index is presented in Table 1.

2.10. Statistical Analysis

In the statistical analysis, a Kruskal-Wallis Test and Mann Whitney Test was made using GraphPad Prism software.

3. RESULTS

The objective of this project is to evaluate and compare the histological level of surgical wound healing using traditional sutures (Monocryl®), tissue adhesive (Histoacryl®), mice MSC's (Control, $2.5x10^4$, $5x10^5$, $1x10^6$ MSC) and PAMM (Control, 25, 50 and 100 ng/uL). The histological evaluation of each wound was scored according to the parameters of the WHI observed at 10X and 40X magnification with H&E and Masson's trichrome staining. In Figure 1a the WHI of the simple suture and tissue adhesive reached a score of 12.5 13 respectively. In Figure 1d it is presented the overall results where the simple suture reached a score of about 12.5, medio reached a score of 13, the MSCs concentrations of $2.5x10^4$, $5x10^5$, $1x10^6$ MSC a score of about 14, 13 and 15 respectively and the mitochondria concentrations of 25, 50 and 100 ng/uL a score of 15, 14 and 15 respectively. $1x10^6$ MSCs, 25 and 100 ng/uL of mitochondria had the higher WHI score.

3.1. Nonparametric analysis

For Figure 1a, first a Shapiro Wilk nonparametric analysis with a confidence level of $\alpha = 0.05$ was performed to evaluate Monocryl® and Histoacryl®, it was found that the data do not follow a normal distribution $p = 0.006$. For Figure 1b, c and d, a Kruskal Wallis nonparametric analysis was performed with a $\alpha = 0.05$ and it was found that the data do not follow a normal distribution $p = 0.001$.

3.2. Non-Parametric Mann Whitney test analysis

The Mann-Whitney mean difference test was used with a confidence level of $\alpha = 0.05$ and it was found that there is no difference in WHI between Monocryl® and Histoacryl® $p = 0.74$. Monocryl® achieved a score of about 12.5 and Histoacryl® a score of 13.

For regenerative agents, it was found that there is significant difference for a confidence level of $\alpha = 0.05$ between the single point and $2.5x10^4$ MSCs with a WHI score of about 12.5 and <15 ($p = 0.0047$). Also, significant difference was found to exist for a confidence level of $\alpha = 0.05$ between the single point and $1x10^6$ MSCs with a WHI score of 12.5 and 15 approximately ($p = 0.0139$). However, no difference was found for a confidence level of $\alpha = 0.05$ between the single point and 500 000 000 MSCs ($p=0.1172$). Regarding mitochondria concentrations, it was found that there is significant difference for a confidence level of $\alpha = 0.05$ between the single point and 25 ng/uL Mito with a WHI score of 12.5 and <15 respectively ($p=0.0001$). Also, significant difference was found to exist for a confidence level of $\alpha = 0.05$ between the single point and 100 ng/uL Mito with a WHI score of 12.5 and >15 respectively ($p = 0.0290$).

4. DISCUSSION

4.1. Histology

Under normal conditions, immediately after wound formation, the skin repair process starts. During homeostasis, the bleeding is stopped by a clot formation led by blood platelets, then inflammation and swelling start and a release of chemoattractant and inflammatory cytokines is produced. The injured cells promote the infiltration of inflammatory cells and leukocytes, neutrophils, and macrophages to prevent infection. At the same time, wound contraction and fibroplasia start with fibroblast proliferation and differentiation and the formation of the extracellular matrix and granulation tissue formation (Guillamat-Prats, 2021). Fibroblasts release growth factors, interleukins, and cytokines such as Interleukin 1 (IL-1), platelet-derived growth factor (PDGF), transforming growth factor beta TGF-β1, tumor necrosis factor alpha (TNF-a), epidermal growth factor (EGF) and others which activates fibroplasia among other processes of the immune response. Then, the angiogenesis, endothelial cell proliferation, nerve repair, regeneration and reinnervation start. (Cañedo-Dorantes & Cañedo-Ayala, 2019). And, in the last phase of wound healing, granulation tissue formation, endothelial cells and vascular components decrease, the extracellular matrix starts the maturing process until a restored skin integrity is achieved (Guillamat-Prats, 2021).

In histology, dyes are usually used to contrast and differentiate structures in the sample. H&E and Masson's Trichrome staining are useful to visualize key wound healing components such as collagen, structures, measure angiogenesis, re epithelization, granulation tissue formation connective tissue synthesis and scar formation (Cardiff, Miller, & Munn, 2014; Fischer, Jacobson, Rose, & Zeller, 2008). Masson's Trichrome stains young and mature collagen, collagen deposition and granulation tissue formation. Both stains are complementary in wound healing evaluation, due to the complex processes and multiple cell infiltration that display in this. Another dye that can be included in this assessment is Red Syrius which allows to visualize collagen and collagen

level of organization, this could provide better punctuation in analysis of later time points. See Fig.2.

4.2. Effect of the MSCs and Mitochondria in wound repair

A higher WHI punctuation was obtained for wounds with $2.5x10^4$ and $1x10^6$ MSCs and 25 and 100 ng/uL compared to those closed with sutures and adhesive tissue. MSCs are stromal stem cells that can secrete various agents with therapeutic potential. They can produce chemokines, cytokines, growth factors and molecules that participate in the extracellular matrix formation. During wound healing, MSCs secrete signals involved in healing, tissue regeneration, proliferation, and differentiation. Therefore, the use of allogeneic MSCs in wound healing can improve this process as external stem cells can interact with the wound environment. Several studies have shown that the use of MSCs participates and stimulates each step of the wound repair process (Dash et al, 2009; Lei et al, 2018; Mazini, 2020). In homeostasis, it has been seen that they can promote coagulation by the expression of phosphatidylserine and tissue factor which prompts a thrombotic response (Guillamat-Prats, 2021). In the inflammatory phase, administered MSCs can migrate to the damaged sites of the wound and moderate the leukocyte response. Additionally, the secretion of factors can regulate the immunological response, promote proinflammatory factors and reduce inflammation which leads to a faster regeneration (Mazini, Rochette, Admou, Amal, & Malka, 2020).

On the other hand, a smaller concentration of mitochondria (25ng/uL) showed the same WHI punctuation of higher cellular concentrations of MSC. Naturally, mitochondria transfer occurs from healthy cells to damaged cells trough tube formation, micro vessels, cell fusion or isolated mitochondria incorporation, this can modulate cellular bioenergetics and maintain mitochondrial number to achieve homeostasis (Paliwal, Chaudhuri, Agrawal, & Mohanty, 2018). During the oxidative phosphorylation of mitochondria, a partial reduction of oxygen and reactive oxygen species (ROS) occurs, consequently inflammation is reduced and this can promote the healing

process (Cano Sanchez, Lancel, Boulanger, & Neviere, 2018). The introduction of allogenic mitochondria to the wound may boost native cells to induce the healing process. Other studies using external electrical stimulation have also shown faster and smoother wound healing (Kai et al., 2017). Therefore, stimulation by external or internal electrical supply can improve the redox based reaction that occurs in wound healing.

There are some limitations related to the administration of MSCs, some studies have reported an induced thrombosis due to an overexpression of the allogeneic MSCs, this can be explained by the little or no control over the cell quality that enters the wound. Also, the isolation process of MSCs is invasive and under *in vitro* conditions it presents a gradual loss of potency. Additionally, under stress conditions almost 99% of the MSCs result in death, therefore huge amounts of cells are needed to achieve positive results (Guillamat-Prats, 2021; Nour et al., 2019). These disadvantages could be overcome using new therapeutic agents such as mitochondria which has shown promising applications as therapeutic agents as it can promote cells in wounds to achieve faster homeostasis.

4.3. Closing methods vs therapeutic agents in mice

Figure 1a showed no difference in the WHI between traditional closing methods Monocryl® and Histoacryl. It is well known that the use of tissue adhesives are less invasive closing techniques than traditional sutures or staples. The approximation of incisional wounds can relieve the tension and ease healing. Studies in guinea pigs have shown that silk sutures promote wound healing. Other studies performed in dogs using subdermal plexus skin flaps secured with sutures and cyanoacrylates showed that cyanoacrylates allowed a good healing and outcome of the wounds. Results suggest that both methods can be used to close wounds in small mammals (Villagomez et al., 2021). Even though these techniques are commonly used, it is time to improve and overcome its disadvantages with new active or therapeutic agents. Results (Figure 1d) showed significant differences between closing methods, MSCs and mitochondria. The use of therapeutic agents can

improve and accelerate wound healing processes in small mammals. Also, it could be interesting to evaluate combined methods such as cyanoacrylate with therapeutic agents to provide a physical and biological boost in skin repair. Therapies such as active hydrogels with active compounds showed an inflammatory relief and promotion of stem cell proliferation (Bai et al., 2020). Hence, the use of therapeutic agents combined with other novel methods such as active dressing could better overcome wound healing and reduce the risk of infection in acute and chronic wounds.

4.4. Other animal models for wound healing

Multiple in vitro and in vivo wound healing models have been described in medical literature. Mice models are cost effective, easy to maintain, rapid to reproduce and can present human pathological conditions and genetic mutations. Hence, mice models are extensively used to develop new therapies. There is still a drawback in the ability to predict how wound healing treatment would be in humans. Human studies are difficult to perform as there are multiple variables related to the wound characteristics and the patient's medical history (Sullivan et al., 2001). To identify enough patients to perform a complete randomized trial is challenging and the need for biopsies and control samples such as an untreated wound are limiting human studies due to ethical considerations (Harn et al., 2019; Sullivan et al., 2001).

Animal models are good candidates for experimentation under animal welfare standards and replacement, reduction, and refinement principles (Rodan & Heath, 2016). Hence, good results in animal experimentation encourage researchers to make more investigations to achieve clinical validation (Sami, Heiba, & Abdellatif, 2019). Also, to identify molecular pathways in small mammal’s models, non-human primates and primates can provide information to compare and further understand how wound healing converge and diverge among species. As a solution to overcome these limitations, other animal models can be used such as the pig. The porcine skin is more similar to human skin at an anatomical and physiological level which suggests it would be an excellent animal model for wound healing (Sullivan et al., 2001).

5. CONCLUSIONS

- No difference was found between the use of suture Monocryl® and adhesive tissue Histoacryl, this suggests that both closing methods can be used in small mammals.
- Wounds treated with $2.5x10^4$ and $1x10^6$ MSCs showed a higher skin restoration compared to Monocryl®. This can be explained by the nature of MSCs and its ability to interact with the wound and secrete several factors that can improve and accelerate wound healing.
- Mitochondria have shown the same grade of regeneration of $2.5x10^4$ and $1x10^6$ MSCs. This demonstrates that the power cellhouse can also interact with the environment of the injury. The introduction of allogenic mitochondria improves the homeostasis of damaged cells by participating in its bioenergetic processes and influence in wounds.
- New therapeutic agents can improve and accelerate wound healing process compared to traditional closing methods such as sutures and adhesive tissue. Further analysis should be done to further understand the interaction of mitochondria, the injury, and its healing process, for example essays in bigger mammals and molecular analysis.
- This preclinical model shows promising histological evaluation results for the use of therapeutic agents in wound healing, this encourages researchers to achieve clinical evidence of the efficacy and safety of this s regenerative therapy.

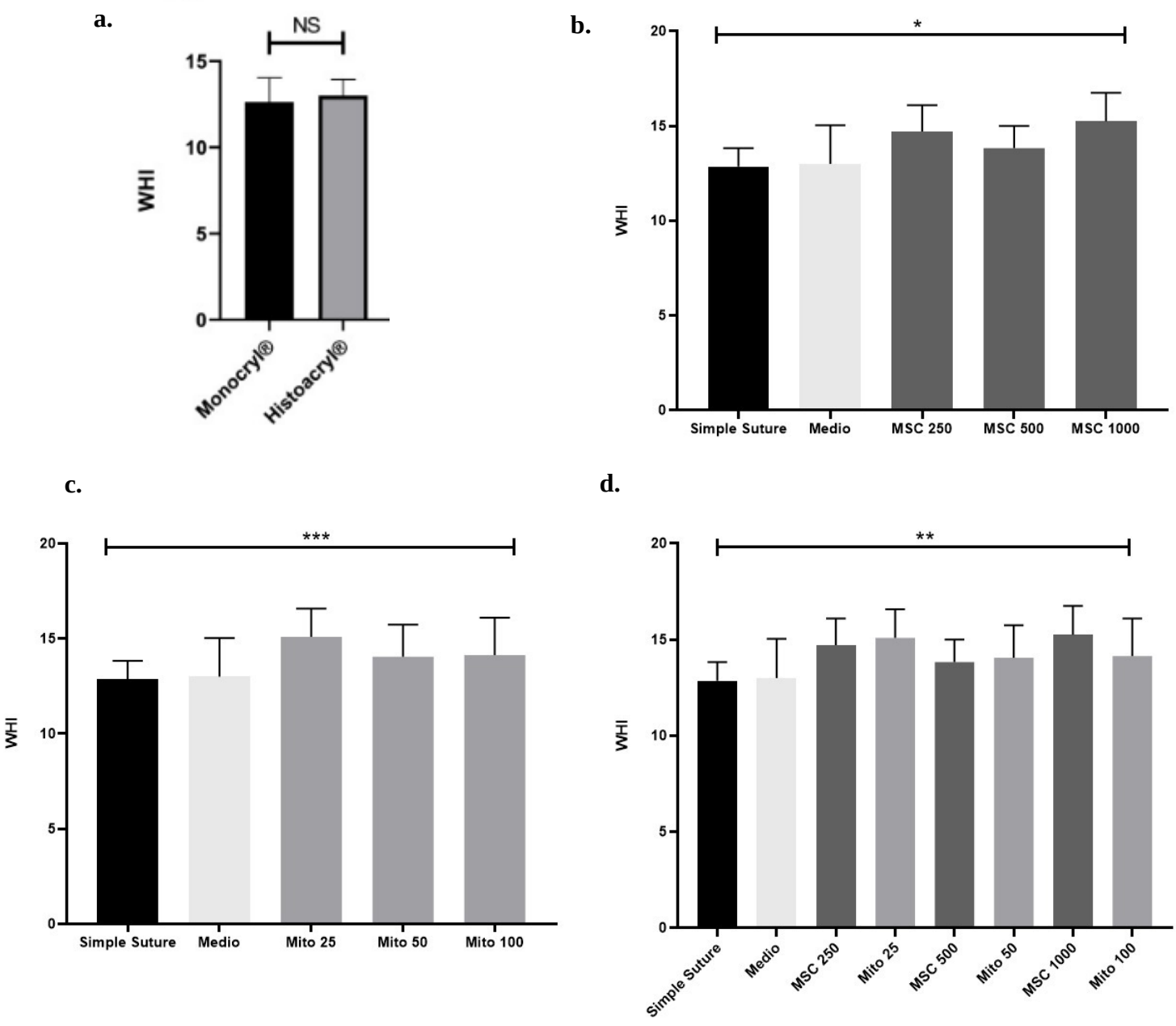
6. TABLES

6.1. Table 1. Wound Healing Index (WHI).

5 Histological Parameters are presented: granulation tissue, inflammatory infiltrate, collagen pattern, amount of early collagen and amount of mature collagen. Each parameter with a design score to punctuate the level of regeneration.

Wound Healing Index (WHI)		
	Histological Parameters	**Score**
1	Granulation Tissue	1 Profound 2 Moderate 3 Scarce 4 Absent
2	Inflammatory infiltrate	1 Profound 2 Moderate 3 Scarce
3	Collagen pattern	1 Reticular 2 Mixed 3 Fascicular
4	Amount of early collagen	1 Profound 2 Moderate 3 Scarce 4 Absent
5	Amount of mature collagen	1 Profound 2 Moderate 3 Scarce

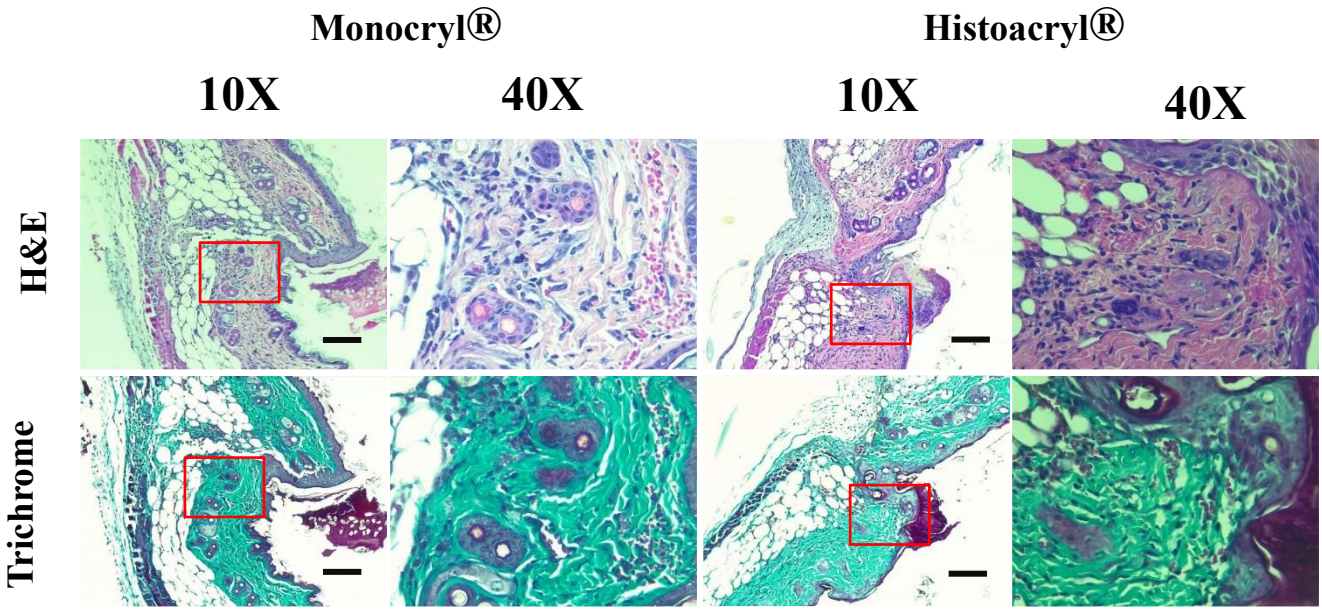
7. FIGURES



7.1. **Fig. 1.** Wound Healing Index (WHI) and 48h Histological analysis of murine skin treated with Histoacryl®, Monocryl®, MSCs and mitochondria, stained with hematoxylin and eosin (H&E).

a. Monocryl® and Histoacryl® data did not show a normal distribution, no differences among treatments were found (Shapiro-Wilk test $\alpha = 0.05$, Monocryl® $p = 0.045$, Histoacryl® $p = 0.006$; Mann-Whitney test $\alpha = 0.05$, $p = 0.74$). **b.** The evaluated data did

not show a normal distribution (Kruskal Wallis test α = 0.05; p = 0.001). The analysis of the non-parametric Mann-Whitney test resulted in no significant differences to conditions MSC 500 and Simple Suture (p = 0.1172); significant differences were found between conditions MSC 250 and simple suture (p = 0.0047); and MSC 1000 and Simple Suture (p = 0.0139). **c**. The evaluated data did not show a normal distribution (Kruskal Wallis test α = 0.05; p = 0.001). The Mann- Whitney test resulted in significant differences to conditions Mito25 and Simple Suture (p < 0.0001); and Mito100 and Simple Suture (p = 0.0290). **d.** The evaluated data did not show a normal distribution (Kruskal Wallis test α = 0.05; p = 0.001). Mann-Whitney test analysis in b and c.



7.2. **Fig.2.** Representative images of Monocryl® and Histoacryl samples at 10X and 40X magnification stained with H&E and Masson's Trichrome.

Images of the stained tissue samples with H&E and Masson's Trichrome, fixed with 10% buffered formalin. To analyze differences, two images' magnifications were taken (10X and 40X, 250µm scale). The red square in each image represents the 40X magnification of each

sample. In both images, similar parameters were observed among conditions and samples. No evident differences were found among the two staining's.

8. BIBLIOGRAPHY

Asadi, N., Pazoki-Toroudi, H., Del Bakhshayesh, A. R., Akbarzadeh, A., Davaran, S., & Annabi, N. (2021). Multifunctional hydrogels for wound healing: Special focus on biomacromolecular based hydrogels. *International Journal of Biological Macromolecules, 170*, 728–750. https://doi.org/10.1016/j.ijbiomac.2020.12.202

Bai, H., Kyu-Cheol, N., Wang, Z., Cui, Y., Liu, H., Liu, H., … Li, Z. (2020). Regulation of inflammatory microenvironment using a self-healing hydrogel loaded with BM-MSCs for advanced wound healing in rat diabetic foot ulcers. *Journal of Tissue Engineering*, *11*, 2041731420947242. https://doi.org/10.1177/2041731420947242

Borie, E., Rosas, E., Kuramochi, G., Etcheberry, S., Olate, S., & Weber, B. (2019). Oral applications of cyanoacrylate adhesives: A literature review. *BioMed Research International*, *2019*, 8217602. https://doi.org/10.1155/2019/8217602

Cano Sanchez, M., Lancel, S., Boulanger, E., & Neviere, R. (2018). Targeting oxidative stress and mitochondrial dysfunction in the treatment of impaired wound healing: A systematic review. *Antioxidants (Basel, Switzerland)*, *7*(8). https://doi.org/10.3390/antiox7080098

Cañedo-Dorantes, L., & Cañedo-Ayala, M. (2019). Skin acute wound healing: A comprehensive review. *International Journal of Inflammation*, *2019*, 3706315. https://doi.org/10.1155/2019/3706315

Cardiff, R. D., Miller, C. H., & Munn, R. J. (2014). Manual hematoxylin and eosin staining of mouse tissue sections. *Cold Spring Harbor Protocols*, *2014*(6), 655–658. https://doi.org/10.1101/pdb.prot073411

Dash, N. R., Dash, S. N., Routray, P., Mohapatra, S., & Mohapatra, P. C. (2009). Targeting nonhealing ulcers of lower extremity in human through autologous bone marrow-

derived mesenchymal stem cells. *Rejuvenation Research*, *12*(5), 359–366. doi: 10.1089/rej.2009.0872

Ellis, S., Lin, E. J., & Tartar, D. (2018). Immunology of wound healing. *Current Dermatology Reports*, *7*(4), 350–358. https://doi.org/10.1007/s13671-018-0234-9

Fischer, A. H., Jacobson, K. A., Rose, J., & Zeller, R. (2008). Hematoxylin and eosin staining of tissue and cell sections. *CSH Protocols*, *2008*, pdb.prot4986. https://doi.org/10.1101/pdb.prot4986

Grada, A., Mervis, J., & Falanga, V. (2018). Research techniques made simple: animal models of wound healing. *The Journal of Investigative Dermatology*, *138*(10), 2095-2105.e1. https://doi.org/10.1016/j.jid.2018.08.005

Guillamat-Prats, R. (2021). The role of MSC in wound healing, scarring and regeneration. *Cells*, *10*(7). https://doi.org/10.3390/cells10071729

Harn, H. I.-C., Ogawa, R., Hsu, C.-K., Hughes, M. W., Tang, M.-J., & Chuong, C.-M. (2019). The tension biology of wound healing. *Experimental Dermatology*, *28*(4), 464–471. https://doi.org/10.1111/exd.13460

Hu, H., & Xu, F.-J. (2020). Rational design and latest advances of polysaccharide-based hydrogels for wound healing. *Biomaterials Science*, *8*(8), 2084–2101. https://doi.org/10.1039/d0bm00055h

Joseph, B., George, A., Gopi, S., Kalarikkal, N., & Thomas, S. (2017). Polymer sutures for simultaneous wound healing and drug delivery - A review. *International Journal of Pharmaceutics*, *524*(1–2), 454–466. https://doi.org/10.1016/j.ijpharm.2017.03.041

Kai, H., Yamauchi, T., Ogawa, Y., Tsubota, A., Magome, T., Miyake, T., … Nishizawa,

M. (2017). Accelerated Wound Healing on Skin by Electrical Stimulation with a Bioelectric Plaster. *Advanced Healthcare Materials*, *6*(22). https://doi.org/10.1002/adhm.201700465

Kanji, S., & Das, H. (2017). Advances of stem cell therapeutics in cutaneous wound healing and regeneration. *Mediators of Inflammation*, *2017*, 5217967. https://doi.org/10.1155/2017/5217967

Lei, Z., Singh, G., Min, Z., Shixuan, C., Xu, K., Pengcheng, X., … Lin, Z. (2018). Bone marrow-derived mesenchymal stem cells laden novel thermo-sensitive hydrogel for the management of severe skin wound healing. *Materials Science & Engineering. C, Materials for Biological Applications*, *90*, 159–167. doi: 10.1016/j.msec.2018.04.045

Liang, Y., He, J., & Guo, B. (2021). Functional hydrogels as wound dressing to enhance wound healing. *ACS Nano*. https://doi.org/10.1021/acsnano.1c04206

Mazini, L., Rochette, L., Admou, B., Amal, S., & Malka, G. (2020). Hopes and Limits of Adipose-Derived Stem Cells (ADSCs) and Mesenchymal Stem Cells (MSCs) in Wound Healing. *International Journal of Molecular Sciences*, *21*(4). https://doi.org/10.3390/ijms21041306

Nour, S., Baheiraei, N., Imani, R., Khodaei, M., Alizadeh, A., Rabiee, N., & Moazzeni, S. M. (2019). A review of accelerated wound healing approaches: biomaterial-assisted tissue remodeling. *Journal of Materials Science. Materials in Medicine*, *30*(10), 120. https://doi.org/10.1007/s10856-019-6319-6

Paliwal, S., Chaudhuri, R., Agrawal, A., & Mohanty, S. (2018). Regenerative abilities of mesenchymal stem cells through mitochondrial transfer. *Journal of Biomedical*

Science, *25*(1), 31. https://doi.org/10.1186/s12929-018-0429-1

Robson, M. C., Hill, D. P., Woodske, M. E., & Steed, D. L. (2000). Wound healing trajectories as predictors of effectiveness of therapeutic agents. *Archives of Surgery*, *135*(7), 773–777. https://doi.org/10.1001/archsurg.135.7.773

Rodan, I., & Heath, S. (2016). Feline behavior and welfare. In *Feline behavioral health and welfare* (pp. 12–22). Elsevier. doi: 10.1016/B978-1-4557-7401-2.00002-7

Rodrigues, M., Kosaric, N., Bonham, C. A., & Gurtner, G. C. (2019). Wound healing: A cellular perspective. *Physiological Reviews*, *99*(1), 665–706. https://doi.org/10.1152/physrev.00067.2017

Sami, D. G., Heiba, H. H., & Abdellatif, A. (2019). Wound healing models: A systematic review of animal and non-animal models. *Wound Medicine*, *24*(1), 8–17. https://doi.org/10.1016/j.wndm.2018.12.001

Shekho, H. A., Humadi, S. K., & Basim, H. (1994). Cyanoacrylate versus silk suture microscopy of 10- and 15-day skin healing in sheep.

Singh, P. K., Degala, S., Shetty, S., Rai, V. S., & Das, A. (2019). To Evaluate the Efficacy and Effectiveness of N-butyl-2-cyanoacrylate glue (TRU SEAL) in Closure of Oral and Maxillofacial Laceration and Surgical Incisions. *Journal of Maxillofacial and Oral Surgery*, *18*(1), 131–138. https://doi.org/10.1007/s12663-018-1111-6

Sullivan, T. P., Eaglstein, W. H., Davis, S. C., & Mertz, P. (2001). The pig as a model for human wound healing. *Wound Repair and Regeneration*, *9*(2), 66–76. https://doi.org/10.1046/j.1524-475x.2001.00066.x

Tottoli, E. M., Dorati, R., Genta, I., Chiesa, E., Pisani, S., & Conti, B. (2020). Skin wound healing process and new emerging technologies for skin wound care and

regeneration. *Pharmaceutics*, *12*(8). https://doi.org/10.3390/pharmaceutics12080735

Villagomez, A., Borja, T., Pontón, P., Segnini, G., Barba, P., Chiliquinga, A., … Caicedo, A. (2021). Histological and microbiological evaluation of surgical wound closure in mouse skin with cyanoacrylate (Histoacryl®) in comparison to poliglecaprone (Monocryl®) traditional suture. *Veterinary and Animal Science*, 100180. https://doi.org/10.1016/j.vas.2021.100180

Yousef, H., & Sharma, S. (2018). Anatomy, skin (integument), epidermis. In *StatPearls*. Treasure Island (FL): StatPearls Publishing.

Zomer, H. D., & Trentin, A. G. (2018). Skin wound healing in humans and mice: Challenges in translational research. *Journal of Dermatological Science*, *90*(1), 3–12. https://doi.org/10.1016/j.jdermsci.2017.12.009