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Do Bioactive Sutures Improve Wound Healing?

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ABSTRACT OF THE THESIS

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Suturing is a primary technique for wound closure, and it is used to keep body tissues together after injuries or surgeries. There is a wide variety of suturing including skin suturing, blood vessel suturing and tendon suturing. Despite of new developments and suture modifications, the outcome of the suturing needs further improvement. One of the challenges impacting the outcomes of suturing is the prolonged inflammatory process of healing when standard of care suturing is used. Tendons, for example, are usually repaired using sutures, and despite new developments and suture modifications, the outcomes of these repairs are not satisfactory as 25% of patients complain about their clinical outcomes and 7.7% need further surgeries as the repairs, re-rupture. It has been shown in variety of studies using histological assessments of repaired tendon, that there is a distinct acellular zone in the tissues surrounding the sutures that could be the cause of the prolonged inflammatory activity leading into the weakening of tendon repair site. The purpose of this article is to focus

primarily on MSC-coated bioactive suture enhancing the process of wound healing by providing sufficient resources to the wounded area. In this article, literature studying the effect of MSCs bioactive sutures on wound healing will be discussed, and further analysis and recommendations for future work will be provided.

1. INTRODUCTION

Suturing is a primary technique for surgical wound closure, and it has been around for thousands of years [1]. The main purposes of suturing are to approximate wound edges and provide mechanical relief to the wound until the healing process is complete. More modern approaches, like glues and unique bandages serve similar functions, but they still rely on the natural healing process at the wound edges for long-term healing [1]. Suturing is used to keep body tissues together after injuries or surgeries, and there are different types of sutures for different body parts, mechanical approximations, and resorbabilities. Skin suturing, blood vessel suturing, and tendon suturing are just a few common types. Tendons, for example, are usually repaired using sutures, and despite new developments and suture modifications, the outcomes of these repairs are not satisfactory as 25% of patients complain about their clinical outcomes and 7.7% need further surgeries as the repairs, re-rupture [24, 25]. As an example, rupture of Achilles tendon is a common type of injury among athletes with recovery periods lasting from 6 to 12 months [30]. The recovery period can be even longer in the case of tendon re-rupture (4 to 13 weeks post operation), with the rate of re-rupture is between 1.7% - 5.6% [26]. The re-tear rate is much higher (25% to 90%) in patients with rotator cuff tears, which also require further surgery [28]. Therefore, new materials and techniques are required to improve the outcomes of tendon suturing, as well as other types of suturing, in terms of increasing the strength of the repair site, reducing healing time, and preventing re-ruptures.

One of the challenges that has not been fully addressed in suturing is the prolonged inflammatory process as compared to normal healing, which results in a delay in recovery period and therefore, delay in the overall repair [33, 35-38]. The normal inflammatory stage begins only a few hours after the onset of the lesion and can remain present up to 6 days [32]. The

inflammatory stage plays an important role in wound healing by contributing to infiltration of the wound site by neutrophils, whose main task is to prevent infection [32, 33, 34]. Since inflammation is activated by the lesion and causes tissue destruction in the early stage facilitated by neutrophils, prolonged activity of neutrophils can increase the inflammatory period, and therefore can delay healing [32, 33]. A delay in this transition, from inflammatory stage to proliferative stage, would result in severe healing disturbances and could result from poor wound debridement, delayed in fibroblast maturation, and delayed angiogenesis which will all lead into a more weakly repaired wound [29, 33, 35-38].

It has been shown in variety of studies using histological assessments of repaired tendons, that there is a distinct acellular zone in the tissues surrounding the sutures that could be the cause of the prolonged inflammatory activity leading to the weakening of tendon repair site [6, 29, 39]. The acellular zone is thought to be caused by mechanical disruption between cells, whereas the cell-to-cell or cell-to-matrix contacts are disrupted by tying of a suture [6, 39]. Through the loss of cell-to-cell contacts in the acellular zone, cells lose their tension. This loss to tenocytes can impact the surrounding matrix dramatically [29]. The cell anchoring will also be disrupted by this process, which causes the cells to migrate away from the site of injury [6]. This acellular zone forms rapidly within 6 hours and can persist for one year with no sign of cellular repopulation of the matrix [29]. While cells are migrating away from the acellular zone, they are being retracted to the surrounding tissues with peak cellularity at 24 hours and remains elevated for up to 84 days [6, 29]. The biphasic cellular response to tissue injury, where there is an acellular region at the suture site and an elevated cell density in the surrounding tissues will result a delay in healing process as a result of the prolonged inflammatory response period in the surrounding tissues.

There are new developments in suturing aimed at enhancing the process of wound healing by providing sufficient resources to the wounded area [2]. Examples of recent approaches include combining the sutures with antimicrobial agents, coating them with insulin-like growth factor, and more recently introducing mesenchymal stem cells to sutures [2,3,4,5]. The purpose of this article is to highlight previous work on suturing aimed at improving the healing process, and to propose new potential techniques that could help reducing inflammation and accelerating healing while maintaining the suture's mechanical strength. Although there are numerous suture developments using MSCs, the main goal of this article is to focus primarily on MSC-coated bioactive suture.

1.1 What are mesenchymal stem cells and their functions?

Mesenchymal stem cells are self-renewing, meaning that they can replicate as undifferentiated cells [11]. This type of cells contributes to the regeneration of mesenchymal tissues such as bone, cartilage, fat, tendon, muscle, and marrow stroma [21]. MSCs have broad anti-inflammatory and immune-modulatory properties, and they can produce and secrete a great variety of growth factors and cytokines [11]. Based on previous studies, MSCs can, directly or indirectly, contribute to healing [16, 17, 21]. The application of MSCs, whether through injection or cell-scaffold construct, has shown promise in accelerated healing, tissue-specific gene expression, and more organized repair structures [14-20]. Although there are variety of techniques to deliver MSCs to the wounded sites, some techniques have shown more promising results than others. The simplest delivery method is by injecting MSCs to the wound site after suturing. In a rotator cuff repair study, while the cells were viable in the repair site, no improvements were detected in tendon structure and mechanical strength when MSCs were injected [44, 45]. This might be due to the fact that the injected cells will not be preserved for the

entire healing process to be complete, or that they are simply not helpful to the healing process. Other techniques such as fibrin glue, collagen gel and coated sutures have shown promise in improving healing and mechanical strength of the repair [44]. For instance, MSCs seeded onto polyglycolic acid (PGA) sheets used to repair rotator cuff defects in a rabbit model showed improvements on mechanical properties of the repair as well as producing greater collagen 1 in the site of injury [44, 46]. Employing suture as a scaffold for MSCs delivery will have the advantage of delivering the viable cells more directly to the site of injury and may be preserved for the entire healing process. In addition, since the cells are already combined with sutures, the suturing will only be a single step procedure [3]. Since introducing MSCs into wounded tendon tissues has shown increases in the initial repair strength of the tissue in rabbit models, we hypothesized that introducing MSCs into the tissue via coated sutures may enhance and accelerate healing process in humans by increasing repair strength [10].

1.2 What does the literature say about MSCs coated sutures?

New sutures, known as bioactive sutures, are being developed to enhance the wound healing process reportedly by releasing collagen content into the wounded area [21, 22]. They may also facilitate accelerating wound healing by delivering various cell lines from the suture to the wounded areas [7]. As mentioned earlier, one of the issues arising from suturing is the formation of the acellular region which causes prolonged inflammation, leading to a delayed healing. Therefore, it is crucial to deliver enough cells to the wounded area to promote healing. There are variety of techniques that have been implemented to incorporate MSCs onto the sutures- MSC bearing, MSC filled and MSC coating suture are just a few techniques [2, 9, 10, 40]. The adherent characteristic of MSCs to plastic surfaces encouraged scientists to coat them on to a special type of sutures, and then observe if they are effective in accelerating the wound

healing processes. For example, Casado et al. determined the most compatible sutures to use as a bioactive suture's base material, which effectively can carry MSCs, and investigated whether pretreated sutures impact cell adhesion [2]. Casado et al. hypothesized that the bioactive suture could play an important role in providing biomechanical support while preventing MSCs from spreading [2]. Firstly, they showed that pretreating sutures with NaOH enhanced the MSCs adhesion capacity significantly employing Hoechst Staining assay. Hoechst staining is a fluorescent dye for fixed and live cell fluorescent staining of DNA and nuclei in cellular imaging technique. [2]. However, this resulted in a weakened suture tensile strength. [2] In this study, murine bone marrow derived MSCs were isolated from femurs of euthanized animals. MSCs were cultured for 48 hours in culture medium Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum until the 80-90% confluence was reached, and cell counts were up to 5000-6000 cells/ cm^2 [2]. Vicryl, an absorbable suture composed of a copolymer made from 90% glycolide and 10% L-lactide, was proven to have the best cells scaffold characteristics [2]. Poly-lactic-co-glycolic acid (PLGA) is common component for temporary scaffolds in tissue engineering that provides sufficient support to the implanted cells [2]. To enhance interactions between cells and scaffold, the PLGA needs to be chemically modified [2, 41]. Hence, Casado et al. selected Vicryl suture that has the cell scaffold capability, and then pretreated with NaOH to promote cell-scaffold interactions leading to a more hydrophilic surface, altered porosity, and greater roughness in the surface [2, 42]. Importantly, the results showed that pretreating Vicryl with NaOH and Poly-L-Lysin significantly increases the cell adhesion, quantified by Hoechst Staining assay. The mechanical strength of each pretreated suture was compared to the control suture with no pretreatment, and it was shown that the tensile strength of NaOH-treated sutures was significantly reduced, which consequently made these

sutures unsuitable for surgical procedures [2]. Although Casado et al. was able to show the most effective suture that can carry MSCs and investigate the effect of pretreatment on cell adhesion, there are some limitations to be addressed. Casado et al. investigated the mechanical strength of the pretreated sutures in the absence of MSCs to eliminate the potential changes due to cell growth. This can potentially be a limitation to this study as the purpose of validating bioactive suture is to investigate the effect of pretreated suture in the presence of MSCs. Eliminating MSCs from this part of the experiment makes the whole argument regarding the effectiveness of pretreated sutures untestable. Another limitation is that there is no evidence showing the bioactive suture enhanced the healing processes as mechanical strength of the wounded tissue was weaken based on this experiment.

In another study, Yao et al. took a similar approach to first, show the pluripotential cells are adherent to sutures and second, show the cells are alive once they pass through tissue (tendinous tissue here) [9]. Yao et al. believed the cells that adhered to commercially available sutures would survive while passing through the tissues and would infiltrate and repopulate the acellular region within the site of injury [9]. In this study, the mouse embryo fibroblast cells were cultured in DMEM with 10% fetal bovine serum, and the cells were incubated at 37°C in humidified air containing 5% CO_2 until cells reach 2×10^6 counts. FiberWire braided poly-blend sutures were cut into segments and coated with pretreatment PLL and seeded on to the sutures and incubate overnight [9]. DAPI nuclear staining technique was employed to show the cells were present on the sutures, and a Live/Dead fluorescent microscopy technique was used to investigate whether the cells are viable after passing through the tissue [9]. Although Yao et al. took the essential steps needed to prove the hypotheses presented in his work, substantiated proof was not provided as there are number of weaknesses in this study. To address whether MSCs are

alive on the sutures using Live/Dead assay, it is not possible to distinguish between the cells and suture segments as the sutures are also auto fluorescent. For this reason, the Live/Dead assay failed to show the viability of the cells on the sutures. Another limitation is the in-vitro nature of this study; in-vivo study is required to show how bioactive sutures react in tissues, and how effective they are in enhancing repairs. Because of in-vitro nature of this study, it cannot be concluded that bioactive sutures promote healing.

In a subsequent article by the same authors, Yao et al. aimed to investigate whether the sutured-based delivered MSCs remained metabolically active and whether MSCs coated suture strengthened tendon repair in rat model [10]. Along with their previous work, Yao et al. believed that MSCs offer greater advantages than introducing growth factors to the sutures which have been proved to enhance healing by strengthening tendon repair [10]. It is believed that since MSCs can secrete a great variety of growth factors as well as differentiate into tenocyte precursors, introducing them to repair sites can increase tendon repair strength leading into a faster healing period [10, 43]. In this study, bone marrow derived stem cells (BMSCs) were harvested from male Sprague-Dawley rats aged from 8 to 12 weeks, and the cells were cultured in DMEM in the same manner as their previous work [10]. The cell viability was above 95% prior to the in-vivo experiment [10]. Mechanical analysis was performed on specimens by placing them under tensile load until failure [10]. Yao et al. claimed a significant increase in ultimate load strength for coated suture repairs in comparison to no-coated sutures at the 7- and 10-day time points [10]. However, this claim is not accurate since the reported data and the published plot in the paper are not consistent (different numbers). A reconstructed plot from their data (Data Thief program) shows that the coated suture performs significantly worse than the control group at day 7. In other words, the repaired tendon showed weaker ultimate load once it

was under tensile load. Based on the inaccuracy of showing true results, further analysis is required to investigate the author's claims.

Besides MSC-coated bioactive sutures, there have been other studies combining stem cells with sutures to promote healing. One example of this is filling sutures with adipose-derived stem cells. This technique was an alternative to bioactive coated sutures to improve wound healing process. Reckhenrich et al. believed that adipose-derived stem cells (ASCs) attach to the suture material and distribute equally throughout the tissue while remaining viable in the suture [40]. Because of the ability of ASCs to release variety of cytokines and endothelial growth factors, Reckhenrich et al. believed that combining ACSs with surgical sutures allows the treatment and fixation of wounds in a single step [40]. The steps that were taken to prove their hypothesis are as follows. The ASCs were isolated from human tissues from donors ranging from 24 to 67 years old. 8 samples (7 female and 1 male) were taken from different body areas (abdomen, flanks and legs). The samples were digested with 0.1% collagenase A solution in PBS incubated at 37°C. The culture medium, DMEM, 10% fetal calf serum, 10 U/ml penicillin, 10 mg/ml streptomycin sulfate, and 25 ug/ml amphotericin, was used to prevent collagenase digestion. The supernatants were aspirated after centrifugation and the pellet was suspended in 5 ml PBS. The pellet was then suspended in culture medium and the culture medium was kept at 37°C and 5% CO_2. Once the confluence of the cells reached 80-90%, ASCs (5×10^5) were suspended in 8 ul culture medium and injected with a 100 ul HPLC syringe into the cavity of a 3 cm long suture which was cut from a biodegradable Vicryl suture. Scanning electron microscopy (SEM) and laser scanning microscopy (LSM) were performed after 10 days under standard culture conditions to evaluate cellular distribution and attachment to the suture. Biomechanical characterization was also performed after 10 days the ASC-filled sutures were incubated at 37°C

and 5% CO2. A tensile test was applied using a uniaxial system where the sutures were clamped on both ends, and the sutures were loaded at a constant rate of 5 mm/min until failure. The elastic limit, maximum force and channels stiffness were determined using the force and displacement sensor. Other assays were performed in this study such as cytokine release, and an *in vitro* wound healing assay. Employing LSM, they were able to show the attached cells along the suture surface, but whether the cells are viable or how many of them were alive are unknown. The metabolic activity of ASCs after being injected into the sutures was evaluated by MTT metabolic assay, but it was only shown that the cells were metabolically active at day 1 and day 8 after seeding. The biomechanical properties assay showed that the ASC-filled sutures have lower values for stiffness and maximum force, but elastic limit is identical to the control group. This result shows that the ASC-filled sutures can tolerate a lower mechanical force which consequently made these sutures unsuitable for surgical procedures. However, there were some advantages to using ASC-filled sutures. They allowed for retention of cells at the wound site and protected them from the mechanical stress induced by suturing. The cell implantation is a single step procedure since the cells are already in the core of the suture, which is an advantage over injecting after repair. The viability of the cells will not be affected by the suturing process because the main source of the cells is within the core of the suture. The main limitation to this study is the reduction of maximum force and stiffness in the ASC-filled suture in compare to control. This characteristic of the sutures filled with stem cells is problematic when dealing with tendons or non-elastic tissues. In addition, they need to prove that wound healing process improved somehow, whether through a shorter healing period or stronger mechanical properties of the tissue compared to the experimental control.

In another study, Yokoya et al. investigated a technique where they combined bone marrow derived MSCs (bMSCs) on polyglycolic acid (PGA) sheet to promote the rotator cuff regeneration in a rabbit model [46]. Yokoya et al. hypothesized that PGA sheet scaffold with seeded MSCs enhance the expression of type I collagen and eventually increase the mechanical strength of the tendon in vivo. The PGA sheet was used to make the scaffold for the bMSCs. The bMSCs were isolated from the tibia of 34 Japanese white rabbits. The culture medium used in this study was DMEM with 10% fetal bovine serum and penicillin-streptomycin-amphotericin. The cells were suspended in the culture and incubated in a humidified CO_2 atmosphere at 37°C. The cells were removed with 0.25% trypsin and 0.02% EDTA and rinsed twice with culture medium. The cells were then seeded onto each PGA scaffold in aliquots of 100 ul in a PBS solution for MSC group, and PBS seeded onto each PGA for the control group. The cell-polymer complex was incubated at 37°C with 5% CO2 for 30 minutes. The mechanical properties were evaluated by measuring ultimate failure loads and Young's moduli of 8 shoulders in each group at 4 and 16 weeks after surgery. There were no significant differences between the MSC group and the control group in terms of the ultimate failure load, tensile strength, and Young's modulus. In fact, employing PGA has some negative points. One of the negative points is that the degradation of the synthetic polymers is acidic and toxic to implanted cells and host tissues if they accumulate [47]. Although Yokoya et al. was able to observe the production of type I collagen after the implantation of MSC onto the PGA sheets for the purpose of tendon regeneration, the enhancement of mechanical properties in the regenerated tendon was not statistically significant. For this reason, the PGA sheet seeded with bMSCs will most likely not increase the strength of the tendon in human model as well, and alternative methods are yet to be determined.

Therefore, there remains a need to investigate whether MSCs bioactive sutures can be effective in the wound healing process as previous studies failed to provide substantiated evidence. To further investigate on this matter, the cell adherence to the sutures, cell viability after the implantation of the bioactive sutures, and wound healing rate and strength need to be determined. Currently, it is not fully understood whether the cells are preserved on the sutures while passing through bone and cartilage. Also, the percentage of viable cells after the suture is being passed through the tissue is yet to be determined. The cells proliferation and wound healing of the repair sites need to be analyzed and confirmed in order to determine if they have positive impacts in wound healing process. Once it is confirmed that MSCs do adhere to sutures, the viability of cells is unaffected after suturing, and the cell proliferation and metabolic activity of the cells are appropriate, the next main objective is to study the impact of MSCs coated sutures on wound healing by comparing the mechanical properties of the repaired site to the control group where standard sutures without coating are used. In the following section, a new set of experiments is proposed that address all of these aims; 1) investigating whether MSCs are adherent to sutures and whether they are viable, 2) investigating whether the cells are still viable on sutures while passing through a tissue and, 3) investigating whether the bioactive sutures promote wound healing by assessing the mechanical properties of repaired site post-operation.

2. Materials and Methods

2.1 Cell Adhesion and Cell Viability Before Suturing

The aim for the first experiment is to determine if co-culturing (suture and cells) time influences MSC adherence to sutures and whether the cells are viable on sutures. The hypothesis for this aim is that cocultured sutures with MSCs for 24 hours are more effective in retaining the

cells, and the number of alive cells is higher in compare to sutures cultured for 1 hour. These time points are relevant for determining if suture-cell constructs could be made intraoperatively. In order to investigate on this matter, the following procedures and preparations are required.

Isolation and Culturing of MSCs

Bone marrow derived stem cells will be extracted from New Zealand rabbits using bone marrow cavity puncture as described in previous studies [23]. DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) is used to flush out the cells. In order for the cells to repopulate and grow, a supplemented media is required. 89% DMEM with addition of 10% fetal bovine serum, and 1% Penstrep will make a populatable environment for cells to grow. The stem cells with the solution will be added to 12-well culture plates and will be incubated at 37°C with 95% air and 5% CO_2. After 96 hours of incubation, the cells will be washed twice using phosphate buffered saline solution (PBS) and transferred to 96-well culture plate and incubated at 37°C with 95% air and 5% CO_2. This process will be repeated until 80% confluence is reached [23].

Cell Seeding Procedure

Based on previous studies, the most adherent suture was found to be Vicryl® (Size 2-0 or 3-0) which showed greater number of cells on the suture [1]. The suture will be cut into 10-cm segments and rinsed with PBS 1X. 0.25% trypsin solution will be used to detach the cells from the well. The cell count should reach $2x10^6$ cells/ml measured by hemocytometer. In 10 ml of DMEM containing 10% fetal bovine serum, $20x10^6$ cells are placed in a tube together with the sutures. To transfer the cells on to the sutures, a culture container with grooves for sutures is provided. Group 1: sutures are placed in the designated groove cocultured with cells for 1 hour and group 2: sutures are placed in the designated groove cocultured with cells for 24 hours and

group 3: sutures are not coated with cells as negative control. After coculturing the sutures with cells, the sutures will be rinsed with PBS and transferred to a 14 ml polystyrene round-bottom tubes. The sutures will be incubated for 1 hour at 37°C with 95% air and 5% CO_2 in a humidified chamber.

Staining Procedure and Fluorescent Microscopy

To measure cell adherence onto sutures, the initial step is to label the cells and trace them. The first step is to label cells with DAPI staining. DAPI staining is a fluorescent stain for labeling DNA in fluorescence microscopy. Since this type of staining can bind to both live and fixed cells, it is a good method to check for the presence of the cells (whether live or fixed) and whether they are adherent or not. The important note for this part of the experiment is to use a suture material that is not auto fluorescent, like Vicryl. The sutures are rinsed with 1x PBS and covered with 10 uL of the DAPI staining on the slide. All the sutures, including the non-coated suture, will be incubated for 24 hours at room temperature in a dark place for complete polymerization before the confocal microscopy is performed. This adhesion assay will be performed on all three suture groups and the result will be compared.

CCK-8 assay

Another staining technique will be used in order to verify that the cells that do adhere, also survive. Cell Counting Kit-8 (CCK-8) assay will be performed to measure cell activity via mitochondrial activity. To perform CCK-8, suture segments will be placed on a 24-well plate with 300 µL of DMEM medium and 30 µL of CCK-8 dye and incubated for 24 hours. The absorbance of supernatants at 450nm is measured using a microplate reader. The same procedure will be done for all three groups (Group 1, Group 2 and Group 3) and the absorbance of the supernatants will be compared.

Statistical and analytical technique

For the adherence assay analysis, three groups will be evaluated. The first group consists of sutures with cell co-cultured for 1 hour, the second group consists of sutures with cell co-cultured for 24 hours, and last group consists of sutures with no cell co-cultured. The outcome variable is the number of adherent cells per millimeter of suture, and the analysis technique would be one-way ANOVA with post-hoc-Tukey test. For the cell viability assay analysis, the same groups will be used, and the outcome variable is the number of live adherent cells per millimeter of suture.

2.2 Cell Adhesion and Viability after Suturing

For the second experiment, the aim is to investigate whether the suture can retain its cells and their viability while passing through a tissue. The hypothesis for this aim is that the number of alive cells immediately after suturing and 24 hours after suturing is maintained. To perform this experiment, the cells are isolated, the sutures are pretreated, and the cells are coated in the same way as experiment 1. The following procedure is required to investigate the cell viability on coated sutures.

CCK-8 Assay

In this assay, the main objective is to verify that the cells are still viable after being pulled through the tissue. To verify this, the co-cultured sutures will be pulled through the B6D2 mice skin, and the suture will be evaluated for the cell viability employing fluorescent microscopy. The CCK-8 technique is used for this assay before, immediately after and 24 hours after suturing (incubated), and the result will be compared. This assay will determine whether the cells are alive onto the sutures or not.

Statistical and Analytical Technique

For the cell adhesion and viability after suturing, three groups will be evaluated. The first group is viability of cells before suturing, the second group is viability of cells immediately after suturing, and the last group is the viability of cells 24 hours after suturing. The outcome variable for this experiment is the number of cells and number of live adherent cells per millimeter of suture, and One-way Repeated measures ANOVA would be used as the analysis technique.

3. Summary

The concept of MSC bioactive suturing for improved wound healing is appealing and has some scientific merit. MSCs have broad anti-inflammatory and immune-modulatory properties, and they can produce and secrete a great variety of growth factors and cytokines. Based on previous studies, MSCs can, directly or indirectly, contribute to healing. MSC based bioactive sutures are being developed to enhance the wound healing process reportedly by releasing collagen content into the wounded area. They may also facilitate accelerating wound healing by delivering various cell lines from the suture to the wounded areas. Currently available research supporting the concept of improved wound healing with bioactive suturing has serious limitations that preclude the adoption of these materials in clinical practice. These limitations arise from not providing substantiated proof on whether the cells can adhere to sutures, or whether the adhered cells can survive passing through a tissue, or whether these types of sutures can enhance healing. Therefore, there remains a need to investigate whether MSCs bioactive sutures are effective in the wound healing process. Hence, a set of rationale experiments is proposed to fill these gaps, which will inform practice in a meaningful way.

Appendix:

A.1. Regulatory Issues

i. FDA Guidelines

It is important to consider the regulatory guidelines when implementing a new technique on an existing application. Since what we intend to achieve is to implement MSCs on suture for a better clinical outcome, some regulatory steps need to be completed. For this matter, MSC bioactive sutures need to go through some guidelines that FDA appointed. In the case of surgical sutures, FDA only requires manufacturers who intend to market a device of this type to follow the general controls of the Federal Food, Drug & Cosmetic Act (FDA Act) requirements, address the risks associated with surgical sutures devices, and eventually, acquire a substantial equivalence determination from FDA before marketing the device. [52] FDA believes that it will be sufficient to provide reasonable safety and effectiveness assurance of these devices, and that is the reason surgical sutures were reclassified from FDA premarket approval (class III) to FDA special controls (class II). [52, 53] Besides the FDA regulations about different sutures usage in human body, there is regulatory considerations for human cells, tissues, and cellular and tissue-based products (HCT/P) that needs attention as well [66]. These regulations are intended for products containing human cells or tissues to be transferred or transplanted into a human recipient [66]. If the product meets the HCT/P criteria, it will not be regulated as a drug, device, and/or biological product, and premarket review will not be required [66]. The following criteria needs to be met: 1) The HCT/P is minimally manipulated; the relevant biological characteristics of cells is not altered, 2) HCT/P is for homologous use only, meaning the repair or reconstruction of a recipient's cells with an HCT/P, performs the same function in the recipient as in the donor, 3) The product does not involve the combination of cells with another cells or chemicals except

for water or preserving agents, and 4) The HCT/P is not dependent on the metabolic activity of living cells, or it is dependent on metabolic activity of living cells for its function, and: i) is for autologous use, ii) is for allogenic use in first-degree or second-degree blood relative, or iii) is for reproductive use [66]. Since the purpose of the final product is to use allograft, it will be regulated as drug, device, or biological product, and premarket review will be required. Once approved that the product can be used on a human, it is also required to get approval whether the product can improve healing faster which needs a substantial investment of time and money.

ii. Allograft versus Autograft Source Material

FDA recommends manufacturers to provide as much details as possible about their new product. This information includes but not limited to the identity and percentages of all the material of the suture, the material of coating and additives, the size of the suture and color additive to the sutures. [52] Since the suture used for this application is already FDA approved, there is no need for it to be approved again. The suture used for this new development is Vicryl suture which is an absorbable, synthetic, and braided suture manufactured by Johnson and Johnson, and it is already an FDA approved item. The next item to consider for FDA regulation is the coating material which is MSCs in this case. As mentioned previously, there are different types of MSCs (bone marrow MSCs, blood cord MSCs, etc.) in both allograft and autograft forms. Since the purpose of this study is to investigate the effect of MSC taken from one animal and implement that on to suture used in another animal to prevent the complications in the operating rooms later, we would choose allograft MSCs over other options. For this matter, we found a company called Hansa Biomed Life Sciences, as mentioned before, that offers human Mesenchymal Stem Cells from adipose tissues in vials of 100ug. FDA approval is required for

stem cell products, and it is important to note that the only stem cell products that are FDA-approved in the US are derived from umbilical cord blood to be used in patients with disorders that affect the production of blood. [54] All other cases, such as treatment of any orthopedic conditions, need to get FDA approval first before going into the market [54].

iii. Culturing and Expanding Issues

As mentioned before, the MSCs capacity for multi-lineage differentiation has attracted clinical interests, and for that reason, it has a good potential for commercial therapeutic development. Since we're using the prepared vials of hMSCs from Hansa Biomed Life Sciences, that include greater than $1x10^{10}$ cells, the issues coming from culturing and expanding cells would not apply. These vials have enough cells to be coated on to sutures and we do not need to worry about how to expand them. As a matter of fact, the MSC coating to sutures would be the only procedure that requires attention.

iv. Prior Art

As mentioned in the introduction section of this study, there are variety of sutures available for medical purposes, such drug -eluting sutures like antimicrobial sutures, and stem cells containing sutures [55]. To have a better understanding of what the process of regulatory pathway is, we could take a closer look at a medical product similar to MSC bioactive sutures that is FDA-approved. In 2002, FDA approved triclosan-coated sutures for the first time, and the main purpose of using this drug-eluting suture was to reduce the wound infection during the treatment [55]. This could be a good medical product candidate to follow its regulatory pathway for 2 reasons: 1) the underlying goal of both products is to enhance the quality of suturing and 2) the drug-eluting suture uses a similar technique in delivering materials to the wounded areas as MSC coated sutures. One specific example of drug-eluting sutures is antibacterial sutures.

Johnson & Johnson has cleared FDA approval of the Vicryl Plus antibacterial sutures coated with triclosan that can help reduce the infections [55].

A.2. Cost Analysis

One of the main aspects that needs to be analyzed in the context of improving a technique such as suturing are the costs. It is important to know the average cost of regular sutures currently being used as a reference, and it is crucial to estimate the cost of the new sutures to understand the feasibility of the proposed approach, and ultimately to establish value (outcome/cost). There are several factors that influence the cost of suturing; these factors are included but not limited to surgical times, material costs, suture costs, and costs associated with complications of suturing [49]. The average total cost per case for suturing in the United States is between $200 to $3000 depending on the injury and complexity of the repair [48]. In orthopedic surgeries, which is our primary focus, suturing usually costs between $200 to $300 per case [48, 64]. That includes the average price of the suture, the packaging and handling, as well as the time allocated for the process of the suturing in the operating room [48]. Closure of the wound could be a time-consuming process, and it plays an important role in determining the cost of suturing [49]. That means the new suture developments should not impact the suturing time performed by the surgeon in the operating room unless there is a clear advantage in terms of outcomes. The total average cost for one minute of operating rooms (OR) amounted to $16.21 in the United States, and this does not include the surgeons or other professional persons' fees in OR [50]. The mean cost for one minute OR time in California hospitals was $37.45 including all the cost and within the variety of the operations [50]. For developing the new bioactive sutures that can carry MSCs, the following costs need to be taken into considerations:

Since the MSCs need to be added along with our final product as an allograft of human bMSCs, reducing the complications in operating rooms and eventually reducing the cost of the operation, the cost of extracting human bMSCs as well as the storage and distribution need to be investigated. The idea of using autograft instead of allograft will consume more time and energy during the operation and will not be an efficient way to approach this issue. So, the overall cell coating procedure will be using allograft cells and complete the manufacturing process somewhere else, which has its own costs associated with it, and store those samples onsite ready to use which has other cost associated with it.

Hansa Biomed Life Sciences is a leading company dedicated to research and development of products in the Extracellular Vesicles field. This company offers human Mesenchymal Stem Cells from adipose tissue in vials of 100ug. The number of cells per each vial is estimated to be greater than $1x10^{10}$ cells. These cells can be stored up to 3 years at 4 degrees Celsius in the lyophilized form and can be stored at -20 degree Celsius for up to one month or -80 degree Celsius for up to 6 months in the reconstituted form. The cost of human bMSC from Hansa Biomed Sciences is estimated to be $290.40 per vial. Based on the previous studies and effectiveness of their methods, at least 20 million cells need to be coated per each suture. Hansa Biomed Sciences mentions that there are 10^{10} cells per each vial which is enough for 500 sutures. That will bring up the cost of each suture to additional $1.72 not including the packaging, storage and handling, and manufacturers cost.

As mentioned earlier, one of the other main costs associated with bioactive coated sutures new developments is the manufacturing cost. This cost consists of extracting human bMSCs and implementing them onto the determined sutures. The suture used for this new development is Vicryl suture. Vicryl is an absorbable, synthetic, and braided suture manufactured by Johnson

and Johnson which can hold its tensile strength for up to three weeks in tissues and will be absorbed completely within 56 to 70 days [62]. This type of suture is made from 90% glycolide and 10% l-lactide, and the main reason this type of suture is used is because the studies have shown great attachments between MSCs and the suture compared to other types of sutures [2]. The average cost of Ethicon Coated VICRYL (polyglactin 910) Suture, Synthetic Absorbable, (13 mm), 3/8 Circle Needle, Size 4-0, 18" (45 cm), Undyed is $13.67 per suture.

In terms of the storage cost, a sub-80 Celsius cooling box is required to keep the bioactive sutures effective up to 6 months. These storages are not cheap, and they can go from $10,000-$20,000. Many hospitals do not have the space or the facility to store at ultra-cold temperatures, and these storages need to be purchased. These freezers can last from 12 to 15 years. [65] By calculation, the average cost of the cooling box ($15000) working for an average of 13.5 years will result in an additional storage cost of $0.0063 per minute of surgery assuming the operating rooms are only active for 8 hours per day. For the shipping and handling of this product, it is safe to compare the delivery cost of these sutures to delivery of the novel Corona virus Covid 19 vaccines. These vaccines should be delivered in a similar fashion to Bioactive sutures as they need to be transferred within a specific temperature (between -40 to -70 degree Celsius). Total financial cost per dose of vaccine was estimated to be US $1.66 which 57% of that total amount estimated to be the in-country outreach and fixed site delivery [63]. This comes down to $0.946 per dose of vaccine which can be estimated as our preliminary bioactive suture delivery cost per suture. Shipping, handling and storage of bioactive sutures will bring up the cost of the suturing to a total amount of $0.95 per suture.

	bMSC vial per suture	Type of Suture	Shipping and Handling	Storage	Total
Bioactive Suture	$1.72	Vicryl - $13.67	$0.94	0.006	$16.34
Regular Suture	0	Ethibond - $4.95	Not considerable	0	$4.95

This total cost is to be compared with the regular suturing which costs $4.95 per suture on average excluding the manufacturing costs associated with the bioactive suturing, such as facility and labor costs. The effectiveness of new bioactive sutures will determine whether it is financially feasible to implement this technique or not. If the marginal improvement in wound healing is high in operations such as rotator cuff injury, which shows a higher failure rate in orthopedic surgeries, then it would be necessary to implement this technique despite its higher cost.

A.3 Proposed Mechanisms of Action

First, the process of normal wound healing will be discussed and the factors that play major role in the process. In the second part, the process of healing when sutures are used, and how sutures would impact on wound healing mechanism will be discussed. In the last part, the proposed idea of MSC bioactive sutures will be discussed, and how these new sutures can change the stereotypic responses.

i. Normal Wound Healing Process

Wound healing is a complex set of mechanisms, and it involves more than just the three phases of inflammation, proliferation, and maturation [57]. Wound healing involves complex processes between numerous cell types, mediators, and cytokines [67]. In this section, a thorough

explanation of each phase of wound healing, the important factors affecting wound healing mechanism, such as inflammatory mediators and growth factors, and future directions on wound healing will be discussed.

When an injury occurs, the initial stage is always outpouring of blood where the adequate hemostasis is reached [67]. The arterial vasoconstriction plays an important role in stopping blood loss, which is a short process followed by vasodilation to allow the influx of white cells [67]. The next stage is called inflammatory stage where the collagen released during the wound formation starts the clotting cascade [57]. The formed clot consists of collagen, platelets, fibronectin, and thrombin (which is an enzyme in blood plasma facilitating the clotting of blood), and these factors release growth factors and cytokines that initiates the first stage of wound healing, which starts immediately after the injury or surgery, and it can take up to 4 to 6 days [57]. Immediately after the clot formation, a cellular distress signal will activate the vasodilation of the nearby blood vessels which will draw the neutrophils into the injured area [57]. About 48 to 96 hours after the injury, the monocytes in the surrounding tissues and in the blood will be attracted to the site of injury and will be transformed into macrophages. The activation of macrophage, which facilitates angiogenesis, is a crucial step for the transition from the inflammatory stage to proliferation stage [57]. Variety of enzymes will be secreted by macrophages, including collagenases, which remove damaged tissues from the wound, and TNF, which stimulate fibroblasts and promote angiogenesis, and the second stage of wound healing will begin [57]. Angiogenesis, which is the migration of endothelial cells and formation of capillary into the wound, is a critical process for a proper healing in the proliferation stage which starts from day 4 and can take up to 14 days [57]. Epithelial cells on the skin edge will proliferate to build a protective barrier against fluid losses and bacterial invasion [57]. This step will shortly

start after the injury, and it is stimulated by inflammatory cytokines [57]. The final step of proliferation stage is the granulation tissue formation; fibroblasts will be activated at wound site and begin synthesizing collagen [57]. The last phase of wound healing, maturation, and remodeling stage is all about the deposition of collagen content in an organized manner [57]. This stage starts at day 8 and will continue to up to a year [57]. There is a difference in production of collagen from an injured sample compared with an uninjured sample [57, 58]. Granulation collagen is biochemically thinner than collagen from an uninjured skin for example, and the collagen in the scar will never be as organized as an uninjured skin even after a several months [57, 58].

ii. Wound Healing when Sutures are used

The wound healing process, as mentioned in the beginning of this section, remains a challenging clinical issue which seeks efficient solution. The wound closure techniques have developed significantly, and there are different types of techniques for different types of wounds; there are simple sutures in form of absorbable and non-absorbable, staples as well as adhesive compounds [59, 60]. The primary function of sutures is to bring and keep the wound edges together until the healing process is complete [59]. Keeping the wound edges together, the mechanical support the tissue needs during the repair will be provided, and the stages of wound healing process will be facilitated [61]. In addition to providing mechanical support, sutures can improve healing by a variety of factors as they are the components at wounding sites. As mentioned before, there are new developments in improving wound healing by providing sufficient resources to the wounded area such as antibacterial sutures and MSC coated bioactive sutures [2,3,4,5]. In the next paragraph, how MSC coated sutures might affect the healing process will be discussed.

iii. Impact of MSC Bioactive Sutures in Healing

In addition to providing mechanical support while healing, which is the primary function of suturing, the new developed MSC bioactive sutures may enhance wound healing. MSCs have broad anti-inflammatory and immune-modulatory properties, and they can produce and secrete a great variety of growth factors and cytokines [11]. Based on previous studies, MSCs can, directly or indirectly, contribute to healing [16, 17, 21]. The application of MSCs, whether through injection or cell-scaffold construct, has shown promise in accelerated healing, tissue-specific gene expression, and more organized repair structures [14-20]. It has been reported that MSCs can improve wound healing by promoting the release of collagen content into the wounded area [21, 22]. They will also reduce the prolonged inflammation period of wound healing causing the delay in healing by supplementing enough cells to the acellular regions [6, 29]. The distinct acellular zone in the tissues surrounding the sutures is a potential cause for the prolonged inflammatory activity leading to the weakening of tendon repair site [6, 29, 39]. Based on the previous studies surrounded this area, we hypothesize that MSC bioactive sutures can supplement these acellular regions with enough MSCs to reduce the inflammatory stage period and enhance healing more efficiently.

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