

Regenerative Treatment of Stress Urinary Incontinence by Autologous Macrophage Therapy

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Abstract

Stress urinary incontinence (SUI), is the leakage of urine by vigorous activities such as coughing and sneezing when pressure is exerted on the urethra. SUI is prevalent as 2 out of 3 women are affected. To solve this common problem, many hospitals use mid-urethral and polypropylene slings in an estimated 750,000 patients/year, but these slings are not ideal due to high erosion rates which cause the mesh to break in the body. Thus have been subjected to a significant amount of negative publicity in recent years. Developing an advanced biomaterial to promote a healing response may instead be an effective way to improve outcomes in tissue reinforcement surgeries that often result in SUI. For this project, collagen, a primary protein in many tissues, was selected as the biomaterial for testing, and was embedded with macrophages. An electromagnetic wheel and plate were used in order to generate collagen threads and then wound into scaffolds with collagen mesh. Scaffolds made from these fibers were then sterilized, seeded with macrophages, and cultured for up to 3 days in vitro. Genipin, a plant-derived compound with known anti-inflammatory properties, was used to crosslink the collagen fibers prior to seeding macrophages on the mesh. Specifically M2 macrophages were used due to their regenerative capabilities. The goal is to develop a collagen cross-linked mesh seeded with macrophages that treats SUI while simultaneously healing tissue in the surrounding area, as part of a platform to facilitate pro-regenerative responses.

Introduction

Stress urinary incontinence (SUI) occurs during vigorous movement and activities such as coughing or sneezing. When this happens, pressure is placed on the urethra which causes the sphincter muscles to open and urine leakage occurs (Biomedicines). The more severe the SUI the more likely leaking will occur with less pressure placed on the urethra. SUI is also more common in women as two out of three women suffer from the condition (*Journal of mid-life health*). A current solution is polypropylene (PP) meshes. However, the erosion rates associated with polypropylene meshes are high (~10 %) which means 1/10 women inserted with this mesh experience erosion. Erosion is when the mesh breaks, starts infecting surrounding tissue, and overtime weakens tissue. Many existing meshes regarding SUI also lack robustness, elasticity, and overall have a lack of regeneration and anti-inflammation (*Biomaterials*).

Macrophages, along with monocytes, are immune cells that are classified as components of the mononuclear phagocyte system (*Biomaterials*). This system is where bacteria, viruses, and anything foreign to the body are destroyed. In the cardiovascular network, monocytes produced in the bone marrow circulate to supply tissue macrophages whose phenotypes/polarization states depend on changes in their microenvironment. Macrophages can instigate accumulative inflammatory responses, depending on their polarization state. Macrophage polarization, although each group has additional subtypes, is mainly classified as pro-inflammatory (M1) and anti-inflammatory (M2) (*Biomaterials*). In general, it is well known that M1 macrophages predominate in the initial stages of inflammation, and M2 macrophages take over in the longer term to promote wound remodeling (Scientific Reports volume 6).

It is also established that M2-macrophages are pro-regenerative, which specifically promotes collagen deposition in vivo (Scientific Reports volume 6). The production of an advanced biomaterial that encourages monocytes' polarization to M2 macrophages may be an

effective way to improve surgical results in mesh applications regarding SUI. Collagen deposition is necessary, as collagen is a protein that makes up almost 35% of our body (J Leukoc Biol. 1987 Jan;41(1):83-91.) and is the main component in strengthening tissue and cells. The ideal mesh should include collagen and specific load-bearing tissue supporting possibilities, including hernia, urogynecology tissue, or musculoskeletal soft tissue repair, where rapid collagen deposition is needed to achieve sufficient durability and strength. This delivery strategy requires a scaffold that will allow cell seeding and embedding into the scaffold mesh, demonstrate structural robustness, and most importantly, be able to exhibit a pro-regenerative response while also maintaining M2 macrophage polarization as it is anti-inflammatory.

In a permeable system that can handle cell seeding, woven bio textiles of electrochemically aligned collagen (ELAC) threads provide structural integrity. The collagen threads made to use these scaffolds were crosslinked with a plant extract known as genipin. Previous research has indicated that genipin has been the most suitable host for a greater amount of M2 macrophages compared to other crosslinked threads for a mesh (Adv Funct Mater). This overall suggests collagen crosslinked threads promote M2 polarization (the pro-regenerative macrophage subtype), however, collagen scaffolds also contributing to an ideal response remain unclear. Previous research also suggests that elongating the cytoskeleton of macrophages can also promote M2 macrophage polarization (Adv Funct Mater). The issue of whether genipin and cell topography contribute to the polarization of M2 macrophages needs to be addressed to create an ideal mesh for SUI. It is hypothesized that delivery of autologous-blood-derived M2 macrophages via genipin crosslinked collagen meshes will result in faster regeneration, e.g. earlier deposition of collagen, earlier inception of neovascularization (formation of new blood vessels), and earlier attainment of mechanical robustness than the collagen meshes which are implanted without M2 macrophages.

Material & Methods/Results

Scaffold fabrication

Electrochemical compaction was used to transform collagen solutions on spools into aligned threads of continuous length. Bovine Collagen solution was dialyzed against DI (deionized) water 24 hours prior to electro compaction at pH 7.0. The collagen solution was then loaded into a syringe and dispensed in the space between a specialized equipment, electrode wheel, in a regulated manner. The wheel spun the collagen and used electro compaction, a process that compresses the collagen using electrical potential, to create the solution into threads. The threads were collected in a solution of 80% isopropanol and 20% water.

After the collection of the collagen threads, around 50-100 threads were twisted together to form a yarn to increase mechanical robustness of the structure. To combine the collagen threads, L-ascorbic acid solution (2.7 mg/ml in DI water) was used to stick the ends of the thread together to create the yarn. The yarn was then carefully spun onto a spool and dried in a fume hood before it was stored at 4 °C.

In order to turn the yarn of collagen threads into scaffolds a computer numeric controlled machine (CNS; Sherline Inc. CA), was used to make and position the structure of the scaffolds. The CNC machine used a software (LinuxCNC software, v. 2.6.11) programmed to create two axes to wind the yarn around a mandrel. The two axes created a pore pattern to create the collagen scaffold which were 5 mm in length and 1 mm in width. During the fabrication of the scaffold making process the yarns were fused to each other by applying L-ascorbic solution, followed by a drying period after formation of the complete structure. Overall the dimensions of the rectangular scaffold were approximately 20 x 5 x 0.5 mm.

Collagen scaffolds were crosslinked in genipin solution (2% w/v in 90% ethanol) for 72 h at 37°C. Scaffolds were sterilized prior to further use by treatment for 3 h in peracetic/ethanol solution (1%/22.5% v/v in DI water).

This student researcher assisted the postdoctoral scholar during these steps, but can perform all independently.

Immunofluorescence

Macrophages were seeded on glass bottom 35mm fluoro dishes at 5×10^4 cells. They were incubated with three different antibodies: mouse anti-rat CD68, mouse anti-rat NOS2, and mouse anti-rat arginase overnight. The next day the samples were incubated with secondary antibodies: Alexa Fluor 488, Alexa Fluor 555 and Alexa Fluor 595. After being rinsed twice with PBS the samples were placed under fluorescent microscope and immunofluorescence viewed. The images produced were used to see the embedding of the macrophages onto the collagen scaffolds.

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Results

Once the mesh was created it had to be figured out whether the macrophages were polarized correctly. To test this Dapi staining was used to see the cell nuclei, and to see if they are present in the cells or not. If the nuclei are present and seen it means the cells are alive, seeded properly, and have been polarized. CD68, NOS2, and ARG1 are the markers for M0, M1, and M2 respectively (Figure 1). The images were then composited to create the merge image. Immunocytochemical identification was used to determine that the cells were polarized correctly.

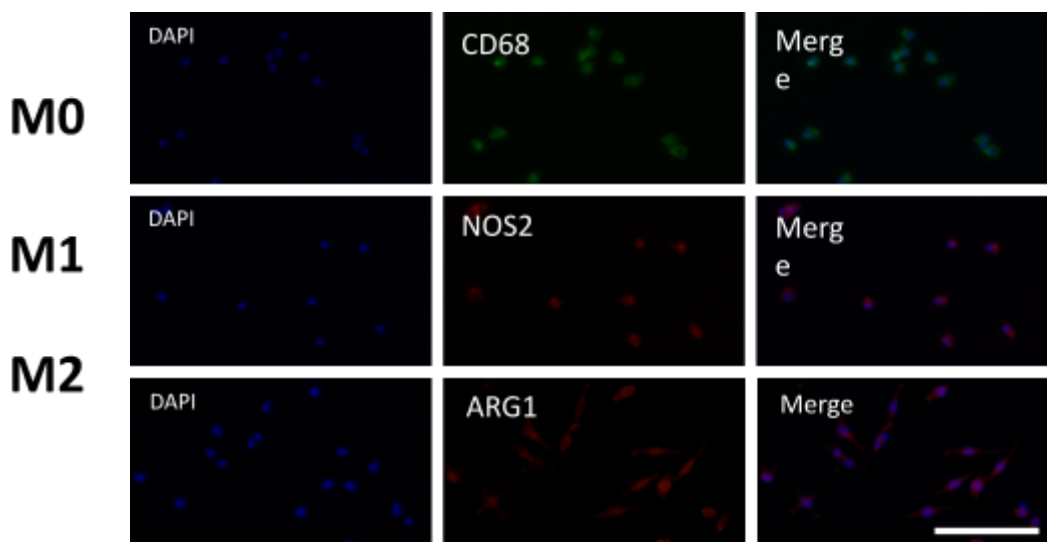


Figure 1: Immunocytochemical identification of macrophages seeded on collagen scaffolds.

After determining polarization the amount of collagen produced also known as collagen disposition to be tested. H and E staining (Figure 2) is a basic staining method to look for the cell integration and new collagen deposition around the material. The stars are collagen threads, and the arrows indicate that there is new cell integration around the material. The M2 cell material suggests that there is more cell integration and decreased fibrose encapsulation. This means when the mesh is inserted inside the vagina material will degrade overtime and will be replaced by new collagen and the body will produce its own collagen. This helps to strengthen and renew tissue while also helping treat SUI.

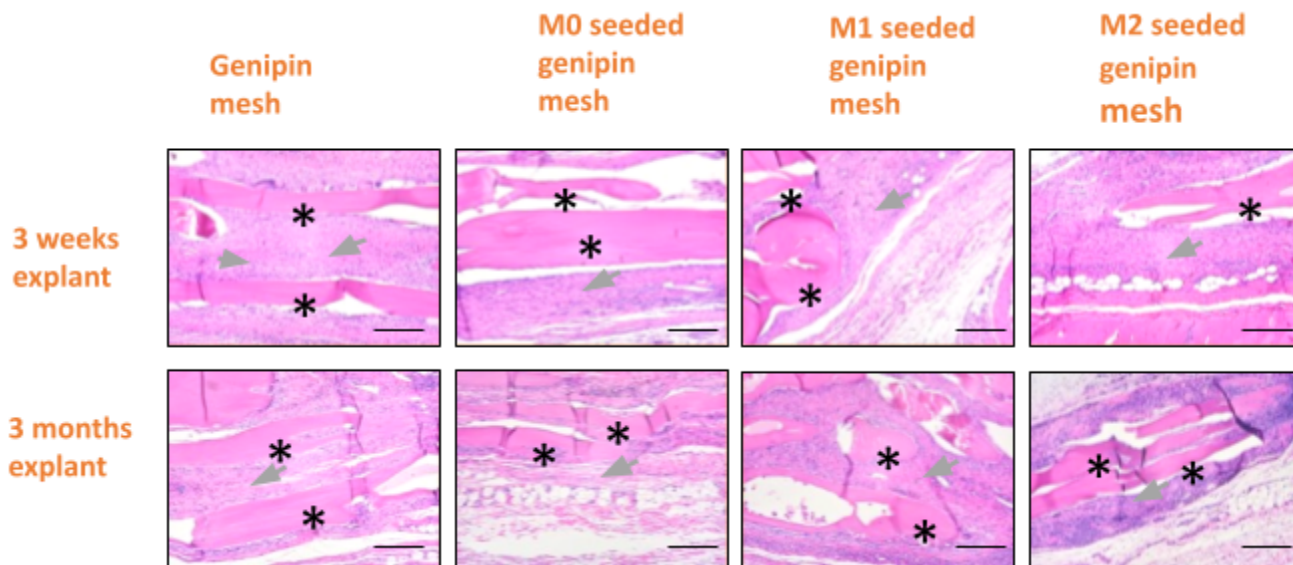
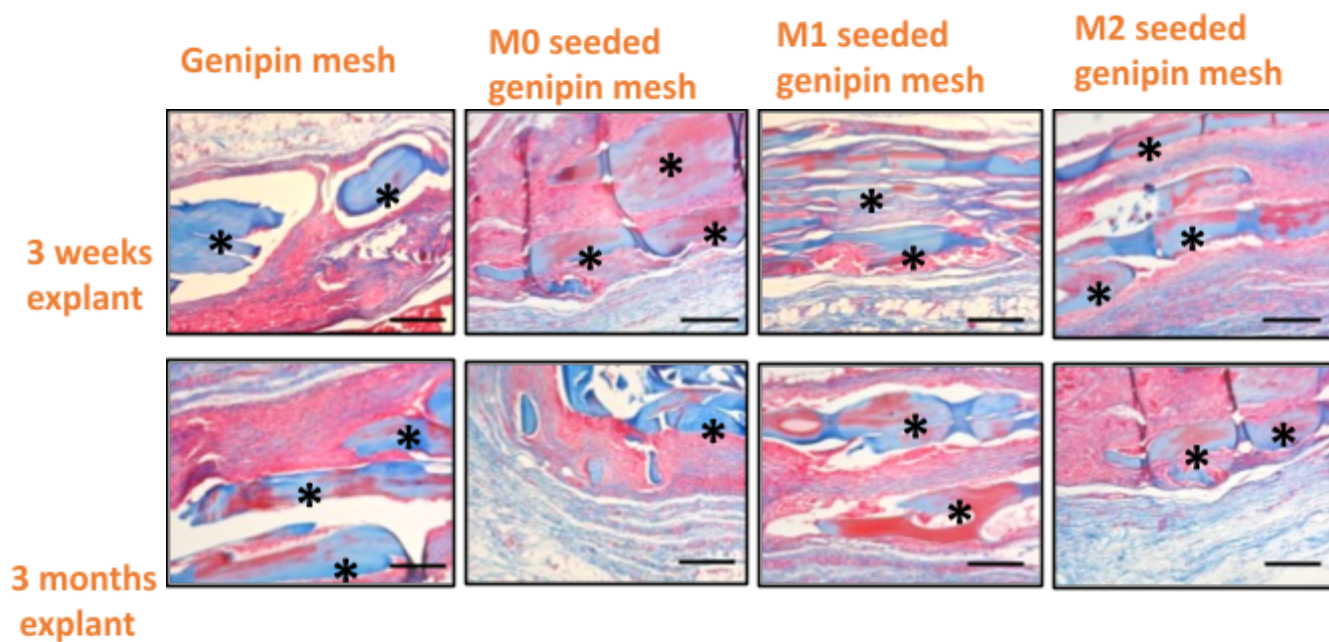


Figure 2: H&E Staining of Explanted Meshes. High magnification (100 µm) images of H&E-stained mesh sections over time. Stars denote the respective mesh materials and arrows indicate areas of new collagen deposition in and around mesh materials. Scale bars are 100 µm.

Afterwards, Masson Trichrome staining (Figure 3a) was done to see if there was more collagen deposition happening or not. Masson Trichrome is a staining for collagen, and the blue (thin) and red (thick) color indicate that there is more collagen deposition. The quantification analysis (Fig. 3b) indicated that M2 seeded genipin scaffolds are responsible for more collagen deposition, as shown in the quantitative data. Quantification analysis showed that there was more collagen deposition at 3 months on genipin scaffolds. This is an extension of Fig 2 and also shows the difference between various meshes and which one produced the most collagen. It also compares collagen thickness, and this is important because the images with the most red show how much collagen the rat's body was able to produce on its own with the assistance of the mesh.

a)



b)

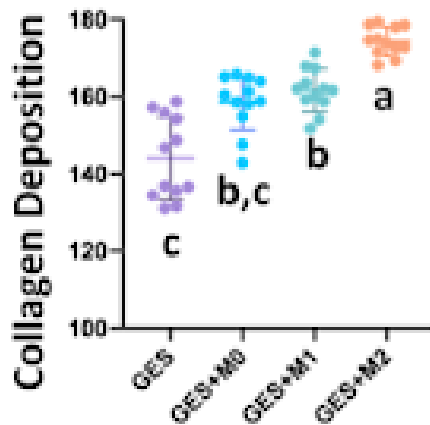


Figure 3: Masson's Trichrome Staining of Explanted Meshes. a) High magnification images of Masson's Trichrome stained mesh sections over time. Stars denote the respective mesh materials. Scale bars are 100 μ m. 9. b) GES-genipin scaffolds GES+M0 -genipinscaffold +M0 cells like others.

In order to see if the mesh was structurally robust it was tested using Modulus. Since erosion is a common problem in vaginal meshes the collagen cross linked mesh was tested to see how strong it was along with various macrophage polarizations. Fig 4 shows that the mesh with M2 macrophages seeded was the structurally the strongest, and further proves that its not only effective for promoting regeneration but also structure.

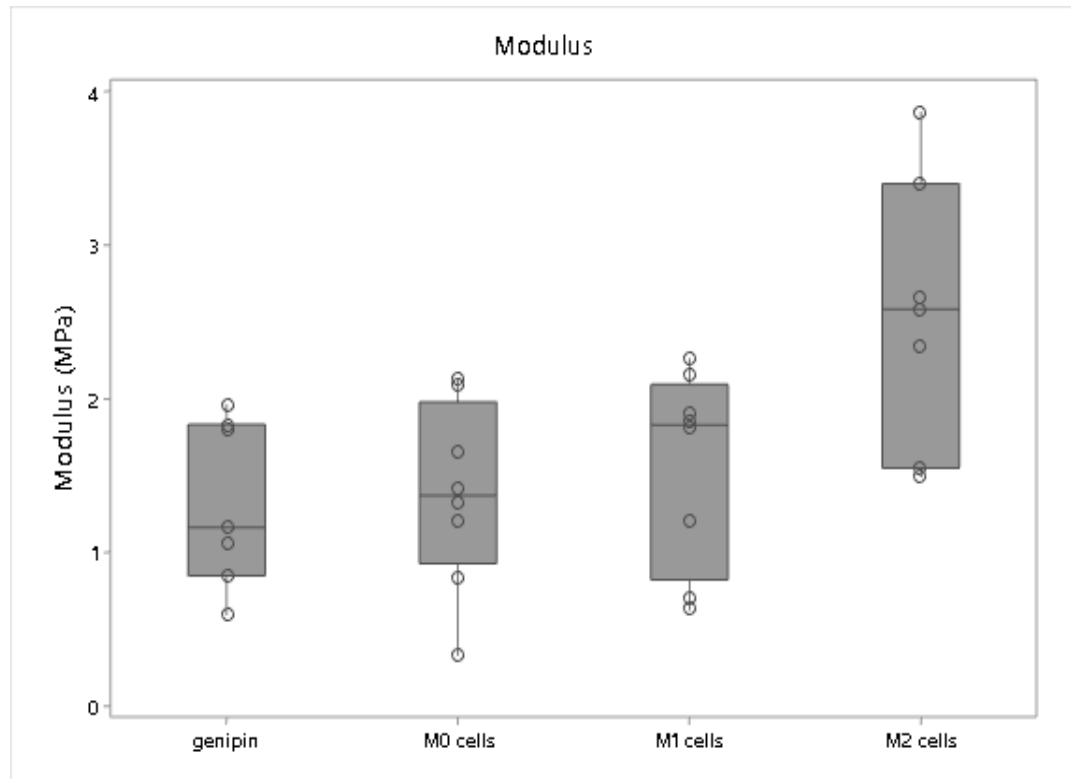


Figure 4: Modulus of the explanted samples at 3 months. Measure strength at MPa and shows why M2 macrophages are ideal for structure alongside regeneration.

The images in Figure 5 relate back to Figure 4. The following shows the meshes being stretched out to test its structures. This is because the material strength should be similar to vaginal tissue strength. At three months modulus was significantly higher. M2 seeded genipin meshes had significantly greater modulus values (almost 4 Mpa) than genipin and M0 seeded genipin mesh at three months. Mechanically the material should be robust and similar to the environment.

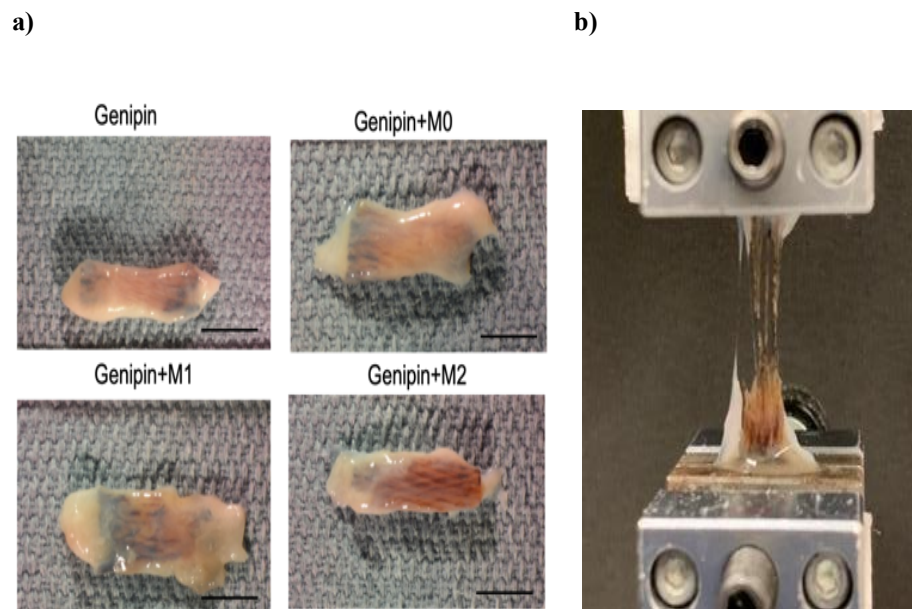


Figure 5: Mechanical Testing of Harvested Samples. This shows the collagen mesh being stretched after explanation to see if the tissue would break in the vagina.

Discussion/Conclusion

The findings of this research illustrate the significance of recognizing the macrophage response to genipin crosslinked collagen scaffolds in order to have a positive effect on the regenerative response after implantation of biomaterials. So far, the research suggests: A) Genipin-crosslinked collagen meshes induce polarization of M0 macrophages to an M2 phenotype. B) M0 and M2-seeded genipin-crosslinked collagen meshes support sufficient macrophage attachment and survival for implantation. C) Delivery of bone marrow-derived M2-macrophages improves the healing. D) The mechanical properties (modulus) of M2-seeded scaffolds were higher compared to genipin scaffolds. Limitations to consider regarding future work are sample size, subcutaneous model and need functional repair to fully assess biomechanics and tissue integration, and long-term evaluation. The mesh also led to investigating the appropriateness of genipin crosslinked collagen scaffolds for tendon repair. The proposed technology may impact a wide-ranging set of clinical problems such as hernia repair, pelvic prolapse, cardiac repair patches, and other soft tissue reinforcement applications.

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