HEROVICI'S STAINING: A USEFUL DIFFERENTIAL STAINING METHOD FOR EVALUATING COLLAGEN DISTRIBUTION IN BIOMATERIAL-MEDIATED HERNIA REPAIR*

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Abstract

Natural biomaterials are gaining momentum in tissue engineering because of many reported cases of recurrence in ventral hernia, repaired with synthetic mesh materials. Collagen is the main component of the extracellular matrix of the abdominal wall and their distribution and ratio alter during hernia formation. Therefore, a differential staining method for evaluating collagen distribution after biomaterial implantation known as Herovici's polychrome staining was employed in this study. Here, abdominal wall defects were created in rat models (six animals/ group) and grafted with jejunum-derived scaffold (JDS) and a reference material, Surgisis (C-SIS) scaffold. The implanted scaffold samples were retrieved at 4 weeks post-implantation and subjected to Herovici's staining. Data analysis revealed a significant ($p<0.05$) and favorable type I: III collagen ratio in C-SIS group compared to JDS group. This study shows Herovici's staining to be a better differential stain that can be used for studying the efficacy of newly introduced biomaterial for ventral hernia repair in human and veterinary patients.

Keywords: *Biomaterials, ventral hernia repair, collagen, Herovici's staining*

Abdominal ventral wall hernia is still a major health concern and large abdominal defects results from a variety of pathological events such as trauma, congenital defects, tumor ablation or denervation. Ventral hernias are currently repaired either with synthetic or natural biomaterials (Bilsel and Abci, 2012). Synthetic mesh materials available for repair behave like passive scaffolds without proper host integration and demonstrated to have limited biocompatibility. Further, they can trigger vigorous inflammatory responses followed by infection. The success rate of synthetic mesh materials is only about 60-80 per cent (Ayubi *et al*., 2008).

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More recently, decellularized matrices have been used to address hernia repair because of their higher biocompatibility and improved host integration. Even though, these bioscaffolds are effective in the repair of hernia, their poor mechanical strength results in hernia recurrence (Petter-Puchner *et al*., 2007). Hence, an efficient bioscaffold material with better biocompatibility and mechanical strength is highly desired.

The extracellular matrix (ECM) is a unique platform consisting of structural and functional proteins, growth factors and hidden peptides and plays a major role in tissue repair (Badylak, 2002). The extracellular matrix has been explored for the repair of hernia in clinical practice. Bioscaffold-mediated repair provides enhanced neovascularisation, stem cell recruitment, release and removal of scaffold degraded products and repair of injured tissues (Badylak and Gilbert, 2008). Collagen is the main component of the extracellular matrix of the abdominal wall and hernia formation was accompanied by their altered ratio or metabolism (Franz, 2006). Therefore, a differential staining method for evaluating collagen distribution and ratio after biomaterial implantation known as Herovici's polychrome staining was employed in this study.

Materials and Methods

Bioscaffolds

Small intestine (jejunum)/JDS, a cross-linked material, was prepared from slaughtered Large White Yorkshire pigs by a non-detergent and non-enzymatic method (Anilkumar *et al*., 2014). Surgisis (C-SIS), a non cross-linked material was used as the reference bioscaffold (Figure1A and 1B).

In vivo model

Twelve Sprague-Dawley rats of either sex, weighing 200-250g (SABS, College of Veterinary and Animal Sciences, Mannuthy) were divided in two groups: JDS and C-SIS (n=6 each). The rats were anaesthetized with ketamine hydrochloride (Aneket – 70mg/kg, Neon laboratories limited, Mumbai) and xylazine hydrochloride (Xylaxine – 5mg/kg, Indian Immunologicals, Hyderabad) by intraperitoneal injection. A ventral rat abdominal wall injury model (2cm X 2.5cm) was made for the surgical implantation of test scaffolds.

Following the surgical procedure, the animals were monitored until they fully recovered and became active. The study was performed in conformity with the guidelines established by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests (Animal Welfare Division), Government of India. All procedures were approved by the Institutional Animal Ethics Committee (IAEC) at the College of Veterinary and Animal Sciences, Mannuthy.

Tissue sample collection and Herovici's staining

In order to understand and characterize the types of collagen in the implanted bioscaffold grafts over time, implants were explanted at 4 weeks post-implantation. Representative interface tissue specimens were fixed in 10 per cent neutral buffered formalin for histological examination. The formalin fixed explanted interface tissue specimens were stained with Herovici's polychrome stain (Herovici, 1963;

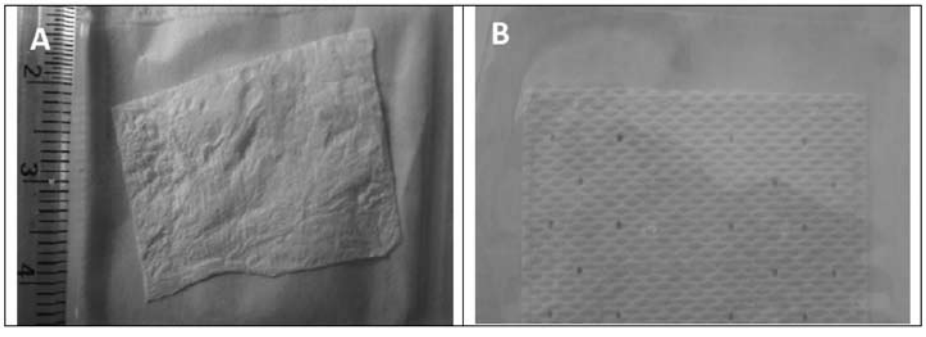

Figure1. Macroscopic appearance of **A.** jejunum-derived scaffold (JDS) **B**. Surgisis graft (C-SIS)

Fitzgerald *et al*., 1996) as follows. Herovici's stain combination had acid fuchsin (0.1 per cent w/v) in picric acid and methyl blue (0.05 per cent w/v) in acetic acid solutions (1 per cent v/v) for 5 min. Later, these slides were differentiated in 1% acetic acid for 2 min, followed by a 5 min wash in 100% isopropanol. These slides were then mounted and examined under microscope.

Statistical analysis

Results are represented as mean ± Standard Error (Mean ± SE). Student's t-test was used to test statistical significance. The confidence interval was fixed at 95 per cent ($P < 0.05$).

Results and Discussion

Excess fibrous tissue formation or encapsulation due to the deposition of newly secreted extracellular matrix by fibroblasts plays a significant role in graft failure. The majority of the newly deposited extracellular matrix consists of particularly, types I and III collagen during progression of tissue repair. It is characterized by the primary accumulation of type III collagen, followed by type I collagen in wound repair (Fitzgerald *et al*., 1996).Those biomaterials, which have the ability to form minimal fibrosis are usually preferred. The commonly available histochemical special stains such as Masson's trichrome, Verhoff's Van Geison or Movat's pentachrome may not be suitable to identify the types and collagen distribution separately.

In this study, abdominal wall defect model was created successfully in the abdominal wall in SD rats. All the animals survived the 4weeks study period after the surgical implantation procedure with JDS and C-SIS scaffolds. There were no signs of infection or rejection of the bioscaffold materials in both the groups. All rats showed normal feeding and voiding habits throughout the course of the study. Both scaffolds were found integrated and highly vascularized at 4 weeks post-implantation. The implanted scaffolds were retrieved and subjected to Herovici's staining. Herovici stained sections revealed purple coloured type I and blue coloured type III collagen (Figure 2A and 2B). This was quantified morphometrically in Herovici-stained tissue sections for the determination of ratio of type I: III collagen (Figure 3). Later, the assessment of the collagen types I: III ratio revealed that C-SIS induce more deposition of type I collagen compared to JDS scaffold. This may be attributed to the fact that JDS is a cross-linked scaffold while C-SIS is a non-cross-linked scaffold. Higher concentration of type I collagen is preferred in hernia repair with bioscaffolds because occurrence of hernia has been associated with a lower type I: III collagen ratio in human patients (Henriksen *et al*., 2011).

The implantation of biomaterial induces an inflammatory response followed by healing mechanisms (Badylak, 2002). This host response may lead to either acceptance of the biomaterial with the adjacent host tissue with minimal scar tissue formation or impairment of function by excessive connective tissue deposition (Reing *et al*., 2009). Initial inflammatory response could be studied with haematoxylin and eosin stain while individual collagen components could not be assessed with available connective tissue special stains. Here, Herovici's polychrome could be of use in distinguishing types I and III collagen (Herovici, 1963). ECM scaffolds promote tissue repair and *de novo* skeletal muscle formation with degradation of scaffolds around the host microenvironment of implants (Valentin et al., 2009). On the other hand, repair with cross-linked JDS scaffolds resulted in excessive connective tissue deposition.

Rats are the most widely investigated animal models and frequently used to study hernia repair. This experimental data will form the basis for clinical studies, using C-SIS as an interesting hernia-repair substitute compared to JDS group. Further studies are needed to assess the potential of C-SIS to improve hernia repair and validate the potential clinical relevance of this substitute. Thus, type I: III collagen ratio determines the clinical outcome of patients with newly introduced biomaterial for ventral hernia repair.—

The objective of this study was to employ Herovici's polychrome technique to compare collagen distribution within the repaired

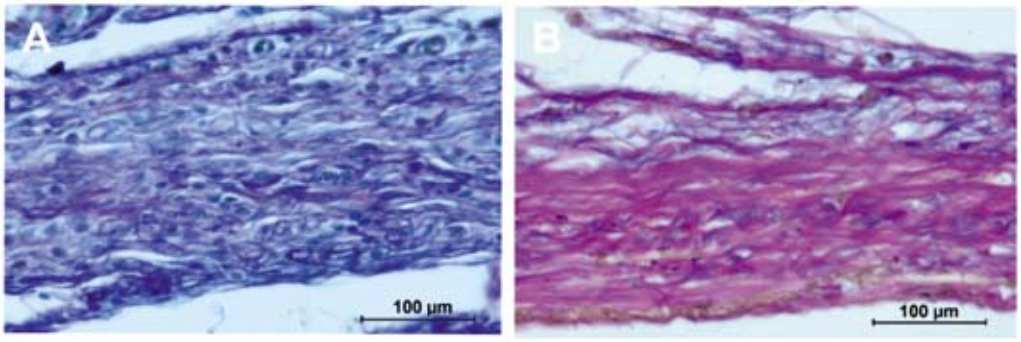

Figure 2. Herovici staining showed extracellular matrix components of implanted JDS and C-SIS biscaffolds at 4 weeks. The JDS-grafted wound showed increased type III collagen distribution (blue) at 4 weeks (A) compared to C-SIS-grafted wounds (B). C-SIS grafted wounds had more type I collagen (purple). Jejunum-derived scaffold (JDS), Surgisis graft (C-SIS).

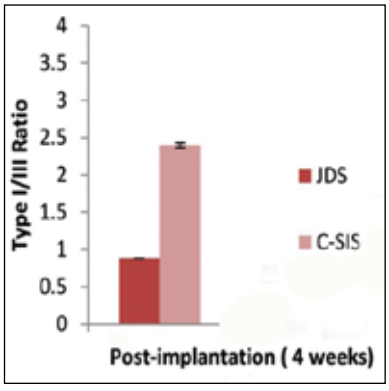

Figure 3. C-SIS explants revealed enhanced types 1: III collagen ratio when compared to JDS at 4 weeks (p value <0.05) Jejunum-derived scaffold (JDS), Surgisis graft (C-SIS).

tissue in a hernia repair model. These findings indicate that C-SIS can be successfully used to reduce fibrosis and enhance constructive remodeling in repairing hernia in both human and veterinary patients. Here, CSIS showed better type I: III ratio compared to JDS group. This study shows that Herovici's polychrome staining is a better differential stain and can be used for studying bioscaffold mediated hernia repair.

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