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Rohu fish swim bladder as a sustainable biomaterial: decellularisation and crosslinking for scaffold fabrication#

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Abstract

Decellularised fish swim bladders are emerging as promising natural biomaterials for tissue engineering owing to their rich extracellular matrix composition. Despite this potential, species-specific variations in swim bladder structure remain underexplored, which may influence their suitability as scaffolds. In this context, the present study investigated the histological characteristics of the rohu fish (Labeo rohita) swim bladder and evaluated its structural suitability as a scaffold following decellularisation and crosslinking. Histological analyses revealed distinct architectural differences between the two chambers of the swim bladder. The anterior chamber consisted of sparsely arranged, wavy collagen fibres, while the posterior chamber exhibited abundant, thick and parallel-aligned collagen bundles interspersed with elastin fibres. Based on its dense and well-organised ECM network, the posterior chamber was selected for further processing by means of decellularisation and crosslinking. Decellularisation was employed to eliminate cellular and immunogenic components, thereby minimising potential immune responses and crosslinking was done to enhance the structural stability and durability of the resultant matrix. Subsequent evaluation unveiled that bovine bile mediated decellularisation efficiently removed cellular residues while preserving the native ECM organisation and EDC crosslinking helped to maintain tissue integrity. Collectively, the findings suggest that the posterior chamber of the rohu swim bladder could serve as a promising and sustainable natural source for future biocompatible scaffold fabrication and tissue engineering applications, warranting further investigation.

Keywords: Swim bladder, rohu fish, decellularisation, bovine bile, biomaterial, scaffolds

Tissue engineering and regenerative medicine depend on suitable biomaterials capable of restoring tissue function and architecture. Traditionally, extracellular matrix (ECM)-derived scaffolds have been sourced from mammalian tissues. However, these raise concerns of zoonotic disease transmission, ethical issues and immunogenic responses. This has led to growing interest in alternative, sustainable biomaterials, particularly those derived from aquatic sources. By-products from the fishing industry, such as fish skin, scales, swim bladders, fins, bones and cartilage are rich in collagen and ECM components but are often discarded as waste. Their utilisation not only mitigates environmental burden but also transforms low cost waste into high value biomaterials (Liu et al., 2012). Among these, fish swim bladders are especially

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promising due to their abundance of collagen and elastin, which impart favourable mechanical and biochemical properties for scaffold fabrication (Zhang et al., 2019). Despite this potential, only a few studies have investigated freshwater fish swim bladders such as those of rohu (*Labeo rohita*) for scaffold preparation. Their histological features and ECM organisation have not been systematically characterised, leaving their biomedical applicability largely unexplored. Understanding the structural and compositional attributes of rohu swim bladders could thus provide insights into their suitability as natural biomaterials for scaffold development. Scaffold fabrication often involves two critical processes: decellularisation and crosslinking, which are essential for transforming naïve tissues into biocompatible and stable matrices. Decellularisation removes cellular and immunogenic components while retaining ECM structure and composition (Blaudez et al., 2020) and bovine bile has recently emerged as an efficient decellularising agent capable of preserving ECM integrity (Vasudevan et al., 2016). Crosslinking plays a vital role in improving the physicochemical and mechanical stability of the decellularised scaffolds (Zhang et al., 2020). Among various crosslinkers, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is particularly advantageous because it strengthens collagenous matrices through zero-length amide bond formation without introducing cytotoxic residues (Powell et al., 2006). However, the combined use of bovine bile mediated decellularisation and EDC crosslinking in freshwater fish swim bladders has not been previously examined. Therefore, the present study investigates the histological organisation and ECM composition of the rohu swim bladders, with emphasis on assessing the efficacy of bovine bile mediated decellularisation and the effect of EDC crosslinking, to assess its potential as a promising candidate for bioscaffold fabrication.

The study involved histological characterisation, decellularisation and crosslinking treatments of fish swim bladder tissues for scaffold fabrication and subsequent evaluation of ECM preservation and DNA removal efficiency. Fresh rohu fishes (*Labeo rohita*) were collected from Meenkara Dam, Palakkad, Kerala and their swim bladders were excised, washed thoroughly in cold distilled water to remove fat, blood and debris and stored at -20°C until further processing. To establish the baseline tissue architecture and ECM organisation, histological analyses of the swim bladder tissues were carried out. Representative tissue samples from both anterior and posterior chamber of the swim bladders were fixed in 10 per cent neutral buffered formalin, processed and sectioned at a thickness of 5μm. The sections were stained with haematoxylin and eosin (H&E) for general histological evaluation (Suvarna et al*.,* 2018). Additionally, Masson's trichrome, Picrosirius red and Verhoeff-Van Gieson stains were employed to visualise collagen and elastin (Luna, 1968). This preliminary histological characterisation served as a reference for assessing structural alterations following decellularisation and crosslinking. Following histological profiling, scaffold fabrication was undertaken through sequential decellularisation and crosslinking steps, with the posterior chamber selected owing to its dense ECM. Swim bladders were defatted using chloroform and methanol mixture (2:1, v/v) for 48 hours at 4°C with intermittent stirring. The defatted scaffolds were subjected to decellularisation using 80 per cent aqueous bile (v/v) in a shaking incubator for two hours (Fig. 2.). This was followed by sequential washing with one per cent phosphate buffered saline (PBS, pH 7.2) for 30 minutes (repeated 4 times) and deionised water for 30 minutes (repeated 4 times) (Megha et al., 2022). To stabilise the decellularised matrix, crosslinking was carried out using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Kumar et al., 2013). The scaffolds were immersed in one per cent (w/v) solution of EDC in PBS (pH 7.2) and placing it in a shaking incubator for 10 minutes under agitation, followed by thorough rinsing with cold distilled water to remove residual reagents and dried under laminar airflow. The efficiency of the decellularisation was assessed both histologically and quantitatively. Haematoxylin and Eosin staining used to verify the removal of cellular components in decellularised tissues. In addition, histological evaluation aided in assessing the influence of EDC crosslinking on ECM organisation, identifying any possible alterations in the arrangement of collagen and elastin fibres. To complement histological findings, quantitative assessment of residual DNA was performed as a molecular indicator of decellularisation efficacy. In this study, the residual DNA content of fish swim bladder tissue before and after decellularisation was extracted using the DNeasy Blood & Tissue Kit (*QIAGEN, Germany*) and the total DNA concentration was determined spectrophotometrically at λ=260nm (*Nano drop 1000, Techno Scientific, CELBIO, Milan, Italy*). Data are expressed as mean ± standard error of the mean (n = 3) and statistical comparison between decellularised and non-decellularised groups was performed using an unpaired t-test, with $p < 0.05$ considered statistically significant

The swim bladder of rohu fish consisted of two distinct chambers, anterior and posterior, each displaying unique histoarchitectural features with significant compositional differences. Haematoxylin and eosin staining revealed scanty, wavy collagen fibres interspersed with muscle fibres in the anterior chamber, while the posterior chamber exhibited abundant, thick, parallel collagen bundles. Masson's trichrome and Picrosirius red staining confirmed dense collagen deposition in the posterior chamber, whereas Verhoeff-Van Gieson staining indicated the presence of elastin fibres, forming a well organised collagen-elastin network that confers both tensile strength and elasticity, which are essential properties of scaffolds intended for tissue engineering (Audelo et al., 2020). The organisation of rohu swim bladder tissue observed in the study aligns with previous descriptions of swim bladder composition rich in collagen and elastin (Morris and Albright, 1979; Liu et al., 2020). However, interspecies

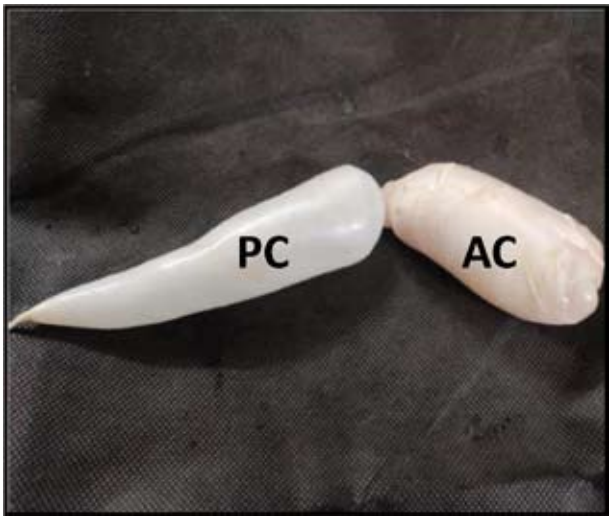

Fig. 1. Swim bladder from rohu fish AC: Anterior chamber, PC: Posterior chamber

Fig.2. Decellularisation in a shaking incubator

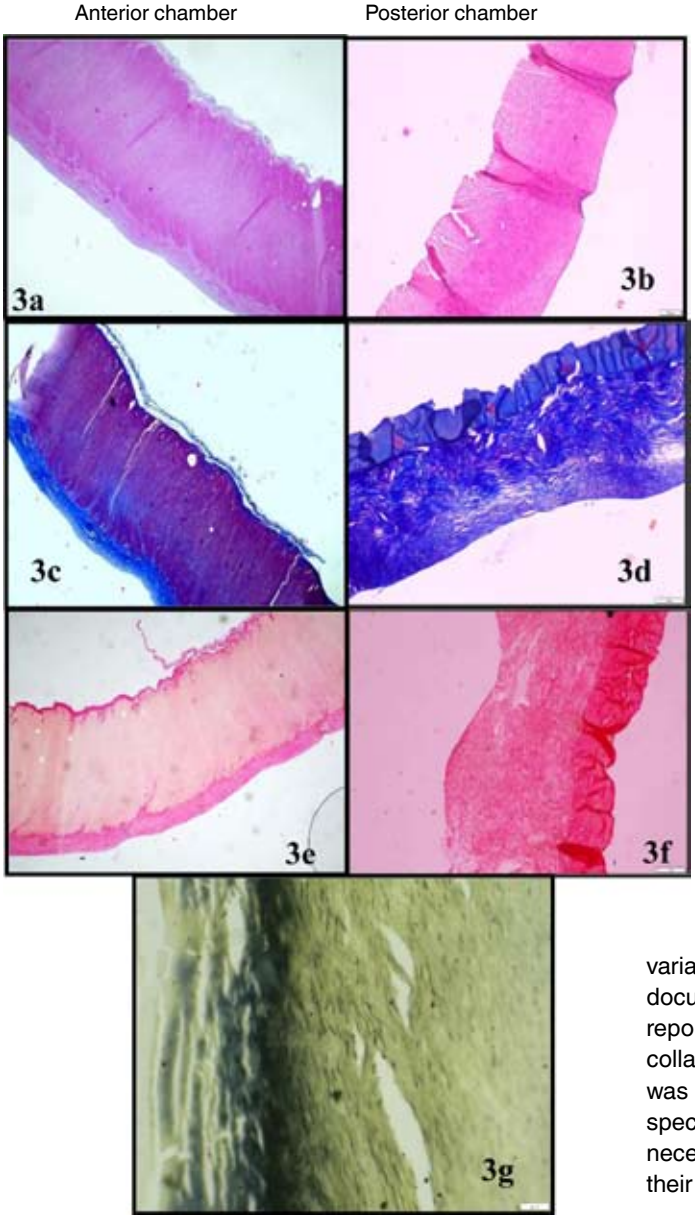

Fig.3. Histology of fish swim bladder

a. Anterior chamber of fish swim bladder with wavy collagen fibres in tunica externa (H&E X 100)

b. Posterior chamber of fish swim bladder with wavy collagen fibres throughout (H&E X 100)

c. Anterior chamber of fish swim bladder with blue stained collagen fibres and red colour muscle bundle in between (Masson's Trichrome X 100)

d. Posterior chamber of fish swim bladder with blue collagen fibres throughout tissue (Masson's Trichrome X 400)

e. Anterior chamber of fish swim bladder with red stained collagen fibres and yellow muscle bundle in between (Picrosirius red X 100)

f. Posterior chamber of fish swim bladder with red collagen fibres throughout tissue (Picrosirius red X 100)

g. Posterior chamber of fish swim bladder showing black stained elastin fibres against yellow background (Verhoff Van-Gieson X 400)

variations in swim bladder architecture have been documented. For instance, Morris and Albright (1979) reported that in goldfish, the posterior chamber lacked a collagen rich tunica externa, while the anterior chamber was more collagen dense. These observations highlight species-specific structural adaptations, underscoring the necessity of characterising individual fish species before their utilisation in biomaterial applications.

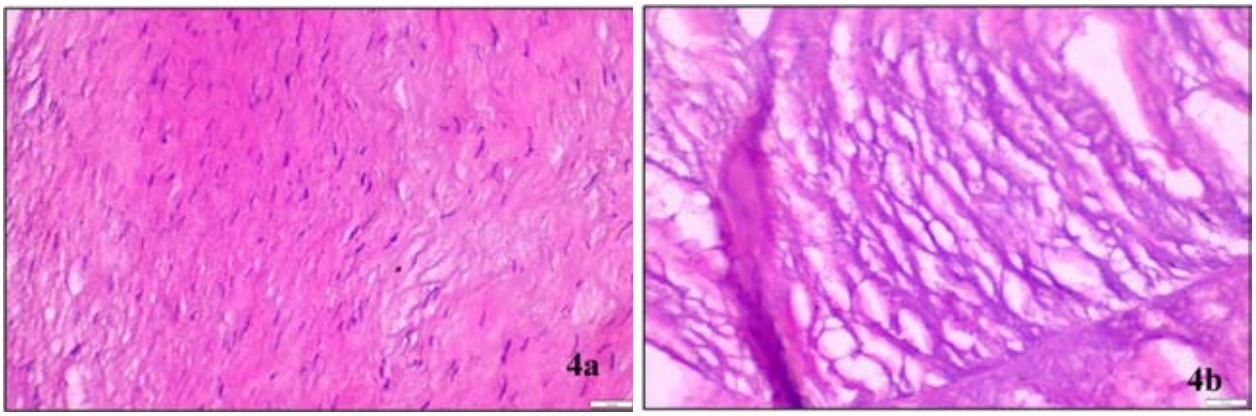

Fig.4a. Non-decellularised swim bladder, containing blue stained nuclei

Fig.4b. Decellularised-crosslinked swim bladder showing absence of nuclear remnants and preservation of the ECM components

Table 1. The DNA concentrations of d-FSB and n- FSB (n=3)

DNA concentration (ng/µl)	
d-FSB	**n-FSB**
12.1	213.7
2.8	189.5
23.1	230.8

Table 2. The mean DNA concentrations of d-FSB and n- FSB

Scaffolds	DNA concentration (ng/µl)
d-FSB	$12.67^{b} \pm 5.87$
n-FSB	$211.3^{a} \pm 11.98$
t-value (P-value)	14.89** ($p < 0.001$)

** Significant at 0.01 level

Means having different small letter as super script differ significantly within row

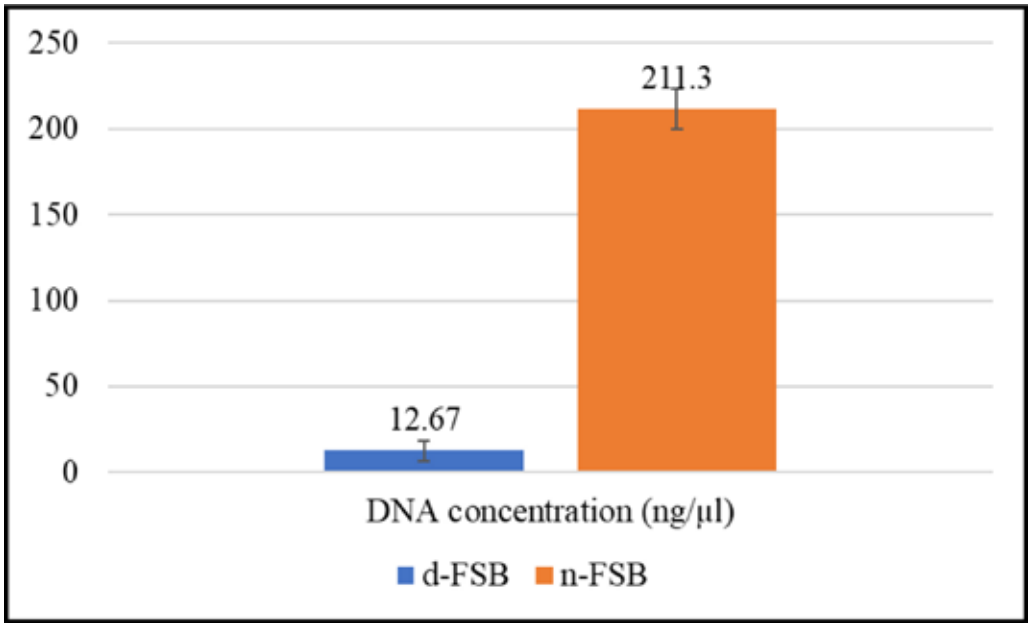

Fig.5. Comparison of DNA concentration of d-FSB and n-FSB

The posterior chamber, owing to its rich ECM composition, was selected for decellularisation using bovine bile and EDC mediated crosslinking. Histological evaluation demonstrated effective removal of nuclear and cytoplasmic components in the decellularised and crosslinked (d-FSB) scaffolds compared to naïve (n-FSB) tissue, while preserving the collagenous matrix architecture. The collagen bundles in the d-FSB remained well-aligned and intact, with no evidence of fibre disruption or matrix shrinkage, suggesting that EDC crosslinking successfully stabilised the ECM without compromising its innate ultrastructure (Fig. 4a., 4b.). Quantitative analysis of DNA content corroborated the histological findings, showing a reduction in DNA concentration from 211.3 ± 11.98 ng/µl in n-FSB to 12.67 ± 5.87 ng/µl in d-FSB (Table. 1 and 2., Fig. 5.), which was well below the recommended threshold of 50 ng/mg tissue, satisfying the decellularisation criteria outlined by Crapo et al. (2011),

which include dsDNA content <50 ng/mg tissue, fragment length <200 base pairs and absence of visible nuclei in H&E or DAPI stained sections. (Crapo et al., 2011). These outcomes validate that bile-mediated decellularisation effectively removed cellular residues with minimal ECM alteration (Vasudevan et al., 2016; Megha et al., 2022), while subsequent EDC crosslinking reinforced the matrix through covalent bonding between collagen carboxyl and amine groups (Jiang et al., 2022). Collectively, these results confirm that the integrated bovine bile mediated decellularisation and EDC crosslinking approach yields structurally stable and biochemically preserved scaffolds from rohu swim bladder, supporting its potential utility as a natural biomaterial for tissue engineering applications.

Summary

The present study demonstrated that the posterior chamber of the rohu fish swim bladder possesses superior structural organisation and extracellular matrix composition, characterised by a dense collagen network and well-defined elastic components. Histological evaluation revealed a well preserved ECM architecture with distinct collagenous and elastic layers, indicating its inherent structural robustness. Bovine bile mediated decellularisation effectively removed cellular and nuclear residues while maintaining key ECM constituents such as collagen and elastin. Subsequent EDC crosslinking further enhanced the mechanical stability of the scaffold without compromising its innate structure. Collectively, these findings establish the posterior chamber of rohu swim bladder as a promising and sustainable source of natural biomaterial with potential applicability in scaffold fabrication and other biomedical uses.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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