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Short Communication

AgNOR STAINING: A DIAGNOSTIC TOOL FOR DETERMINING THE PROLIFERATION ACTIVITY OF FIBROBLASTS IN BIOSCAFFOLD ASSISTED HEALING

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ABSTRACT: A battery of *in vivo* evaluations is done on bioscaffolds to ascertain its biocompatibility and host responses. Fibroblast proliferation in a controlled manner is integral part in scaffold assisted wound healing and tissue regeneration. Therefore, AgNOR staining was employed in this study for quantification of fibroblast proliferation and therefore evaluating the host bioscaffold response. Here full thickness skin wound healing studies were done in adult male New Zealand White rabbits using decellularized porcine cholecyst derived scaffolds (dPCS), scaffold supplemented with autologous bone marrow cells (dPCSM) and open wound control group (OW). The scaffolds were explanted seven and forteen days post implantation and were subjected to AgNOR staining. Statistical analysis showed significant variations between scaffold assisted and open wound healing. The study concluded that AgNOR could be used as a good quantitative marker involving fibroblast proliferation and could be used in primary screening of bioscaffold assisted healing and regeneration studies.

Key words: AgNOR, Bioscaffold, Fibroblasts, Wound healing.

Biomaterials constitute parts of medical implants, extracorporeal devices and disposables that have been utilized in medicine, surgery, dentistry, and veterinary medicine as well as in every aspect of patient health care. Naturally occurring biomaterials and scaffolds derived from different animal origin have shown to have great significance in the welfare of human and veterinary patients (Thampi *et al*. 2013, Anilkumar *et al.* 2014, Suvaneeth *et al*. 2016). The processed bioscaffolds undergoes a battery of *in vivo* evaluations as per ISO 10993:6 prior to clinical or commercial productions. Some of these involve host tissue responses to the implanted or in contact bioscaffolds *in vivo.* A variety of factors results in considerably varying host tissue responses to the scaffolds. Fibroplasia or fibroblast proliferation in a controlled manner is essential for bioscaffold applications like wound healing and tissue regeneration.

The actively dividing fibroblasts have a higher rate of protein translation. Nucleolar organizer regions (NORs) are defined as nucleolar segments containing proteins which are having high affinity to silver, naming argyrophilic nucleolar organizer region (AgNOR) proteins. NORs are the loops of DNA that transcribe genes for ribosomal RNA. AgNORs have been used as histochemical pointers of cell multiplication and is corresponding to the proliferative action of the cell. Hence AgNOR staining is employed in this study for quantification of fibroblast proliferation and therefore evaluating the host bioscaffold response.

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Study pattern

Bioscaffolds

Decellularised porcine cholecyst derived scaffolds (dPCS) were produced from gall bladders collected from freshly slaughtered Large White Yorkshire pigs (of about 90 kg weight average) from Meat Technology Unit, Department of Livestock Products Technology, CVAS, Mannuthy using a non-enzymatic process with mechanical delamination as described by Anilkumar *et al*. (2014). The implantation studies were done using the scaffold alone (dPCS), scaffold supplemented with autologous bone marrow cells (dPCSM) and open wound control group (OW).

***In vivo* studies**

The study was performed in conformity with the guidelines established by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), GOI. The animal experiments were done with the approval and as per requirements of the Institutional Animal Ethics Committee of College of Veterinary and Animal Sciences, Mannuthy (BT/4/12/DVM/2013/PA).

Twelve adult male New Zealand White rabbits of about 1.5 Kg were randomly assigned to three groups *viz.* dPCS, dPCSM and OW (N=6). The surgical procedures were done under general anaesthesia (Markowitz 1964) using a combination of 80 mg/kg ketamine hydrochloride (Aneket, Neon laboratories limited, Mumbai) and 5mg/kg xylazine hydrochloride (Xylaxin, Indian Immunologicals, Hyderabad). The scaffolds were grafted on full thickness square shaped excision wounds of 2X2 cm made dorsally, on either side of the spine at thoracic region (N=6) with polyamide suture material (Ethilon™ 3-0, Johnson and Johnson India).

On day 7 and 14 post-surgery, six animals from experiment (six on each occasion) were humanely sacrificed using carbon dioxide chamber. The skin graft along with the surrounding normal skin at the site of implantation were explanted and fixed in 10 per cent NBF and were processed to make paraffinized sections of 4-5µm thickness (Bancroft and Gamble 2008).

AgNOR staining

The paraffinized tissues sections were dewaxed and cleared in three changes of xylene, rehydrated in serial grades of isopropyl alcohol and then in distilled water. Slides containing the sections were then placed on a staining rack and two drops of gelatine solution (two per cent gelatine solution in de-ionised water containing one per cent formic acid) were added on the section. Over this one drop of silver nitrate solution (50 per cent solution of AR grade silver nitrate in de-ionised water) was overlaid and the sections were incubated in a dark chamber at room temperature for 45 min (Umashankar 2001, Vishnu 2015). The slides were then air dried and subjected to microscopic examination.

The AgNORs were visualized as brownish black discrete dots of variable size within the nuclei. AgNOR counts of 100 fibroblast nuclei were carried out under oil immersion objective and were expressed as percentage.

Histopathology

Histopathological evaluations of the paraffinized tissue sections were done using H&E staining and Masson's Trichrome staining (Bancroft and Gamble 2008) to compare the fibroblast proliferation and remodelling status of the explanted tissue.

Statistical analysis

Results were represented as Mean ± Standard Error (Mean ± SE). Student's t-test was used to test statistical significance using IBM Statistical Package for the Social Sciences software version 20 (SPSS 20). The confidence interval was fixed at 95 per cent ($p< 0.05$).

Results and discussion

Proliferating fibroblasts were counted by the presence of nucleolar spots when stained with silver nitrate based special staining. Positively stained fibroblasts contained on an average of two nucleolar spots within each nucleus. More fibroblasts were seen positive on day 7 Post implantation (PI) (Fig. 1) compared to day 14 PI (Fig. 2). The AgNOR counts in the fibroblasts of the control group were significantly lower on day 7 PI, compared to dPCS and dPCSM groups (Table 1).

Histopathological examination confirmed increased number of open nucleated fibroblasts indicating increased fibroblast proliferation and wavy collagenisation on day 7 PI in dPCS and dPCSM compared to OW groups. More spindle shaped fibroblasts were seen by day 14 PI suggesting diminishing fibroblast proliferation and maturation and organization of collagen fibers in all treatments.

AgNOR count was selected as a preliminary tool in quantifying fibroblast proliferation (Umashankar 2001, Vishnu 2015). The staining represents actively transcribing nucleolar organizing regions (NOR) and hence well expressed in replicating or proliferating cells with active ribosomal activity. More number of proliferating fibroblasts were observed during day 7 PI in all the animals. dPCS and dPCSM implants showed a

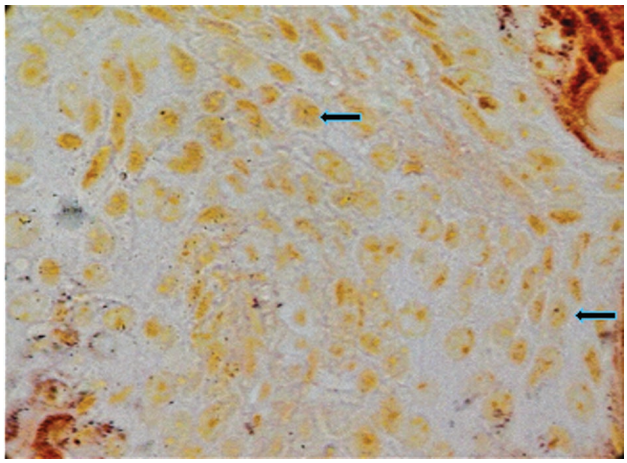

Fig. 1. dPCS (Day 7): Round nucleated fibroblasts with nucleolar spots (arrows) (AgNOR X 1000).

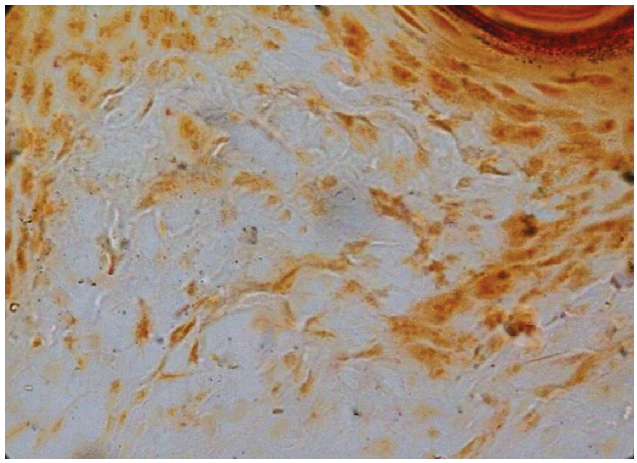

Fig. 2. dPCSM (Day 14): Spindle shaped fibroblast nuclei with multiple black nucleolar spots (AgNOR X 1000).

significantly higher fibroblast proliferation compared to open wound on day 7 PI. Fibroblast proliferation reduced gradually over the remaining period of the experiment. Thereafter there was no difference in fibroblast proliferation between any of the treatments on day 14. The initial higher fibroblast proliferation suggests that the scaffold application promoted the migration of fibroblasts thereby accelerating the collagenization and faster remodeling of the tissue. AgNOR count indexed active fibroblast proliferation in tissue remodeling and relates to keratinocyte activity in epidermal repair and regeneration. Fibroblasts directly affects the wound healing as it secretes the remodeling matrix, growth factors, cytokines and even proliferation and differentiation of keratinocyte resulting in neo-epidermal formation (Ghahary and Gaffari 2007). NORs in the active fibroblasts were small pin head sized and were commonly two in numbers and rarely three were seen. Vishnu (2015) have observed similar sized NORs with same dispersion frequencies while analyzing the biocompatibility of differently processed de-cellularised bovine pericardium, while Hussain (2017) observed similar sized NORs with a higher dispersion frequency (up to five per nucleus) while analyzing subcutaneous tissue reactions of differently treated bovine omentum. Bukhari *et al.* (2007) observed that the NORs varied in size and numbers depending on mitotic activity. Studies done by Akhtar *et al.* (2004) and Khan *et al.* (2006) confirmed that AgNOR counts increase in the dermis, but size and dispersion variations are observed in malignancy. In this experiment, it is found that the NOR expression relating the smaller size and lesser numbers denotes a controlled increased proliferative activity in scaffold assisted healing against a relatively lower proliferation in open wounds. Histopathological observations also confirmed that cholecyst derived scaffold favors fibroblast proliferation in a regulated manner compared to open wound control groups, ensuring proper remodeling which was in accordance with the gross and histological evaluations of the wound healing processes observed by Thampi *et al.* (2013), Ali (2013) and Vishnu (2015) in subcutaneous implantation studies; Anilkumar *et al.* (2014) in cutaneous wound healing studies; Suvaneeth *et al.* (2016) and Hussain (2017) in intramuscular implantation studies.

Table 1. Mean AgNOR count for fibroblast proliferation.

Treatment	Mean AgNOR count (%)	
	Day 7	Day 14
dPCS	2.148 ± 0.009[a]	1.858 ± 0.009[a]
dPCSM	2.125 ± 0.016[a]	1.835 ± 0.016[a]
OW	2.008 ± 0.026[b]	1.846 ± 0.019[a]

Means bearing different superscripts in a column differ significantly $p<0.05$.

AgNOR count is a simple and efficient technique used since long for detecting proliferative activity of cells, especially tumour cells. Bioscaffold based regeneration depends on host tissue remodeling based on fibroblast activity. Fibroblast proliferation index in bioscaffold assisted healing was quantitatively measured using AgNOR count. The result of the study suggests that AgNOR is a good quantitative marker of initial proliferative changes involving fibroblasts and can be used as primary indexing tool for bioscaffold assisted controlled healing and regeneration studies.

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REFERENCES

Akhtar GN, Chaudrhy NA, Tayyab M, Khan SA (2004) AgNOR staining in malignant and benign effusion. Pak J Med Sci 20: 29-32.

Ali A (2013) Evaluation of decellularised bovine pericardium and jugular vein in abdominal regeneration model in rat. M.V.Sc. thesis, Kerala Veterinary and Animal Sciences University, Thrissur. 68.

Anilkumar TV, Vineetha VP, Revi D, Rajan A (2014) Biomaterial properties of cholecyst-derived scaffold recovered by a non-detergent/enzymatic method. J Biomed Mater Res 102(7): 1506-1516.

Bancroft JD, Gamble M (2008) Theory and practice of histological techniques. 6th. edn. Churchill Livingstone, United States of America. 725.

Bukhari MH, Niazi S, Khan SA, Hashimi I, Perveen S *et al*. (2007) Modified method of AgNOR staining for tissue and interpretation in histopathology. Int J Exp Path 88: 47-53.

Ghahary A, Ghaffari A (2007) Role of keratinocyte-fibroblast cross-talk in development of hypertrophic scar. Wound Repair Regenr 15(1): S46.

Hussain KVS (2017) Tissue response of decellularised bovine omental implants in rat model. M.V.Sc thesis, Kerala Veterinary and Animal Sciences University, Thrissur. 85.

Khan SA, Chaudhry NA, Khalid AW, Akhtar GN, Ibne- Rasa SN (2006) Patterns of argyrophilic nucleolar organiser regions in pleural and peritoneal effusions. J Coll Physicians Surg Pak 16: 412-415.

Markowitz J, Archibald J, Downie HG (1964) Experimental surgery. 5th. edn. The Williams and Wilkins Company, Baltimore. 643.

Suvaneeth P, Nair ND, Umashankar PR, Vijayan N, Mammen, JA *et al*. (2016) *In vitro* characterization of decellularised bovine pericardium. J Livestock Sci 7: 13-18.

Thampi P, Nair ND, Lalithakunjamma R, Vijayan N, Venugopal S *et al*. (2013) Pathological effects of processed bovine pericardial scaffolds - a comparative *in vivo* evaluation. Artificial Organs 37(7): 600-605.

Umashankar PR (2001) Efficacy of argyrophilic nucleolar organizer region count test for *in vivo* biomaterial evaluation. M.V.Sc thesis, Kerala Agricultural University, Thrissur. 68.

Vishnu S (2015) Effect of differently processed decellularised bovine pericardium on tissue remodelling in rat subcutaneous model. M.V.Sc thesis, Kerala Veterinary and Animal Sciences University, Thrissur.

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