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Tissue engineering approach to repair abdominal wall defects using cell-seeded bovine tunica vaginalis in a rabbit model

T. Ayele · A. B. Z. Zuki · B. M. A. Noorjahan · M. M. Noordin

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Abstract The aim of this study was to engineer skeletal muscle tissue for repair abdominal wall defects. Myoblast were seeded onto the scaffolds and cultivated in vitro for 5 days. Full thickness abdominal wall defects (3 × 4 cm) were created in 18 male New Zealand white rabbits and randomly divided into two equal groups. The defects of the first group were repaired with myoblast-seeded-bovine tunica vaginalis whereas the second group repaired with non-seeded-bovine tunica vaginalis and function as a control. Three animals were sacrificed at 7th, 14th, and 30th days of post-implantation from each group and the explanted specimens were subjected to macroscopic and microscopic analysis. In every case, seeded scaffolds have better deposition of newly formed collagen with neovascularisation than control group. Interestingly, multinucleated myotubes and myofibers were only detected in cell-seeded group. This study demonstrated that myoblast-seeded-bovine tunica vaginalis can be used as an effective scaffold to repair severe and large abdominal wall defects with regeneration of skeletal muscle tissue.

T. Ayele (✉) · A. B. Z. Zuki (✉) · M. M. Noordin
Faculty of Veterinary Medicine, University Putra Malaysia, 43400 Serdang, Selangor, Darul Ehsan, Malaysia
e-mail: ayutadei@yahoo.com

A. B. Z. Zuki
e-mail: zuki@vet.upm.edu.my

T. Ayele
Faculty of Veterinary Medicine, University of Gondar, P.O. Box 196, Gondar, Ethiopia

B. M. A. Noorjahan
Faculty of Biotechnology and Bimolecular Sciences, University Putra Malaysia, 43400 Serdang, Selangor, Darul Ehsan, Malaysia

1 Introduction

Tissue engineering represents a scientific approach that attempts to mimic neo-organogenesis [1]. The creation of skeletal muscle tissue using tissue engineering methods holds promise for the treatment of a variety of muscle diseases; including skeletal myopathies such as muscular dystrophy. In addition traumatic injury, aggressive tumour ablation and prolonged denervation are also common clinical situations that often result in significant loss of muscle tissue requiring subsequent surgical reconstruction [2, 3].

One of the strategies for muscle tissue engineering involves the harvesting of satellite cells, their expansion in vitro, and their subsequent autologous implantation in vivo into the sites requiring repair or replacement. One of the main obstacles in the formation of new muscle tissue is the lack of an adequate support for expanded satellite cells. To overcome this obstacle, many researcher groups are trying to develop adequate synthetic and biological delivery systems for implanted cells [4].

In recent years, there has been an increasing demand of biological biomaterial using as scaffolds due its compatibility, availability, suitability for cell attachment, proliferation, differentiation and migration to facilitate tissue ingrowths, which may help long term maintenance of mechanical strength [5]. To this effect the present study has used bovine tunica vaginalis which, is a serous membrane formed by an out-pouching of the parietal peritoneum of the abdominal cavity during testicular descent [6].

Previous study has reported that reduction in post-operative pain, length of recovery period and the number of recurrence and transformed into fibrous tissue when bovine tunica vaginalis alone used for repair of abdominal wall defects in a rat model but failed to recover skeletal muscle tissue [7], which indeed require the use of novel technology

like tissue engineering to recover or regenerate muscle tissue. Therefore, the aim of this study was to engineer skeletal muscle tissue by combing satellite cells with biological scaffolds.

2 Materials and methods

2.1 Animal

New Zealand White rabbit were purchased from East Asian Private Limited Company, Malaysia. One 5-day-old rabbit served as donors of myoblast and eighteen adult rabbits served as recipient of implantation. All the experiments were performed in compliance with the principles and guide to ethical use of laboratory animals approved by University Putra Malaysia, Faculty of Veterinary Medicine's Animal Care and Use Committee.

2.2 Scaffold preparation

Fresh bovine tunica vaginalis sacs were collected from abattoir. Immediately the adherent fat and fascia were removed and washed thoroughly under running tape water, disinfected in 0.05% sodium hypochlorite and shaken three times in serial changes of sterile normal saline for 20 min each. The sacs were then spread on freeze-drier steel dishes and stored in a deep freezer overnight at −80°C. The steel dishes containing the grafts were transferred into Christ Loc-1 freeze-drier (Braun Diessel Biotech, Germany) adjusted at −40°C for 24 h under vacuum pressure of 0.12 mbar. The freeze-drier condenser temperature and shelf temperature during freeze-drying process were −40 and −30°C respectively. The freeze-dried graft were cut into identical pieces of 3 × 4 cm, sealed in airtight double-layered polyethylene bags and sterilized by 25 kGy gamma rays (Cobalt 60, JS8900, IR-174) and stored at room temperature before implantation.

2.3 Myoblast harvest and isolation

Primary myoblasts were isolated from Soleus muscles of one 5-day-old rabbit. Enough phosphate buffered saline (PBS) was added to keep the tissue moist, and the muscle tissue was minced with razor blades in the petri-dish. Approximately 2 ml of Collagenase/dispase/$CaCl_2$ solution per gram of tissue was added and the mincing activity was continued for 2 min. The minced tissue was transferred to a sterile tube and incubated at 37°C for approximately 20 min until the mixture became fine slurry and filtered through a piece of 80 μm nylon mesh in a sterile funnel to remove large pieces of tissue. The cells were centrifuged for 10 min at 1,000 rpm. The pellet were re-suspended into F-10-based primary myoblast growth medium (Ham's F-10 nutrient, 20% Fetal bovine serum, 25 μg/ml basic fibroblast growth factor and 1% 100× penicillin/streptomycin) and plated in 75 cm^2 tissue culture flasks (Greiner bio-one, Germany) and incubated in a 37°C, 5% CO_2 incubator and the medium was changed every 2 days. When the cells were ready to be split, the cells were removed from the dish using PBS to isolate myoblast from fibroblast. The medium was changed to F-10/DMEM-based primary myoblast growth medium (Ham's F-10 nutrient, Dulbeccos Modified Eagle Medium (DMEM), 20% Fetal Bovine Serum, 25 μg/ml basic fibroblast growth factor and 1% 100× penicillin/streptomycin) and the medium was again changed every 3 days until the required amount of myoblast cells were obtained. It took only 3 weeks to harvest the pure myoblast using this modified cell culture technique [8, 9]. Myoblast harvested from the primary culture using 0.25% Tryple (GIBCO, USA) were frozen in liquid nitrogen using freezing medium (10% dimethyl sulfoxide (DMSO) and 90% Fetal bovine serum) until required.

2.4 Immunocytochemistry

Purity of the harvested myoblast was determined by an immunocytochemical analysis (desmin staining). Briefly, myoblasts were fixed with 2% paraformaldehyde for 5 min, washed two times for 5 min each and permeabilized using 0.2% Triton X-100 for 10 min, and washed three times for 5 min each. All further incubations were carried out at room temperature and an initial blocking step was performed with a blocking solution consisting of 2% horse serum and 1% bovine serum albumin/PBS to block non-specific antibody binding and the cell were then incubated with the primary antibody for 1 h. Primary antibody was a mouse monoclonal antibody against desmin (mAb DE-U-10, ab6322, Abcam, UK) used at a 1:400 dilution in 1% bovine serum albumin/PBS. The cells were then washed three times with PBS and incubated for 30 min at room temperature in the dark with the FITC-secondary antibody, which was a Fluorescein isothiocyanate-conjugated antimouse IgG (goat polyclonal to Mouse IgG, ab6785 Abcam, UK) diluted 1:100 in 1% bovine serum albumin/PBS. The negative controls were carried out by similarly treating myoblast but omitting primary antibody. The percentages of myogenic cells were determined by flow cytometry (BD Biosciences, USA).

2.5 Labeling of myoblast

Prior to seeding the myoblast onto collagen-based scaffold, myoblasts were labelled with PKH26 red fluorescent (Sigma, USA) according to the manufacturer's instructions. In brief: myoblasts were washed with culture medium without serum and centrifuged and 1,000 μl of Diluent C

(Sigma, USA) were subsequently added to the cell suspension. 4 µl of the PKH dye were added to 996 µl of Diluent C to make 1 ml of 4×10^{-6} M PKH26 dye. Immediately 1 ml of cells mixed with 1 ml of PKH26 dye and incubated for about 5 min at room temperature, then after 2 ml of serum was added to stop further dye incorporation. The cell suspension was washed and centrifuged three times using complete cell culture medium.

2.6 Myoblast seeding protocol

The 3 × 4 cm sized sterilized scaffolds were placed on petri dishes and pre-wetted with culture medium and placed into a 37°C/5% CO_2 incubator for 2 h prior seeding of cell and the scaffolds were blot-dried and transferred to the new petri dishes. PKH26 labelled myoblasts were suspended in a growth medium at a density of 1.0×10^7 cells/ml and subsequently, the scaffold were seeded with myoblast at a density of 1.0×10^7 cells/scaffold and left for 30 min to allow their full attachment to the scaffolds and F-10/DMEM-based primary myoblast growth medium was added. The following day, the culture medium was switched to fusion medium (Dulbeccos Modified Eagle Medium (DMEM), 5% Horse serum, and 1% 100× penicillin/streptomycin) and then changed every 2 days. Cultivation was conducted up to 5th day. Cell morphology and growth in the scaffolds were studied at 1st, 3rd and 5th day of post-seeding using Scanning Electron microscopy (SEM).

2.7 Surgical procedure

The study was conducted on eighteen clinically healthy New Zealand White Rabbits. The animals were randomly divided into two equal groups (I and II) of nine animals each. The rabbit were withheld food and water for 6 h prior to operation. The animals were anesthetized by intramuscular injection of a mixture of 35 mg/kg Ketamine hydrochloride (TROY, Australia) and 5 mg/kg of Xylazine hydrochloride (TROY, Australia). The anaesthesia was maintained by 1%/min Isoflurane throughout the entire procedure. The animals were secured in dorsal recumbency and the abdominal wall was shaved and washed using 4% chlorhexidine (Pharmaniaga, Malaysia), scrubbed with povidone-iodine solution, and draped with the aperture of the fenestrated drape at the intended operation site. A long 6 cm mid-ventral skin incision was made (Fig. 1a) and the 3 × 4 cm whole layer of abdominal wall defects at the mid-ventral abdominal region was created (Fig. 1b). In group I (treatment), the defects were repaired with myoblast-seeded-bovine tunica vaginalis scaffolds, and in group II, the defects were repaired with non-seeded bovine tunica vaginalis scaffolds and served as a control group. The implants were placed in a direct contact with visceral organs, subcutaneous tissue and sutured to the edges of the defect with a simple continuous suture pattern using 3-0 silk (DemeTECH, USA) suture material, except at the corners where interrupted suture pattern were applied (Fig. 1c). The skin were apposed over the operated area in

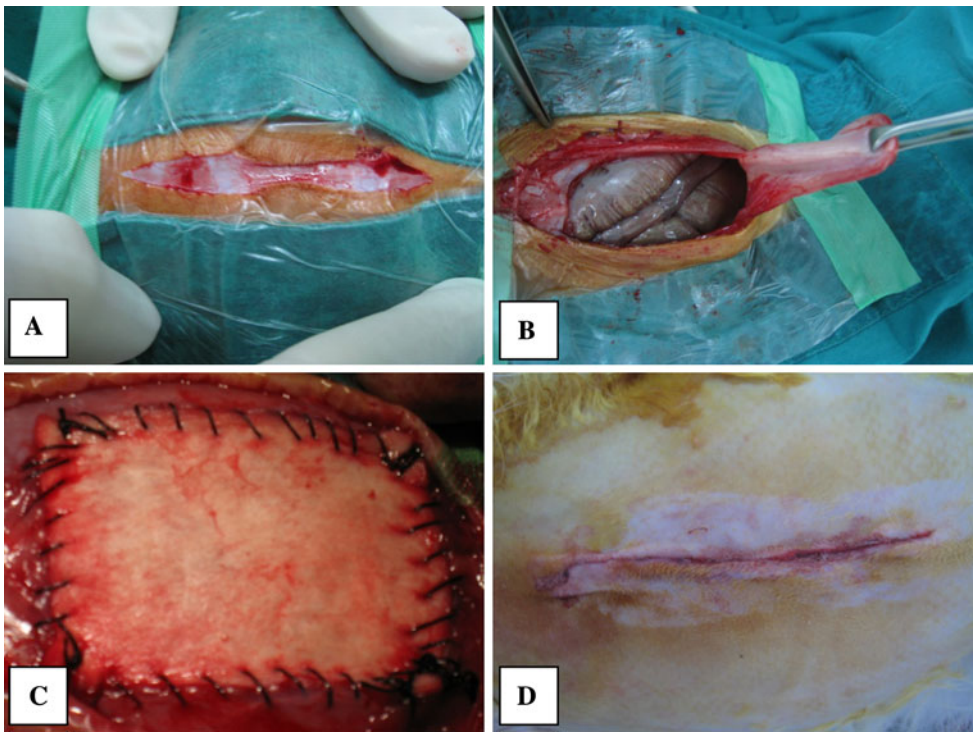

Fig. 1 Implantation of the graft. **a** 6 cm Long mid-ventral abdominal skin incision. **b** Removal of 3 × 4 cm whole abdominal wall. **c** 3 × 4 cm abdominal wall defect repaired by the same size scaffolds stitched with a continuous suture pattern with single interrupted suture pattern at the corner. **d** Skin flap is apposed together by intradermal suture pattern

all operated rabbits and sutured with intra-dermal continuous suture pattern using 3-0 silk (DemeTECH, USA) suture material to avoid self-mutilation of the suture (Fig. 1d). Post-operatively, the operated rabbits were kept in individual cages after they recovered from the general anaesthesia. Analgesia, Tramadol hydrochloride at rate of 4 mg/kg (UNICHEM, India) was given once a day for 3 consecutive days via intramuscular and antibiotics, Streptopenicillin at the rate of 100 mg/kg body weight were administered intramuscularly for 5 consecutive days in all animals. Postoperative follow up were carried out daily up to the end of experiment and the rabbits were subjected to a continuous and closed observation for detection of any post-surgical clinical complication, general health problem and abnormality in food and water intake. Three rabbits from each group were sacrificed at 7th, 14th, and 30th days of post-implantation by using an intra-cardial injection of sodium pentobarbital (CEVA, France) at rate of 100 mg/kg to assess the integration of the implant within the recipient tissue, formation of neo-peritoneum and skeletal muscle tissue in general. Time of sacrification has been chosen based on its critical inflammatory transition stages occurring during the specified time and also due to inflammatory, proliferative, and remodeling phases of wound healing process in a rabbit. Indeed, a similar point of time has been also used in previous studies [10, 11].

2.8 Macroscopic analysis

Macroscopically, the abdominal wall defect areas were examined carefully for detection of postoperative healing complications and to estimate the degree of adhesion formation between the implant and the abdominal viscera. The skin covering the abdominal wall was gently detached and cut off. The abdominal wall including the operated area was cut, lifted gently and examined carefully on both inner and outer surfaces for the development of new peritoneum, vasculature, connective tissue, muscle tissue and for the detection of healing complications such as infection, hernia, fistula, adhesion, seroma, implant shrinkage and graft lost. The adhesion strength was evaluated and graded on the basis of consistency according to the scoring system as described in Table 1 [10, 12, 13].

Table 1 Scoring system for estimating the adhesion degree

Degree	Adhesion classification
0	No adhesion
1	Minimal adhesions that could be freed by slight pull
2	Moderate adhesion that could be freed by aggressive blunt dissection
3	Strong adhesions that required sharp dissection to freed

2.9 Microscopic analysis

2.9.1 Light microscopy analysis

The specimens collected from operated area at 7th, 14th and 30th days of post-implantation were fixed in 10% formalin for 24 h. The specimens were dehydrated in ascending grades of ethanol, cleared in xylene, embedded and blocked in paraffin wax, and then cut into 4 μm thick sections by a rotatory microtome (Leica 2045, Germany). The sections were stained with haematoxylin and eosin for general histology and also Masson's trichrome staining for the demonstration of collagenous tissues and muscle tissue. The stained sections were examined under light microscope (Olympus BX51, Japan).

2.9.2 Fluorescence microscopy analysis

The specimens were fixed in 4% paraformaldehyde mounted in O.C.T. compound (Merck, UK) and snap frozen in liquid nitrogen before cutting frozen sections of 10 μm thick on a cryostat used for fluorescence microscopy to demonstrate the existence of the implanted myoblasts. Additionally, sections were stained with 1,000× dilution of 10 mg ml^{-1} (stock solution) 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Sigma, USA) to show the presence of several nuclei in fused myoblasts. Finally, the sections were mounted with cover slips using aqueous fluorescence mounting media (Merck, USA) and viewed under fluorescence microscope (Olympus BX51, Japan) with the aid of image analyzer.

2.9.3 Scanning electron microscopy analysis

Specimens trimmed into 1 cm^2 blocks and fixed in 4% glutaraldehyde buffered solution 12–24 h at 4°C. The samples were washed three times in 0.1 M sodium cacodylate buffer at pH 7.3 for 10 min each and then post fixed for 2 h with 1% osmium tetroxide at room temperature and washed with 0.1 M sodium cacodylate buffer for 3 changes of 10 min each. The samples were dehydrated in ascending grades of acetone solution and brought to critical point drying by critical point drier (CPD 030, Bal-TEC, Switzerland) for about 30 min. Then, the specimens were affixed to a metal SEM aluminium Stub and sputter coated in gold by using SEM coating unit (E 5100 Polaron, England). The coated specimens were the viewed by Scanning Electron Microscopic (LEO 1455 VPSEM attached with OXFORD INCA ENERGY 300 EDX, UK) at accelerating voltage of 20–30 kV.

2.10 Statistical analysis

Mann–Whitney U test was used to compare adhesion formation and score between treatment and control groups. $P < 0.05$ was considered to be significant.

3 Results

3.1 Myoblast isolation and immunocytochemistry

Myoblasts were successfully isolated from a soleus muscle, digested mechanically as well as enzymatically and cultured as described in Sect. 2.3. During the initial stages of culture, the myoblast showed a rounded morphology. After the first 24 h, the cells attached to the culture surfaces and also spindle-shaped cells started to sprout out from isolated muscle fibers and then proliferated. It took only 3 weeks to harvest the ideal number of a pure myoblast and to get rid of the fibroblast cell. Based on desmin immunocytochemistry staining and flow cytometry, more than 97% of isolated skeletal muscle cells had a myogenic phenotype. The remaining cells had a fibroblastic appearance (desmin negative).

3.2 Macroscopic analysis

Clinically, none of the animal died during the surgical procedure and no post-implantation mortality was recorded. However, the operated rabbits in both groups were appeared dull, depressed and partially anorectic for 1–2 days of early stage post-operation. Food and water intake normalized by 2nd–3rd post-operative day. The surgical wounds appeared healthy throughout the period of observation in both groups.

Macroscopically, the implant were well tolerated by the recipient animals, with no episodes of rejection, infection, seroma, sign of herniation, and also neither the experiment nor the control group have showed fluid leakage through the implanted scaffolds. The host-graft junctions were clearly filled with white fibrous tissue, and were relatively firmer and intact than control group.

The peritoneal surfaces of the implanted grafts in group I were completely covered by newly developed white fibrous connective tissue, fatty tissue as well as neo-peritoneum with smooth and shinny appearance (Fig. 2a), which later on accompanied with the numerous fine new blood vessels (neovascularisation) (Fig. 2c). However, in control group, the peritoneal surface appeared relatively irregular as a result of excess accumulation of fibrous tissue due to mild type of adhesion (Fig. 2b, d). In fact, it was on day 30th, the peritoneal surfaces of the implanted grafts in group I were completely covered by newly formed neo-peritoneum with overwhelming blood vessels over the surface, and the fatty tissue was completely resorbed (Fig. 2e). In control group, the implanted grafts were also almost transparent despite newly developed white fibrous tissue and neo-peritoneum with very few blood vessels present which was not sufficient to bear the weight and pressure of abdominal viscera (Fig. 2f) and therefore, even though none of the control group has showed herniation but the constructed area showed pouching and distension appearance.

The result concerning adhesions scoring of both in treatment and control groups are shown in Table 2. Over all none of the animal in treatment group has showed adhesions, thus, the adhesion degree were 0 as it is apparent from the table. However, in control group a single minor type of adhesion was recorded at 7th and 14th days of post-implantation. Interestingly, caecum was the only organ found to be adhered with the implanted tissue (Fig. 2b).

3.3 Microscopic analysis

3.3.1 Light microscopy analysis

Myoblast-seeded scaffolds at 7th days post-implantation has showed a narrow presence of blood cells such as neutrophils and macrophages, some fibroblasts and neo-formed capillary vessels (Fig. 3a), whereas at 14th days there was a marked presence of fibroblasts, collagen fibers with neovascularisation and newly formed young muscle (Fig. 3c). At 30th days of implant this fibrillar tissue was replaced by a reparative tissue constituted by a neo-formed mesothelium with a mild inflammatory reaction, abundance of newly formed blood vessels and skeletal muscle tissue (Fig. 3e). However, in control group, collagen fibers with few neovascularisations without a single muscle fiber were detected at all time-point (Fig. 3b, d, f).

3.3.2 Fluorescence microscopy analysis

Fluorescence microscopic examinations demonstrated that red-PKH26-labeled myoblast were found exclusively on all over the implanted graft at 7th day of post-implantation, however fusion of myoblast was not clearly seen rather aggregation of myoblast were detected which was an initial stage for fusion cells as it was shown on Fig. 4a. On day 14th myotubes were clearly detected (Fig. 4b). On 30th day of post-implantation, the double-fluorescence micro-photography clearly demonstrated the well developed red-PKH26-labeled myofibers which were found on the myoblast-seeded implanted graft (Fig. 4c). The control implants did not contain structures similar to those in the constructs described above. Neither single myoblast nor fluorescent spots were detected.

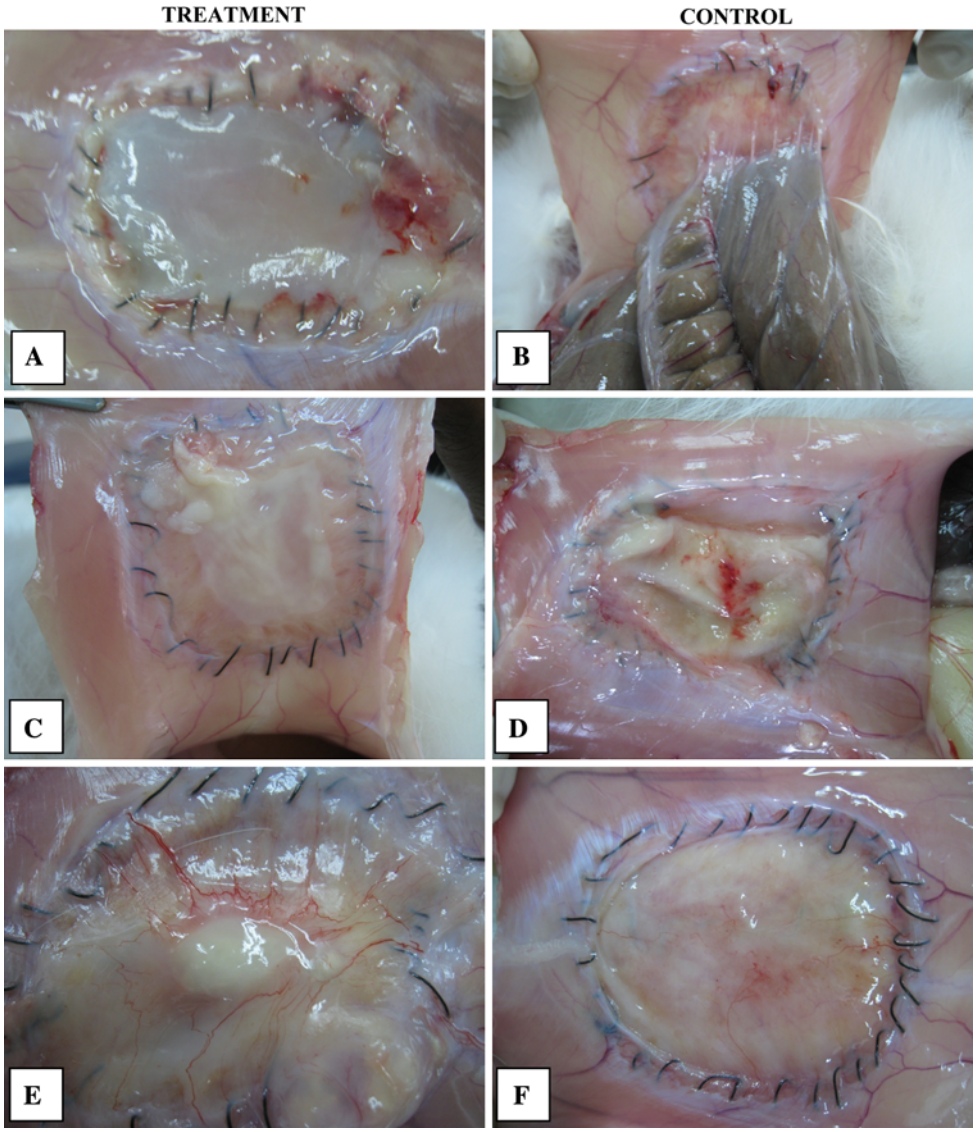

Fig. 2 Macroscopic view of the grafts from peritoneal sides in both group I (treatment) and group II (control). **a** and **b** at 7th day: **a** the surface is completely covered by neo-peritoneum with smooth and shiny appearance in group I; **b** minor type of adhesion between the implant and caecum in group II. **c** and **d** at 14th day: **c** neo-peritoneum with sprouting small blood vessels in group I; **d** shrinkage of the construct tissue in group II. **e** and **f** at 30th day: **e** neo-peritoneum with numerous blood vessels and white fibrous tissue at host-graft junction in group I; **f** thinning and overstretched of the graft in group II

Table 2 Scoring of adhesion formation at 7th, 14th, and 30th days of post-implantation

Adhesion degree	Date of euthanizing					
	7th day		14th day		30th day	
	T	C	T	C	T	C
0	3	2	3	2	3	3
1	0	1*	0	1*	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0

Note: *T* myoblast-seeded scaffold (treatment), *C* non-seeded scaffold (control)

1*—Showing minimal/minor adhesion observed between the implanted and the underlying visceral organ

Based on Mann–Whitney test, $P = 0.145$, since $P > 0.05$, there is no significant difference in adhesion formation between treatment and control groups

3.3.3 Scanning electron microscopy analysis

The SEM microphotographs of in vitro findings showed that bovine tunica vaginalis scaffolds were able to support myoblast growth and differentiation, which were evidenced by few myoblast began to cover the whole surface of the scaffolds and fused into myotube within 24 h. At the 3rd day of post-seeding, the myoblast continues to fuse and form a series of uniformly arrayed myotube (Fig. 5a). In fact at 5th day post-seeding densely packed myotube with morphology reflecting myofibers were also observed (Fig. 5b). In addition, the explanted grafts in both group I and II revealed infiltration of spherical shape of inflammatory cells with scattered polygonal and spindle shapes of cells of on the peritoneal surface indicating the beginning of mesotheliazation as depicted on Fig. 6a and b at 7th day of post-implantation. On 14th day only the treatment group

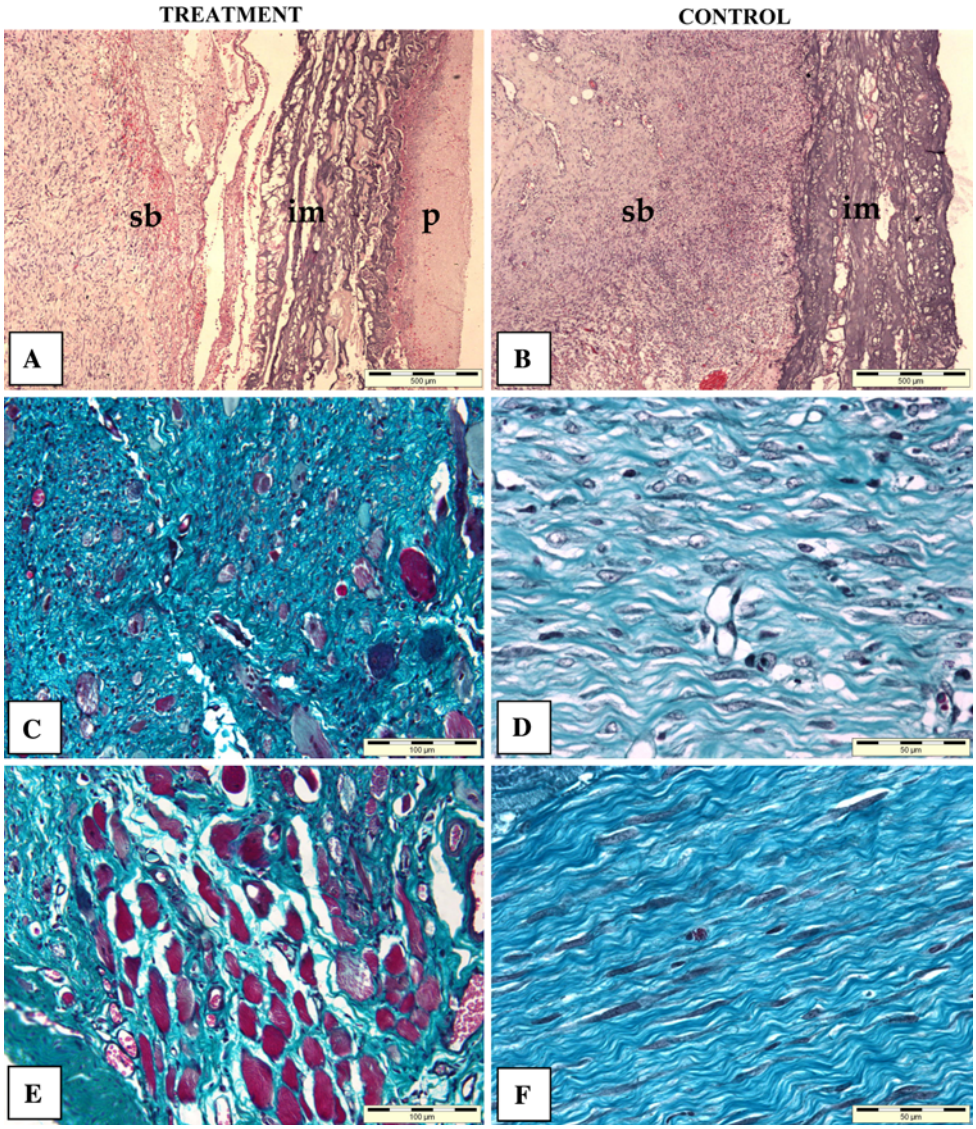

Fig. 3 Microphotographs of the explanted grafts in both group I (treatment) and group II (control). **a** and **b**: H&E stain at 7th day, scale bar: 500 μm: **a** presence of neo-peritoneum (p), implanted scaffolds (im) and subcutaneous tissue (sb) in group I; **b** only implanted scaffolds (im) and subcutaneous tissue (sb) in group II. **c** and **d**: Masson trichrome stain at 14th day, scale bar: 100 and 50 μm: **c** small delicate muscle fibers (*red color*) collagen fiber (*green color*) in group I; **d** only collagen fiber in group II. **e** and **f**: Masson trichrome stain at 30th day, scale bar: 100 and 50 μm: **e** well developed muscle fibers (*red color*) and collagen fiber (*green color*) in group I; **f** only well-organized collagen fibers (*green color*) in group II

have showed well-organized mesotheliazation with polygonal shapes of cells covering all over peritoneal surfaces (Fig. 6c) which later on became smooth (Fig. 6e). However, in control group the peritoneal surfaces were filled with irregularly arranged fibrous tissue with few mesothelial cells (Fig. 6d, f).

4 Discussion

The success of tissue engineering and regenerative medicine approaches depends crucially on the creation of suitable structures/scaffold where appropriate biochemical signals guide the growth of functional neo-tissue [14] because proliferation of most mammalian cell types is anchorage dependent [15], and thus, the utilized scaffold must provide a suitable surface for cell attachment, proliferation, differentiation and migration [16]. In addition, the scaffold material should be easy to handle and apply with biocompatible and non-toxic character, while also being cost effective.

Currently, the repair of large soft tissue defects especially abdominal wall defects is a complex challenge for surgeons and continues to be a significant problem for patients [5, 10]. In fact, several kinds of naturally derived biomaterials scaffolds such as bovine pericardium [17, 18], porcine small intestinal submucosa [19] and acellular dermal matrix [20] have been employed to repair abdominal wall defects in experimental animal models and the biomaterial used in this study have also been reported to be used similarly without myoblast seeding [7, 13]. However, fail to recover muscle tissue which is the main component of abdominal wall, and lack of strength over time is also a concern for clinical applications in which adequate tensile

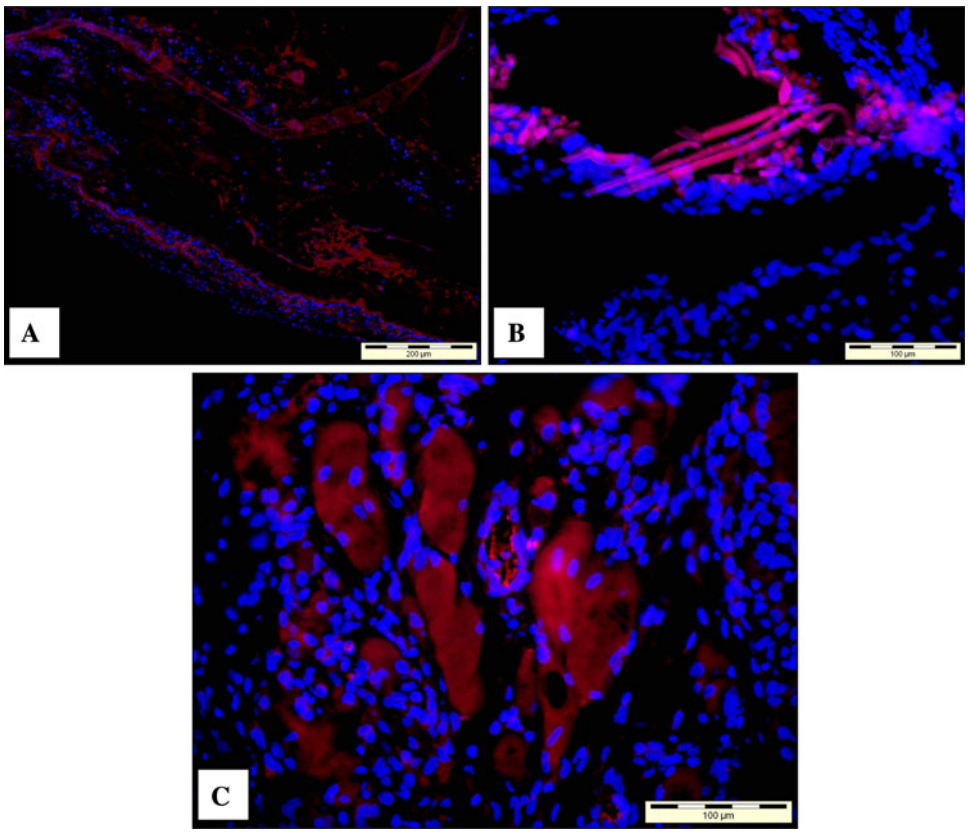

Fig. 4 Double-fluorescence microphotograph of graft from group I showing the red-PKH26-labeled and DAPI-stained nuclei. **a** Myoblasts at day 7th, **b** myotubes at day 14th, **c** myofibers and its DAPI-stained nuclei at day 30th of post-implantation (scale bar for **a**: 200 µm; **b** and **c**: 100 µm)

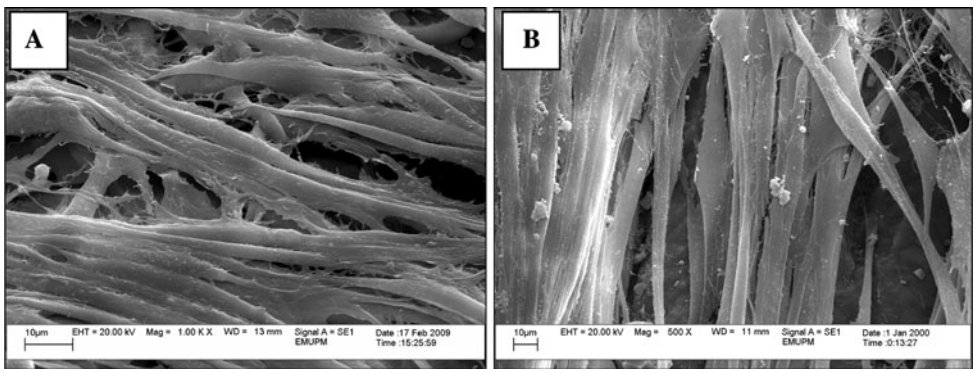

Fig. 5 SEM electron micrograph of the myoblast-seeded-bovine tunica vaginalis scaffold demonstrate. **a** Myotube with unidirectional pattern at 3rd day of post-seeding. **b** Fusion of myotube to form myofibers at 5th day post-seeding

properties are necessary. To this effect, free muscle transfer from local or distant sites is commonly employed for the surgical repair of muscle tissue defects, but this practice is frequently associated with significant donor-site morbidity [21]. To the best of our knowledge, the present research is a preliminary study, which utilize non-edible bovine offal's of collagenous nature for the development of cheap, cost effective and safe surgical patches for clinical use and further enhanced by the in vitro development of a functional three-dimensional skeletal muscle in order to direct myogenesis at the target site and ultimately to engineer skeletal muscle tissue.

Myoblast were successfully harvested and isolated from primary cell culture which was obtained using enzymatic digestion from soleus muscle fibers. According to desmin immunocytochemistry and flow cytometric analysis more than 97% of the isolated skeletal myoblast cells have got myogenic phenotype. Hence, the technique appears to give better result than previously used to isolate myoblast cells [5] where approximately 80% of the isolated skeletal muscle cells had a myogenic phenotype in desmin immunocytochemistry analysis. Our in vitro study clearly demonstrated that the harvested myoblasts can proliferate, differentiate and mature into myotubes on bovine tunica vaginalis scaffolds.

Clinically, neither during surgical process nor post-implantation mortality was recorded which possibly explained by satisfactory anaesthetic technique, thus

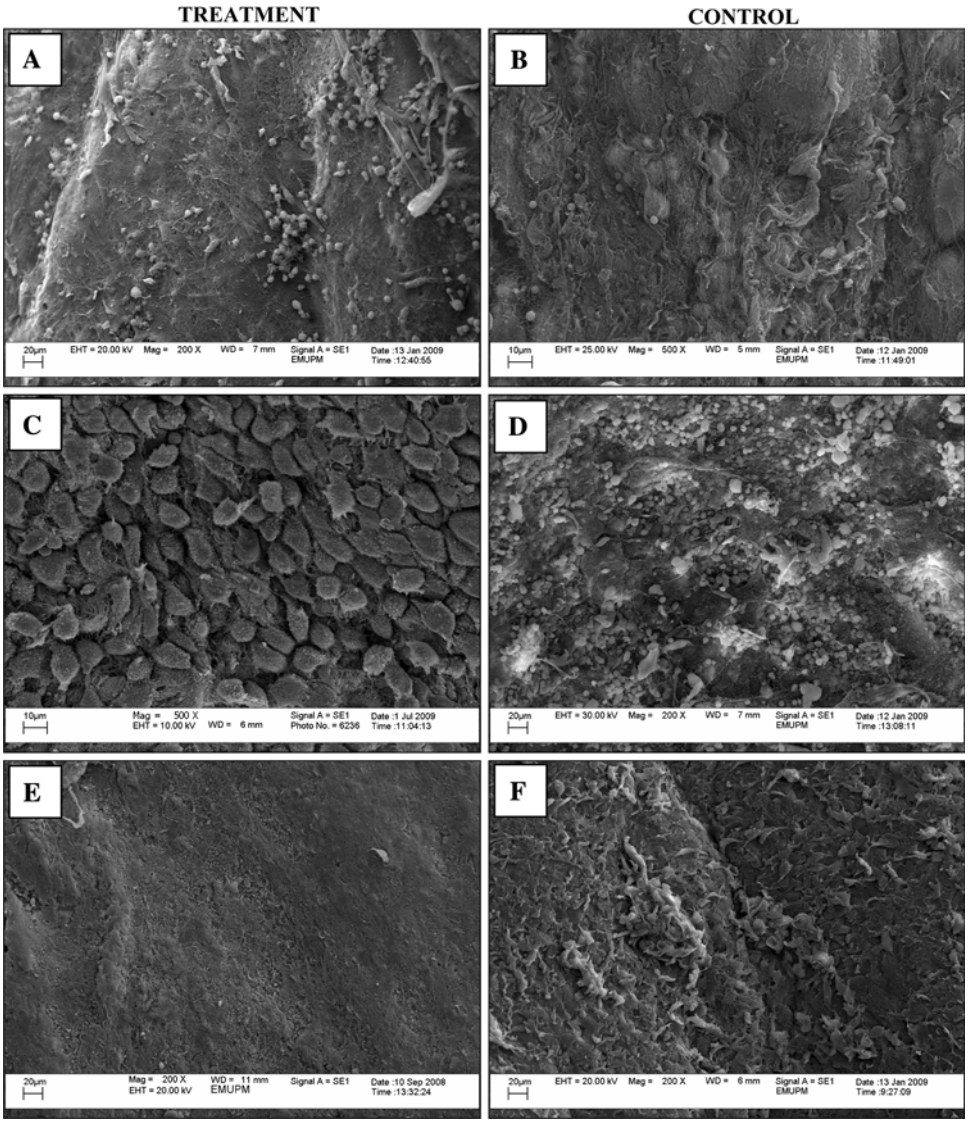

Fig. 6 SEM electron micrographs of the peritoneal surface. **a** and **b** at 7th day: **a** irregular appearance of the surface with infiltrated spherical shape of inflammatory cells in group I; **b** irregular appearance of the surface in group II. **c** and **d** at 14th day: **c** well-organized mesotheliazation of the surface with polygonal shape mesothelial cells in group I; **d** irregularly arranged fibrous tissue infiltrated with spherical shape inflammatory cells in group II. **e** and **f** at 30th day: **e** smooth appearance of the surface in group I; **f** irregular appearance of the surface in group II

leading the animal to tolerate well to the surgical procedure. In fact, dullness, depression and partial anorexia in the immediate postoperative period was attributed to surgical trauma and inflammation at the site of reconstruction [20].

Macroscopically, the ex-implanted myoblast-seeded grafts have shown early neo-peritoniazation with overwhelming angiogenesis than the non-seeded grafts. By the day 30 post-implantation non-seeded bovine tunica vaginalis have showed thinning and fascial weakness which was evidenced by pouching and distension appearance of the abdominal wall. However, none of the treatment group has showed similar appearance rather various degree of tissue regeneration was noticed, which was evidenced by graft opacity, and integrations of overwhelming blood vessels, fatty tissues, fibrous tissue and neo-peritoneum. Report had proven that scaffolds covered with cells had better mechanical properties than non-seeded scaffolds. Moreover, acellular collagen-based matrix alone may be in-sufficient scaffold for abdominal wall reconstruction [5, 22]. The vascular change at reconstructive site is a part of normal body response to injury. It is an attempt to increase resorption and removal of clot and debris from the wound site and finally helping in the laying down of fibrous tissue [23]. Although variable degree of angiogenesis were a common feature of the treatment and its control group of the scaffolds, and hence overwhelming neo-angiogenesis were recorded in the treatment group which might be attributed by seeding of myoblast.

Most interestingly, absence of adhesion was noticed in myoblast-seeded-bovine tunica vaginalis which perhaps elucidated by early mesotheliazation or peritoniazation of the graft. However, in control group, mild types of adhesion were recorded which could be explained by the delay

of mesotheliazation. Despite the presence of mild adhesion only in control group, our data suggest that there was no significant difference between treatment and control group.

Histologically, the inflammatory response in treatment and control groups of animals were significantly high during the 1st week. It was indeed an immediate response initiated by surgical trauma when the abdominal wall defects were created which perhaps correlated with the inflammatory phase of wound healing process [10, 11, 24]. In fact this study has also revealed that the inflammatory process gradually decreased in every advancing period in treatment and control groups of bovine pericardium scaffolds. These findings suggest that the processing method to produce fibro-collagenous scaffolds is better to removes antigenic proteins and maintain graft integrity. It might also be associated with early degradation and resorption of the implant. Moreover, absences of foreign body giant cells were noted in both treatment and control group. This could suggest the biocompatibility of the scaffolds with the host tissue. The presence of PKH26 labelled multinucleated myotube which later on differentiated into mature striated muscle fibers and its existence till the end of the study period showing the superiority of this study to the previous case reported [25]. However, a comparable result has been also reported in the successful regeneration of skeletal muscle tissue [4, 26]. Neither PKH26 labelled myoblast nor even a single skeletal muscle tissue has been detected in the control group.

In conclusion, myoblast-seeded-bovine tunica vaginalis holds a promise to become a suitable scaffold for surgical repair of abdominal wall defects to regenerate skeletal muscle tissue. Over all tissue-engineered-construct may be an alternative device for body wall replacement. However, orientation and innervations of skeletal muscle tissue should be addresses in the future research.

References

1. Mooney DJ, Mikos AG. Growing new organs. Sci Am. 1999;280:60–5.
2. Law PK, Goodwin TG, Fang Q, Deering MB, Duggirala V, Larkin C, et al. Cell transplantation as an experimental treatment for Duchenne muscular dystrophy. Cell Transplant. 1993;2:485–505.
3. Guettier-Sigrist S, Coupin G, Braun S, Warter JM, Poindron P. Muscle could be the therapeutic target in SMA treatment. J Neurosci Res. 1998;53:663–9.
4. Conconi MT, Coppi PD, Bellini S, Zara G, Sabatti M, Marzaro M, et al. Homologous muscle acellular matrix seeded with autologous myoblasts as a tissue-engineering approach to abdominal wall-defect repair. Biomaterials. 2005;16:2567–74.
5. Lai JY, Chang PY, Lin JN. Body wall repair using small intestinal submucosa seeded with cells. J Pediatr Surg. 2003;38:1752–5.
6. Frandson RD, Wilke LW, Fails DA. Anatomy and physiology of farm animals. 6th ed. Philadelphia: Lippincott Williams and Wilkins; 2003.
7. Hafeez YM, Zuki ABZ, Loqman MY, Noordin MM, Norimah Y. Comparative evaluations of the processed bovine tunica vaginalis implant in a rat model. Anat Sci Int. 2005;80:181–8.
8. Springer ML, Rando T, Blau HM. Gene delivery to muscle. In: Boyle AL, editor. Current protocols in human genetics. Unit 13.4. New York: Wiley; 1997. p. 13.4.1–19.
9. Pavlath GK. Isolation, purification and growth of human skeletal muscle cells. In: Jones GE, editor. Methods in molecular medicine: human cell culture protocols. Totowa: Humana Press; 1996. p. 307–17.
10. Gangwar AK, Sharma AK, Kumar N, Kumar N, Maiti SK, Gupta OP, et al. Acellular dermal graft for repair of abdominal wall defects in rabbits. J S Afr Vet Assoc. 2006;77:79–85.
11. Singh J, Kumar N, Sharma AK, Maiti SK, Goswami TK, Sharma AK. Acellular biomaterials of porcine origin for the reconstruction of abdominal wall defects in rabbits. Trends Biomater Artif Organs. 2008;22:34–44.
12. Jenkins SD, Klamer TW, Parteka JJ, Condon RE. A comparison of prosthetic materials used to repair abdominal wall defects. Surgery. 1983;94:392–8.
13. Tung WS, Zainol J, Pillay AG, Yusof N, Yusof LM. Processed bovine tunica vaginalis as a biomaterial for the repair of large abdominal wall defects in surgical treatment. Science. 2002;2:7–11.
14. Mattioli-Belmonte M, Lucarini G, Zizzi A, Alhuwalia A, Vozzi G. A comparative study of porous and engineered biomaterials. Biomed Pharmacother. 2008;62:487–502.
15. Prokop A, Rosenberg MZ. Bioreactor for mammalian cell culture. Adv Biochem Eng Biotechnol. 1989;39:29–71.
16. Kim MS, Ahn HH, Shin YN, Cho MH, Khang G, Lee HB. An in vivo study of the host tissue response to subcutaneous implantation of PLGA- and/or porcine small intestinal submucosa-based scaffolds. Biomaterials. 2007;28:5137–43.
17. Zuki ABZ, Hafeez YM, Loqman MY, Noordin MM, Norimah Y. Effect of preservation methods on the performance of bovine pericardium grafts in a rat model. Anat Histol Embryol. 2007;36:349–56.
18. Decurtins M, Buchman P. Bovine pericardium-A new graft material for hernial repair. Res Exp Med. 1982;180:11–4.
19. Badylak S, Kokini K, Tullius B, Simmons-Byrd A, Morff R. Morphologic study of small intestinal submucosa as a body wall repair device. J Surg Res. 2002;103:190–202.
20. Chung S, Hazen A, Levine JP, Baux G, Olivier WA, Yee HT, et al. Vascularized acellular dermal matrix island flaps for the repair of abdominal muscle defects. Plast Reconstr Surg. 2003;111:225–32.
21. Wei CY, Chuang DC, Chen HC, Lin CH, Wong SS, Wei FC. The versatility of free rectus femoris muscle flap an alternative flap. Microsurgery. 1995;16:698–703.
22. Fauza DO, Marler JJ, Koka R, Forse RA, Mayer JE, Vacanti JP. Fetal tissue engineering: diaphragmatic replacement. J Pediatr Surg. 2001;36:146–51.
23. Silver IA. Basic physiology of wound healing in horse. Equine Vet J. 1982;14:7–15.
24. Gamba PG, Conconi MT, Piccolo RL, Zara G, Spinazzi R, Parnigotto PP. Experimental abdominal wall defect repaired with acellular matrix. Pediatr Surg Int. 2002;18:327–31.
25. Van Wachem PB, Brouwer LA, van Luyn MJA. Absence of muscle regeneration after implantation of a collagen matrix seeded with myoblasts. Biomaterials. 1999;20:419–26.
26. Kamelger FS, Marksteiner R, Margreiter E, Klima G, Wechselberger G, Hering S, et al. A comparative study of three different biomaterials in the engineering of skeletal muscle using a rat animal model. Biomaterials. 2004;25:1649–55.