Effect of Acellular Bovine Pericardium and Urinary Bladder Submucosa Matrixes in Reconstruction of Ventro-Lateral Hernias in Bucks; Molecular Evaluation

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Summary

The present study aimed to estimate the efficiency of both a cellular bovine pericardium and bovine urinary bladder matrix sheets in the reconstruction of large ventro-lateral hernias in Iraqi bucks by using of molecular evaluation depending on real time-polymerase chain reaction technique to investigate the level of basic-fibroblast growth factor and vascular endothelial growth factor genes during the healing process and reconstruction of the abdominal defects. Under sedation and local anesthesia, (6cm X 8cm size) of ventro-lateral hernias were induced in 24 of Iraqi bucks. The animals were divided randomly into two main equal groups. In bovine pericardium-treatment group, the hernias were treated with onlay implantation of bovine pericardium. While, the hernias in UBM-treatment group were treated with onlay implantation of urinary bladder matrix, 30 days post-inducing of hernias. The molecular evaluation along the period of following-up recorded a significant up-regulation of the level of basic-fibroblast growth factor gene specific for presence of fibroblasts, myofibroblasts and collagen deposition in urinary bladder matrix -treatment group in comparison to bovine pericardium -treatment group with significant difference even at the end of the study. While, a significant up regulation of the levels of angiogenesis classic gene vascular endothelial growth factor were recorded in the bucks of bovine pericardium -treatment group compared to urinary bladder matrix -treatment group. In conclusion; molecular detection of the level of growth factors in target tissue can be used as an important criterion.

Keywords: Abdominal wall Hernia, Bioscaffolds, Growth factors, Wound healing, Bucks.

Introduction

Abdominal wall defects (hernia) correspond a difficult trouble, is a common acquired condition in ruminant's which has some harmless effects, such as lowering the productivity and reproductively of there (1and 2). Surgical intrusion (herniorrhaphy) is a practical in these cases but wide ranging loss of abdominal wall may need a hernioplasty by provide biosynthetic mesh (Mesh repairs) by which angiogenesis and deposit new collagen formation produce by fibroblasts, as well as, decrease the strain that must be place on the abdominal wall in order to coat the hernia and are considered favorable for treatment of incisional hernias in general (3 and 4). Although, the medical technology continues to improve the reconstruction of abdominal wall defects, especially for complex defects, remain a surgical challenge (5). The detection of prosthetic meshes has significantly diminish the rate of hernia recurrence, however, these implants are non-absorbable and can cause infection, chronic pain and hernia recurrence to the surgical area which can lead to more complex operations (6). Furthermore, they may also take part to the malfunction of other organs, such as the adherence of intestine, obstruction and creation of fistula (7). Therefore, in excess of the days, surgeons from all branches have tried for ways to substitute missing tissues or repair damaged ones. With the advance in clinical and sciences researches, biomaterial was revealed a material that interacts with biological systems for therapeutic purposes (8), these biomaterials scaffolds which are derived from different mammalian tissues, such as porcine small intestinal submucosa, acellular dermal matrix, urinary bladder matrix, human dura mater and pericardium have been employed to repair of

musculotendinous, dermal, cardiovascular, gastrointestinal, lower urinary tract structures, esophageal, myocardial, musculoskeletal, as well as, the abdominal wall defect repairs (9 and 10). Preclinical and clinical evidences reveal that not all biologically derived scaffolds are the same and that host responses in tissue regeneration contrast among scaffold products (11).

In Iraq, many of studies were tried to treat the large abdominal hernias using different techniques. Some researchers were observed the superiority of tension-free sewing technique from other techniques like; polypropylene mesh and external rectus abdominis sheath (12). Founded that Vypro meshes enable to obliterate the abdominal hernial ring completely during four months of study better than polypropylene meshes in sheep (13). Other studies tried to compare between the efficacies of sewing by silk and by polypropylene mesh in the healing of the ventral hernias in bucks (14). Due to lack of study regarded the studying of efficacies of different biological scaffolds on repair of large abdominal wall hernias, as well as, the variation of performance of different tissue sources scaffolds in tissue repair and regeneration, this study was designed to evaluate the effect of the level of both growth factors in the study of efficiency of both acellular bovine Pericardium(BP) and urinary bladder matrix (UBM) in the repair of experimentally induced large abdominal wall hernias in Iraqi bucks.

Materials and Methods

Twenty four apparently healthy adult local bucks aged 1-2 years and weighing 25-30 kg were used in the current study. Two weeks before the starting of research, all animals were housed in pens of farm of animals at the College of Veterinary Medicine/ University of Baghdad. The animals were numbered and classified according to the experimental design. The animals of the study were dewormed with 0.2mg/Kg., B.W. ivermectin (KEPRO) and to avoid the enterotoxaemia, they were injected subcutaneously with a dose of 5ml of Co-Baghdad vaccine.

Inducing and treatment of latero-ventral abdominal wall Hernia: Pre-creation of hernia, all the animals were medicated with penicillin-streptomycin in a dose of 10.000 IU and 10mg/kg B.W., respectively, with holding of food for 24 hours and water for 12 hours. Right lower flank of 24 bucks were prepared for aseptic surgical technique which has been done under sedation by intramuscular injection of 0.2 mg/kg., B.W. of 2% xylazine hydrochloride with lateral recumbancy. Under the effect of local anesthetic of 2% lidocaine hydrochloride at a dose of 10mg/kg, B.W. vertical straight incision of 10cm was done throughout the skin and subcutaneous fascia through inverted (L) shape local infiltration technique. To create a hernia the skin was separated bluntly from the subcutaneous tissue and from the muscles of the abdominal wall. Full-thickness of (6X8 cm) of these muscles was elliptically resected with avoiding the opening of peritoneum. Finally, the skin and subcutaneous tissue closed with interrupted horizontal matrix pattern by silk (No.1) and hernia were recognized immediately after surgery then, the wounds were covered with dressing. According to the method of the treatment of hernia, the animals of this study were divided randomly into two groups (12 bucks/group) as following: Bovine Pericardium (BP) Treatment Group: In this group, the hernias were treated by covering the hernia ring with onlay implantation of sheet of a cellular xenogeneic BP sheet, 30 days post-inducing of latero-ventral hernia. Urinary Bladder Matrix (UBM) Treatment Group: In this group, the hernias were treated by covering the hernia ring with onlay implantation of sheet of acellular xenogeneic acellular UBM, 30 days post-inducing of latero-ventral hernia.

Post-inducing of hernias, all animals were housed in a separated pens. The animals were received a half quantity of food associated with intramuscular administration of a combination of penicillin and streptomycin in a dose of 10.000 IU and 10mg/kg B.W., respectively for first five consecutive days.The biopsies of one gram of native tissue were collected from implanted area in each animal

of the study, 2, 8 and 16 week's post-treatment (four animals/each period) and single biopsy of normal abdominal muscle tissue was collected from the same animal before operation which regarded as a control group. These samples were used for b-FGF and VEGF gene expression as mentioned below؛

In this study, three primers were used including; the specific gene of muscle tissue in goats according to the method described by (15) which include anterior reverse glyceraldehydes-3-phosphate dehydrogenase (AR-GAPDH) gene primer that was used as Housekeeping gene and AR-FGF and AR-VEGF gene primers that were used as target genes (Table,1). The primers were used in quantification of gene expression by using RT-qPCR techniques based BRYT Green DNA binding dye (Promega-USA).

Total RNA extraction: The biopsies from the muscle tissue at the site of operation were used to extraction of total DNA. The tissue was mixed directly with TRlzol® reagent and processed according to the steps mentioned in kit filter.

Determination of RNA Concentration: Quantus Florometer (Promega, USA) was used to detect the concentration of extracted RNA (ng/µl) in order to detect the quality of samples for downstream application. For 1µl of RNA of diluted Quantity Flour Dye was mixed. After 5 mins incubation at room temperature, RNA value was recorded.

Quantitative real-time PCR (qPCR) master mix preparation :Quantitative PCR (qPCR) master mix was ready with one step of RT-qPCR kit that dependant BRYT Green dye for discovering of amplification of gene in RT-PCR system and its contents were listed in (Table, 2 and 3).

After that, the smart PCR tubes were loaded in the smart cycle instrument using the reaction protocol mentioned in(Table, 3).

Table,1: The details of primers used in the study.

Primer		Sequence	A
AR-GAPDH	F	GCAAGTTCCACGGCACAGTC	219 (bp).
	R	CCCACTTGATGTTGGCAGGA	
AR-FGF	F	GCAGAGTGGGCATCGGTTT	211 (bp).
	R	CTGAACTTGCAGTCATC	
AR-VEGF	F	GTGCGGGGGCTGCTGTAATGA	213 (bp).
	R	TCACCAGGAAAGACTGACACA	

F: Forward R: Revivers bp: base pair

Table,2: The quantitative PCR (qPCR) master mix preparation

qPCR master mix	Conc.	Volume
Master Mix	2X	10 µL
RT mix	1X	0.5µL
Forward primer(10pmol)	10 uM	1 µL
Reverse primer (10pmol)	10 uM	1 µL
Nuclease free water		5.5 µL
RNA	10-40 ng/ul	2 µL
Total		20 µL

Table,3: The protocol real-time PCR system.

qPCR step	Temperature	Time	Repeat cycle
cDNA Synthesis	37 °C	15min	1
Initial Denaturation	95 °C	5min	1
Denaturation	94-96 °C	20 sec	45
Annealing \ Extension Detection (scan)	60-65ºC	30 sec	
Melting	72 °C	0.5 sec	1

Data analysis of qRT-PCR: The PCR assay was performed to amplify the synthetic cDNA of FGF and VEGF gene in bucks. To calibrate the target genes values, GADPH gene was used as endogenous control. The data of outcome of RT-qPCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) which depicted by (16). The relative quantification method, quantities that taken from RT-qPCR experiment essential to be normalized by an approach that the data became biologically meaningful. In this technique, three of the experimental samples were checked up, as control samples; each of the normalized target values (Ct values) was divided by the calibrator normalized target value to generate the relative expression. Later, the ΔCt method with a Reference Gene was used as in the next equations;

Gene	Test (Treatment)	**Cal. (Control)**
Target gene	Ct (target, test)	Ct (target, cal)
Reference gene	Ct (ref, test)	Ct (ref, cal)

Firstly, normalize the Ct of the reference (ref) gene to that of the target gene, for calibrator sample: ΔCt (calibrator) = Ct (ref, calibrator) – Ct (target, calibrator). Secondly, normalize the Ct of the reference (ref) gene to that of the target gene, for the test sample:
ΔCt (Test) = Ct (ref, test) – Ct (target, test)
ΔΔCt= ΔCt (test) – ΔCt (calibrator)
Fold change =
2- ΔΔCt Ratio (reference/target) =
2Ct (reference) – Ct (target).
So that, the relative expression was divided by the expression value of a selected calibrator for every expression ratio of the tested sample.

Results and Discussion

The results in table (4) showed the presence of significant differences P<0.05 in the mean values of VEGF gene within BP and UBM-treatment group in different periods of following-up in current study. It was clear enough that there were high mean values of this gene at 2nd week after treatment in the animals of both groups with a significant differences (P<0.05) between these values compared to the 8th week and continues to decrease at 16th week. In addition, this table showed a significant differences (P<0.05) between the mean values of BP and UBM treated groups compared to normal tissue along the experiment. The mean value was significantly different (P<0.05) between BP and UBM-treated group at 2nd, 8th and 16th week with a higher mean value in BP-treated animals group compared to UBM-treated group along the study. As well as, the results were detected (Table, 5), the presence of significant differences (P<0.05) of the mean values of b-FGF gene, which was higher in both treatment groups (with superiority of UBM- treatment group) compared to normal tissue along the period of study.

The molecular results of gene expression in the current study detected an important changing in quantity of growth factors (b-FGF

and VEGF) which were retained in BP and UBM implants and their liberation after implantation by degradation process of bioimplants through inflammatory cells infiltration (monocytes/macrophages) that bind to ECM proteins, this binding stimulates phagocytosis leading to further break down of ECM fragments under the effects of proteolysis of collagenase enzymes from these inflammatory cells. The retaining of GFs in implant was recorded by some investigators whom estimated the levels of VEGF and b-FGF in the pericardium mesothelium and they observed that VEGF, b-FGF and TGF-β were retained in (17) (18). In addition, other researcher estimated the level of FGF in UBM and found that the level was higher than that of other type of GFs (19). IN addition, the change actually related mainly to the nature of biological scaffolds and its molecular compositions (20). The variation in the level of GFs may be related to the ultrastructure of these biomaterials which vary depending on multiple factors including; tissue source, animal species source, the method of manufacturing, the efficiency of decellularization and post-processing modification (21).

The molecular results of the level of VEGF showed significant difference between normal and treatment group along the period of study. The level of VEGF was high at 2nd week and then gradual decreasing with time which was at 8th and more decreased at 16th week for both groups. This fact may be related to the healing process progress and gradually degradation, initially granulation tissue formed to replaced degraded part of implant (degradation was continuous along the follow up period). The VEGF was responsible for angiogenesis (new blood vessels formation to transmit cells necessary for healing) to enhance granulation tissue formation and remodeling that require less vascularity due to tissue maturity. This elevation in the level of VEGF after implantation was discussed by researchers whom confirmed that the increasing in the expression of VEGF is correlated with increasing of protein expression (collagen synthesis by fibroblasts) and vessel density which cause increase the progression and then reduces with the progress of healing process (22 and 23). Some researchers have demonstrated that the degradation of ECM will release both b-FGF and VEGF (10 and 24) and cryptic peptides (25) which activate the receptors of cell surface that are necessary for viability, motility and differentiation of cell (26 and 27).

The level of VEGF expressed in BP implants was more than UBM implant but both liberated this growth factor, this change may be related to the quantity of this GF that retained in. This fact was showed by some studies whom estimated the level of VEGF in pericardium mesothelium and observed that VEGF was higher than those found in other types of bioscaffolds (17 and 18). In addition, other study has confirmed the ability of BP implants in enhancing angiogenesis when used in the repairing of hernias in rats (28). While, others were recorded that UBM preserves (VEGF and FGF) after decellularization (29).

The molecular results of the level of b-FGF showed bell shape elevation of it in both groups with significant difference between treatment groups and superiority in UBM than BP treatment group along the studied period. This result may be related to the quantity of gene that retained in each implant. In addition to, each implants effects on host issue cells response after implantation. This result agree with other confirmed, that the level of preserved FGF in UBM was more than the level VEGF compared to BP when measured by RTPCR (30), as well as, other researcher who estimated the level of FGF in UBM and found that the level was higher than that of other types of GFs (19), they were; TGF-β: 0.9 pg/cm2 in an ECM sheet, b-FGF: 11.6 pg/cm2 and VEGF 1.4 pg/cm2. Some studies were explained that the levels of GFs in the implantation area depend on the amount of the GFs which were reserved in each biological matrix after decellularization, as well as, the activity of degradation process (18).

The elevated level of b-FGF in UBM treated group meaning more fibroblasts proliferation and collagen deposition in this group compared to BP group at 2 and 8 week after treatment. These results are closed to study used UBM for repairing of hiatal hernia in human which showed that this type of scaffold facilitate the restoration of normal site

appropriate tissue (31). Other study was showed the formation of dense collagen fibers at treated site compared to control group at 8th week after implantation of UBM for tenorrhaphy (32). While, other researcher was confirmed that the use of UBM-implant for wounds closure will induce additional satellite cellular activation and differentiation by its own physical structure and bioactive components in cellular and molecular studying (27). In conclusion, the current study was confirmed that retained growth factors (especially b-FGF and VEGF in BP and UBM implants, were liberated during implant degradation and their ratio related to the source of implant.

Table,4: The means±SE values of VEGF in Control, BP and UBM groups at different period post-treatment.

Group	2 Week Post-implantation	8 Week Post-implantation	16 Week Post-implantation
Normal	A 1.00±0.16c	A1.00±0.16c	A1.00±0.16c
BP	A 3.77±0.58a	B 3.30±1.31a	C 1.95±1.74a
UBM	A 2.44±0.42b	B 2.01±0.14b	C 1.52±6.12b
LSD	0.3334		

*Different small letters mean significant differences (P<0.05) within group. Different capital letters mean significant differences among groups.

Table,5: The means ±SE values of b-FGF in Control, BP and UBM groups at different period post-treatment.

Group	2 Week Post-implantation	8 Week Post-implantation	16 Week Post-implantation
Normal	A 1.47±0.25b	A 1.47±0.25c	A 1.47±0.25b
BP	A 3.80±0.15a	A 4.20±2.73b	A 2.77±4.78ab
UBM	AB 4.01±0.63a	A 6.01±2.44a	B 3.81±2.28a
LSD	2.288		

* Different small letters mean significant differences (P<0.05) within group. Different capital letters mean significant differences among groups.

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تأثير مصفوفتي التامور وتحت مخاطية المثانة البولية البقرية اللاخلوية في إعادة بناء فتوق جدار البطن في المعز: تقييم جزيئي

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الخلاصة

هدفت هذه الدراسة لتقييم تأثير غشائي التامور والمثانة البولية اللاخلوية البقرية على شفاء فتوق جدار البطن الكبيرة في الماعز العراقية تحت تأثير التسكين والتخدير الموضوعي. تم استحداث فتوق جدار البطن الوحشية (قياس10 سم × 8 سم) في 24 من ذكور الماعز العراقية. استنادا إلى طريقة علاج هذه الفتوق، قسمت حيوانات التجربة عشوائيا إلى مجموعتين رئيسيتين متساويتين (12حيوان/مجموعة). في مجموعة غشاء التامور البقري المنزوع الخلايا تم علاج الفتوق بطريقة ال (Onlay implantation) لغشاء التامور البقري منزوع الخلايا بعد مرور أربعة أسابيع على استحداث الفتوق البطنية، في حين تم في المجموعة غشاء المثانة البولية البقري المنزوعة الخلايا علاج الفتوق بنفس طريقة العلاج في المجموعة غشاء التامور البقري المنزوع الخلايا ولكن باستخدام غشاء المثانة البولية البقري منزوع الخلايا. تمت متابعة التغييرات جزيئيا طول مدة 16 أسبوع والتي سجلت ارتفاعا ملموسا في مستوى الجين الخاص بتواجد الخلايا الليفية والليفية العضلية وترسب الكولاجين (b-FGF) في مجموعة الفتوق المعالجة بغشاء المثانة البولية البقري مقارنة بمجموعة غشاء التامور البقري. بينما سجلت النتائج نفسها فيما يتعلق بمستوى الجين التقليدي المولد للأوعية الدموية (VEGF) في المجموعة المعالجة بغشاء التامور البقري وبفروقات مهمة إحصائيا مقارنة بالمجموعة المعالجة بغشاء المثانة البولية البقري.

الكلمات المفتاحية:فتوق جدار البطن،المصفوفة البايولوجية،عامل النمو،التئام الجرح،المعز.