

Role of acellular bovine urinary bladder submucosa on skin wound healing in Iraqi goats

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Summary

This study is designed to assess the effectiveness of bovine urinary bladder submucosa on healing of cutaneous wound in Iraqi goats. A 32 (2X2) cm of full-thickness cutaneous wounds were induced in eight goats, two on each side of the lateral thoracic region. The wounds were divided into two equal groups (16 wounds/ group); treatment group included the wounds on the right side which were treated by covering the wound beds with strips of acellular sterilized bovine urinary bladder matrix. While, the wounds on the left side were left without any treatment (control group). The results were evaluated clinically (along) and histopathologically on 7, 14, 21, and 35 days post-inducing of wounds. The clinical evaluation of treated wounds showed that the wound healing process contraction%, re-epithelization % and total wound healing % were $P < 0.05$ significantly than that of control wounds at 35 days of the study. The histopathological results confirmed that urinary bladder matrix treated wounds have enhanced cellularity, increased vasculature, thick and large granulation tissue suggesting enhanced cutaneous healing, than those in untreated wounds. Depending on the clinical and histopathological findings, this study concluded that a cellular bovine urinary bladder matrix play an important role in stimulation of cutaneous wound healing of goats without signs of immuno-rejection.

Keywords: Skin, Cutaneous wounds, Urinary bladder matrix, Urinary bladder sub-mucosa.

Introduction

Advances in the treatment of problematic wounds and tissue repair have led to the development of materials that might assist with optimizing the wound bed environment. Appropriate wound care is critical, and various treatment modalities are used to improve the wound bed (1). The aim of wound care is to promote wound healing in the shortest time possible, with minimal pain, discomfort and scarring to the patient and must occur in a physiologic environment conducive to tissue repair and regeneration (2). The term biomodulation has been used to describe the process through which materials affect cell activity in a wound undergoing the repair process. Collagen-derived xenografts are of particular importance as they may have a therapeutic effect on wounds, especially those characterized by high levels of inflammation. Acellular biological tissue like; small intestine submucosa (SIS), equine pericardium (EP) or urinary bladder matrix (UBM) have been proposed to be used as natural biomaterials for a various tissue repair. Natural biomaterials are composed of extracellular matrix (ECM)

proteins that are conserved and can be serve as scaffolds for cell attachment, migration and proliferation (3).The present study conducted at evaluated the efficacy and fate of bovine acellular UBM for the acceleration and reconstruction of skin defects in goats.

Materials and Methods

The whole fresh urinary bladders were collected from a slaughtered cows and UBM will be prepared as a decellularized scaffold (4 and 5). The intraluminal water pressure was used to expand and stretch the bladder to facilitate the removal of urinary bladder layers except the submucosal layer. The bladder was then dissected on one side from the opening to the apical region forming a sheet. By using of a sharp knife, the tunica serosa, tunica muscularis and mucosal layers were removed by genital mechanical delamination, and finally prepared a flattened rectangular sheet. The remaining tissue (submucosal layer) was then soaked in phosphate buffered saline (PBS) pH 7.4 containing penicillin 100 IU/ml, streptomycin (100 ug/ml) and amphotericin

100ug/ml. The risk of host rejection (immuno-rejection) of UBM was minimized by disruption of cellular and DNA materials. The remaining tissue was treated with a 0.1% peracetic acid (PAA) and 4% ethanol solution for two hours at room temperature on a shaker. Traces of peracetic acid were removed and the pH was returned to approximately 7.4 by rinsing the UBM at room temperature, with shaking, in PBS one time, in water twice, and again in PBS one time. Each rinse lasted for 15 min. The resulting decellularized ECM scaffolds were terminally sterilized by immersion in 0.1% PAA solution titrated to pH 7.0 at room temperature for five hours and the disinfected and decellularized scaffold was maintain in sterile PBS containing antibiotics and antifungal drugs and preserved at 4 °C (6). Eight healthy local breed adult male goat, age from 2-3 years and weighing 33 to 38 kg, were used. After injected the animals with broad spectrum antibiotic, and deeply sedated with intramuscular injection of 2% xylazine hydrochloride (XYL-M2-Belgium) at a dose of 0.2 mg/kg, B.W., the lateral thoracic sides were prepared aseptically for the creation of 2X2 cm four square full-thickness skin wounds under local anesthesia with 2% lidocaine hydrochloride (OUBARI-Pharma-Syria), two wounds on each side, one cranial and one caudal with a distance of 10cm between each. Enough quantity of sterilized acellular UBM strips were covered the wound beds of the treatment group (Fig. 1), while the wounds of control group were left without treatment.



Figure, 1: The covering of wound bed of treatment group with strips of UBM.

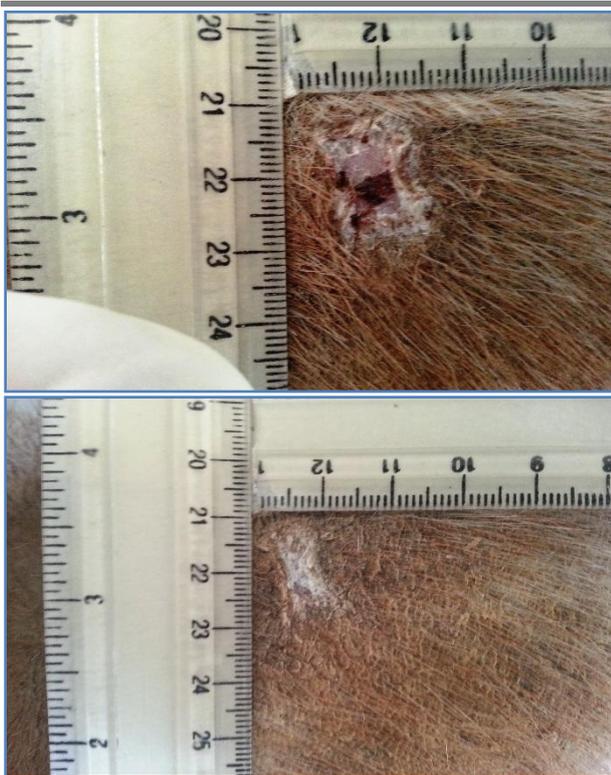
Post-operatively, all wounds were left without suturing and bandaged which had been changed three times a week and wounds have been gently cleaned. The wound healing processes in treatment and control group were evaluated clinically, morphometrically and histopathologically during five weeks study, as follows;

Evaluation: A complete clinical examination was performed on all animals every three days along the period of the study. Digital photographs were taken for all wounds after the area had been carefully shaved to visualize the wound margins. The scab of each wound was carefully removed for better visualization of area of epithelialization and granulation tissue using saline. The percentages of epithelialization, wound contraction and total wound healing were calculated for each wound, depending on the parameters (7). The histopathological examination was performed on 7, 14, 21 and 35 days post-creation of wounds for each treatment and control group (four wounds for each period). A full-thickness incisional biopsies were obtained 5-6 mm in width, and they included approximately 3-4 mm of unwounded skin on both side of the wound, and fixed in 10% neutral formalin solution, embedded in paraffin, sectioned in 5 micron sections on a rotary microtome and staining with hematoxylin-eosin stain (8).

The Statistical Analysis System SAS (9), was used to effect on different factors (treatment and days) in study parameters (percentage). The least significant difference (LSD) test was used at the comparative between percentages.

Results and Discussion

In general, all wounds of treatment and control group decreased rapidly in size along the study, but the close inspection of wounds images revealed that the rate of wound closure in UBM treated wounds were significantly ($P < 0.05$) more along the period of the study as compared to untreated wounds. The enhancement appeared in the UBM treated wounds on day 7th post-wounding and continuous until the end of the study (Fig. 2).



Figure, 2: The significant differences in rate of wound closure between control (C) and treatment (T) wounds, 35 days post-wounding.

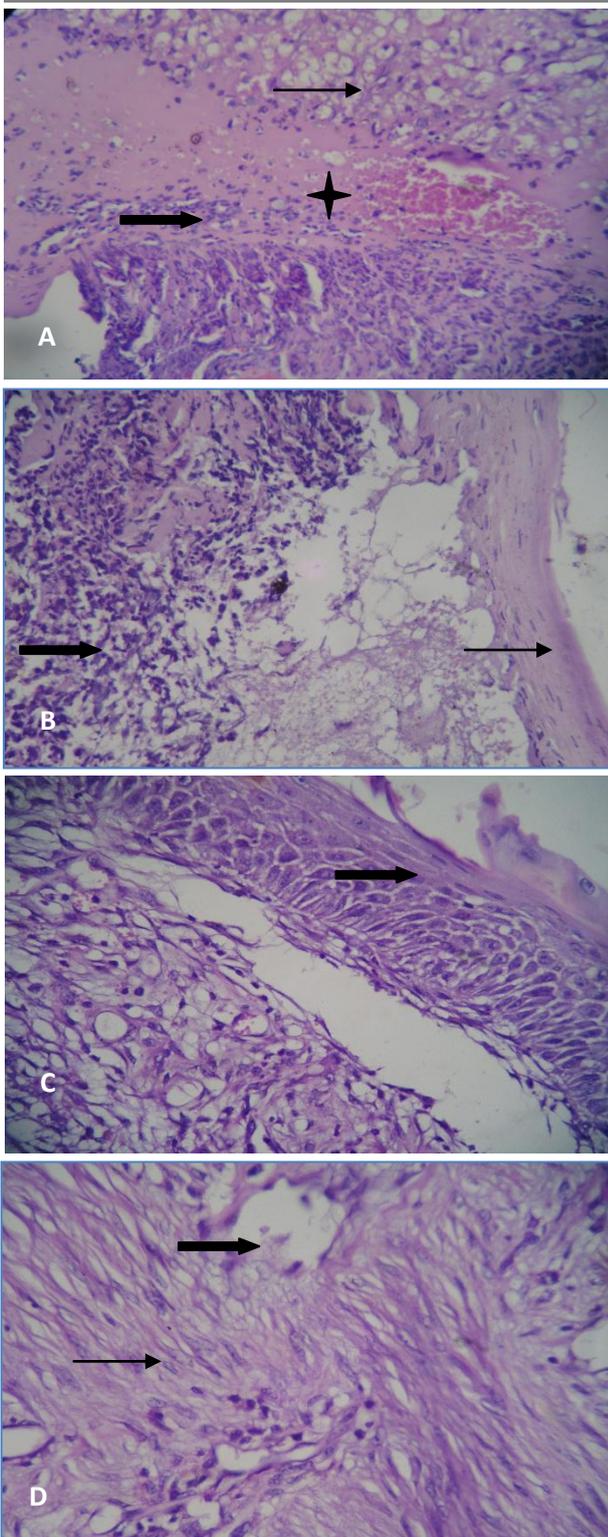
Pending on the data in (Table, 1), clinical morphometrical investigation of wound healing process along five weeks of the study showed that wound re-epithelization percentages were increased in treatment group faster than control group, 88.8 ±3.79 in treatment group and 75.31 ±2.84 in control group, on day 35 post-wounded, with a significant difference (P<0.05). The contraction percentages was noticed on day 7th post-wounding in both control and treatment group with no difference between them. After that,

these percentages were started to increase with significant difference (P<0.05) until the end of the study 80.85 ±3.73 in treatment group and 52.17 ±2.76 in control group. While, the percentages of total wound healing were 90.75 ±5.07 in the treatment group and 78.86 ±2.89 in the control group, with a significant difference (P<0.05). The histopathological examination of wound biopsies from the peripheries and beds revealed that the main differences between treated and untreated wounds were started on day 7th post-wounding. The sections of control group were showed the main microscopic findings, 14 days post-wounding which characterized by the presence of inflammatory exudates covering the wound bed which consisted of polymorphous nuclear cells (PMNCs) and fibrin deposition with congested blood vessels together with minimal re-epithelization, and the newly formed thin flatted epithelium consist of developed stratum corneum accompanied in other section with intense PMNCs aggregation. Also the result showed that the increase space appeared to build up with moderate granulation tissue formation that contain of immature B.Vs and mild fibroblasts producing collagen fibers (irregular fibrils) (Fig. 3, A and B). While, the histopathological changes in UBM treated wounds showed moderate to complete re-epithelization and the incision lined with mature newly epithelial consist of stratified sequamous epithelial cells developed over the highly cellular granulation tissue.

Table, 1: The % of wound epithelization, contraction and total wound healing (%) in both treatment and control wound, goats. (M±S.E)

Parameters	Days	Treatment wounds	Control wounds	LSD Value
Epithelization	7	28.22± 0.52	19.35± 0	10.133
	14	58.45± 1.25	30.67± 0.73	7.343*
	21	75.61± 2.68	55.44± 1.59	7.551*
	35	88.80± 3.79	75.31 ± 2.84	9.876*
Wound Contraction	7	22.60 ± 0.81	15.87± 0.63	8.232
	14	48.70± 1.68	35.65± 1.56	7.788*
	21	65.43± 2.53	40.82± 1.64	6.790*
	35	80.85± 3.73	52.17±2.76	6.665*
Total Wound Healing	7	25.69± 0.64	20.34± 0.47	6.212
	14	54.22± 1.84	45.23± 1.73	5.888*
	21	77.33± 2.70	65.56± 1.95	4.890*
	35	90.75± 5.07	78.86± 2.89	8.675*

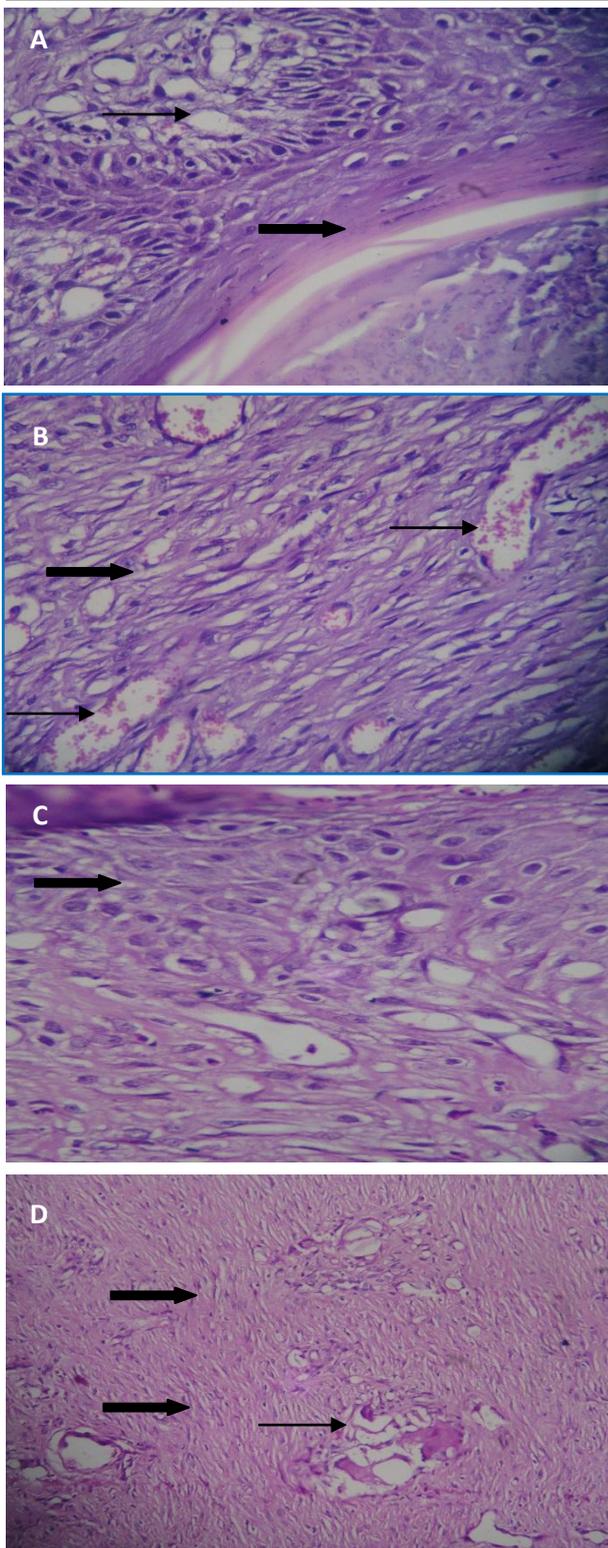
* (P<0.05), Significant.



Figure, 3: The sections of control wounds, 14days post-wounding, appears the presence of PMNCs (thick arrow) and fibrin deposition (thin arrow) with congested B.Vs together with minimal re-epithelization (star) (A), with newly formed thin flattened epithelium consist of developed stratum corneum (thin arrow) accompanies in other section with intense PMNCs aggregation (thick arrow) (B). The sections of treated wounds, 14days post-wounding, shows the presence of moderate to complete re-epithelization and the incision lined with mature newly epithelial consist of stratified squamous epithelial cells (thick arrow) developed over the highly cellular granulation tissue (thin arrow) (C), and moderate number of B.Vs containing polymorphous leukocytes (thick arrow) with young fibroblasts producing collagen fibers (thin arrows) (D) (H and E 10,40X).

Other section showed the presence of moderate number of B.Vs containing polymorphous leukocytes with young fibroblasts producing collagen fibers. Other observation showed increase dermal collagen density in the mature granulation tissue with evidence of perivascular MNCs aggregation (Fig. 3, C and D). At the end of the study (35 days post-wounding), the sections of control group (Fig.4, A and B) appeared that the incision lined with slight keratinized mature epithelial layer that consist of stratum corneum, stratum spongiosum and stratum basali with proliferation of fibrous connective tissue containing of congested B.Vs. Other section showed few number of myofibroblasts scatter through the fibrous tissue.

In 9 comparison to the microscopical appearance of treated UBM wounds (Fig.4, C and D), they showed more mature epithelial layer lining the incision (Normal epidermal appearance of stratified squamous). In addition, large amount of mature granulation tissue fill the dermis, associated with number of myofibroblasts plus giant cells formation, also the other section showed immature fibrous C.T fill the dermis which consist mainly mature fibroblasts and new of B.Vs. Along the period of the study, no signs of immune rejection were detected in all sections of treated wounds (no accumulation of inflammatory cells or immune cells (lymphocytes) at the site of implantation, no foreign giant cells and no fibrous encapsulation. Many studies have confirmed that biological scaffolds are composed of ECM which has been investigated as inductive templates for functional tissue reconstruction in a number of anatomic locations including; the lower urinary tract, skin, musculotendinous tissues, dura mater, esophagus and cardiovascular structures in both preclinical animal studies and in human clinical applications (10). But, during the clinical applications, the composition and three-dimensional ultrastructure of these bio-implants plays a greater role in determining the outcome of the interaction of cells with the scaffold material, at the same time, is highly related to cell phenotype and the required functions of the tissue or organ from which it is derived (11).



Figure, 4: The sections of control wounds, 35days post-wounding, the lining of incision with slight keratinized mature epithelial layer (thick arrow) that consist of stratum corneum, stratum spongiosum and basali (thin arrow) (A),with proliferation of fibrous connective tissue (thick arrow) containing of congested B.Vs. (thin arrows) (B). More mature epithelial layer lining the incision (thick arrow) (C), large amount of mature granulation tissue fill the dermis (thick arrows), associated with number of myofibroblasts plus giant cells formation (thin arrow) (D) are notice in the sections of treated wounds, 35days post-wounding (H and E, 10,40X).

In this study, an xenogenic, collagen rich membrane scaffold derived from the bovine urinary bladder sub-mucosa has been used to evaluate the effectiveness of UBM on skin wounds healing. The clinical inspection wound healing along the study showed rapid significant decreasing in wound size with a minimum scar tissue formation in treated wounds compared to untreated once. While, the histopathological evaluation of treated wound sections appeared a high incidence of mature granulation tissue, myofibroblasts and new B.Vs, at the same time, few myofibroblasts were scattered trough fibrous connective tissue containing congested B.Vs were notice in the sections of control wounds.

The results of this study might be related to the effect of implanted UBM which could be play an important role in the enhancement and acceleration of cutaneous wound healing. This conclusion is in a harmony with other many studies, in which the acellular matrix was used to repair tissue defect directly. They have shown that acellular matrix (in different forms) could induce specific tissue regeneration *in vivo*. They have reported that implanted ECM proved tissue healing through promote progenitor cell infiltration, adhesion and proliferation association with acceleration of angiogenesis at the wound site, as well as, enhancing of granulation tissue formation and deposition of host derived neomatrix (collagen contents) that results in tissue remodeling with minimal scar tissue formation (4 and 11). Brown (12), concluded that in tissue engineering and regenerative medicine applications, the cells that participate in the processes of tissue reconstruction require 'instruction' for proliferation morphogenesis and differentiation. The sources of this 'instruction' are the cellular microenvironment and the scaffold or matrix surfaces with which these cells interact. The mechanism action of UBM in promote of wound healing was described (13) who explained that the positive effects of these bioimplant could be obtained either directly by ECM molecules or indirectly by their bioactive signal molecules within the UBM; like growth factors, cytokines, chemokines and hormones.

In vivo studies, (14 and 15) have revealed that during the biodegradation process of ECM

components, a peptides molecules like collagen, elastin, laminin and fibronectin will be released and participate in mellaprotease matrix metalloprotease (MMP) expression, cellular activity modulation, growth factor signaling and tumor vascularization and angiogenesis, therefore, they helps in the recruitment of cells to the remodelling site, and help in the tissue specific differentiation. In addition, (16) showed that ECM shows an attractive property towards circulating bone marrow-derived cells and they will remain in the remodelled tissue. It confirmed that ECM also helps in the stem cells differentiation and maintain the phenotype of the differentiated cell line in a tissue specific manner. As a result, these events or reactions have an important role in determining the eventual clinical outcome.

Allogeneic and xenogeneic grafts are limited by the risk of immune rejection or infection diseases in comparison to autologous once (17). Depending on the clinical or histopathological observation during this study, it has been noticed that UBM was typically associated with tissue acceptance and no signs of immune rejection were detected despite the xengeneic characteristic of the implant. This result might be related to the composition of the implant which formed mainly from acellular, non-immunogenic, resorbable collagen-based biomaterial. Previous studies discuss the immunogenic response of the target tissues after implantation of bio-implants and indicated that the implanted scaffold (ex. Small Intestine Submucosa (SIS)) elicits an immune lymphocytic response that is predominately T-helper lymphocyte-2-like which stimulates the production of interleukins (IL-4, IL-5, IL-6 IL-10), and as a result promote graft acceptance and prevent the activation of neighboring inflammatory macrophages (18 and 19).

The absence of the infections during this study could be due to good and suitable pre and post-operative care and may be connected to the characteristic of UBM to resistance of micro-organisms, as mentioned by many preclinical and clinical studies which explained that ECM scaffolds shown resistance towards deliberate and spontaneous bacterial contamination (20 and 21). Sarikaya

(22) Appeared that after the transplantation of scaffold into body, it will start degradation and during the biodegradation process, small peptides (5 kDa to 16 kDa) will be released from the fibers of the scaffolds like collagen, these molecules mimic some peptides that inhibit the growth of gram positive and gram negative bacteria in vitro.

In conclusion, the qualified healing process of treated wounds comparison to untreated wounds confirmed that using of bovine acellular UBM promoted and enhanced skin wounds healing.

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دور غشاء تحت المخاطية للمثانة البولية البقرية في شفاء جروح الجلد في الماعز العراقي

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الخلاصه

صُممت هذه الدراسة لتقييم دور غشاء الطبقة تحت المخاطية للمثانة البولية البقرية في شفاء الجروح الجلدية في الماعز العراقي. استُحدث 32 جرح مربع الشكل بقياس 2×2 سم في الجانب الوحشي للمنطقة الصدرية. قُسمت الجروح إلى مجموعتين متساويتين (16 جرح/مجموعة)، مجموعة المعالجة والتي شملت على الجروح في الجانب الأيمن للمنطقة الصدرية والتي عولجت بتغطية الجروح بشرائط غشاء المثانة البولية البقرية المنزوعة الخلايا، في حين تركت الجروح في الجانب الأيسر بدون علاج (مجموعة سيطرة). تم تقييم نتائج الدراسة سريرياً طيلة مدة التجربة ونسجياً مرضياً (7 و 14 و 21 و 34) يوماً بعد استحداث الجروح. أظهرت المتابعة السريرية أنّ معدلات النقائص، إعادة تكون الخلايا الظهارية وشفاء الجرح الكلي كانت مرتفعة وبفارق إحصائي ($P<0.05$) مقارنة بالجروح غير المعالجة في مجموعة السيطرة طيلة مدة الدراسة. في حين اثبتت المتابعة النسيجية المرضية وجود تحسنا ملحوظا في التفاعل الخلوي، التجهيز الوعائي الدموي وكثافة النسيج الحبيبي في جروح مجموعة المعالجة مقارنة بجروح مجموعة السيطرة. استناداً إلى نتائج المتابعة السريرية والنسجية المرضية استنتجت الدراسة أن غشاء تحت المخاطية للمثانة البولية البقرية قد أدى دوراً في تحفيز شفاء الجروح الجلدية في الماعز وبدون علامة للرفض المناعي.

الكلمات المفتاحية: الجلد، الجروح الجلدية، غشاء المثانة البولية، تحت المخاطية المثانة البولية.