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EFFICACY OF AMNION AND PRP FOR TREATMENT OF EXPERIMENTALLY INDUCED WOUNDS IN DISTAL EQUINE LIMBS

By

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ABSTRACT

Twelve healthy adult donkeys (7 males and 5 she-donkeys) aging from (5-10 years old) and weighing from (150-200kg) were used in this study. They were equally divided into two groups. Each group was then subdivided into two subgroups (3 animals each). All animals were subjected to application of four full thickness circular skin incisions using punch devise with 1,5 cm diameter.They were created at medial aspect of the metacarpal / metatarsal regions. The first sub group was used as control, its wounds were treated by local dressing soaked with saline only while the second subgroup wounds were treated by equine amniotic membrane. On the other hand, the third subgroup acted as control, its wounds were treated by injection of saline only while the last subgroup wounds were injected by PRP. All animals were kept under observation. Tissue samples for histopathological examination were taken at 1, 5,9,14 days post wounding from control and treated animals.The specimens were fixed, dehydrated and paraffin blocks were prepared, sectioned and stained with haematoxylin iodine. The gross clinical and histopathological findings declared a relatively delayed and incomplete healing in both the control subgroups. Meanwhile the amnion treated wound subgroup displayed faster healing process than PRP subgroup treated wounds at 14 days (p.w.).

In conclusion, the gross and histopatological investigations of comparative experimental studies on the use of fresh amniotic sheathes and autologous PRP for treatment of equine distal limb wounds indicated that equine amniotic membrane was found better and more efficient in acceleration of cutaneous wounds of equine limbs specially those of the distal limb region .

Key words:

Amniotic membrane (AM), platelets rich plasma (PRP).

INTRODUCTION

Distal limb wounds often result in a large loss of soft tissue, precluding primary closure. When possible, primary closure is ideal but prone to failure because of excessive contamination, limited availability of overlying soft tissue, and dehiscence due to poor blood supply or excessive tension and/or motion at the wound edges **(Christopher *et al.*, 2012)**. Most of distal limb wounds must heal by second intention, requiring prolonged, intensive wound care to obtain a desirable outcome. Therefore, new and improved wound management products and techniques are needed. The use of PRP in vitro and in vivo has been shown to facilitate and accelerate healing in equine models by enhancing fibroblast proliferation and migration **(Tablin *et al.*, 2008)** and by accelerating epithelial differentiation **(Carter *et al* ., 2003).** The use of PRP in clinical or contaminated wounds has not been evaluated, so the effects of these environments on PRP solutions are unknown. Therefore, the use of PRP in a clinical or contaminated wound should likely be reserved until after the wound has been sufficiently debrided. PRP is relatively expensive and may be cost prohibitive; however, the cited experimental models show promise, warranting consideration of the use of PRP in selected clinical cases when a fast recovery is a top priority. The other treatment that offers a solution for a cost-effective biological wound dressing is equine amnion. Amniotic membrane (AM) has been used as a skin substitute for treatment purpose **(Kesting *et al.*, 2008; Lo and Pope, 2009)**. Equine amnion also had beneficial effects in vivo experimental settings include increased rates of contraction and epithelialization, faster overall healing, and reduced granulation tissue formation compared with other dressings **(Goodrich *et al.*, 2000)**. The objective of the present work is to study and evaluate the use of PRP and amnion implantation for treatment of distal limb injuries in equine experimentally.

MATERIALS AND METHODS

Twelve healthy adult donkeys (7male and 5 female) aging from (5-10 years old) and weighing from (150-200kg) were used in this study. All donkeys were apparently clinically free from systemic and musculoskeletal disease, and were also protected against the external and internal parasites using doramectin (Dectomax@ zoitites, USA) at dose (1ml /50 kg) 10 days before the experiment. All were kept on room with supplement of proper diet and water during the whole experiment period at the Faculty of Veterinary Medicine, South Valley University, Egypt.

The animals were equally and randomly divided in to two groups:

Group A) divided into two subgroups:

Subgroup I (Control group) (n=3) that were treated by using dressing soaked with saline.

Subgroup II (Treated group) (n=3) that were treated by amniotic membrane

Group B) divided into two subgroups:

Subgroup I (Control group) (n=3) that were treated by using saline.

Subgroup II (Treated group) (n=3) that were treated by PRP.

Material used:

Fresh amniotic membrane, platelet rich plasma (PRP),0.9%saline,cooling centrifuge, micro pipette, laminar airflow device, Wassermann glass tubes, sodium citrate 10% ,calcium chloride 10% and surgical instrument (biopsy punch device, scalpel, tissue forceps, dressing bandage, gauze and cotton) were used on this study. Placentas were gathered from recent healthy live births from she-donkey. Under sterile conditions, the amnion was removed from the chorion by blunt dissection and washed with phosphate buffered saline (PBS) with 1% antibiotic (Oxytetracycline), then carefully cut into 4 x 4 cm pieces, placed into sterile containers containing 3% gentamycin. The amniotic sheath was refrigerated at 4 C° **(Bigbie *et al.*, 1990 and Lange Consiglio., 2012).** PRP was collected and prepared according to the technique described by **Bosch *et al.* (2010).** Blood was collected from the jugular vein using sterile syringe that containing sodium citrate 10% anticoagulant in a ratio (6 cubic ml sodium citrate for 34 cubic ml blood.under complete aseptic condition, divided into 10 sterile Wasserman glass tubes (4 cubic ml / tube). Then putting at cooling centrifuge (The first at 4800 rpm/20 min. 4C°and the second at 2400 rpm/15 min. 4C°) all steps of manual separation was performed under the laminar airflow device to provides a sterile condition for all steps of preparation and activated by addition of calcium chloride 10% in ratio (0.1 cubic ml calcium chloride to 1 cubic ml PRP) **Farghali *et al.*, 2017**).

Details of the experiment:

All donkeys received xylazine 2 mg /kg.b.w intramuscular prior to surgical procedure. A high palmer nerve lock was performed using lidocaine2%**.** After aseptic preparation of the site of procedure, four full thickness circular skin incisions induced by a punch device with 1.5 cm diameter were created on medial aspect of metacarpal / metatarsal region. The first defect was created 2cm below the carpometacarpal / tars metatarsal, the distance between each wound

was 2cm then marked the proposed circular skin defect and then remove it. Then applied a 4 cm^2 piece amniontic sheath on dressing bandage and wrapping the wound while the control one received saline which was placed on dressing bandage.Concerning the other experimental group, by using sterile syringe infiltrated the PRP subcutaneously at the boundaries of the circular skin defect then bandage the wounds while the control one was injected by saline only subcutaneously. The wound healing was observed, photographed, recorded the measurements was collected and biopsies 1^{st}, 5^{th}, 9^{th} and 14^{th} days post-wounding from all groups for histopathological examination.

Histopathological examination:

Skin specimens from all groups were fixed in 10% neutral buffer formalin (NBF) after incision at 1^{st} , 5^{th} , 9^{th} , 14^{th} days post-wounding, then dehydrated in alcohol and prepare a paraffin blocks. Five millimeter thickness were made, and stained by Harri's hematoxylin and eosin, for histopathological examination **(Drury and Wallington, 1980).**

RESULTS

A) Macroscopical (clinical presentation of the wounds of the experiment):

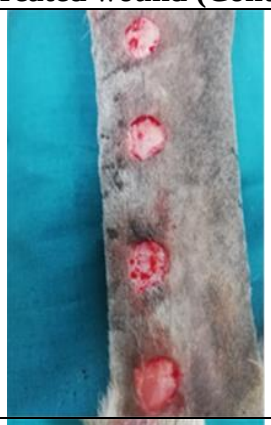
The gross evaluation of healing of the created wounds in the control and experiment wounds treated with amnion for 14 days post operation. After 1 day post wounding, there was swelling and hyperemia of the wound edges in the control (gpI).Five day's post wounding, swelling and hyperemia relatively diminished with a degree of mild wound contraction. However, at 9 day post wounding the wound gap reduced with presence of rosy granulation tissue formation filling nearly half of the wound gap. At 14 day post wounding, a comparative increase in the granulation tissue with a relatively increased of wound contraction but without complete closure of the wound gap (Fig. 1).The corresponding group of the wounds treated with amnion group A sub- group II after 1 day post wounding showed nearly the same clinical picture of the above group. Five days post wounding, a relatively narrow wound gap with healthy granulation tissue formation was seen. However, even at 9 day post wounding an increased wound contraction was detected and the wound begin to close. Additionally, at 14 day post operatively the experimental wound were seen completely closed and the skin return to normal peculiarities (Fig.1). The gross appearance of the healing of the created wounds in the control and treated wound with PRP were recorded for 14 day post-surgery. The gross appearance of the healing of the control wounds (g I) the same gross clinical pictures was seen as previously mentioned above 1, 5, 9 and 14 days post operatively (Fig.2).

The gross clinical picture of the corresponding group of wounds treated with PRP group B subgroup II revealed that, after 1 day post wounding, the wound gap showed blood clots with starting granulation tissue formation. Five days post wounding, an increased granulation tissue formation with reduction of the wound gap was detected .Meanwhile , at 9 day post wounding , the wound diameter decreased with partial closing of it. At 14 day (Are illustrated in (Fig.2) from Fig. (1),(Table 1) and graph one and two) post wounding in complete wound closure was detected with scar formation (Fig.2)

Fig. (1)**:** Group A, control (subgroup I) and treated wounds Subgroup II (Treated group)with amnion at the days 1&5&9 and14.

Day	Untreated wound (Control)	Treated wound by amnion
Day 1		
Day 5		
Day 9		
Day 14		

Fig. (1): Group B control (subgroup I) and treated wounds Subgroup II with PRP at the days 1&5&9 and14.

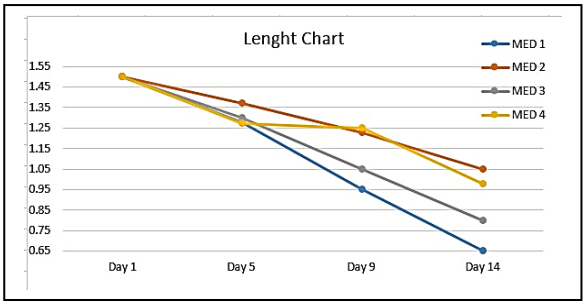
Day	Untreated wound (Control)	Treated wound by PRP
Day 1		
Day 5		
Day 9		
Day 14		

Fig, (2): Group B control (subgroup I) and treated wounds Subgroup II with PRP at the days 1&5&9 and14.

Table (1): Showing the differences in wound length and width in the experimental and control sub group using AM and PRP.

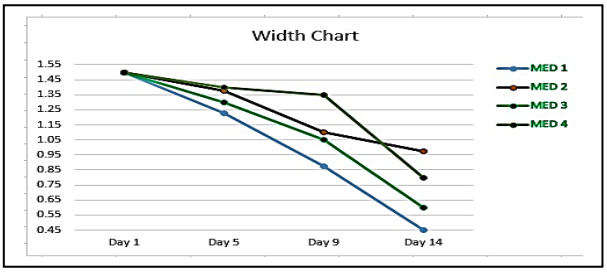
Day	Amnion		Control		PRP		Control	
	Length (CM)	Width (CM)	Length (CM)	Width (CM)	Length (CM)	Width (CM)	Length (CM)	Width (CM)
1	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
5	1.3	1.2	1.5	1.3	1.4	1.3	1.3	1.4
	1.4	1.25	1.3	1.4	1.2	1.3	1.2	1
	1.2	1.2	1.4	1.3	1.3	1.4	1.4	1.3
	1.2	1.3	1.3	1.5	1.3	1.2	1.2	1.2
Mean	1.275	1.23	1.37	1.375	1.3	1.3	1.27	1.4
9	1	0.8	1.3	1.1	1.2	1	1.2	1
	1.1	0.9	1	1.2	1	1.1	1.3	1.2
	0.9	1	1.4	1.1	1.1	1.2	1.4	1.3
	0.8	0.8	1.2	1	0.9	0.9	1.1	1.2
Mean	0.95	0.875	1.225	1.1	1.05	1.05	1.25	1.35
14	0.7	0.4	1.1	1	0.8	0.6	1	0.9
	0.6	0.3	1	0.9	0.9	0.6	1.1	0.9
	0.7	0.6	1.2	1	0.7	0.4	0.9	1.1
	0.6	0.5	0.9	1	0.8	0.5	0.9	0.8
Mean	0.65	0.45	1.05	0.975	0.8	0.6	0.975	0.8

	MED 1 (AM)	MED 2 (Control)	MED 3 (PRP)	MED 4 (Control)
Day 1	1.5	1.5	1.5	1.5
Day 5	1.275	1.37	1.3	1.27
Day 9	0.95	1.225	1.05	1.25
Day 14	0.65	1.05	0.8	0.975



Graph(1): Showing the differences in wound length between all experimental and control subgroups

	MED 1 (AM)	MED 2 (Control)	MED 3 (PRP)	MED 4 (Control)
Day 1	1.5	1.5	1.5	1.5
Day 5	1.23	1.375	1.3	1.4
Day 9	0.875	1.1	1.05	1.35
Day 14	0.45	0.975	0.6	0.8



Graph(2): Showing the differences in wound width between all experimental and control.

B) Histopathological findings of experimental animals:

The artificial skin wounds of untreated (Control) and treated with amnion and PRP, in groups (1,2) (Fig. 3, 4) at 1st day post-wounding (p.w.) showed aseptic wound gap deeply to basal cells at the bottom of the epidermis with few hemorrhage. The skin injury at 5th day (p.w.) displayed angiogenesis, collagen fiber with inflammatory reaction in necrotic cells debris in the wound bed in all groups. Angiogenesis of blood vessels with collagen fibers and inflammatory reaction still showed at 9th day (p.w.) in untreated animals but decreased in treated animals with formation in the inner layer of the epidermis. The wound of animals which left without treatment at 14 day (p.w.) still not completely healing in both groups (1,3) while in the treated animals completely healing in amnion group was seen, while granulation tissues appeared above the surface in PRP group.

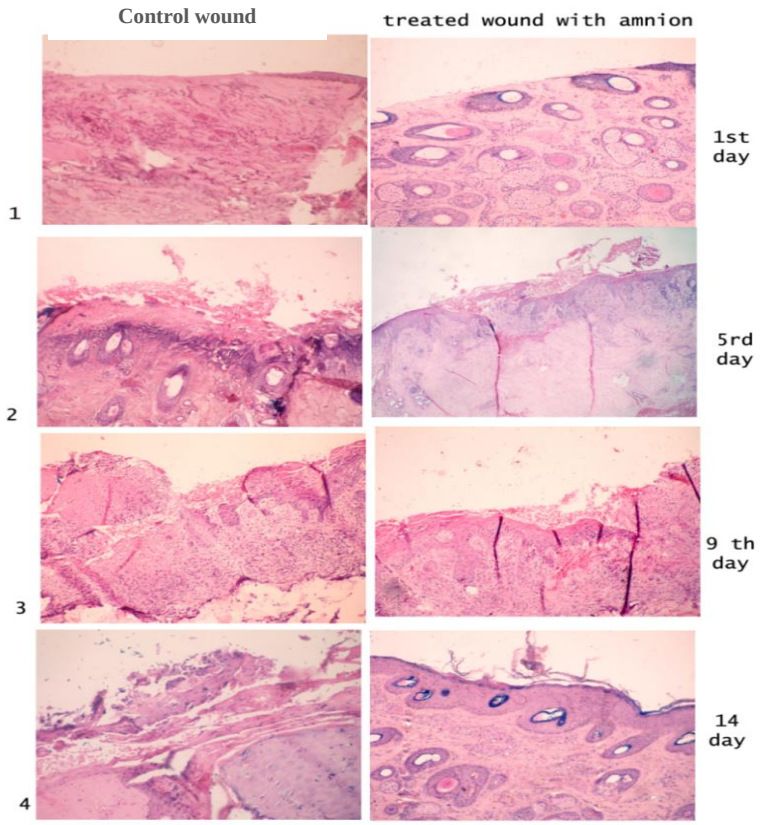


Fig. (3): Untreated (control) and treated artificial wound with amnion, group A (1, 2) showing the differentiation between the injury of the limb of donkey, which left without treatment, the wound still not completely closed while which treated with amnion the wound completely healing occurred at 14 day post wounding. **(H&E., x 150).**

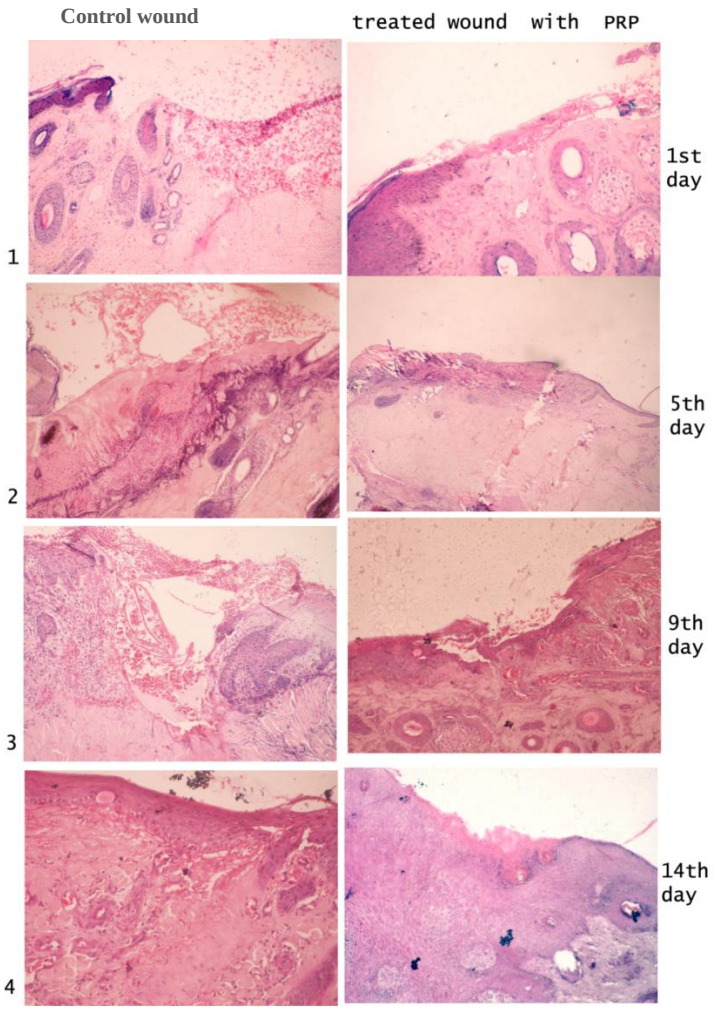


Fig. (4): Control and treated artificial wound with PRP group B (3, 4) showing the differentiation between the injury of the leg of donkey, which left without treatment, the wound still not completely healing while which treated with PRP the wound appeared with scar formation above the surface at 14 day post wounding. **(H&E., x 150)**

Histological scoring systems for skin wounds repair between the experimental groups (Control and treated group):

Table (2): Scoring for evaluate the healing period among the different treated wounds (gps 2, 4) and control wounds (gps 1, 3).

Gps	No of animals	Treated substance	% Clotting blood in gap wound				% Angiogenesis and inflammation reaction				% Collagen fiber and Granulation Tissue				% Scarring formation				% Complete remodeling			
			Time of sampling				Time of sampling				Time of sampling				Time of sampling				Time of sampling			
			1	5	9	14	1	5	9	14	1	5	9	14	1	5	9	14	1	5	9	14
G1	3	untreated	++	-	-	-	-	++	+++	-	-	-	++	+ +	-	-	-	+	-	-	-	+
G2	3	amnion	+++	-	-	-	-	+++	++	-	-	-	+++	-	-	-	-	-	-	-	-	+++
G3	3	untreated	++	-	-	-	-	++	+++	-	-	-	++	+ +	-	-	-	+	-	-	-	+
G4	3	PRP	+++	-	-	-	-	+++	++	-	-	-	+++	+ +	-	-	-	+++	-	-	-	++

+= Mild ++ = Moderate +++= Severe

DISCUSSION

The results of the present study reflect the importance of wounds management as they constitute the most surgical affection in animals especially in equine and particularly drafting donkey. It may impair the physiological functions of tissues or organs in addition to the complication and the cost of treatment in case of presence of infection. Also it reflects the efficacy of some biological materials like amnion sheathes and platelet rich plasma (PRP) for accelerations of wound healing.

Lazarus *et al.* (1994) ; Wetzler *et al.* (2000) ; Velnar *et al*. (2009) and Wang *et al*. (2018) mentioned that, wound healing is a dynamic, complex and natural process including a regular interaction between diverse immunological and biological system, and consist of the overlapping stages which arebleeding,coagulation,initiation of an acute inflammatory response, regeneration, migration and proliferation of connective tissue. The experiments in the present study were directed to investigate the potential effect of amnion sheaths and PRP on cutaneous wound healing in equine and giving a scientific assessment and clinical evidence for the more effective one that stimulating rapid repair with minimal scar formation.

The wounds were measured and photographed. Biopsies from all groups were taken at the time periods 1, 5,9,14 days post wounding. skin specimens were fixed in 10% neutral buffer formalin (NBF),dehydrated in alcohol and paraffin block were prepared which sectioned at 5 micro meter thickness, and stained by Harri's hematoxylin and eosin for histopathological examination **(Drury and Wallington, 1980).**

The gross evaluation of wounds in the first control group wounds with amnion for 14 days post wounding revealed that, after 1 day post wounding, there was swelling and hyperemia of the wound edges in the control (gpI). Five days post wounding, there was swelling and hyperemia which relatively diminished with a degree of mild wound contraction. However, at 9 day post wounding the wound gap reduced with presence of rosy granulation tissue formation filling nearly half of the wound gap. At 14 day post wounding, a comparative increase in the granulation tissue with a relatively increased wound contraction but without complete closure of the wound gap.

The corresponding group of wounds treated with amnion (gpII) after 1 day post wounding showed, nearly the same clinical picture of the above group. Five days post wounding, a relatively narrow wound gap with healthy granulation tissue formation was seen. However, at 9 day post wounding an increased wound contraction was detected and the wound begin to close. Additionally, at 14 day post operatively, the experimental wound were seen completely closed and the skin return to its normal peculiarities. The gross appearance of the healing of created wounds in the second control for 14 day post-surgery. The gross appearance of the healing of the control wounds (g I) the same gross clinical pictures was seen as previously mentioned above at 1, 5, 9 and 14 days post operatively. The gross clinical picture of the corresponding group of wounds treated with PRP (g II) revealed that, after 1 day post wounding, the wound gap showed blood clots with starting granulation tissue formation. Five days post wounding, an increased granulation tissue formation with reduction of the wound gap was detected .Meanwhile , at 9 day post wounding , the wound diameter decreased with partially closed of it. At 14 day post wounding, in complete wound closure was detected with scar formation. The results of gross clinical pictures of both amnion sheath and PRP indicated that they gave a relatively better wound healing concerning the healing time and granulation tissue formation, the amount of scar tissue and wound epithilization compared with both the control groups. These results nearly agreed with **Goodrish *et al.* (2000);**

Dylund *et al.* (2012) and Favaron *et al.* (2015) concerning the use of the amniotic sheath for wound healing in general. On the other hand our results of application of PRP for wound healing agree with **Wilmink *et al.* (2001); Gomez *et al.* (2004); Chen *et al.* (2010) Lopez and Carmona, (2014)** and disagreed with **Monterio *et al.* (2009); Moradi *et al.* (2013)** they reported PRP were relatively less effective not effective on equine distal limb because PRP induce a production of over granulation tissue formation. Meanwhile, the present studies declared that, amnion proved to be a relatively more effective than PRP with a comparatively lesser healing time with rapid wound closure with lesser scar formation. Regarding the histopathological findings of the experimental animals, the artificial skin wounds of untreated and treated with amnion and PRP, in groups (1, 2) at 1st day post-wounding (p.w.) showed aseptic wound gap deeply to basal cells at the bottom of the epidermis with few hemorrhage. The skin injury at 5^{th} day (p.w.) displayed angiogenesis, collagen fibers with inflammatory reaction in necrotic cells debris in the wound bed in all groups. Angiogenesis of blood vessels with collagen fibers and inflammatory reaction still present at 9th day (p.w.) in untreated animals but decreased in treated animals with formation in the inner layer of the epidermis. The wound control of animals which left without treatment at 14 day (p.w.) still not completely healed in both groups (1,3) while those in the treated animals were completely healed in amnion group over granulation tissues appeared was encountered in PRP group. The aforementioned histological picture proved that, the use of both amnion and PRP gave a comparatively better effect concerning angiogenesis, collagen formation and orientation and epithlization compared with both the control groups.These results nearly agreed with **Rumsey *et al.* (1995)** in wounds treated with amniotic sheath and with **Carter, (2003)** concerning the use of PRP for treatment of wound in general. Meanwhile amnion is comparatively over coming PRP.

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