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Evaluation of the Efficacy of Microvesicles Derived from Mesenchymal Stem Cells on Healing of Experimentally Induced Full-Thickness Skin Wound in Donkeys

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ABSTRACT

The present study aimed to evaluate efficacy of microvesicles (MVs) derived from mesenchymal stem cells on healing of experimentally induced full-thickness skin wound in donkeys.6 adult healthy donkeys were subjected to two full thickness skin wounds on the back region.Hence; two groups were evaluated; wounds on the left side were treated with injection of saline solution 24 hrs post wounding (PW)and repeated at day 7 PW as control group and wounds on the right side treated with MVs solution 24 hrs PW and repeated at day 7 PW. Wounds were clinically for determination of wound surface area and percentage of wound contraction. Histopathological evaluation was performed at days 21 and 42PW. Immunohistochemical evaluation was performed at 21 days PW for detection of the activity against CD31 and alpha smooth muscle actin (α-SMA) markers. The wound surface area progressively decreased in both MVs and control groups and it was significantly lower in MVs group than the control group. The percentage of wound contraction was significantly higher in MVs group than the control group. Histopathological evaluation revealed better findings in MVs group compared to the control group. MVs group showed high immunreactivity against both CD31 and α-SMA antigensand the control group showed no immunreactivity against both antigens.In conclusion, mesenchymal stem cells derived microvesicles could promote neovascularization, re-epithelialization and collagen deposition at wound sites and thus accelerate wound healing in experimentally induced full thickness skin wounds in donkeys when compared to the control group.

Key words: Wound, Healing, Microvesicles and Donkey.

INTRODUCTION

Skin is considered the largest organ in the animal body. It serves as a protective barrier against foreign pathogens and chemical compounds, regulates body temperature, prevents dehydration, and sensing the external environment (Brandner and Jensen, 2008). It consists of three major layers; the epidermis (outermost layer), the dermis (middle layer), and the subcutis (innermost layer) in addition to other components including skin appendages (such as hair and hooves), and subcutaneous muscles and fat (Scott, 2003). Skin wounds are common causes of substantial economic losses in equine industry. wounds usually associated with tissue and contamination avulsion, which prevent primary closure. Consequently, these wounds are left to heal by second intention (El-Sayad et al., 2004).

The process of wound healing is a complex process and consists of three overlapping phases; the inflammatory phase involves

hemostasis and acute inflammation, the proliferative phase during which tissue formation occurs and the remodeling phase during which the healing tissue regains its strength (Franz, 2007). Acute wounds proceed through these steps in a timely manner and achieve functional and anatomic integrity. In contrast, chronic wounds will similarly begin the healing process, but will have prolonged inflammatory, proliferative or remodeling phases, resulting in tissue fibrosis and non-healing ulcers, which is not an uncommon outcome in horses (Mast and Schultz, 1996; Wilmink et al., 2002).

Mesenchymal Stem Cells (MSCs) are undifferentiated cells and can divide during self-renewal and differentiate into different cell types and tissues (Smirnov et al., 2007). The Soluble factors secreted from MSCs is another way that plays a role in the homeostasis of various tissues. There is compelling evidence that the effects of MSCs are mainly mediated by paracrine mechanisms and particularly by the secretion of extracellular vesicles (EVs) (Brown et al., 2019).

EVs are lipid bilayer-delimited particles that are naturally released from most eukaryotic cells under both normal and pathophysiological conditions and are present in all body fluids. They can be generally classified into exosomes, MVs and apoptotic bodies according to their cellular origin. The apoptotic bodies are produced by dying cells during apoptosis while exosomes and MVs are released by all cells. Recent studies showed that EVs played pivotal roles in tissue physiology such as modulation of inflammatory response and promotion of cutaneous wound healing (El-Tookhy et al., 2017; Connor et al., 2019).

MVs are membrane vesicles that are released by many types of cells and have recently been considered important mediators of cell-to-cell communication. It serves as a vehicle to transfer proteins and messenger RNA and microRNA (miRNA) to distant cells, which alters the gene expression, proliferation, and differentiation of the recipient cells (Chen & Chen, 2015).However, several studies described the effect of mesenchymal stem cells derived microvesicles on wound healing in dogs (El-Tookhy et al., 2017; Bahr et al., 2021) and in mice (Trinh et al., 2016; Ren et al., 2019). There was little information in the literature about the use of mesenchymal stem cells derived microvesicles in management of wounds in equine. Therefore, the aim of the present study is to evaluate efficacy of MVs derived from mesenchymal stem cells on healing of experimentally induced full-thickness skin wound in donkeys.

MATERIALS AND METHODS

Animals

The present study was approved by Animal Welfare and Ethics Committee, Faculty of Veterinary Medicine, University of Sadat City. Six adult healthy donkeys (5-6 years old with average body weight180-220 kg) were used in this study. The animals considered normal based on physical examination (not suffering from any skin diseases) and hematological analyses. During the experiment, the animals were housed in a covered stable containing several partitions and they were fed twice daily with wheat straw and a one kilogram of concentrates and allowed free choice of water.

Study groups

In the present study, each animal was subjected to two full thickness skin wounds (one on each side of the back region). Hence, 2 groups were evaluated as follows:

- Control group: wounds at the left side of the back region treated with injection of saline solution at 24 hrs and repeated at day 7 post wounding.
- MVs group: wounds at the right side of the back region treated with mesenchymal stem cells derived microvesicles solution 24 hrs after

wounding and repeated at day 7 post wounding.

Surgical operation

Before the operation, all animals received a prophylactic dose of antitetanic serum. The skin of the back region was clipped and aseptically prepared with povidone-iodine on both sides then the animals were sedated using xylazine hydrochloride at a dose of 1.1 mg/kg, IV with local infiltration of Lidocaine HCL at dose of 1ml/cm. In each animal, one full-thickness excisional square skin wound (3 x 3 cm) (Sadek et al., 2020) was created bilaterally on the back area using sterile template. Then, the wounds were dissected from the underlying tissue using scissors and tissue forceps and bleeding was controlled by placing sterile gauze on wound surface. Each wound was subsequently treated with one of the two tested treatments in this study (figure 1). The day of wound creation was designed as day 0.

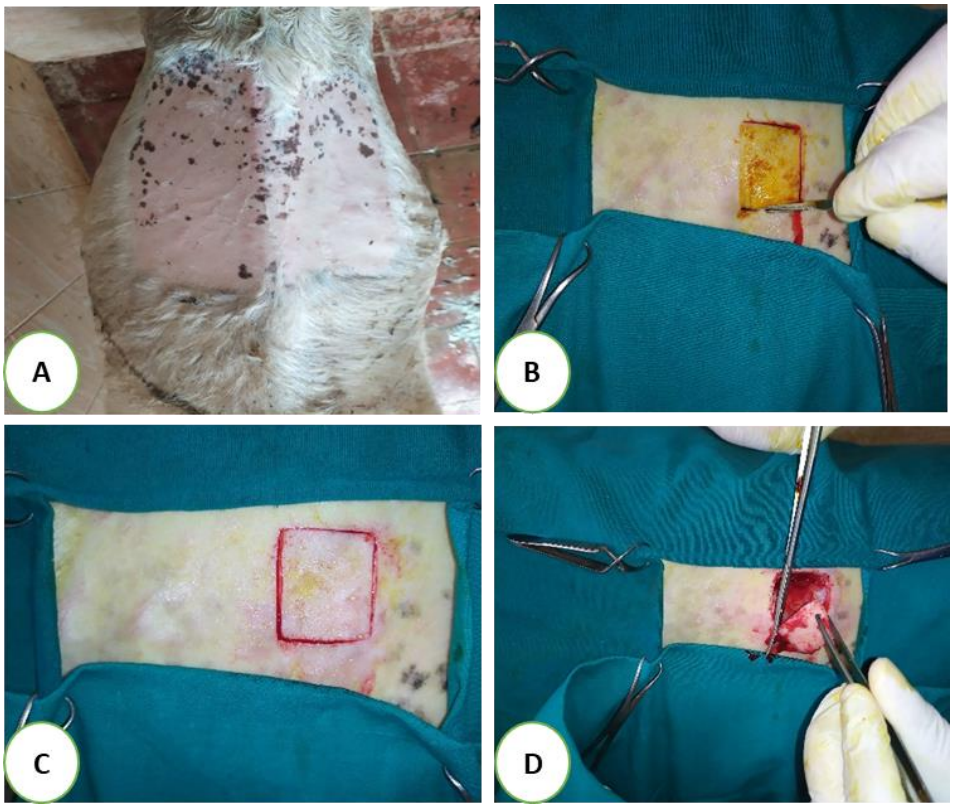

Figure (1): Surgical operation for induction of skin wounds; A) Preparation of the area of the back region. B) Incision of the skin after marking of the wound area by sterile template. C) The wound area after incision. D) Dissection of the skin covering the area using scissors.

Preparation and application of Stem cells derived microvesicles

Stem cells derived microvesicles vial (Stem- Sol®; StemVie Lab Giza-Egypt) containing 3 gm lyophilized material derived from 3x106 mesenchymal stem cells. The vials were dissolved in 6 ml of distilled water to give about 6 ml of injectable microvesicle solution. 4 ml of stem cells derived microvesicles solution were subcutaneously and intradermally injected around each wound 24 hrs after wound creation and repeated at day 7 post wounding. The amount of the injected microvesicle solution equal to the amount produced by 2×10^6 MSCs/wound (El-Tookhy et al., 2017).

Estimation of wound healing

Clinical evaluation of the wounds

Control and MVs groups were clinically evaluated for 42 days through determination of the wound surface area, percentage of wound contraction. The width and the length of wounds were

measured using digital caliber after the wound had been carefully cleaned by saline to visualize wound margins. The wound surface area, percentage of wound size, and wound contraction percent were calculated using the following equations (Zaid et al., 2017).

Histopathological evaluation

Tissue biopsies from skin wounds (at days 21 and 42) were fixed in 10% neutral-buffered formalin overnight, then washed with 70% ethanol, and processed with several processes of paraffin sectioning. Sections were then stained with hematoxylin and eosin to evaluate morphological features of tissues and were stained with Masson's trichrome to highlight the formation collagen tissue during proliferation and remodeling phases of wound healing.

Immunohistochemical evaluation

CD31 and alpha smooth muscle actin (α-SMA) markers were detected by immunoperoxidase technique and evaluated on light microscope for skin wound samples taken from wound margins at day 21 post wounding. For immunoperoxidase staining, skin samples from the wound sites were fixed in 10% formalin, dehydrated with different grades of alcohol series, embedded in paraffin, and sectioned into 4-μm-thick sections on positive slides.

Statistical analysis

Statistical analysis was performed with SPSS 20 software (SPSS Inc., Chicago, IL, U.S.A.). Different variables were analyzed by Student's t-test. P values <0.05 were considered statistically significant.

RESULTS

Clinical evaluation of the wounds

Throughout the observation period, the wound surface area gradually declined in both control and MVs groups. At 42 days post wounding, the Mvs group showed complete wound healing evidenced by complete disappearance of the open wound area while at the same time point, wounds of the control group were not completely healed (Figure 2). From 7 up to 42 days post wounding, the wound surface area was significantly lower in Mvs group than the control group (Figure 3).

The wound contraction percentage progressively increased in both control and MVs groups whereas it was significantly higher in MVs group than the control group from 14 up to 42 days post wounding and reaching 100 % in MVs group (Figure 4).

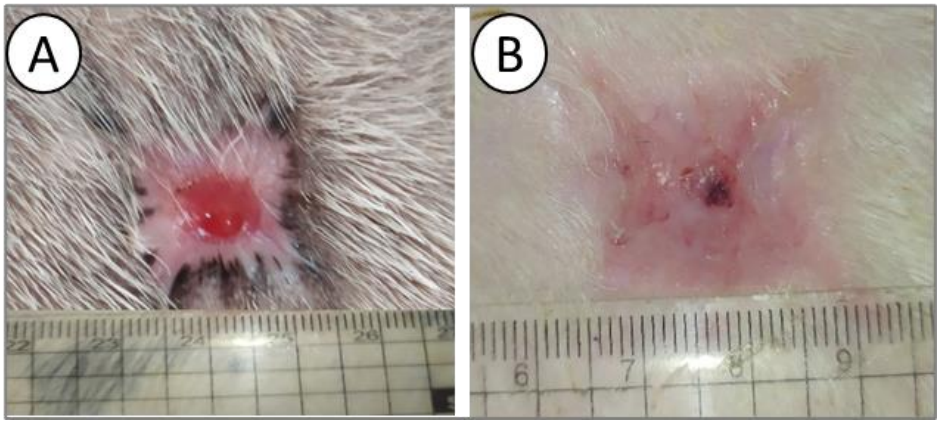

Figure (2): Photographs showing the wound area in the two study groups at 42 days post wounding: (A) Saline treated group showing incomplete closure of the wound area compared to (B) MVs treated group showing complete closure of the wound area.

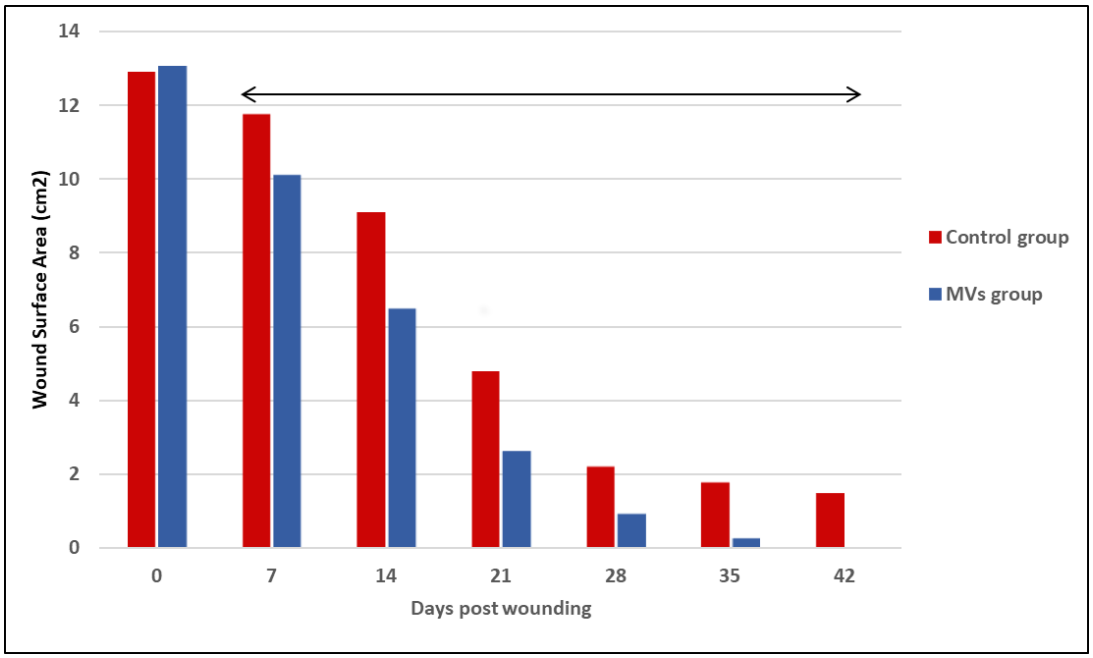

Figure (3): The mean of wound surface area in control and MVs groups.
↔ Significant difference between groups.

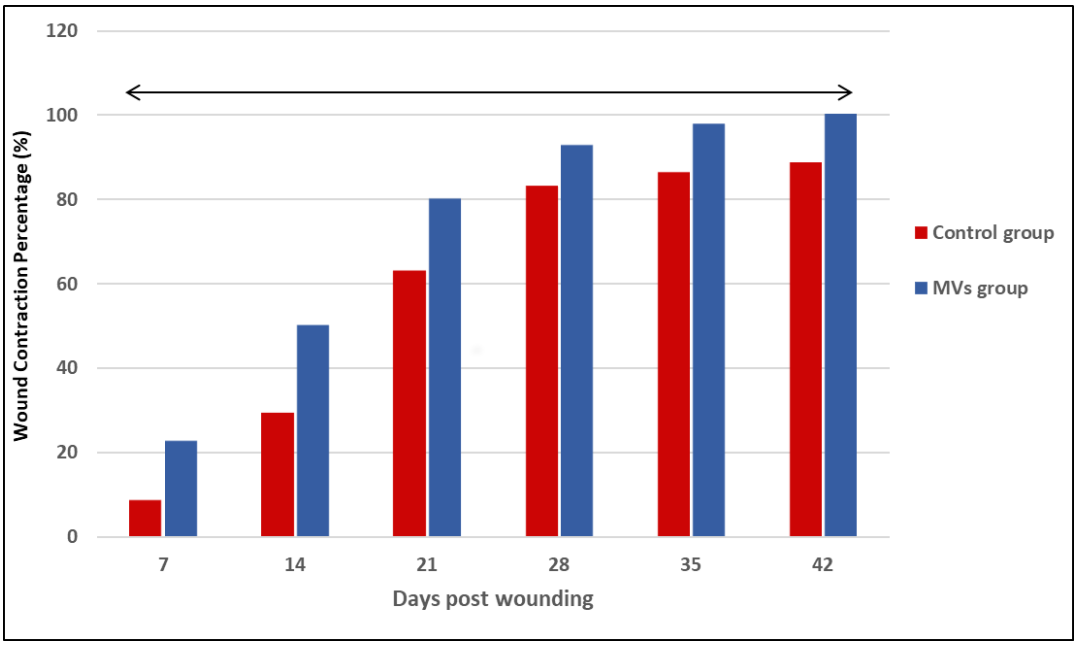

Figure (4): The mean of wound contraction percentage in control and MVs groups.
↔ Significant difference between groups.

Histopathological Evaluation

At day 21 post wounding, histopathological examination of control wound samples revealed high proliferation of fibrous tissue with very high congestion and hemorrhage. At 42 days showed mature fibrous tissue with hemorrhagic granulation tissue (figure 5).MVs group showed high proliferation of epidermal cells with migratory islets of epidermal cells to the surface and the dermis is mainly hypercellular condensed immature collagen at 21 days post wounding and thickening of the epidermal layer with a thin layer of keratin and increased production of collagen in the dermis layer at 42 days post wounding (figure 6).

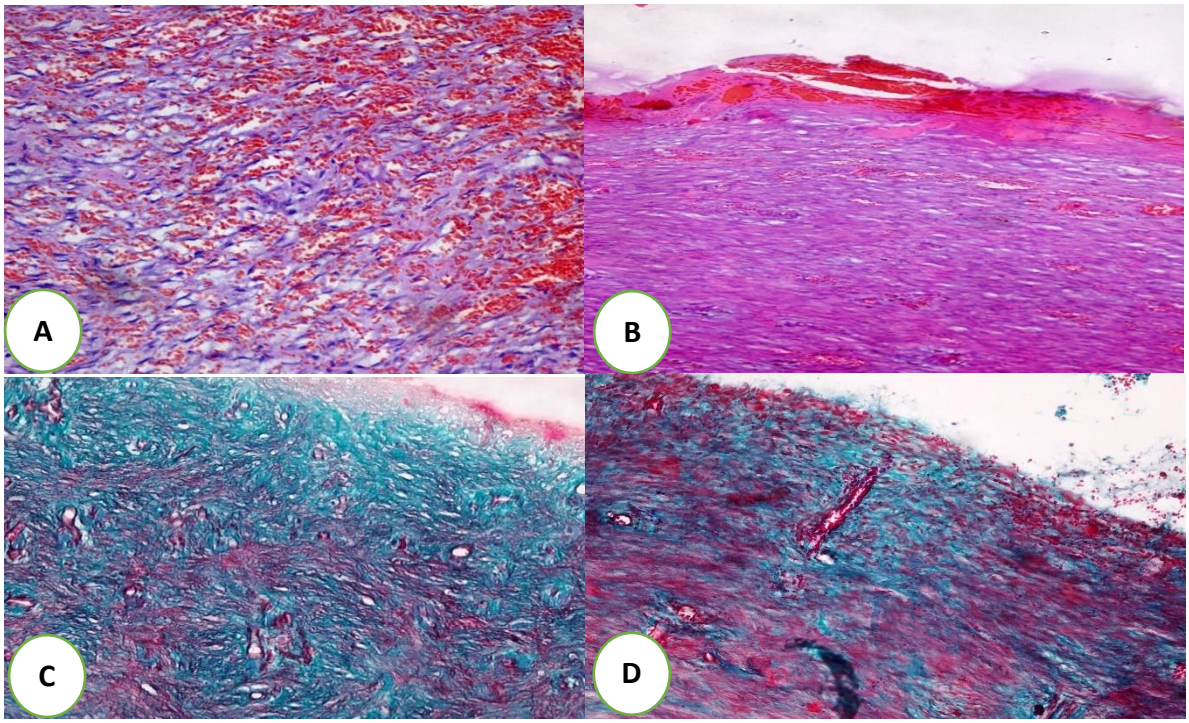

Figure (5): Photomicrograph showing the wound healing site of control group; A) at 21 days post wounding showing high proliferation of fibrous tissue with very high congestion and hemorrhage H&E 200X. B) At 42 days showing mature fibrous tissue with hemorrhagic granulation tissue H&E 100X. C) At 21 days showing high proliferation of fibrous tissue with very high congestion and hemorrhage MTC 100X. MTC 100X. D) At 42 days showing mature fibrous tissue with hemorrhagic granulation tissue MTC 200X.

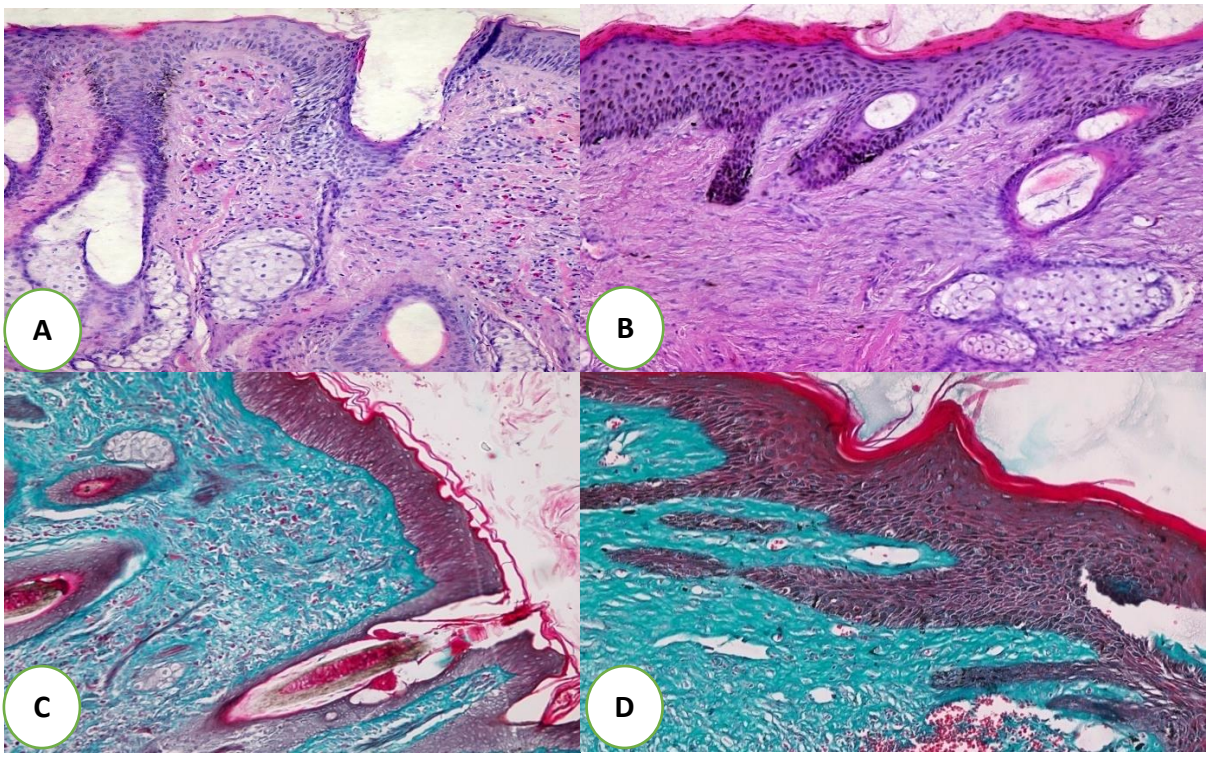

Figure (6): Photomicrograph showing the wound healing site of MVs group; A) at 21 days post wounding showing high proliferation of epidermal cells with migratory islets of epidermal cells to the surface and the dermis is mainly hypercellular condensed immature collagen H&E

200X. B) At 42 days showing thickening of the epidermal layer with a thin layer of Keratin and increased production of collagen in the dermis layer H&E 200X. C) At 21 days showing high proliferation of epidermal cells with migratory islets of epidermal cells to the surface and the dermis is mainly hypercellular condensed immature collagen MTC 200X. D) At 42 days showing thickening of the epidermal layer with a layer of keratin and increased production of collagen in the dermis layer and few fibrous tissue MTC 200X.

Immunohistochemical evaluation

Immunohistochemical examination of skin wound sections at 21 days post wounding revealed no immunreactivity against both CD31 and α-SMA antigens in control group (figure 7) while the MVs group showed high immunreactivity against both CD31 and α-SMA antigens (figure 8).

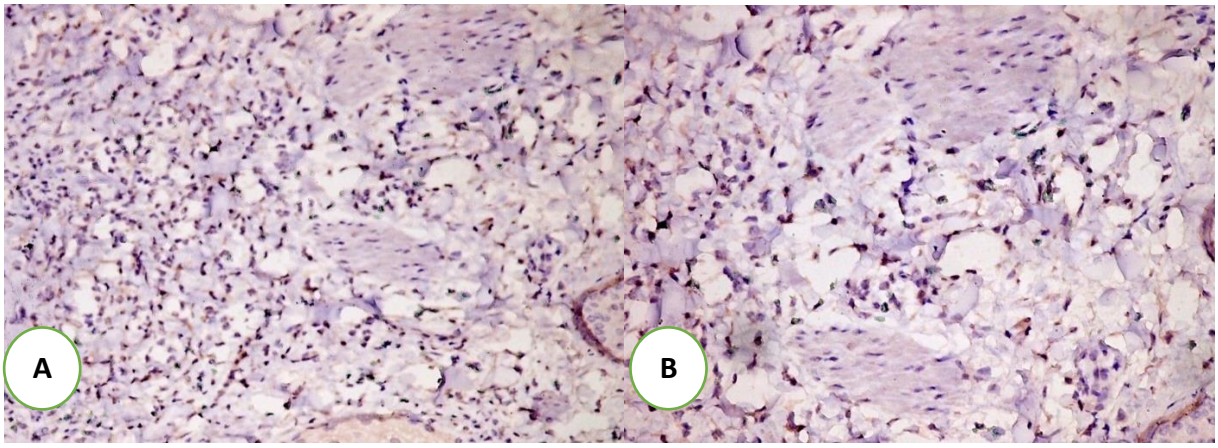

Figure 7: Immunohistochemistry of skin wound section of control group 21 days post wounding; A) no immunreactivity against CD31 antigen. Avidinbiotin immunoperoxidase complex x400. B) no immunreactivity against α-SMA antigen. Avidinbiotin immunoperoxidase complex x200.

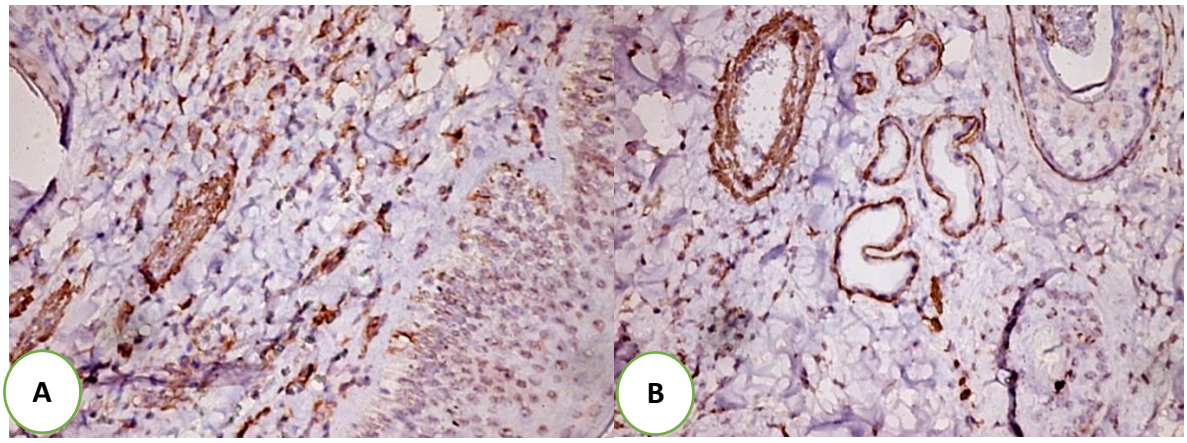

Figure 8: Immunohistochemistry of skin wound section of MVs group 21 days post wounding; A) High immunreactivity against CD31 antigen. Avidinbiotin immunoperoxidase complex x400. B) High immunreactivity against α-SMA antigen. Avidinbiotin immunoperoxidase complex x400.

DISCUSSION

Exosomes are one of the many secretory factors released by the mesenchymal stem cells (MSCs) (Tögel et al., 2007; Rani et al., 2015) and the most effective ingredients that play an important role in cell-to-cell communication (Kinnaird et al., 2004; Simons and Raposo, 2009). Consequently, it was hypothesized that if the beneficial

actions of stem cell therapy are mediated by its exosomal secretions, then direct treatment with these exosomes may overcome the limitations and risks associated with stem cell therapy (Rani et al., 2015; Zhang et al., 2015).

The correlation between the number of cells applied per each cm2 of the wound surface and the subsequent decrease in wound size has been reported by several studies. It was suggested that at least 1×106 cells/cm2 of wound should be used for a significant therapeutic effect (Falanga et al., 2007). In the present study, the amount of microvesicle solution used equal to the amount obtained from 2×106 MSCs/wound similar to (Fu et al., 2006; Basiouny et al., 2013; Abd-Allah et al., 2015). The route of administration also varied. Some studies demonstrate that the treatment could be achieved by intra-dermal injection (Kim et al., 2013; Abd-Allah et al., 2015), local and systemic injection (Tark et al., 2010; Basiouny et al., 2013), or spray (Falanga et al., 2007). In this study, the MVs solution was subcutaneously and intradermally injected around each wound similar to (El-Tookhy et al., 2017; Chen et al., 2008).Moreover, in this study, the MVs solution was injected two times with one-week interval starting 24hr after wound creation in contrary to the study reported by El-Tookhy et al. (2017) in which the solution is used as a single injection 24 hrs after wound creation.

The present study demonstrated better and faster healing of microvesicles treated wounds compared with the control wounds. Wounds treated with MVs showed faster wound contraction than observed in the control group and the percentage of wound contraction was significantly higher in MVs group than the control group from 7 days post wounding till the end of the observation period. Similar results reported by El-Tookhy et al. (2017) and Bahr et al., (2021).

In the current study, histopathological evaluation of skin wound sections revealed better healing quality in MVs group compared the control group. Mvs treated wounds showed high proliferation of epidermal cells with migratory islets of epidermal cells to the surface and the dermis is mainly hypercellular condensed immature collagen at 21 days post wounding and thickening of the epidermal layer with a thin layer of keratin and increased production of collagen in the dermis layer at 42 days post. In contrary, the saline treated wounds showed high proliferation of fibrous tissue with very high congestion and hemorrhage at 21 days and showed mature fibrous tissue with hemorrhagic granulation tissue at 42 days post wounding. These findings are similar to that reported by El-Tookhy et al. (2017) and Bahr et al. (2021).

New-vascularization is an essential step in the wound healing process, angiogenesis and maturation of the new blood vessels is necessary to maintain a healthy granulation tissue (Borena et al., 2010; Singer and Clark., 1999). To study MVs induced angiogenesis during the healing process, immunoreactivity against CD31 and alpha smooth muscle actin (α-SMA) antigens were detected at 21 days post wounding. The newly formed vessels and capillaries were detected by CD31 positive staining, while mature vessels were characterized by CD31 and α-SMA double-positive vascular structures (Zhang et al., 2015; Kim et al., 2013;Hattori et al., 2009). In this study immunohistochemical examination of skin wound sections of the MVs treated wound revealed high immunreactivity against both CD31 and α-SMA antigens. Wound sections of the control group showed no immunreactivity against both CD31 and α-SMA antigens. These findings indicate quicker angiogenesis and proper collagen deposition in the MVs treated group than the control group.

In conclusion, mesenchymal stem cells derived microvesicles could promote neovascularization, re-epithelialization and collagen deposition at wound sites and thus accelerate wound healing in experimentally induced full thickness skin wounds in

donkeys when compared to the control group. Hence, mesenchymal stem cells derived microvesicles appeared to be a superior candidate for management of cutaneous wounds in equine and represent a promising opportunity to develop a new cell-free therapy that might overcome the risks associated with the use of engineered stem cells transplantation therapy.

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