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Potential Regenerative Effect of Mesenchymal Stem Cells-Derived Microvesicles on Healing of the Ruptured Achilles Tendon in a Dog Model

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ABSTRACT

Recently, there are increasing number of reports that suggests the microvesicles (MVs) derived from mesenchymal stem cells (MSCs) as a beneficial therapeutic treatment as a cell free regenerative option for multiple diseases so the current study aimed to evaluate the efficacy of MSCs-derived MVsin potentiation of the healing process of surgically repaired,induced Achilles tendon rupture in dog.Study design:Twelve adult mongrel dogs of both sexes weighing 15–18 kg and aged 2-3 years were used in this study and were divided into two groups, Control group: with no additional treatments. MVs group: MSCs-derived MVs were used as additional treatment. During observation period,dogs were observed clinically at 3,6, 8, 10- and 12-weeks post-operation and histologically at 6- and 12-weeks post-operation.Results: the clinical observation revealed a great significant difference between the two groups and the MVs could make complete healing at the end of the observation period with full weight bearing capacity and no lameness during walk and histological examination revealed significantly faster healing rate, better arrangement of the fibers and higher collagen expression.Conclusion: The present study revealed that MVs have a great ability to enhance and accelerate the healing and regeneration processes of the Achilles tendon

Keywords: Achilles tendon rupture, MSCs-derived MVs and Tendon healing.

INTRODUCTION

Achilles Tendon (AT) is the strongest tendon structure of the musculoskeletal system in dogs. It is responsible for the extension of the hock and makes the animal able to stand on the toes. Also, its main function is the forward progression of the rear-limb and passive support of the hock (Spinella et al., 2010). Achilles tendon is the most exposed structure of the musculoskeletal system for injuries in dogs. AT rupture has been frequently demonstrated in dogs and with high incidence in mature middle-aged, middle to large-breed dogs with higher frequency in athletic dogs. Also, in the modern society, AT rupture has become a common problem with increased incidence among athletic people. Its rupture usually associated with an acute trauma: either an impact trauma

resulting in avulsion of the tendon from the calcaneus or a direct sharp trauma to the tendon structure (cut, laceration), or may be secondary to chronic degenerative changes of the tendon that may also be secondary to other causes such as systemic disease (i.e. Cushing's disease), iatrogenic, steroid administration or fluoroquinolone treatment(Hossain et al. 2008, Cervi et al., 2010, Gamble et al., 2017, Allawi, 2019).

To optimize the healing of the tendon to regain its full mechanical and functional strength, angiogenesis, cell proliferation and deposition of extracellular matrix (collagen synthesis) followed by remodeling and maturation of the tendon are consequently required but because of its limited blood supply and slow cell turnover, the ruptured Achilles tendon heals slowly, often requires surgical treatment and several months for full recovery of functional capacities (Sánchez et al., 2007).

Tendon healing after surgical repair generally progresses through a short inflammatory phase, which lasts about a week, followed by a proliferative phase, which lasts a few weeks, followed by a remodeling phase, which lasts many months (Voleti et al., 2012). It generally involves the contributions of cells from multiple sources, including infiltrating inflammatory cells, resident fibroblasts from the tendon surface or midsubstance, and tendon or marrow-derived mesenchymal stem cells(Manning et al., 2014).

As a recent trend in the regenerative medicine, Mesenchymal stem cells (MSCs) proved their regenerative potential for tissue repair in different organs and offers a new attractive field for the cellular and non-cellular approaches of tissue repair, they release two major classes of MVs in the extracellular environment are the exosomes and shedding vesicles (Tetta et al., 2012). These MSCs-derived MVs have the major role of the MSCs potential in the healing process as they act as paracrine or endocrine mediators that interact with tissue cells, modulate immune and inflammatory responses, promote self-repair from cells that survive injury and play important role in communication between stem and injured tissue cells (Camussi et al., 2013).

The released MVs can be up-taken by tissue cells either through surface receptor mediated interaction or by a process of membrane fusion. After interaction MVs can be introduced in the recipient cells and released their content that act as shuttles for selective pattern of ligands, receptors, enzymes, cytokines, transcription factors, messenger RNA, and micro RNA into target cells, moreover they produce high number of GFs in the healing tissue (Ratajczak et al., 2006, Yeo et al., 2013).

Similar to their mother (MSC),MSCs-derived MVs have a great role in repairing tissue damage and reduction of the inflammatory responses while they have some great values over MSCs as they are more stable and reservable, have no risk of aneuploidy, lower possibility of immune rejection after allogeneic administration so they may provide an alternative therapy for various diseases(El-Tookhy et al., 2017).

Microvesicles contain many potential regulatory components including miRNAs, mRNAs, and proteins, which can be transferred as a type of ''physiological lipofection'' to recipient cells to modify their characteristics (Fleissner et al., 2012). They also contain major histocompatibility complex (MHC) class I (as well as MHC class II when derived from antigen presenting cells), co-stimulatory molecules (CD86) 12, tetraspanins (CD9, CD63, CD82) 13- 15, Fas and Fas ligand 16, several cytosolic (heat shock protein 73, annexin II, Gi2, gag) and membrane-bound proteins (Mac-1, MFG-E8) that aid in the healing process of the different tissues (Sabry et al., 2018).

Recently, there are increasing number of reports that suggests the MVs derived from MSCs as a beneficial therapeutic treatment as a cell free regenerative option for multiple diseases (Yu et al., 2020).

This study aimed to evaluate the efficacy of MSCs-derived MVs in potentiation of the healing process of repaired AT rupture in dog.

MATERIAL AND METHODES:

Animals:

Animals used in the present study were twelve adult mongrel dogs of both sexes (6 males, 6 females) weighing 15–18 kg and aged 2-3 years. All animals were physically normal and free from any Musculo-skeletal disorders. Special care was given to the area of the hock and Achilles tendon. AT was examined clinically for

the presence of inflammation, swelling or rupture to ensure its integrity before initiation of the study. Also, complete blood count and serum chemistry profile was performed to all animals. During the study period, dogs were kept in individual cages where they were fed balanced food and allowed free access to water. This study was approved by the Institutional Animal Care and Use Committee of Faculty of Veterinary Medicine, University of Sadat City. Throughout the study, all efforts were exerted to minimize dogs, stress.

The study design:

Animals of the study were divided to 2 equal groups of 6 animals as control group and Mvs treated group. The 2 groups were differentiated as following:

- Control group: Animals of this group were subjected to experimentally induced Achilles tendon rupture of the right hind limb of each animal followed by tendon suture and S.C and skin closure with no additive treatments.
- MVs treated group: Animals of this group were subjected to experimentally induced Achilles tendon rupture of the right hind limb of each animal followed by tendon suture then application of MVs gel was injected between the 2 cut ends and in the proximal and distal stumps of the cut tendon and finally closure of the skin and S.C.

Preparation and application of the MSCs-derived MVs:

Micro vesicles were prepared just before surgery as 3gm powdered heterogenic lyophilized MSCs derived extracellular vesicles solution purchased from (Bioluga® Canada) and reconstituted according to the manufacturer's instructions in 5 ml of sterile normal Saline and mixed manually till obtaining a homogeneous gel-like suspension ready for injection. one milliliter of MVs suspension was injected between the 2 cut ends and in the proximal and distal stumps of the cut tendon after the tendon suture and before closure of the skin and S.C wound in each animal of the MVs treated group.

Anesthetic regimen:

Before the surgery, animals were premedicated with Xylazine HCL (Xylaject® 2% sol. ADWIA Co., A.R.E.) at dose of 0.5 mg/kg bw I.V injection. Diazepam at dose rate 0.4 mg/Kg BW (Neuril® Memphis Co., A.R.E.).and ketamine HCL (KETALITE®, ELITEPHARMA Co., Pakistan) 5 mg/kg BW were used to induce general anesthesia while the surgical anesthesia was maintained using propofol at dose rate 1mg/Kg BW (Propofol® 1% FRESENIUS KABI Co., Germany)with I.V injection as intermittent boluses until the end of the surgery. Meloxicam (Melocam®, AMOUN Co., A.R.E.) at dose rate 0.2 mg/Kg BW was injected subcutaneously during the induction to control postoperative pain.

Surgical approach for induction of AT rupture:

After aseptic preparation of the site of the operation (the medial and lateral aspect of the lower thigh extending across the hock joint till the proximal metatarsal region). The operation was done with the animal in lateral recumbency on its left side. 10 cm longitudinal skin incision was performed on the lateral aspect of the AT just above the point of the hock, blunt dissection of the S.C till visualization and separation of the AT. Complete sharp transverse cut of the AT was done5cm above its calcaneal insertion. Modified locking loopekessler's tenorrhaphy was used for approximation of the cut ends using poly-propylene USP 2 (Prolene®). Routine closure of the skin and S.C tissue was made using polyglactin USP 0 (vicryl®) followed by tarsal immobilization for 3 weeks using complete Fiber glass cast (Tomato® cast Korea.) then replaced by cranial half fiber glass cast for 3 more weeks. after the operation broad spectrum antibiotic course (Cefotax®: EPICO. Co., A.R.E) at dose rate 20 mg/kg b.wt for five days.

Assessment and Evaluation:

Clinical Evaluation:

Based mainly on physical examination, weight bearing capacity and lameness scoring system.

Palpation was used to monitor the continuity of the tendon and presence of inflammatory signs (pain and swelling) and the thickness of the common calcaneal tendon (CCT) was measured using digital caliper after removal of the cast at 3^{rd}, 6^{th}, 8^{th}, 10^{th} and 12^{th}week after surgery in all animals of both groups and all the results were recorded and statistically analyzed.

The weight bearing capacity and the lameness score were done by 2 different assistances blinded of the groups and the results were recorded as means and standard deviations and statistically analyzed.

The lameness scoring was done after removal of the castat 3rd, 6th, 8th, 10th and 12th week after the surgery using the following scoring system as reported by (Saini et al., 2002) (Table 1).

Table (1): Showing the lameness scoring system used in the study:

SCORE	DESCRIPTION
SCORE 1:	Animals that did not put any weight on the operated limb and kept it off the ground or touched the limb to the ground without weight bearing,plantigrade stance on affected pelvic limb.
SCORE 2:	The dog placed the affected limb on the ground and put some weight on it and occasionally kept the limb off the ground, moderate lameness during walk.
SCORE 3:	The dog always kept the affected limb on the ground and started bearing weight, but was still slightly lame during walk.
SCORE 4:	The animal started putting equal weight on the limbs and apparently was not lame during walk. i.e., normal weight bearing

Histopathological Evaluation:

At the 6th and 12th week, the operated tendons were harvested after euthanasia using thiopental sodium at dose rate 35mg/Kg BW IV injection as one shot (6 animals each observation time 3 of each group) just below the musculo-tendinous junction and at its calcaneal insertion then washed with physiological buffer, fixed in buffered formalin 10% for 24 hrs., dehydrated and cleared in xylene then embedded in paraffin. After solidification of paraffin thin longitudinal sections 4-5 µm thickness from the center (site of the cut) and the periphery and stained with H&E and MTC.

Histological Tendon repair assessment score was evaluated on six parameters with grading score 0-3; fiber structure, fiber arrangement, rounding of the nuclei, inflammation, angiogenesis and cell density (Table 2) (Chen et al., 2014).

Table (2): Showing the Histological Tendon repair assessment score used in the study:

	Score 0	***Score 1***	***Score 2***	***Score 3***
Fiber structure	Continuous, long fiber	Slightly fragmented Fiber	Moderately fragmented fiber	Severely fragmented fiber
Fiber arrangement	Compacted and parallel	Slightly loose and wavy	Moderately loose, wavy and crossing each other	No identifiable pattern
Rounding of the nuclei	Long, Spindle-shape cells	Slightly rounded	Moderately rounded	Severely rounded
Inflammation (Areainfiltrated with inflammatory cells)	< 10%	10-20%	20-30%	> 30%
Increased vascularity (neovascular area)	< 10%	10-20%	20-30%	>30%
Cell density	Normal	Slightly increased	Moderately increased	Severely increased

Statistical analysis:

All the results were recorded as mean, standard deviations. The statistical analyses were performed using one-way ANOVA. P-value was considered as statistically significant when $P \leq 0.05$. All the value were analyzed using the SPSS software (version 20.0; IBM, America).

RESULTS

The results of this study demonstrate the clinical and histological findings of the effect of the single use of MSCs-derived MVs gel on the

healing of surgically repaired an experimentally induced AT rupture in dogs in comparison with a control surgically treated group with no additives.

Clinical Evaluation:

Clinical observation revealed that all the animals of both groups showed continuity of the tendon throughout the observation period when examined by palpation and there were obvious inflammatory signs in the control group until the 6th week observation while there were no signs of inflammation in the MVs group after the 3rd week observation. The tendon thickness and lameness score were recorded and statistically analyzed in the following tables (Table 3, 4; Figures 1, 2).

Table (3): Showing the Average and Standard deviation of thickness of the Achilles tendon at the site of the operation of both groups in different examination times:

Group	Surgery day	Week3	Week6	Week8	Week10	Week12	P-value
control	8.29 ± 0.23^{a}	$15.35 \pm 2.76^{b*}$	$16.34 \pm 3.79^{b*}$	$17.07 \pm 2.33^{b*}$	$15.17 \pm 2.83^{b*}$	$14.03 \pm 1.79^{b*}$	**0.006**
MVs	7.53 ± 0.38^{a}	10.2 ± 0.14^{bd} **	$13.04 \pm 0.76^{c*}$	$10.76 \pm 0.82^{b**}$	9.38 ± 1.00^{bd} **	8.69 ± 0.75^{ad} **	**0.000**
P- Value		**0.024**	**0.243**	**0.001**	**0.001**	**0.000**	

*, **: Means and standard deviations with different asterisks superscripts in the same column are significantly different at P <0.05.

A, b, c, d: Means and standard deviations with different small superscripts letters in the same row are significantly different at P <0.05.

The previous data revealed that, the tendon thickness significantly increased in both groups at the 3rd week observation than the preoperative thickness and continued to increase until the 8th week in the control group and 6th week in the MVs group then started to decrease until the end of the study. The MVs treated group showed significantly lower thickness of the tendon than the control group throughout the observation period(P=0.024-0.000) and at the end of the study it had no significant difference with the preoperative thickness of the tendon (P=0.117).

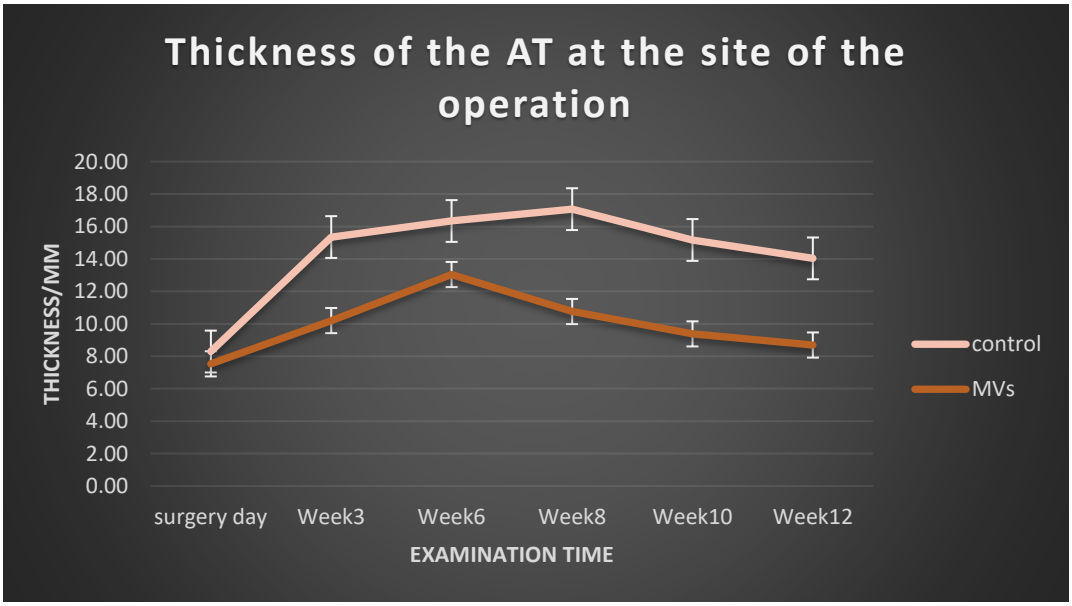

Figure (1): Illustrates the difference of the average thickness of the of the AT at the site of the operation of both groups in different examination times and thechart shows the significant increase in the thickness of the tendon in both groups than the surgery day with the highest increase in the control groups and lowest in MVs group that had significant decrease at the end of the study and the tendon thickness nearly similar to the surgery day.

Table (4): Showing the Median and Ranges of Lameness Score of the 2 groups in different observation times:

Group	Surgery day	Week3	Week6	Week8	Week10	Week12	P-Value
control	4.0 (4.0-4.0)	1.0 (1.0-1.0)^{a*}	2.0 (2.0-2.0)^{b*}	2.0(2.0-3.0)^{c*}	3.0(3.0-3.0)^{d*}	4.0(3.0-4.0)^{e*}	**0.000**
MVs	4.0 (4.0-4.0)	2.0(1.0-2.0)^{a**}	3.0(3.0-3.0)^{b**}	4.0 (3.0-4.0)^{c**}	4.0 (4.0-4.0)^{c**}	4.0 (4.0-4.0)^{c*}	**0.000**
P-Value		**0.141**	**0.017**	**0.011**	**0.017**	**0.195**	

*, **: Median and Ranges with different asterisks superscripts in the same column are significantly different at P<0.05.
A,b,c,d: Median and Ranges with different small superscripts letters in the same row are significantly different at P<0.05.

From the previous data statistical analysis revealed that the MVs treated group had the upper hand regarding the weight bearing capacity and lameness score as it had significant increase of the lameness score from the 2ndobservation till the end of the study (P=0.011-0.017). Moreover, the animals of MVs treated group returned to the normal weight bearing capacity with no obvious lameness as early as the 8thweek while the control group at the end of the study still had slight lameness.

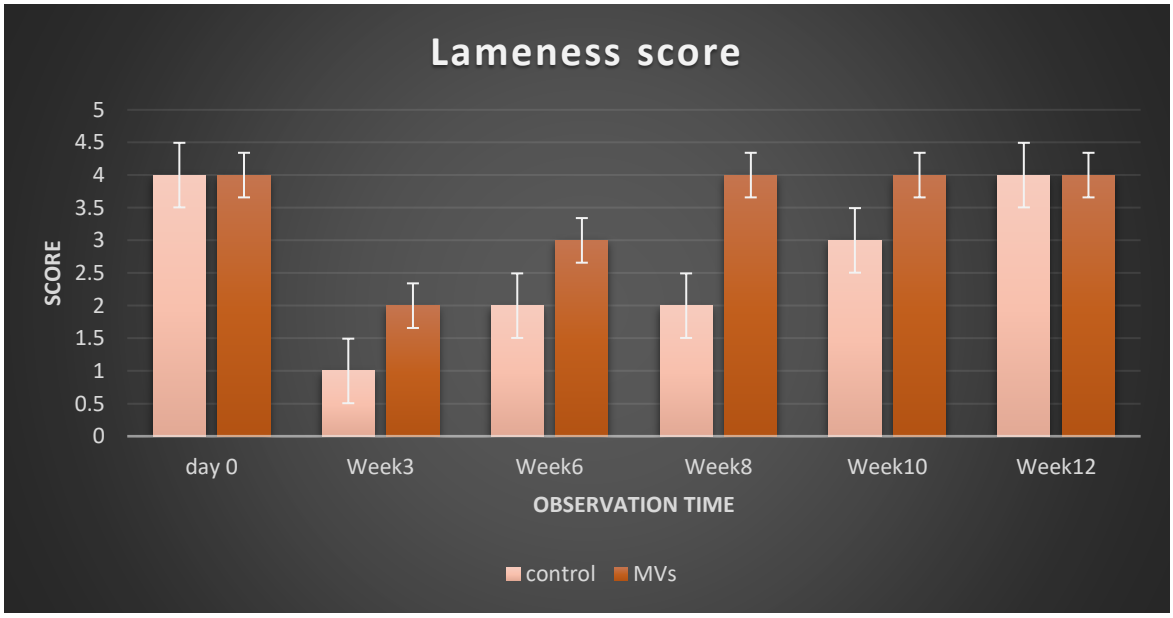

Figure (2): Illustrates the difference of the average Lameness Score of bothMVs and Control groups in different examination times and the chart shows the significant increase of the lameness score in the Mvs group over the control group throughout the observation period the time point where MVs group returned to normal weight bearing with no obvious lameness, score 4 from the 8th weak observation.

Histopathological Evaluation:

Histological evaluation of tendon defects healing with H & E stain showed marked variation in repair parameters, where MVs treated group showed significant improvement compared to the control group; at 6 weeks of evaluation MVs treated group was superior in most of the parameters where cell migration and density, angiogenesis was higher than the control group also, the fiber structure and

arrangement was condensed, regular and parallel with spindle nucleated cells, at 12 weeks the fiber condensation was greater and expressed more collagen fibers and decrease in nucleated cell number compared to the control group (Figure 3).

MTC stain showed high degree of collagen depositionand higher arrangement of the fibers in the MVs treated group than the control group in both the 6th and 12th week observations (Figure 4).

Histological Tendon repair assessment score was evaluated on six parameters with grading score 0-3; fiber structure, fiber arrangement, rounding of the nuclei, inflammation, angiogenesis and cell density. In this score, the normal or ideal tendon will score 0 and the severely affected tendon will score 18. The results listed in (Table 5, 6).

Table (5): Showing the Histological Tendon repair assessment score of both groups at the end of the study (6th week):

Parameters	Control Group	Microvesicles Group	P-Value
Fiber structure	3.0 (3.0-3.0)	1.0 (1.0-2.0)*	0.000
Fiber arrangement	3.0 (3.0-3.0)	2.0 (2.0-2.0)*	0.004
Rounding of nuclei	3.0 (3.0-3.0)	2.0 (1.0-2.0)	0.081
Inflammation	3.0 (2.0-3.0)	1.0 (1.0-2.0)*	0.000
Vascularity	2.0 (2.0-3.0)	2.0 (2.0-2.0)	0.141
Cell density	2.0 (2.0-3.0)	2.0 (2.0-3.0)	0.141
Total Score	16.0 (15.0-16.0)	10.0 (9.0-11.0)*	0.000

*: Median and Ranges withasterisk superscript in the same row are significantly different at P<0.05.

Table (6): Showing the Histological Tendon repair assessment score of both groups at the end of the study (12th week):

Parameters	Control Group	Microvesicles Group	P-Value
Fiber structure	3.0 (2.0-3.0)	0.0 (0.0-1.0)*	0.000
Fiber arrangement	3.0 (2.0-3.0)	0.0 (0.0-1.0)*	0.001
Rounding of nuclei	2.0 (1.0-2.0)	0.0 (0.0-0.0)*	0.000
Inflammation	1.0 (1.0-2.0)	0.0 (0.0-0.0)*	0.004
Vascularity	2.0 (1.0-2.0)	2.0 (1.0-2.0)	0.438
Cell density	1.0 (1.0-2.0)	1.0 (1.0-1.0)	0.438
Total Score	12.0 (12.0-12.0)	3.0 (2.0-3.0)*	0.000

*: Median and Ranges with asterisk superscript in the same row are significantly different at P<0.05.

From the previous data, Statistical analysis revealed that the MVs treated group had the upper hand in most of the parameters of the Histologic tendon repair score where it had significantly higher fiber structure and arrangement with lower inflammatory cell infiltration in both the 6th week and 12th week evaluations (P= 0.000-0.004) while the neovascularization and cell density had insignificant difference (P=0.141- 0.438) but the Rounded Nuclear cells was significantly lower in the MVs group than the control group (P= 0.000) indicating that the higher cellularity was due to replacement of the rounded nuclear cells

with spindle shape fibroblasts. Finally, the Total score was significantly better in the MVs treated group than the Control group in both observation times (P=0.000).

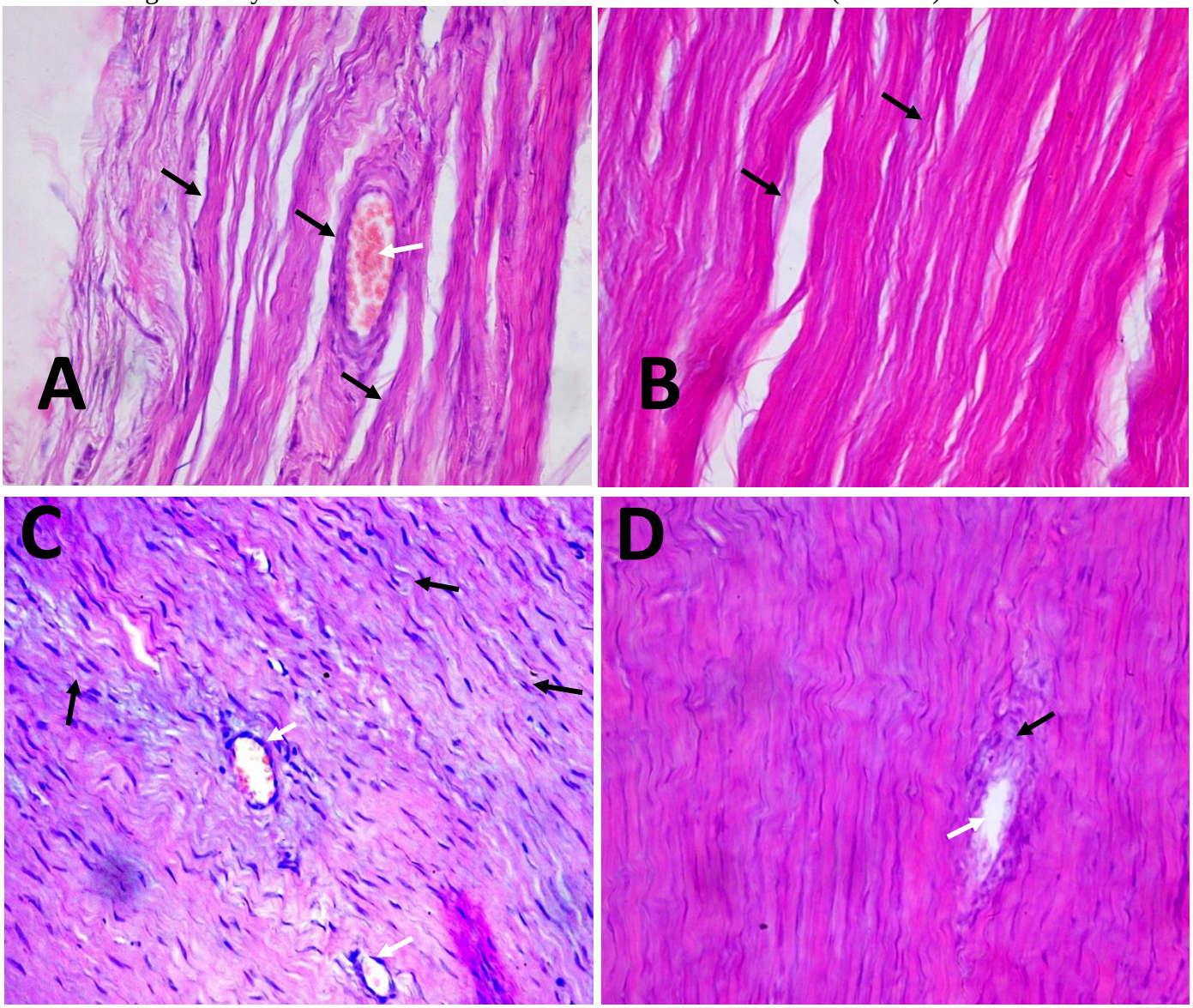

Figure (3):

A- Photomicrograph of tendon defect healing after 6 weeks of the control group showing arrangement of loose fibers (black arrows) with low cellularity around blood vessels (white arrows). H&E stain 20X.

B- Photomicrograph of tendon defect healing after 12 weeks of the control group showing arrangement of loose fibers (Arrows) with low cellularity. H&E stain 20X.

C- Photomicrograph of tendon defect healing after 6 weeks of the MVs group showing arrangement of dense fibers with high cellularity (Black arrows)and newly formed blood vessels (white arrows). H&E stain 20X.

D- Photomicrograph of tendon defect healing after 12 weeks the MVs group showing arrangement of dense fibers with very high cellularity (Black arrows) around blood vessels (white arrow) and darker stained collagen fibers. H&E stain 20X.

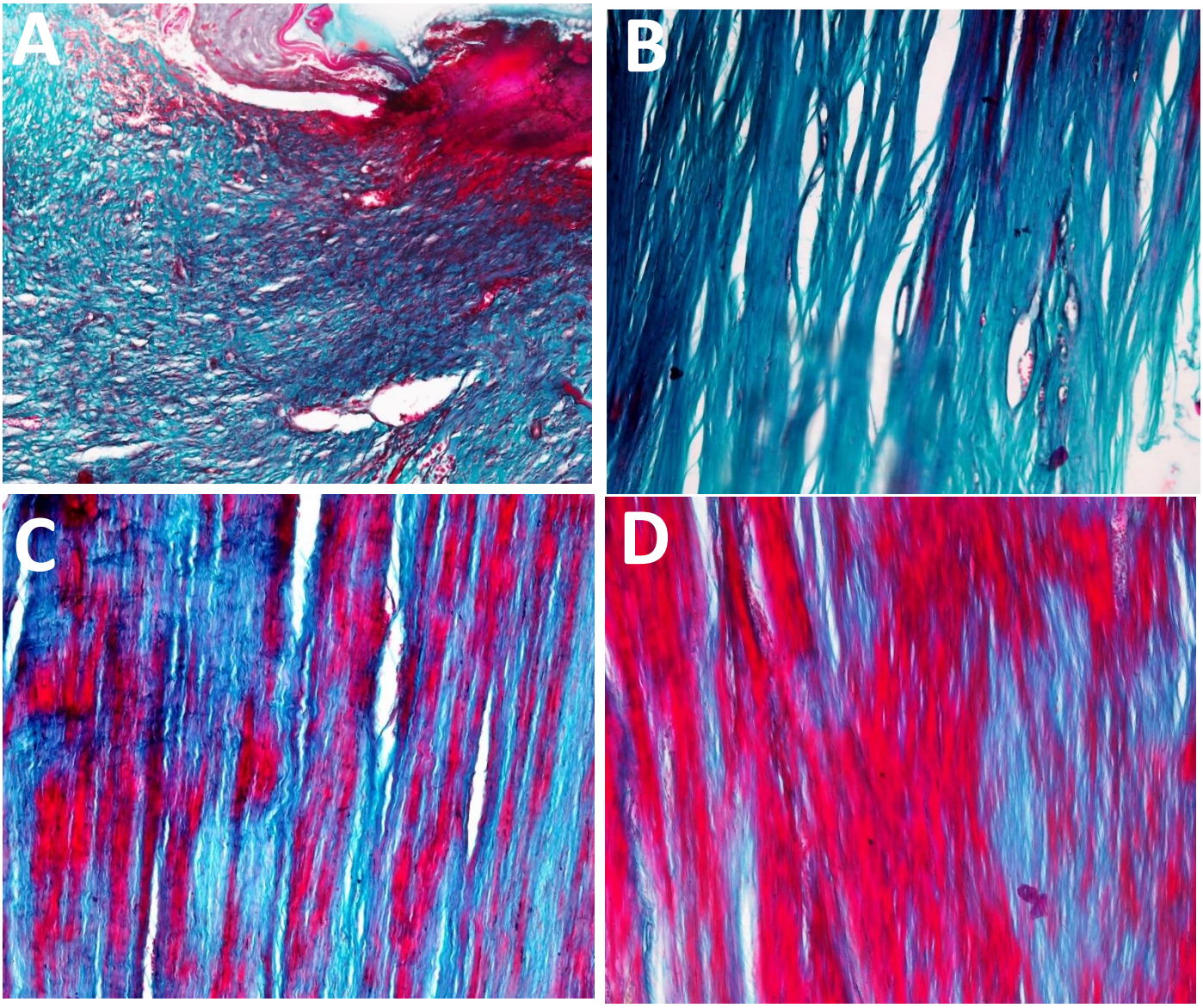

Figure (4):

A. Photomicrograph showing the tendon defect healing after 6 weeks of the control group showing unorganized fibrous tissue with blood vessels (arrows) and very low collagen fibers. MTC stain 10X.
B. Photomicrograph of tendon defect healing after 12 weeks of the control group showing arranged fibrous tissue bundles with very low expression of collagen (arrows). MTC stain 40X.
C. Photomicrograph of tendon defect healing using microvesicles after 6 weeks showing arrangement of condensed hypercellular fibers with moderate expression of collagen (arrows). MTC stain 20X.
D. Photomicrograph of tendon defect healing using microvesicles after 12 weeks showing arrangement of condensed hypercellular fibers with high expression of collagen (arrows). MTC stain 20X.

DISCUSSION

MSCs-derived MVs have the same function of its mother (MSC) in tissue repair and reducing the inflammatory responses while they have some great values over MSCs as they are more reservable, have no risk of aneuploidy, lower risk of immune rejection following allogeneic administration so they may provide an alternative therapy for various diseases (Lui 2021). In this study, MSCs-derived MVs have been used to evaluate their efficacy in potentiation of the healing process of repaired AT rupture in dog.

The results of this study revealed the great potential of the MVs to shorten the period of inflammatory signs (pain, and swelling) that was obvious by palpation of the tendon and through evaluation of the lameness score and weight bearing capacity of the animals of both groups where the MVs treated animals had significantly higher lameness score $P < 0.05$ and greater weight bearing capacity than the control group and returned to the normal score 4 as early as the 10^{th} week observation. That was supposed to be due to the ability of the MVsto suppress the inflammatory response at the early stages of the healing process via modulation of the macrophage inflammatory response throughincreasing the migration of CD163 + cells (markers of anti-inflammatory macrophages) to the tendon regeneration site and that agreed with what reported by (Shen et al. 2020).

In the current study, the thickness of the tendon in the MVS treated group was significantly lower than the control group $P < 0.05$ and was nearly similar to the preoperative thickness of the tendon with no significant difference $P > 0.05$ at the end of the study while the control group still had significantly higher thickness than the MVs group and the preoperative thickness. We postulated that this may be due to increased tissue inflammation and scare tissue formation and that means better efficacy of the MVs in potentiation of the healing of the tendon with normal fibrous tissue instead of scar tissue formation and that agreed with (Gissi et al. 2020) who proved that MVs promoted better restoration of the tendon architecture and arrangement of tendon fibers when used on rat Achilles tendon model.

The results of the histological examination revealed that, MVs treated group showed significant improvement compared to the control group; At the 6^{th} week evaluation MVs treated group was superior in most of the parameters where cell migration and density, angiogenesis was higher than the control group due to the ability of MVs to convert the macrophages to the M2 phenotype that promotes the anti-inflammatory and regenerative responses also increases the angiogenesis through increased migration of the endothelial cells. On the other hand, the fiber structure and arrangement were condensed, regular and parallel with spindle nucleated cells with higher collagen deposition than the control group through expression of the tendon matrix and tenogenic differentiation markers and that agreed with (Shi et al. 2019).

In the current study, the 12^{th} week histological examination revealed greater condensation and arrangement of the fibers and expressed more collagen fibers and decrease in nucleated cell number compared to the control group with significantly higher histological score 3 vs 11 in the control group. Also, MTC stain confirmed high degree of collagen deposition and higher arrangement of the fibers in the MVs treated group than the control group in both the 6^{th} and 12^{th} week observations. That was supposed tobe due to the promoted proliferation of the allogenic tendon stem cells due to increased production of CD146+ cells (marker of tendon stem cells) and enhanced expression of tenomodulin. These results and speculations agreed with that reported by (Yu et al. 2020).

CONCLUSION

The study revealed that MVs have a great ability to enhance and accelerate the heeling and regenerative process of the Achilles tendon of dogs through suppression of the inflammation, increased tenocytes proliferation, enhanced collagen deposition and arrangement and remodeling of the tendon architecture. Moreover, they have a promising value in the field of regenerative medicine specially tendon regeneration both in the animals and humans.

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