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The efficiency of intra-articular injection of silver nanoparticles (AgNPs) on repair of experimentally induced avascular meniscal tear in dogs

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ABSTRACT

Meniscal tears in the inner avascular area have been reported as a common cause of disability in dogs that associated with failure of healing process due to their limited vascularity. In spite of various sterategies were reported for treatment of such tears, their clinical use was limited. Therefore, different biomaterials have been assessed to stimulate regeneration of avascular meniscal tears. Currently, silver nanoparticles (AgNPs) enormously involved in biomedicine including tissue regeneration, drug delivary, and antibacterial applications. Thus, AgNPs was fabricated in the present study to investigate its potential to induce and support meniscal healing process in an avascular meniscal tear model. The nanomaterial was synthesized and characterized using transmission electron microscope (TEM). Next, a full thickness longitudinal meniscal tear was created in the avascular zone and either left empty or treated with AgNPs. Animals were monitored clinically at weeks 3, 6, 9, and 12 weeks after surgery for lameness parameters including lameness during walking, pain on manipulation, range of motion, and functional disability. Additionally, the harvested menisci were examined macroscopically and histologically at 4, 8, and 12 weeks. The designed material revealed improved clinical outcomes compared to control group. The gross and histological observations proved that the meniscal healing was stimulated in the AgNPs-treated group in comparison to control one, where the AgNPs-treated tear sites were filled with reparative tissue. In conclusion, AgNPs nanomaterial has a promoting effect on the process of meniscal tissue healing in the avascular region, proving that AgNPs is a promising material for meniscal tissue regeneration.

Introduction

The menisci are a pair of crescentic fibrocartilaginous discs of the stifle joint positioned between the femur and tibia and held firmly in place to them by various anchoring ligaments. The significance of menisci is attributed to their essential role in normal stifle joint integrity and functions including stabilization, lubrication, load transmission, trauma attenuation, and alleviation of the incongruity between femoral condyles and tibial plateau (Franklin *et al*., 2010; Ballard *et al*., 2014).

Meniscal tears have been reported as a common cause of hind limb disability in dogs that were accompanied with lameness, stifle joint inefficiency, and greater load on the articular cartilage, and were predisposing to degenerative changes of the articular cartilage and later occurrence of osteoarthritis (Franklin *et al*., 2010; Hayes *et al*., 2010; Kawanishi *et al*., 2014; Koch *et al*., 2019; McCready and Ness, 2016; Jeong *et al*., 2021). Meniscal tears have been reported to possess the potential for healing in the outer vascular zone of the meniscus, whereas the inner avascular tears have no capacity to heal spontaneously due to their poor vascular supply. Therefore, the successful repair of these avascular tears represents a significant health problem with financial importance (Ballard *et al*., 2014; Kawanishi *et al*., 2014; Koch *et al*., 2019; Xiao *et al*., 2021).

Several classical repair procedures were recorded to manage meniscal tears including suturing, rasping, debridement, and trephination. However, their clinical applications were restricted due to the limited intrinsic regenerative capacity in the avascular zone. Partial meniscectomy is the alternative standard technique used for this purpose that mostly associated with degeneration of the stifle joint. Further, allogenic grafting is another treatment choice that reduces the incidence of osteoarthritis; however, it has been confined clinically due to the limitations of graft availability, graft preparation, graft rejection, infection, hematoma, arthrofibrosis, and diseases transmission (Kawanishi *et al*., 2014; Yu *et al*., 2015; McCready and Ness, 2016; Koch *et al*., 2019; Stocco *et al*., 2022). The aforementioned shortcomings evoked the demand to fabricate a promising therapy to enhance meniscal regeneration in the inner avascular zone based on tissue engineering that consists of a combination of biodegradable scaffolds, cells, and/or cytokines (Zhang *et al*., 2009; Sadek *et al*., 2023).

Currently, biomaterials have been increasingly reported in tissue engineering of various tissues including meniscus (Shimomura *et al*., 2015; Gopinathan *et al*., 2017;), bone (Sadek *et al*., 2022; 2023), tendon (Kwan *et al*., 2014; Abdelhakiem *et al*., 2023), and skin (Soliman *et al*., 2021). Among these biomaterials, silver nanoparticles (AgNPs) earned a considerable interest in a variety of biomedical uses owing to their unique physicochemical features (Xu *et al*., 2020). AgNPs have been used in drug delivery (Karthik *et al*., 2018; Poudel *et al*., 2018), antimicrobial applications (Elshahawy *et al*., 2022), anticancer therapy (Mukherjee *et al*., 2014; Bandyopadhyay *et al*., 2019), and vaccines preparation (Xu *et al*., 2013). Additionally, AgNPs have been reported to possess a stimulatory impact on healing of bone defects (Zhang *et al*., 2015; Qing *et al*., 2018), tendon laceration (Kwan *et al*., 2014; Abdelhakiem *et al*., 2023), and wounds (Chowdhury *et al*., 2014; Franková *et al*., 2016). It has been proved that AgNPs possesses a stimulatory influence on deposition of collagen fibers, production of proteoglycans, differentiation of fibroblast cells, and proliferation and migration of keratinocyte cells (Liu *et al*., 2010; Kwan *et al*., 2011; Kwan *et al*., 2014). Consequently, our hypothesis stated that AgNPs has a boosting regenerative potential for healing of inner avascular meniscal tears.

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Thus, the main purpose of this study is to evaluate the ability of AgNPs to enhance meniscal tissue repair in the avascular zone.

Materials and methods

Chemicals

Silver nitrate ($AgNO_3$) was purchased from GAMMA Laboratory Chemicals company (Egypt) and soluble starch was obtained from El-Naser company (Egypt). All chemicals were of analytical grade and used without further purification.

Synthesis and characterization of AgNPs

The AgNPs was prepared via green synthesis using soluble starch (Vigneshwaran *et al.*, 2006) (Fig. 1A). Shortly, soluble starch (1 g) was dissolved in distilled water (100 mL). Then, the mixture was heated till total dissolution. After the complete dissolving, the aqueous solution of $AgNO_3$ (100 mM, 1mL) was mixed with the prepared starch solution and shacked thoroughly. Finally, the resulting mixture was autoclaved for 5 minutes at 121ºC and 15 psi pressure.

The size and morphology of the fabricated AgNPs was studied using a transmission electron microscope (TEM; JEOL-JEM- 100CX II, Japan). The concentration of the prepared material was measured using spectrophotometer (Graphite Furnace Atomic Absorption, Model 210VGP, USA).

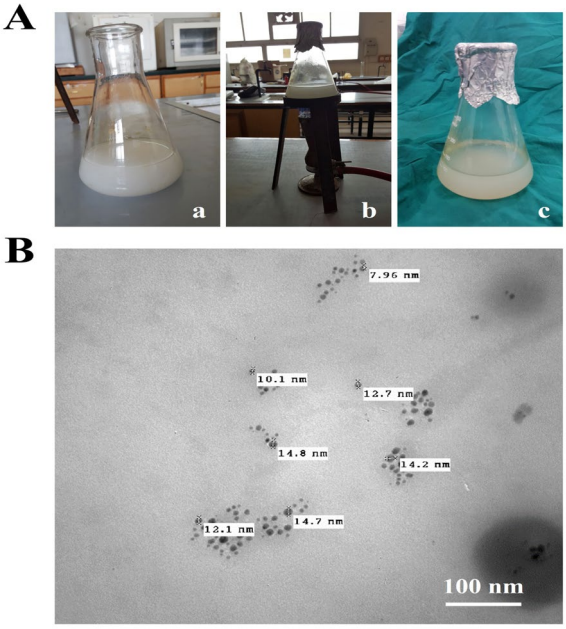

Fig. 1. Synthesis and characterization of AgNPs nanomaterials. (A) Steps of AgNPs fabrication and characterization using TEM (B).

Experimental design

The Animal experiments design were approved by the Institutional Animal Care and Use Committee of Research Facilities, Faculty of Veterinary Medicine, Assiut University, Egypt in compliance with the Egyptian bylaws, OIE standards, and the Animal Research. In this study, 18 clinical healthy adult male Mongrel dogs (2-3 years, 15 – 20 kg) were used. They were housed separately in stainless-steel cages in a well-ventilated room at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Assiut University and fed on a standard dry food diet with constant access to water. Animals were housed for 14 days before the surgery for adaptation.

Animals were checked clinically and radiographically before the study to confirm that all of them had not any degree of lameness. They were randomly divided into control and AgNPs groups; each group had 9 dogs and all animals underwent unilateral induction of meniscal tear.

Induction and management of avascular meniscal tear model

The longitudinal full-thickness meniscal tear model was performed in the inner avascular zone to evaluate the capability of AgNPs to stimulate meniscal healing.

Animals were fasted for 12 hours before the experimental operations. Dogs were subjected to surgery under the influence of general anesthesia induced by a combination of xylazine HCl (1 mg/kg, Xyla-ject: ADWIA Co., Egypt) and ketamine HCl (10 mg/kg, Ketamine 50mg: Sigma-Tec, Egypt). The right hindlimb was prepared aseptically and the animal was restrained in the dorsal recumbency.

Meniscal tears were created in the avascular zone of the medial meniscus through medial approach to the stifle joint as previously described (Xiao *et al.*, 2021) Fig. 2. Briefly, a medial parapatellar skin incision was created and the underlying fascia was subsequently dissected. Then, an arthrotomy incision was made parallel to skin incision followed by lateral displacement of the patella. The stifle joint was fully flexed, and the fat pad was retracted distally to expose the meniscus. A full-thickness longitudinal tear (about 10 mm in length) was performed using a scalpel blade at the anterior pole of the avascular region of each medial meniscus. The joint capsule was sutured followed either by injection of 2 ml of AgNPs or left untreated as a control. At last, the subcutaneous tissue and skin were sutured in a routine manner. After surgery, hindlimbs were immobilized using splint bandage for 2 weeks. Animals received intramuscular injection of Cefepime (4.5 mg/kg, Maxipim: Smith-Kline Beecham Co., Egypt) for the 5 successive postoperative days. Animals were assessed for meniscal tear healing after 4, 8, and 12 weeks of surgery.

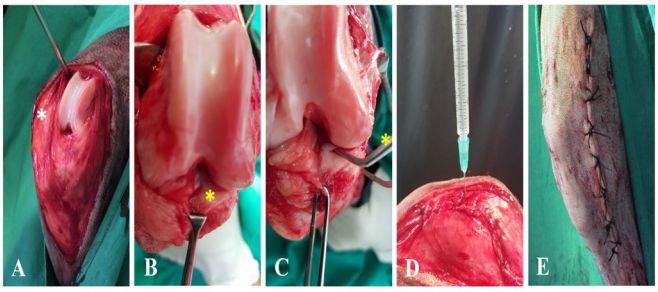

Fig. 2. Surgical creation of longitudinal full thickness meniscal tears. Lateral dislocation of patella "white asterisk" (A) for exposure of medial meniscus "yellow asterisk" (B). Full thickness avascular meniscal tear induction (C) followed by injection of AgNPs (D) and wound closure at last (E).

Clinical observation

All animals were examined clinically every day for post-surgical complications including infection, wound dehiscence, and the level of activity. In addition, animals were subjected to lameness assessment (Table 1) and scoring at weeks 3, 6, 9, and 12 postoperatively including lameness during walking, pain on manipulation, range of motion, and functional disability according to Black *et al.* (2007).

Gross assessment of the harvested menisci

At various evaluation time points, animals were euthanized using xylazine HCl (1 mg/kg) followed by thiopental sodium (85 mg/kg, Anapental: Sigma-Tec, Egypt). Then, medial menisci were resected and harvested for examination. The tear site was assessed grossly for the presence of regenerated tissue, color of tear site, and the surface of the tear site.

Histological examination

Meniscal samples were harvested at different time points (n = 3

Table 1. Clinical lameness assessment score.

Category	Score	Category	Score
Lameness during walking		Pain on manipulation	
Un-detectable	1	No pain	1
Intermittent	2	Mild pain	2
Persistent	3	Severe pain	3
Range of motion		Functional disability	
No limitation	1	Normal activity	1
Pain only at full extension/ flexion	2	Slightly stiff gait	2
Pain at less than extension/ flexion	3	Stiff gait	3
Pain at any attempt	4	Very stiff gait	4
		Unwilling to walk	5

for each time point at each group). The collected samples were fixed in 10% neutral buffered formaldehyde. Then, samples underwent routine processing steps including dehydration in ascending concentration of ethanol, clearance in methyl benzoate, embedding in paraffin wax, sectioning at 5 µm in thickness, and finally stained with Hematoxylin and Eosin (H&E) for general histological inspection of the site of meniscal tear including signs of meniscal healing, neovascularization, and cellular constituents. Furthermore, sections were stained with Crossmon's trichrome stain for histochemical staining of collagen to examine the formation of cross-linked collagen fibers and cartilage plaques within the healed area. Finally, slides were examined using the Olympus CX31 microscope and photographed using a digital camera (Olympus, Camedia C-5060, Japan) for histological interpretation.

Statistical analysis

Data obtained from the results of lameness scores were presented as mean ± standard deviation (SD) with a significant level of $p < 0.05$. The data were analyzed with SPSS statistical software (Version 21: IBM Corp., Armonk, NY). The obtained results were compared by two-way ANOVA, followed by Tukey's post hoc test.

Results

AgNPs characterization

AgNPs solution was fabricated successfully as displayed in Fig. 1Ac. The synthesis of AgNPs revealed in a clear yellow solution. The concentration of AgNPs solution was 157 µM. In addition, the TEM image of AgNPs showed spherical shape particles ranging from 7.96 nm to 14.8 nm in size (Fig. 1B).

Clinical observation

During this experiment period, animals displayed neither surgical nor post-surgical complications. Dogs were able to stand up and move after 48 hours of surgery. In addition, they restored their normal daily activities including food intake, drinking, and grooming within the first 72-96 hours after surgery. Furthermore, animals showed a partial weight bearing on the operated hindlimb for 7 days after surgery in AgNPs-treated group and 14 days in the control group.

Animals in control group showed persistent severe lameness during walking on week 3 after surgery that decreased gradually to moderate lameness at week 6 after surgery and continued up to 9 and 12 postoperative weeks. In AgNPs-treated group, lameness during walking was moderate at week 3 after surgery and reduced in a time-dependent manner till disappeared on week 6 after surgery.

In addition, dogs in the AgNPs-treated group displayed a stiff gait compared to a very stiff gait in the control group on week 3 after surgery. Animals in AgNPs-treated group returned gradually to normal gait at week 6 after surgery, while in control group showed a stiff gait at 9 and 12 weeks after surgery.

On manipulation of the operated limb in control group, severe painful reaction was noticed at week 3 postoperatively that became mild pain at week postoperative 9th week and continued till the end of the study. However, dogs in AgNPs-treated group displayed mild pain in week 3 after surgery that disappeared at week 6 after surgery.

Furthermore, movement of the operated limb on week 3 after surgery revealed pain at less than extension/flexion in control group, while revealed pain only at full extension/ flexion in AgNPs-treated group. In control group pain decreased overtime to became pain only at full extension/ flexion at weeks 9 and 12 postoperatively, whereas dogs in AgNPs-treated group showed no limitation of motion after 6 weeks of surgery.

As shown in Fig. 3, the AgNPs-treated group at postoperative 3,6,9, and 12 weeks showed a significant decrease in lameness during walking, functional disability, and pain on manipulation scores compared to the control group. In addition, the range of motion score was significantly lower at weeks 6,9, and 12 after surgery in AgNPs-treated group than that in control group. Moreover, a significant range of motion scores was reported between week 3 and weeks 6,9, and 12 in AgNPs-treated group.

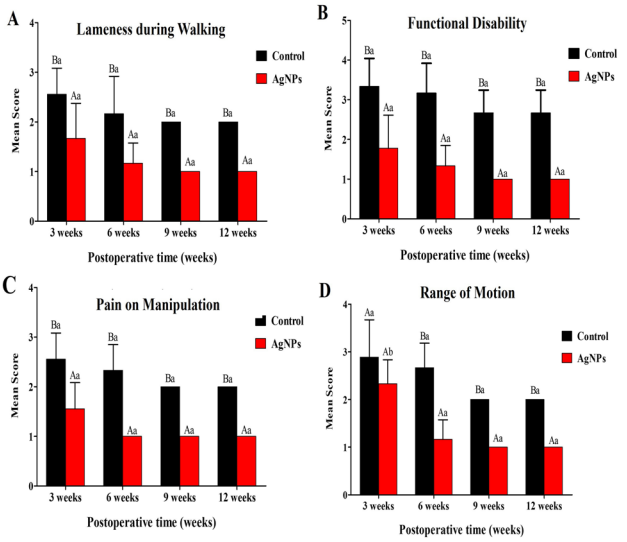

Fig. 3. Lameness score parameters. Percentages of lameness during walking (A), functional disability (B), pain on manipulation (C), and range of motion (D) in AgNPs-treated group compared to control group at weeks 3, 6, 9, and 12 after surgery. Error bars ± SD; n = 3 for each group and time point. Bars with the same letter represent values that are not significantly different (two-way ANOVA followed by Tukey's HSD post hoc test). A and B: significance between groups; a and b: significance between time points within the same group.

Gross assessment of the harvested menisci

As displayed in Fig. 4, the harvested specimens from the experimental groups revealed the absence of obvious signs of infection grossly. The control group displayed a well-detectable tear site with clear space between the rims of the tear indicating no healing at the different evaluation times. At weeks 4 and 8 after induction of tear, a less recognized tear site was observed with the presence of a bridging tissue connecting the rims of the meniscal tear in the AgNPs-treated group indicating partial healing of the tear. In addition, the tear site was white in color with a rough surface on week 4 and a smooth surface on week 8.

On week 12 after surgery, the rims of the tears in the AgNPs-treated group were indistinguishable with a smooth surface and almost as color as the surrounding tissue, indicating complete healing.

Histological examination

Histological inspection of the harvested meniscal tears was per

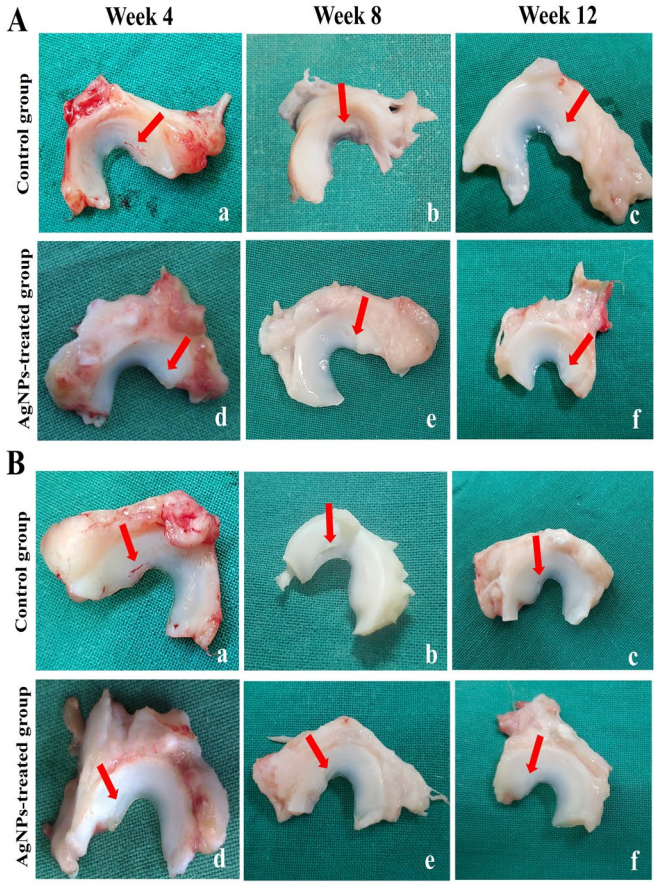

Fig. 4. Gross assessment of meniscal tear sites (red arrow) of control (a-c) and AgNPs-treated (d-f) groups at femoral (A) and tibial (B) surfaces on 4, 8, and 12 postoperative weeks.

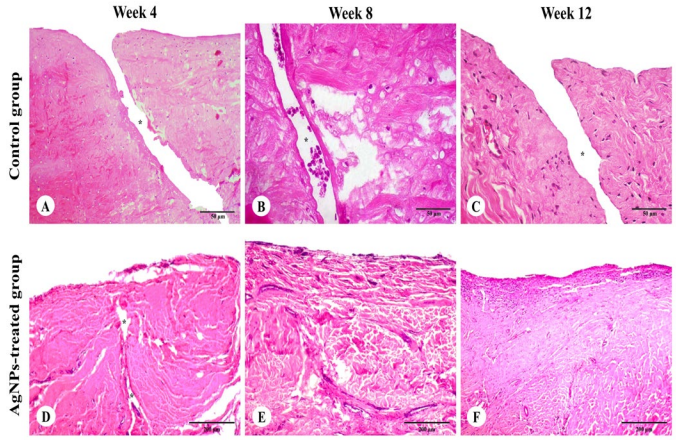

Fig. 5. Histological examination of meniscal tear sites. The repair site of the meniscal tear at week 4 (A, D), 8 (B, E), and 12 (C, F) after surgery in control AgNPs-treated groups was stained with H&E. Black asterisks: tear gap. The scale bars in panels A-C = 50 µm (×400), panels D-F = 200 µm (×100).

formed to evaluate the ability of AgNPs to enhance meniscal healing.

At 4 weeks, in the control group the tear site was empty and lined by a layer of fibroblasts (Fig. 5A). The tear sites in the AgNPs-treated group were occupied by numerous blood vessels, inflammatory cells, and collagen fibers. In addition, fibroblastic proliferation and small gaps were seen at tear sites (Fig. 5D and 6Ac).

At 8 weeks, the control tear sites were still showing empty gaps with few numbers of inflammatory cells, mainly neutrophils within the gap (Fig. 5B). However, the AgNPs-treated sites displayed more tissue formation at the line of healing. Furthermore, the AgNPs-treated group showed inflammatory cells infiltration, neovascularization, deposition of collagen fibers, and fibroblast cells proliferation (Fig. 5E and 6Ab).

At 12 weeks, a non-healed gap in the control group was still observed (Fig. 5C). The tear sites the AgNPs-treated group revealed undetectable tear gap that occupied with collagen fibers, neovascularization, fibroblasts, and fibrochondrocyte aggregations (Fig. 5F and 6Aa).

The collagen deposition and fibrochondrocytes proliferation at the healed areas were further investigated using Crossmon's trichrome staining (Fig. 6B). On week 4 after surgery, irregular collagen fibers deposition was seen in the healed areas of the AgNPs-treated group. However, few fibrochondrocytes and regular collagen fibers were detected at week 8 after surgery. On week 12, the healing areas in AgNPs-treated group showed the presence of regular collagen fibers and fibrochondrocyte plaques.

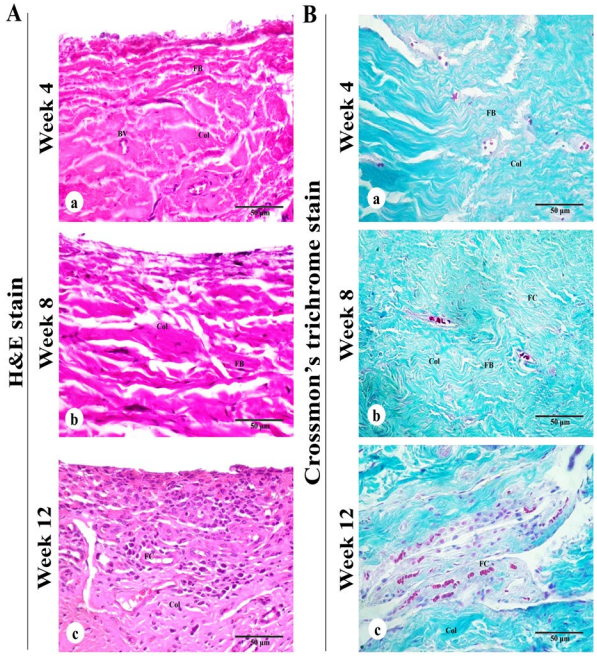

Fig. 6. Histological and Histochemical examinations of the tissue occupied the meniscal tear AgNPs-treated sites. The formed tissue at site of the tears after 4 (a), 8 (b), and 12 (c) weeks of surgery was stained with H&E (A) and Crossmon's trichrome (B) stains. BV: blood vessels, FB: fibroblast cell, FC: fibrochondrocyte, Col: collagen fibers, Black asterisks: tear gap. The scale bars in H&E and Crossmon's trichrome stain panels = 50 µm.

Discussion

Meniscal tears, as a health problem with economic impact, are regarded one of the frequently reported causes of canine hind limb lameness and dysfunction (Franklin *et al.*, 2010; McCready and Ness, 2016). Different attempts were conducted to induce avascular meniscal tears healing including meniscal repair, meniscectomy, and meniscal allografting; however, their clinical use showed a variety of limitations and disadvantages (Koch *et al.*, 2019; Stocco *et al.*, 2022). Recently, tissue engineering represented a promising therapy to overcome these restrictions (Zhang *et al.*, 2009; Kawanishi *et al.*, 2014; Zellner *et al.*, 2014). In this study, we investigated the regenerative capacity of AgNPs to stimulate full-thickness meniscal tear healing in the inner avascular region.

Herein, green synthesis using soluble starch was used to synthesize AgNPs because it is simple process, and the prepared solution showed no signs of nanoparticles aggregation as well as stability at room temperature over a period of three months (Vigneshwaran *et al.*, 2006). The results of TEM revealed the typical spherical-shaped AgNPs that resembles the TEM image showed in other studies (Anandalakshmi *et al.*, 2016; Abdelhakiem *et al.*, 2023).

To investigate the reconstructive capability of the AgNPs, the designed nanomaterial was injected intraarticularly into a well-established inner avascular full thickness canine meniscal tear model (Xiao *et al.*, 2021).

In this study, all experimental animals revealed partial weight bearing on the first week due to the painful reaction resulted from the inflammatory process associated to open arthrotomy procedure (Tomas *et al.*, 2015). However, full weight bearing was achieved at day 7 after operation in AgNPs- treated group compared to day 14 in control group. Furthermore, our data revealed a lower lameness score in the AgNPs-treated group in comparison to control group throughout the experiment. These findings may be related to the anti-inflammatory and antioxidant properties of AgNPs (Wong *et al.*, 2009; Tyavambiza *et al.*, 2021; Bold *et al.*, 2022).

The host cellular immunity reaction against the implanted nanoma-

terials elicits the healing process through influx of various inflammatory cells and formation of fibrous tissue in the surrounding tissues. However, mild inflammatory cells response is crucial for the preservation of the material's in vivo functions and proving its biocompatibility (Vishwakarma *et al*., 2016; Sadek *et al*., 2023). Our findings demonstrated mild recruitment of inflammatory cells in AgNPs-treated tear sites, confirming biocompatibility of AgNPs and providing a suitable microenvironment for stimulation of healing cascades.

The phases of meniscal repair include inflammation, proliferation, and maturation. In addition, the healing mechanism of meniscal injury occurred through extrinsic and\or intrinsic pathways. Extrinsic pathway depends on neovascularization and the progenitor cells differentiation, while intrinsic pathway depends on the activation of the meniscal fibrochondrocyte cells. Furthermore, neovascularization, collagen deposition, fibroblasts proliferation and differentiation, and fibrochondrocytes proliferation and differentiation are considered an indicator of active repair process (de Albornoz and Forriol, 2012; Tarafder *et al*., 2020; Nakagawa *et al*., 2021; Yan *et al*., 2021). Our gross and histological observations in the AgNPs-treated group revealed occupation of the tear sites with reparative tissue formed mainly from new blood vessels, regular collagen fibers, and fibrochondrocyte plaques. These observations may be attributed to the ability of AgNPs to induce and support angiogenesis (Keleştemur *et al*., 2012; Orlowski *et al*., 2018), collagen deposition and alignment (Kwan *et al*., 2011; Kwan *et al*., 2014; Martin *et al*., 2019), proteoglycan production (Kwan *et al*., 2014), and fibroblasts differentiation (Liu *et al*., 2010; Keleştemur *et al*., 2012).

In our study, it is worth mentioning that there were few limitations including inability to induce meniscal tears via arthroscopy in addition to inability to evaluate meniscal tearing using diagnostic imaging tools as contrast computed tomography and magnetic resonance imaging. Moreover, future studies should be conducted on the biomechanical features of reparative tissue. Additionally, the mechanism of initiation of healing should be investigated in future studies.

Conclusion

Intraarticular application of AgNPs revealed clinical improvement of lameness parameters. Furthermore, AgNPs encouraged tear site filling with meniscal tissue, indicating its ability to enhance meniscal regeneration in the inner avascular zone. According to our knowledge, this is the first work to demonstrate the AgNPs as a potential candidate for meniscal tissue repair.

Conflict of interest

The authors declare no competing interests.

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