Journal of Veterinary Medical Research (2021); 27 (2): 109–118
https://doi.org/10.21608/JVMR.2020.34762.1019

ORIGINAL ARTICLE

Effect of Platelet-rich Plasma on MMP-13, ARE and TGF β1 in MIA-Induced Osteoarthritis in Rats

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Received: 05 July 2020 / Accepted: 01 September 2020 / Published online: 02 September 2020

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Abstract
Ankle osteoarthritis (OA) is an inflammatory deterioration chronic disease; yet, OA pathogenesis is obscure. There is no absolute cure from OA and the present pharmacological medication options are constrained and associated with adverse aspect effects. Clinically, Platelet-rich plasma (PRP) is usually used to cure OA. The present study was designed to evaluate the role of PRP in the treatment of experimentally Monosodium iodoacetate (MIA)-induced ankle osteoarthritis in the rat model. Thirty male Wistar rats were divided into three groups (each of 10 rats). Rats of group I were injected with 1 mg MIA in the right ankle joint for two consecutive days, while those of group II were treated with saline instead of MIA; and group III (osteoarthritic +PRP) rats were injected with PRP in the ankle joint at 14, 21, and 28 days after MIA injection. Paw edema, scoring of arthritis, Matrix metalloproteinase 13 (MMP13) level, antioxidant response element (ARE) level, and joint transforming growth factor beta1 (TGF β 1) were evaluated. PRP reduces expression of joint, MMP13, ARE level, and TGF β 1. PRP also significantly reduces the score of arthritis. The administration of PRP decreases paw edema in MIA-induced OA rats. These results suggest that PRP has marked effect against OA in MIA-induced osteoarthritic rats which are mediated through the anti-inflammatory and antioxidant effects.

Keywords ARE induced Osteoarthritis, MMP-13, Platelet-rich plasma, TGF β1

1. Introduction
Osteoarthritis is a whole joint illness. It is a slow progression long-standing inflammatory disorder that produces gradual articular cartilage destruction, accompanied by alterations of articular soft-tissue and bone and finally leading to pain, joint rigidity, and limb dysfunction **(Legrand et al. 2017).** OA is a result of a complicated interaction between biochemical and biomechanical factors. OA might happen due to many reasons such as joint injuries, trauma, genetic, aging, bad shoeing, and the nutritional deficiency **(Souza 2016).** MIA hinders the Krebs cycle leading to the death of chondrocytes as it is a metabolic inhibitor. This, in turn, gives rise to osteophyte formation and degradation of articular cartilage and these changes are considering the greatest characteristic aspects in OA in animals **(Marker and Pomonis 2012).** OA experimental model induced by MIA is often applied to measure pain in animals. This model may be more evaluable of drug efficiency than other models utilized to evaluate OA medications. It is usually used in rats **(Jimbo et al. 2017).** In the past, OA pathogenesis was

mysterious but now, there is abundant research investigated the underlying mechanism of OA. Some studies have recommended that one of the important mechanisms of OA pathogenesis is apoptosis of the chondrocytes of joints or the outcome of losing balance in synthesis and catabolism of cartilage of joint **(Li et al. 2019).** In physiological states, the cartilage of joint plays a critical role as it diminishes friction and makes the movement of joints especially synovial articulation effortless and painless. Normally, dispersed chondrocytes are embedded through the matrix, which consists mostly of proteoglycans, collagen type II and water. Once the process of degeneration is begun, it will motivate the transforming growth factor-β production, which is a cytokine that encourages the formation of PGs and collagen type II. It has been proposed that TGF-β has a double role in OA, i.e., both negative and positive effects **Van Loon et al. 2010; Souza 2016).** In case of OA, many enzymes are released which share in the articular cartilage degradation, such as MMPs and, inflammatory mediators and cytokines. The interaction between cytokines leads to the enzymatic degradation of collagen and proteoglycans of cartilage. This cartilage degradation loses the joint its motion **(McIIwraith et al. 2012).** Matrix metallo-proteinase-13 (MMP-13), the main enzyme that participates in the degradation of extracellular matrix, can degenerate 90% of the collagen in the extracellular matrix. So, MMP-13 expression is linked to OA development **(Xu et al. 2013).** Antioxidant response element (ARE), present in the promoter region of numerous genes regulates cyto-protective proteins and detoxification enzymes. It is an important element, which fundamentally responds to inducers of oxidative stress. Under physiological conditions and in the absence of major cellular stresses, transcription factor Nrf2 (NF-E2-related factor2) is linked to Keap1 in the cytoplasm. When ROS overwhelms the endogenous antioxidant capacity, Keap 1 liberates NRF2 which then moves to the nucleus where it linked ARE **(Bhakkiyalakshmi et al. 2018).** Under the pathological conditions of OA" oxidative stress status", Nrf2 unites to the ARE to make the activation of antioxidant genes which responsible for the incidence and development of OA as demonstrated in the former studies. ARE expressed and actives in the joints of mice with arthritis but not in healthy joints as The Nrf2/ARE signaling pathway is chiefly responsible for cellular defenses versus oxidative stress **(Khan et al. 2018).** The aim of OA treatment is lessening joint pain by minimizing the inflammation and decelerating the cartilage damage progression, resulting in improvement joint flexibility. To achieve these aims, many of traditional pharmaceuticals, experimental therapy, nutraceut-icals, supplements, weight loss, exercise programs, and biological therapy, such as stem cell **(Bland 2015).** Platelet-rich plasma (PRP) is one of the therapies which considered a major potential remedy in the treatment of many medical conditions and is presently one of the hottest topics in regenerative medicine **(Laver et al. 2017).** PRP is a product of blood that includes intense platelets. After activation, the α-granules of concentrated PRP liberate growth factors at concentrations greater than the baseline levels of blood, involving many growth factors such as platelet-derived growth factor, transforming growth factor-β, and other **(Castillo et al. 2011).** The achievement of this therapy lies not only in the qualities of PRP but also in its proper application. Inappropriate utilization of PRP can lead to an ineffective biological response and unsuitable clinical effect. So, the proper using of PRP in treatment is required. PRP has protruded as a biological therapy for the treatment of experimental and clinical musculoskeletal disorders such as articular damage as in OA, chronic tendinitis, muscle injuries, and for intraarticular application to overcome joint pain **(Moraes et al. 2015).** Intra-articular infusions reach the cartilage and the synovial membrane, causing an alteration in the environment of the joint that slows the progress of joint pain and arthritis and adjusts the clinical biological aspects of joint **(Sánchez et al. 2016).** Hence, the target of the existing study is to evaluate the effect of intraarticular injection of PRP on MIA induced ankle OA in the rat model and the effects on MMP-13.ARE and TGF β1 were investigated, in order to obtain better treatment for OA.

2. Materials and Methods

2.1. Animals

Mature male Wistar rats (n = 30) weighting (100-150 g) being 8-10 weeks of age, were purchased from the laboratory animal unit of Helwan Farm-VACSERA, Egypt. Ten days before the beginning of the experiment, animals were kept under monitoring to exclude any inter current diseases. The animals were housed in cages which made of polypropylene with ventilated stainless steel covers in the house of animal of Zoology Department, Science Faculty, University of Beni-Suef, Egypt at (10-12h/day) natural daily lighting cycle, (20-25 °C) good temperature, and were given a balanced diet and water ad libitum.

2.2. Osteoarthritis induction

Rats undergo anesthesia using70 mg/kg ketamine and 7 mg/kg xylazine, OA was induced by intraarticular injecting of 50µL normal saline containing 2 mg MIA (2 mg/50µL) (Sigma-Aldrich, St. Louis, MO) with a 21-gauge needle into the right hind ankle joint on 2 successive days, as previously illustrated by **Möller et al. (2019).**

2.3. Preparation of platelet-rich plasma (PRP)

PRP was prepared using a double spin method according to the manner of **Asjid et al. (2019)** with some amendments

2.4. Animal grouping

The prepared animals were at random divided into 3 groups, each has 10 rats.

2.4.1. Normal group

The right ankle joints of Rats in the normal group were injected with 50µL saline.

2.4.2. Osteoarthritic group

The right ankle joints of rats in the arthritic group were injected with MIA for two successive days. At 14th day, following saline/MIA injection, both of osteoarthritic and normal control rats were treated with 50µL saline in the right hind ankle joint once a week for three weeks.

2.4.3. Osteoarthritic-PRP group

At 14th day, following saline / MIA injection, the right ankle joints of Rats in the MIA-PRP group were treated with 50µL PRP once a week for three weeks. At the end of the experimental periods, the right ankle joints from each rat were extirpated after dissection. Ankles of each group were preserved at - 20°C until exploited in western blot and molecular tests.

2.5. Leg circumference measurement

The right hind leg circumference at the region of the ankle was measured as a guide of paw edema and swelling rate in different groups using a string that was enveloped around the leg at the area of the ankle then the length of the string was measured with a ruler. The measurements were assumed once a week from the zero-days until the end of the experiment after MIA.

2.6. Assessment of clinical signs of inflammation and scoring of arthritis

The rats were monitored for clinical signs of arthritis every week (on the zero-days until the end of the experiment) after MIA injection. The occurrence of MIA was considered when swelling was monitored in at least one joint or paw. The severity of arthritis was scored in each paw according to the prior study **(Wijekoon et al. 2019).** In brief; grade 0: normal, 0.5: light redness of ankle joint or digits, 1. Mild, but distinct redness and ankle swelling, or clear redness and swelling restricted to individual digits, regardless of the affected digits number, 2. Moderate redness and ankle swelling, 3. Severe redness and entire paw swelling including digits, more than two joints involved, 4. Maximally inflamed limb with embroilment of more than one joint.

2.7. Real-Time PCR Test for MMP-13 and ARE mRNA of ankle joint

Molecular analyses have been performed at Laboratory of biochemistry department, Kasr Al Ainy Faculty of Medicine, Cairo University, Egypt Total RNA was separated using Qiagen tissue extraction kit (Qiagen, USA) according to directives of manufacture. The total RNA (0.5–2µg) was used for cDNA conversion utilizing high capacity cDNA reverse transcription kit Fermentas, USA). Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The qPCR assay with the primer sets was optimized at the annealing temperature. The primer sequence was shown in **Table (1).**

2.8. Western blot analysis

Western blot analysis was carried out as previously illustrated **(Mahmoud et al. 2017).** Ankle samples preserved at -20 °C were used to study the effect of PRP on the expression level of transforming growth factor beta1 **(**TGFb1). Briefly, ankle samples were homogenized in RIPA buffer complemented with proteinase inhibitors, centrifuged and the protein content was tested in the clear supernatant using Bradford reagent. 30 mg proteins were separated on SDS-PAGE, conveyed to nitrocellulose membranes, and blocked in 5% skimmed milk dissolved in TBS Tween20 (TBST). The membranes were incubated with primary antibodies against (TGFb1). After washing with TBST, the membranes were probed with the corresponding secondary antibodies and

developed using improved chemiluminescence kit (*BIORAD, USA*).

Table 1: The primer sequence of the studied gene.

Target Gene	Primer sequence
MMP13	Forward primer : 5′-CCCTGGAATTGGCGACAAAG-3′ Reverseprimer:5′GCATGACTCTCACAATGCGATTAC-3′
ARE	Forward primer:5'-TTGTAGATGACCATGAGTCGC-3' Reverseprimer:5'-TGTCCTGCTGTATGCTGCTT-3'

2.9. Statistical analysis

Statistical analysis was achieved by using SPSS v.25. Results were expressed as mean ± standard error (SE); all statistical comparisons were performed by analysis of Duncan's test post hoc. Values of $p< 0.05$ were deemed significant however those of $p> 0.05$ were deem non-significant.

3. Results

3.1. Effect on the right leg circumference as an indicator of the paw edema

The alteration of circumference of the right hind leg at paw region in osteoarthritic rats through six weeks after administration of MIA and as an effect of PRP treatment was illustrated in **Table (2).** The arthritic control rats manifested a significant increase in the hind paw edema; the recorded percentage increases were 95%, 68%, 65%, 64%, 61%and 50 % at the 3rd day, 2nd and 3rd, 4th, 5th, 6th weeks respectively compared to the normal control group. The treatment of the osteoarthritic rats with PRP yielded a significant decrease in the elevated level of paw edema recording percentage changes - 21% at the end of the experiment when compared to the osteoarthritic control animals**.**

Table 2: Effect of PRP treatment on right hind leg circumference of osteoarthritic rats

	Parameter (paw edema)													
	(Zero-day)	%	(3rd day)	%	(2nd wk)	%	(3rd wk)	%	(4th wk)	%	(5th wk)	%	(6th wk)	%
Normal group	1.5± 0.03^{a}	—	1.5± 0.03^{b}	—	1.7± 0.05^{b}	—	1.7± 0.05^{b}	—	1.7± 0.05^{e}	—	1.7± 0.05^{d}	—	1.8± 0.04^{c}	—
Osteoarthritic control group	1.6± 0.05^{a}	5	3.0± 0.05^{a}	95	2.8± 0.04^{a}	68	2.8± 0.05^{a}	65	2.8± 0.03^{a}	64	2.7± 0.03^{a}	61	2.7± 0.03^{a}	50
Osteoarthritic +PRP group	1.6± 0.05^{a}	2	2.9± 0.04^{a}	-2	2.8± 0.05^{a}	1	2.7± 0.05^{a}	-3	2.5± 0.05^{d}	-9	2.5± 0.03^{bc}	-9	2.1± 0.07^{b}	-21

Expressed data mean ± standard error. Number of observed rats for each group is ten

3.2. Clinical score of arthritis

The alteration of Clinical score of arthritis in osteoarthritic rats through six weeks after administration of MIA and as a result of PRP treatment was shown in **(Fig. 1).** In osteoarthritic rats the signs of arthritis started from the first day after administration of MIA with slight swelling and redness, converting to sever swelling and redness of paw reaching a significant level ($p< 0.05$) at Day 3 (acute osteoarthritis) and then occur slight gradually decrease in the clinical score (signs became moderate in severity) until Day 14 (chronic osteoarthritis). After treatment of the arthritic rats with PRP, a significant reduction in the arthritis score was still observed PRP group comparing with that in arthritic.

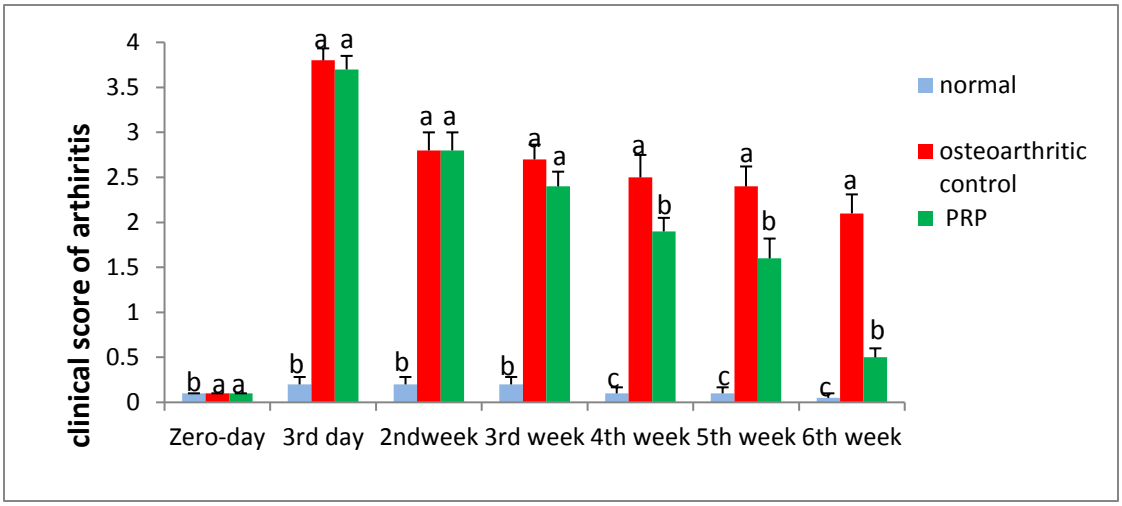

Fig. 1. PRP treatment Effect on clinical score of arthritis

3.4. Effect on Joint MMP-13

Data illustrating the effect of PRP on joint MMP-13 mRNA expression of osteoarthritic rats are shown in **Fig. (2).** The mRNA and protein level of MMP13 elevated sharply in osteoarthritic rats as compared to the normal control group, while in PRP treated group, the levels of joint MMP13 was significantly decreased ($p< 0.05$) when compared to the osteoarthritic group.

3.5. Effect on joint ARE

Data describing the effect of PRP on joint ARE mRNA expressions of osteoarthritic rats were illustrated in **Table (3).** The administration of MIA to rats produced a significant increase in the expression level of ARE recording percentage of 448% in joint tissue when compared to the normal animals. The PRP treated rats showed a significant ($P< 0.05$) decrease in the elevated level of ARE expression as compared to the osteoarthritic control group. Treatment of the osteoarthritic rats with PRP resulted in a significant reduction in the mRNA and protein level of joint ARE and it is still observed in PRP group (change percentage: -06%) comparing with that in MIA arthritic rats.

Table 3: Effect of PRP treatment on joint ARE mRNA expression in joint tissue of osteoarthritic rats.

	Parameters	
	ARE mRNA fold change (relative to control)	**% change**
Normal group	1.0 ± 0.1^{c}	——
Osteoarthritic control group	5.5 ± 0.1^{a}	448
Osteoarthritic + PRP group	2.2 ± 0.4^{b}	-60

Expressed Data Mean ± Standard Error. Number of assessed sample for each group is three for each parameter; Means, which take the similar superscript symbol (s), are not significantly altered. Percentage changes were estimated by comparing osteoarthritic control group with normal and osteoarthritic-treated groups with the osteoarthritic control one

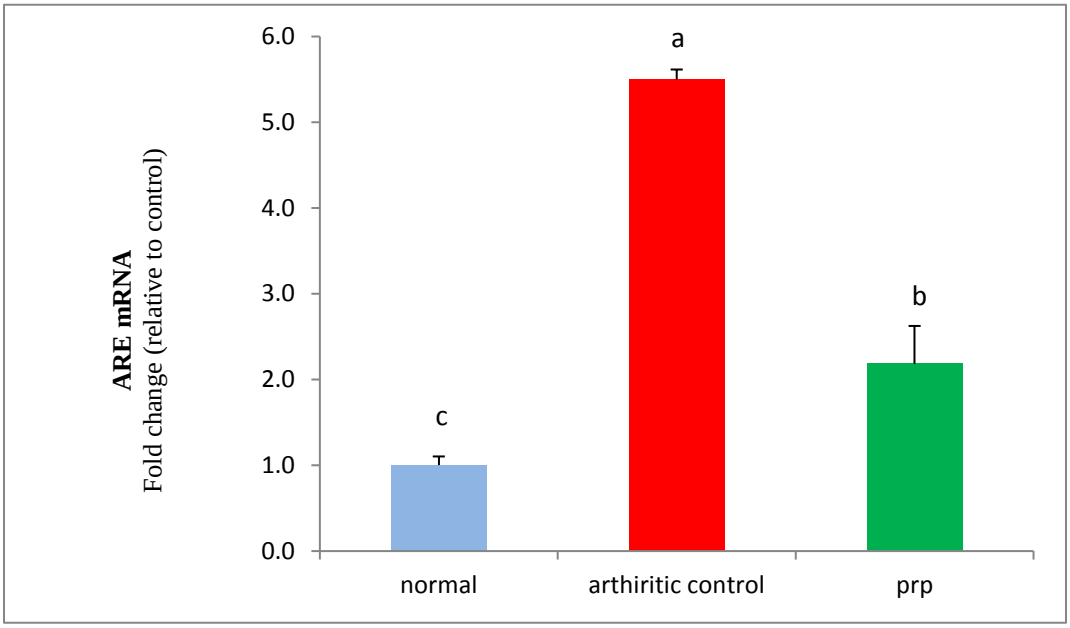

Fig. 2. Effect of PRP treatment on joint ARE mRNA expression levels in joint tissue of osteoarthritic rat. The means, which have the same symbol(s), are not significantly different.

3.6. Effect on Joint TGF-B1

The PRP treatment effect on joint TGF-B1of MIA is represented in **(Fig. 3).** MIA-induced rats showed a significant (P< 0.05) increase in joint TGF-b1 protein expression when compared with the normal control rats. Treatment of the MIA-induced rats with PRP significantly (P< 0.05) decreased TGFb1 protein expression when compared with the osteoarthritic rats but joint TGF-b1 protein expression in PRP treated group is high when compared with the normal control rats.

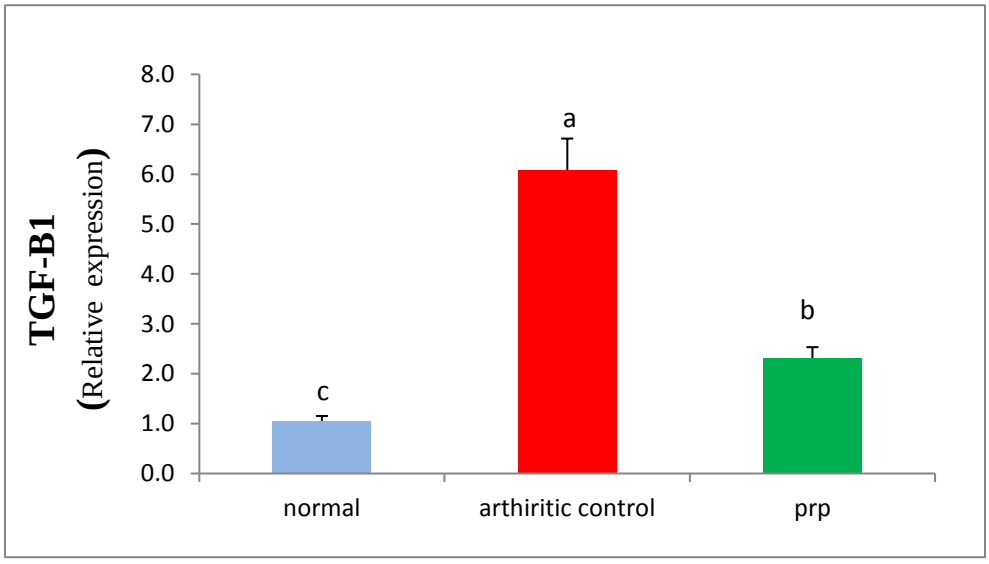

Fig. 3. Effect of PRP treatment on TGF-B1 level in joint tissue of osteoarthritic rat. The means, which have the similar symbol(s), are not significantly different.

4. Discussion

OA is a long-lasting deteriorating joint disorder that is multifactorial in origin and characterized by the progressive wearing of cartilage, subchondral bone changes, and synovitis leading to joint pain, discomfort and lessening joint motility **(Smit et al. 2019).** Routine treatment alternates include analgesics, hyaluronic acid, intraarticular cortico-steroid, PRP, physical therapy, and surgical interferences **(Bruyère et al. 2014).** Therefore, in the current investigation, the effects of intraarticular PRP administered to MIA induced osteoarthritic rats were evaluated where it revealed antiarthritic activity through attenuating TGF-B1, improving arthritic score and diminishing the expression of ARE and MMP-13. Osteoarthritis induced with MIA is a usually utilized experimental model for preclinical investigations. Owing to its short duration of testing, easy measurement, and resemblances to animal and human OA, this model has been used considerably to evaluate therapeutic drugs **(South et al. 2020).** Scores of arthritis were obtained on different observational days depending on the severity of the clinical signs which manifested on rats. The highest mean clinical score for arthritis of injected ankle was observed on day three, followed by day 07 and day 14. With the passage of time, swelling at the site of MIA injection diminished and became at minimum on 6th week. These results are in agreement with the prior study of **Ma et al. (2018).** At the end of the experiment, the treatment of the osteoarthritic rats with PRP yielded a significant decrease in the elevated clinical score when compared to the arthritic control animals. In the present investigation, the circumference of the right hind leg at the region of ankle is used as an indicator of paw edema and swelling rate. Paw volume changes have been used for evaluating anti-inflammatory effects on OA **(Gui et al. 2018).** 3-days after injection of MIA, all of the osteoarthritic and osteoarthritic-treated rats showed a noticeable swelling of the injected ankle manifested as significant edema when compared to the normal control rats. The elevated right hind paw circumference of in osteoarthritic rats was significantly declined as a result of treatment with PRP. The decline in the circumference as a result of treatment with PRP reflect the decline in the swelling rate which may be attributed to the reduction in edema, attenuation of the inflammatory process and the reduction of synovial tissue hyperplasia as described by former publication **(Aniss et al. 2020)** who demonstrated that PRP treatment after CFA-induced arthritis for 6 weeks resulted in suppression of paw swelling. Many factors have been involved in the OA development. The action of MMPs led to degradation of the extracellular matrix which considered the hallmark of OA. MMP13 is a major enzyme that targets cartilage degradation by lessening and destruction of the extracellular matrix. This MMP is induced by inflammatory mediators, such as interleukin-1-beta and TNF-α in tissue and fluid of OA joint **(Yan et al. 2015).** In OA, this mechanism is boosted in animal models. There is an increment in the expression levels of different proteins contributed in OA and simplifying degradation of cartilage matrix, including MMP-13 **(De Santis et al. 2018).** In our work, the MMP13 mRNA expression in joint was examined by qPCR. In the joint of MIA-arthritic control rats, MMP13 mRNA expression was extensively expressed when compared to the normal rats. This is in the line with the former study observation of **Lu et al. (2018)** who documented that the increased MMP13 mirrored the cartilage destruction in MIA-induced OA. The treatment of the osteoarthritic rats with PRP significantly decreased the level of the MMP13 mRNA production in the joint when compared to the elevated level of the osteoarthritic control animals. This is in accordance with the observation of the prior study of **Moussa et al. (2017)** who documented that PRP produced diminishing in MMP-3, MMP-13, and IL-6. Additionally, the proliferation of chondrocytes was augmented and apoptosis was minimized after PRP treatment. In our study, we used qPCR for assessing the mRNA expression of ARE in the joint. In the joint of MIA-osteoarthritic control rats, ARE mRNA expression was significantly expressed when compared to the normal rats. This result is in line with the investigation of **Cai et al. (2015)** who found that ARE is increased in the joints of mice with MIA-OA. The increase in Nrf2/ARE, which occurs in reply to oxidative stress within OA joints, whilst unstressed cells have Nrf2 protein with very low steady-state concentration, these results indicate that oxidative stress induced Nrf2 stimulation during OA. In the present work, we show that treatment of the osteoarthritic rats with PRP make a significant decreasing in the mRNA and protein level of ARE. It was still observed in PRP group, compared with that in the normal rat. These results demonstrate that PRP treatment has shown to play a critical role in protecting against oxidative damage via Nrf2/ ARE signaling pathway activation in cartilage. In the current study, the protein TGF-β1 expression of TGF-β1 in the joint was examined by Western blot assay. **Fang et al. (2016)** documented that various cytokines and growth factors have been shown to be required in

the degeneration of articular cartilage and subchondral bone destruction, which result in OA. TGF-β1 signaling role is one of the key factors in remodeling, and preservation of cartilage and bone formation. TGF-β is a cytokine that plays a vital role in both joints affected by osteoarthritic and normal joints. Yet, the growth factor role in an OA joint differs from its role in a normal healthful joint. In a normal synovial joint, Active TGF-β is present only after joint loading and for a short time. In OA joints, active TGF-β with High and permanent levels are detected **(Van et al. 2018).** Apoptosis of chondrocyte and degeneration of cartilage in OA results from the overexpression of mechanical stress of TGF-β1 from osteoclasts **(Zhang et al. 2018).** Even so, TGF-β role in joints stays argumentative. While TGF-β1 seems to prefer chondrogenesis, intra-articular injections of TGF-β1 may also enhance OA **(Seidel et al. 2019).** In the present study, the osteoarthritic rats showed a significant ($P<0.05$) increase in joint TGF- β1 protein expression when compared with the control rats. This effect was like the effect observed with **Waly et al. (2017)** who reported that serum TGF- β1 was significantly elevated in the OA group after administration of MIA. Treatment of the osteoarthritic rats with PRP significantly declined TGF- β1 protein expression when compared with the osteoarthritic rats and markedly increased TGF-β1 protein expression when compared with the normal control rats. This result is in line with the study of **Wang et al. (2018)** who found that the TGF-β1 level was higher in PRP than normal control. It has been confirmed that platelets have the ability to liberate anti- inflammatory cytokines and many growth factors from the cytoplasmic pool after accumulation. TGF-β1 has been shown to significantly participate in the inhibition of inflammation and cartilage healing in a goat OA model. Former findings propose that the expression of TGF-β1 elevated compared to normal control rabbits after PRP treatment, so TGF-β1 may play an important role in the chondrogenic effect of PRP **(Boakye et al. 2015).**

5. Conclusion

The current results suggest that multiple Intra-articular injection of PRP has obvious antiarthritic effects in osteoarthritic rats which are mediated through anti-inflammatory and antioxidant effects. As it can effectively ameliorate the ankle joint of OA rats, improve clinical score, adjust the expression of MMP-13, ARE mRNA and TGF β 1 in rats, and promote the treatment of rats, which is merited of clinical promotion. However, more in-depth experiments are still required.

6. Conflict of interest

No conflict of interest to declare

7. References

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How to cite this article: Halfaya FM, Ragab GH, Hagag U, Ahmed OM, Abou Elkheir W. Effect of Platelet-rich Plasma on MMP-13, ARE and TGF β1 in MIA-Induced Osteoarthritis in Rats. J Vet Med Res. 2021; 27 (2): 109–118. https://doi.org/10.21608/JVMR.2020.34762.1019