

Comparative study between the effect of stem cells and Metacam® on induced arthritis in dogs

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Abstract

Objective of this project was to study the effect of stem cells on the induced arthritis in the dogs as compared with treatment Metacam®. Incomplete Freund's adjuvant was used to induce arthritis. Twenty five dogs aged 12 month were divided into five groups: 1st group (T1) was negative control, while 2nd group (T2) was induced without any treatment (positive control), 3rd group (T3) was induction arthritis and treated with 0.2 mg/kg B.W. of Metacam® intravenously (I/V) injection, 4th group (T4) was induced and treated with 1ml/kg of B.W of suspension of culture media injected (I/V), and 5th group (T5) was treated with stem cells suspension in a dose of 2.5×10^6 cells / ml / Kg B.W. (I/V) injection. Typical clinical signs of arthritis appeared on all induction groups. The results of Hb and RBCs showed clear reduction in all induction groups as compared with (T1), the WBCs, Erythrocyte sediment rates (ESR) and C- reactive protein (CRP) in (T2) showed clear elevation as compared with (T1), After 15 and 30 days of treatment the RBCs count and HB level showed significant decline in T2, T3 and T4 as compared with T5 and T1, the WBCs ,ESR and CRP significantly elevated in the T2 and T4 as compared with T1. The T3 and T5 showed clear depletion in levels of WBCs, ESR and CRP as compared with T2 after 15 and 30 days of treatment, while the T5 revealed no significant difference as compared with T1 after 30 days of treatment. The histopathological changes in T2 and T4 showed erosion and infiltration of inflammatory cells, T3 showed hypertrophy of synovial lining and mild inflammatory cells in synovium, T5 explained return the bone tissue to normal. In this experiment concluded a successful use of stem cells in treatment of arthritis without side effect as comparison with Metacam®.

Keywords: Stem cells, Metacam®, Rheumatoid Arthritis, Dogs.

دراسة مقارنة بين تأثير علاج الخلايا الجذعية ودواء الميتاكم على التهاب المفاصل المستحدث في الكلاب

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الخلاصة:

هدفت الدراسة إلى بيان تأثير الخلايا الجذعية على التهاب المفاصل المحدث في الكلاب بالمقارنة مع العلاج Metacam®. تم استحداث مرض التهاب المفاصل وذلك بزرق مادة Incomplete freund's adjuvant. قسمت الكلاب (خمسة وعشرين كلب) تتراوح أعمارهم 12 شهرا إلى خمس مجاميع: كانت المجموعة الأولى (T1) ظابطة سلبية ، في حين مجموعة الثانية مستحدثة (T2) بدون أي علاج (ظابطة إيجابية)، عولجت مجموعة الثالثة المستحدثة (T3) بـ Metacam® 0.2 ملغ / كغم من وزن الجسم عن طريق الزرق الوريدي، بينما عولجت المجموعة الرابعة المستحدثة (T4) بالسائل النسيجي 1مل / كغم من وزن الجسم، أما المجموعة الخامسة المستحدثة (T5) عولجت بالخلايا الجذعية $10^6 \times 2.5$ خلية / مل / كغم من وزن الجسم زرق وريدي. ظهرت العلامات السريرية لالتهاب المفاصل واضحة في المجاميع المستحدثة. بينت نتائج خضاب الدم وكريات الدم الحمر انخفاض واضح في المجاميع المستحدثة مقارنة مع (T1). واطهرت نتائج الكريات الدم البيض ومعدل تثقل الكريات الحمر و البروتين نوع سي في (T2) ارتفاع واضح مقارنة مع (T1)، أظهرت عدد كريات الدم الحمر و مستوى خضاب الدم بعد مرور خمسة عشر يوما والثلاثين يوما من العلاج انخفاض كبير في T2، T3 و T4 بالمقارنة مع T5 و T1، والكريات الدم البيض و معدل تثقل الكريات الحمر و البروتين الفعال نوع سي مرتفعة بشكل ملحوظ في T2 و T4 بالمقارنة مع T1. اتسمت المجاميع T3 و T5 بنزول واضح في مستويات الكريات البيض ومعدل تثقل الكريات الحمر و البروتين الفعال نوع سي بالمقارنة مع T2 وذلك بعد مرور 15 و 30 يوما من العلاج، في حين لم تظهر مجموعة T5 اي فرق معنوي بالمقارنة مع T1 بعد مرور 30 يوما من العلاج . أظهرت التغيرات التشريحية المرضية في T2 و T4 تاكل وارتشاح الخلايا الالتهابية للمفصل و بينما أظهرت المجموعة T3 تضخم في بطانة المفصل وارتشاح الخلايا الالتهابية في الغشاء الزلالي ، في حين أوضحت مجموعة T5 عودة أنسجة العظام إلى وضعها الطبيعي نستنتج من هذه الدراسة امكانية استخدام الخلايا الجذعية بنجاح في علاج التهاب المفاصل من دون آثار جانبية بمقارنة مع دواء Metacam® .

Introduction

Researches on stem cells are advance information about how an being grows from a particular cell and how fit cells change damage cells in mature organisms and investigation the possibly of cell-based therapy to treat diseases by regenerative or reparative medication. Stem cells are single of the majority attractive areas of biology, advance in understanding of the biology of adult stem cells have attracted the attentions of the biomedical researches community, including those studying osteoarthritis (1). Autologous adult stem cells are immunological compatible, can be produced from a multiplicity of source, including bone marrow and adipose tissue, which is the most highly characterized, both have demonstrated expansive multipotency with differentiation into a number of cells lines- ages, including adipocytic, osteocytic, and chondrocytic lineages (2).

Arthritis is a sum of conditions effects the health of the bone joints in the

body and considered one of the most widespread chronic health problems worldwide, types of arthritis including rheumatoid arthritis, ankylosing spondylitis or fibromyalgia (3). The most frequent symbols of arthritis are puffiness in the joints, inflexibility around the joint, stable or recur pain with softness in the joint, complexity in moving the affected joint and finally the warmth and redness in the joint, arthritis can appear for a number of reasons including infection injury, abnormality of the immune system and aging (4). The conventional treatments for arthritis are mostly non steroidal anti-inflammatory drugs such as aspirin®, ibuprofen®, Metacam® and voltaren®. This group may cause many side effects and risks such as potential heart attack, stroke, and stomach bleeding (5).

Meloxicam (Metacam®) an oxycam original, is a associate of the enolic acid collection of nonsteroidal anti_inflammatory drugs (NSAIDs) which are a main reason for worry for

their propensities to cause joint weakening in canine getting these drugs for management of pain in arthritis and other severe and continual painful circumstances (6). The aim of this study was designed to comparative healing effects between the stem cells and Metacam[®] after induced arthritis in dogs.

Materials and methods

- **Bone marrow aspiration:** It was carried out from the trochanteric fossa of the femur bone of each dog in 5th group only, according to Wellman and Radin (7), special needle for aspiration of bone marrow with disposable syringe were heparinized and used for this purpose.

- **Preparation of MSCs:** Isolation and purification of MSCs was carried out according to Dodson (8). Bone marrow samples were centrifuged (8°C) on 8000 rpm for 5 minutes, the fat and serum layers were discarded, and the cells pellet resuspended in 5 ml of mesencult[®] (Iscoves Modified Dulbecco's Medium IMDM, Invitrogen-USA) plus 10% fetal calf serum (FCS). Marrow cells were plated at a concentration of 2×10^7 cells per 75cm² culture flask in 20 mL of IMDM containing 10% FBS and 1% Penicillin/Streptomycin (100U/mL). Extra cells were frozen in medium containing 10% Dimethylsulfoxide (DMSO). Non-adherent cells were removed after 72 hours of culture with change of medium. Subsequently, half of the medium was altered twice a week. After 2-3 weeks, cultured cells were separate

using 0.05% trypsin EDTA, washed by centrifugation, and expanded to three 75cm² flasks. After reaching confluency (2-3 weeks), cells were separate using 0.05% trypsin EDTA and tested for MSC function in vitro. Frozen marrow cells were thawed and washed in IMDM, and treated in the same manner as fresh marrow cells (8, 9).

- **Cytometry analysis of canine MSCs:** MSCs dog at the 3rd passage was immunologically examined for surface markers of MSCs. the MSCs were first dissociated from the Petri dishes with Trypsin EDTA and then centrifuged. A total of 200,000 to 300,000 cells were stained with each respective antibody. Rat monoclonal anti-canine CD 90 (AbD serotec, Kidlington, UK) with rabbit anti-rat FITC secondary antibody and monoclonal anti-canine CD44 conjugated with allophycocyanin (APC) (R&D system, Minneapolis, USA) were used as MSC positive markers. Fluorescently-labeled MSCs were finally washed once, fixed with 1% (w/v) paraformaldehyde in PBS and stored in the dark at 4°C until analysis. At least 20,000 MSCs were used to test the presence of each cell surface marker, using flow cytometry (BD Biosciences, Franklin Lakes, USA)(10).
- **Counting of viable cells:** Equal volumes of (0.5 ml) of cells suspension and the trypan blue stain were incubated at 37

°C for 30 minutes. 0.02 ml of the mixture was put in counting chamber slide (hemacytometer) and examined under light microscope (40x). When number of stained cells over 30% , the sample was discarded and re-subculturing of cells was done by using new media (11) .

- **Induction of rheumatoid arthritis:** 0.1 ml of incomplete freund's adjuvant (IFA) was injected in the right tarsal joint of each animal and repeated after 7 days. Arthritis signs appeared after 14 days according to (12).

- **Experimental design :**

Twenty five dogs aged year were equally divided into five groups as below:

First group (T1): was kept on ordinary diet and left without treatment (negative control group).

Second group (T2): was administered IFA to induce arthritis and left without treatment (positive control group).

Third group (T3): was induction arthritis and treated with 0.2 mg/kg body weight intravenously injection given daily Metacam[®] .

Furth group (T4): was induction arthritis and treated with 1ml/kg of B.W injected intravenously suspension of culture media.

Fifth group (T5): was induction arthritis and treated with autologous MSCs suspension of 2.5×10^6 cells / ml / Kg B.W intravenously injection given weekly.

Parameters used in this experiment:

- **Clinical examination of tarsal joints animals**

- **Hematologic parameters:**

- Blood samples were taken from the cephalic veins divided into two tubes from each animal weekly. Tube one contain Ethylene-diamine-tetra-acetic-acid (EDTA) for RBCs counts, WBCs count, Hb, Erythrocyte sediment rates (ESR) according to coles(13) and the tube other without EDTA for C- reactive protein (CRP) level were estimated according to kits commercial abcam[®] Canine CRP ELISA kit, ab157698(PTX1). Assay procedure of kit : all materials were equilibrate and prepared reagents at room temperature previous to use. It is recommended assaying all standards, controls and samples in duplicate. Pipette 100 µL of every standard, including zero control, in duplicate, into pre selected wells then incubated the microtiter plate at room temperature for ten minutes. The keep plate enclosed and level during incubation. Next incubation, aspirate the filling of the wells, then completely fill each well with suitably diluted and washing with buffer and aspirate, this was frequent three periods, for a whole of four washes. This followed by gently striking the wells on absorbent paper to remove residual buffer and Repeated 3 times for a total of four washes. Pipette 100 µL of Enzyme-Antibody Conjugated to every well and incubated at room temperature for ten minutes. The plate covered was reserved in the dark and level during

incubation. After five minutes, 100 μ L of stop solution was further to every well then the absorbance (450 nm) inside of every well was determined.

- **Histopathological analysis:**

One dog was taken from each treated group and then Euthanasia by using high dose of ketamine and zolazepam. The right tarasal joint was cut and specimens fixed in 10% formalin. The bones was passage decalcification process by formic acid performed on bone piesces for three days and then reserved in 10% neutral buffered formalin for fixation, procedured regularly in histoinette, cut at 5mm thickness by microtome and stained with haematoxylin and eosin stain then examined below light of microscope (14).

- **Statistical Analysis :** Analysis of variances (ANOVA) one way and least significant differences (LSD) at a important level of ($p \leq 0.05$) to compare the data of diverse groups throughout the period of trial (15).

Results:

The uses of mesenchymal culture media (mesencult media[®]) with fetal calf serum gave optimum period (7days) to obtain large quantity of cells, also some cells were floating and other cells started to adhere on the flask wall progressively. The MSCs proliferated in culture medium at the

end of the week, they formed colonies of adherent cells, which expanded, adhered and sparse too, they appeared as fibroblast and spindle-like, isolated from canine bone marrow. The adhered MSCs started to proliferate 3 days after sub-culturing in mesencult[®]. The colonizing spindle-like cells were surrounded by the same cells, which gave appearance of more densely cellular masses. These cells adhered to the culture dish by 24 hours of culture. At passage 3, putative MSCs were cultured and analyzed for expression of MSC surface markers and population doubling time. These MSCs were expressed mesenchymal stem cells markers (CD 44 and CD 90) was positive indicator for it.

The animals in (T2, T3, T4 and T5) groups showed signs arthritis seen commonly lethargic, inactive, redness and enlargement of the right tarsal joint, difficult in walking and lameness at 15 day after administration IFA contrast with control group (T1) Also, the consumption of dietary which much less in (T2 and T4 groups) due to the loss of appetite during end of study. While therapeutic stem cells group showed decline clear in clinical signs comparison with treated metacam group.

The results of Hb and RBCs explained significant decline ($P < 0.05$) in all induction groups as compared with negative normal group, in otherwise the WBCs, ESR and CRP in positive groups explained significant raise ($P < 0.05$) as contrast with negative control group (table 1).

Table (1): Comparison the hematological and biochemical parameters between incomplete freunds adjuvant groups (positive control group) and control group (negative) before begin treatment.

Parameters Groups	RBCs count x10 ⁶ /m	Hb g/dl	WBCs count x10 ³ /m	ESR mm/hr	CRP (mg/l)
Negative control	7.5 ±0.32 a	15.7 ±0.20 a	12 ±0.37 a	20 ±0.28 a	7.0± 0.22 a
Positive control	5.3 ±0.16 b	11.3 ±0.22 b	22 ±0.20 b	45 ±0.18 b	50.3 ± 0.31 b
Positive control	4.9 ±0.31 b	10.8 ±0.19 b	23 ±0.17 b	50 ±0.17 b	57.1 ± 0.22 b
Positive control	4.7 ±0.41 b	11.1 ±0.13 b	24 ±0.13 b	49 ±0.25 b	58.7 ± 0.17 b
Positive control	4.5 ±0.38 b	10.9 ±0.24 b	23 ±0.16 b	51 ±0.20 b	52.9 ± 0.21b

* Mean ±SE , number animals 5 / group .

*Different small letters mean significant difference (p <0.05) between column numbers.

After 15 and 30 days of treatment the RBCs and HB level explained significant reduce (P<0.05) in T2, T3 and T4 groups as compared with T5 and control groups while the WBCs ,ESR and CRP significantly elevated in the T2 and T4 groups as compared with negative control group, in otherwise the T3 and T5 groups showed clear depletion in levels of WBCs ,ESR and CRP as compared with T2 group after 15 and 30 days of treatment while the T5 group reveled no significant difference as compared with negative control group after 30 days of treatment (table 2).

Table (2): Effect of stem cells, Metacam® and suspension culture media on the hematological and biochemical parameters levels:

A- after 15 days from treatment.

Parameters Groups	RBCs count x10 ⁶ /µl	Hb g/dl	WBCs count x10 ³ / µl	ESR mm/hr	CRP (mg/dl)
T1	7.4 ±0.31a	15.2 ±0.24a	12 ±0.36a	20 ±0.25a	7.3± 0.20a
T2	5.5 ±0.11b	11.1 ±0.25b	23 ±0.26b	48 ±0.10b	50.9 ±0.39b
T3	5.2 ±0.38b	11.8 ±0.14b	20 ±0.12b	32 ±0.15c	30.6 ±0.29c
T4	4.2 ±0.44b	10.6 ±0.10b	22 ±0.12b	46 ±0.23b	50.3 ±0.18b
T5	6.1	13.4	14	25	15.1

	±0.39c	±0.21c	±0.14c	±0.22d	±0.25d
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B-After 30 days of treatment

Parameters Groups	RBCs count x10 ⁶ /μl	Hb g/dl	WBCs count x10 ³ /μl	ESR mm/hr	CRP (mg/dl)
T1	8.2 ±0.30 a	13.4 ±0.23 a	13 ±0.34 a	21 ±0.25 a	7.1 ±0.20 a
T2	5.3 ±0.16 b	11.3 ±0.22 b	22 ±0.20 b	45 ±0.18 b	50.7 ±0.31b
T3	5.0 ±0.31 b	10.4 ±0.19 b	18 ±0.17 c	29 ±0.17 c	15.6 ±0.24 c
T4	5.5 ±0.41 b	10.1 ±0.13 b	23 ±0.11 b	47 ±0.25 b	50.5 ±0.17 b
T5	7.8 ±0.33 a	12.9 ±0.25 a	15 ±0.10 a	21 ±0.26 a	7.2 ±0.24 a

T1 negative control group, T2 positive control group, T3 induced group treated with metacam, T4 induced group treated with suspension culture media, T5 induced group treated with autologous MSCs *mean±SE, No. of animals =5/ group

*Different small letters mean significant different (p <0.05) between column numbers.

Histological changes:

After 30 days of treatment the histopathological section of dog treated with CFA-injected showed erosion and infiltration of inflammatory cells as in figure(1), histopathological section of dog treated with culture media showed fibrous connective tissue proliferation and infiltration of inflammatory cells (figure 2), while the histopathological section of dog treated with Metacam® showed hypertrophy of synovial lining and mild inflammatory cells in synovial and disappeared of cellular infiltration as in figure (3), in otherwise the histopathological of dog treated with MSCs explained disappearance all infiltration of inflammatory cells and return the bone tissue to normal as in figure (4).

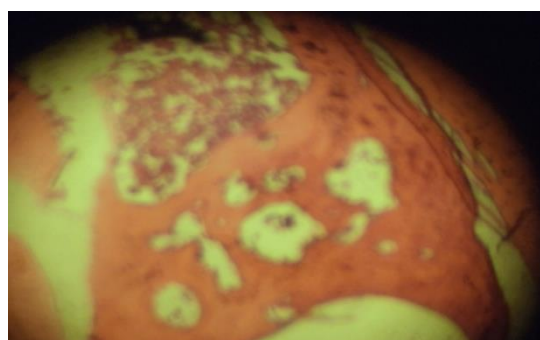


Figure (1): Histopathological section of dog treated with CFA-injected showed erosion and infiltration of inflammatory cells (H&EX400).

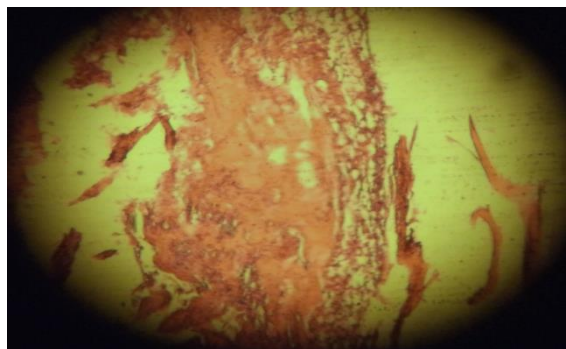
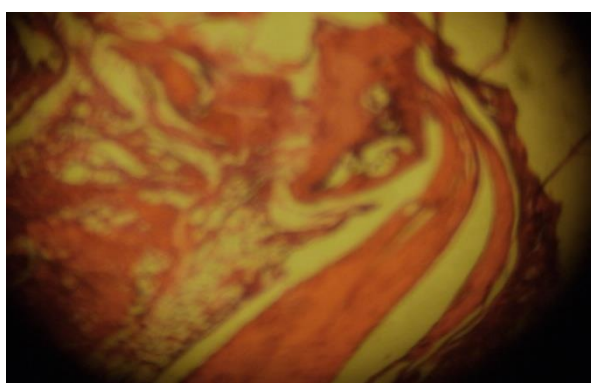


Figure (2): Histopathological section of dog treated with culture media showed fibrous connective tissue proliferation and infiltration of inflammatory cells (H&EX400).



Figure(3): Histopathological section of dog treated with Metacam® showed hypertrophy of synovial lining and mild inflammatory cells in synovium (H&EX400).

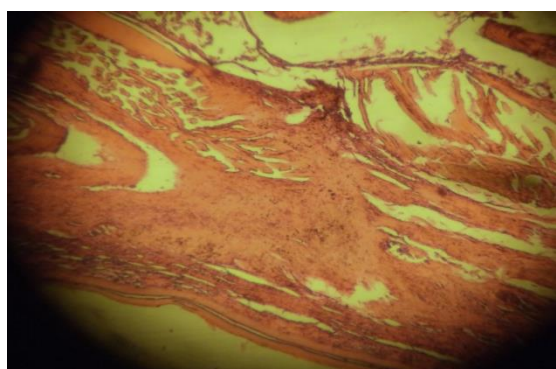


Figure (4): Histopathological section of dog treated with MSCs explained disappearance all infiltration of inflammatory cells (H&EX400).

Discussion:

The non-steroidal anti-inflammatory drugs (NSAIDs) for pattern meloxicam, acetyl salicylic acid, and diclofenac sodium are used to treat

arthritis and this group caused side effect like gastrointestinal irritation, bleeding and ulceration (16), for the above mention reasons MSCs was chosen to treat arthritis, which inhibit

inflammatory process without side effect (17). Canine mesenchymal stem cells was isolated from canine bone marrow, these specialized cells are classified as multipotent stem cells because they have the capability of

differentiation into mesodermal lineage, the results was agreement with (18,19). The isolation, identification and purification of canine MSCs recently become an important issue for the clinical use of MSCs (19). The specificity of canine surface antigen expressed mesenchymal stem cells markers (CD 44 and CD 90). This phenomenon is one of the hallmarks affecting the exploitation of MSCs for clinical use since MSCs are located in the bone marrow, most investigators have isolated MSC using their capacity to adhere to a plastic culture dish, the MSCs can transdifferentiation *in vivo* to extensive multipotency with differentiation into a number of cell lineages, including osteocytic, and chondrocytic lineage (19, 20).

In this study, injection of incomplete Freund's adjuvants in synovial cavity of tarsal joint caused puffy swelling of the joint and redness of joint, this results agreed with results reported by Javeria, *et al* (21) they mentioned that incomplete Freund's adjuvant can cause arthritis and facilitate autoimmune response, lead to inflammatory changes, also this result agreement with result reported earlier by Javeria, *et al* (21) and Butler, *et al* (22). After 15 days of arthritis induction the RBCs and Hb level decreased with increase total white blood cells count, regarded to reticulocytosis and immunostimulant and this result agreed with results reported by Joe and Wilder (23) in addition the adjuvants cause granulocytes were seven times

more than normal level leading to induce anemia and high count reticulocytosis (24).

After 15 and 30 days of treatment the results of depletion RBCs count and Hb level of meloxicam treated group may be attributed to inhibition of stem cell synthesis in the bone marrow or because acute immune hemolytic anemia that resulted from meloxicam metabolites that bound the Hb resulting in hemolysis of these cells this result agreement with (25) who reached to nearly same results in dogs who took this drug and resulted in acute hemolytic anemia with lower RBCs, PCV and Hb, further more the decrease of WBCs, ESR and CRP in meloxicam treated group and observed decrease of clinical signs may be referred to effective in decreasing the local inflammatory response in joint of dogs, this result agreement with (26,27), in addition to Meloxicam is extensively recognized as being single of the first commercially obtainable cyclooxygenase type 2 (COX-2) selective NSAIDs are believed to inhibit many of the inflammatory aspects of arachidonic acid metabolism, they are also believed to spare the functions of prostaglandins and thromboxanes, the same result reported by Van, *et al* (26) and Rainsford, *et al* (27), while the result of enhancement RBCs a count and Hb level of MSC treated group might be indicated to ability of MSC in differentiation potentials, in otherwise the result of WBCs, ESR and CRP in therapeutic stem cells may be referred to that MSC act as transdifferentiation essentially to mesodermal lineages, including osteocytic, and chondrocytic lineages, therefore simultaneous results of clinical signs, hematological and histological changes was decline clear in therapeutic stem cells, this results

was agreement to results reported by Zuk , *et al* (28).

The result of histopathological change in induction group can be explained to fibrous connective tissue proliferation with mononuclear cell infiltration in the membrane of joint due to facilitate autoimmune response and joint damage with progressive inflammatory degenerative changes in joint this results agreed with results supported by (29), whereas the histopathological changes in T3 group may be regarded to the side effect of Metacam® in delayed healing of bone, this results agreed with results recorded by Harder and An (30) when they mention that Metacam® cause delayed in the healing because the mechanism of action inhibition of COX2 leading to inhibitor prostaglandins which essential for bone healing, furthermore the result of MSCs regarded to that MSC act as transdifferentiation essentially to mesodermal lineages, including osteocytic, and chondrocytic lineages lead to increase osteocyte activity and new bone formation , other researches supported this result reported by Zuk , *et al* (28).

Conclusion: This study showed that MSCs could markedly treat arthritis by increase osteocyte and chondrocytic lineages activity lead to new bone formation with reducing the side effect of Metacam® drug.

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