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The Significant Effect of Conditioned Medium of Umbilical Cord Mesenchymal Stem Cells in Histological Improvement of Cartilage Defect in Wistar Rats

Wistar Sıçanlarındaki Kıkırdak Boşluklarının Doldurulmasında Göbek Kordonu Kökenli Kök Hücrelerinin Anlamlı Etkisi

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Abstract

Introduction: Mesenchymal stem cells are multipotent cells present in multiple tissues that have potential for future disease treatment including cartilage damage. This study was aimed to investigate the effect of conditioned medium of umbilical cord stem cells in improving the histological conditions of damaged cartilage in rats.

Materials and Methods: Twenty-four of 3-month old male rats that were divided into 6 groups (3 control groups and 3 treatment groups) with different evaluation time (month 2, month 3, and month 4). Treatment in each group was repeated 5 times. The cartilage defect was induced manually and mechanically at the medial condyle of rats' right femur using a Kirschner Wire (D=1.0 mm; h=1.0 mm). The umbilical cord stem cell cultures were obtained from pregnant rats (19 days). Rats in treatment groups were injected with mesenchymal stem cell-conditioned medium (1 mL/kg BW) 5 times with interval a week after cartilage defect. Histopathological examination of chondrocytes formation and fibrosis tissues was done through Haematoxylin Eosin staining. Data were assessed with O'driscoll scoring and analyzed statistically using Kruskal-Wallis test with SPSS 16.0 software statistically using Kruskal-Wallis Test with SPSS 16.0 software.

Results: The conditioned medium of umbilical cord mesenchymal stem cells was able to repair the damage in cartilage tissue of Wistar rats. It is indicated by the higher score of nature of predominant tissue, surface regularity, structural integrity, bonding with adjacent cartilage and the level of new tissue formed in the treatment group compared to control group. Statistically, there is a significant difference of bonding to the adjacent cartilage score (0.028, $p<0.05$) in treatment group than in control group. There is no significant difference of nature of the predominant tissue score (0.064, $p>0.05$), surface regularity (0.064, $p>0.05$), structural integrity (0.075, $p>0.05$), level of newly formed tissue (0.088, $p>0.05$).

Conclusion: The supplementation of conditioned medium of umbilical cord mesenchymal stem cell ameliorate the repair of damaged cartilage.

Keywords: Conditioned medium, umbilical cord mesenchymal stem cell, cartilage defect, histology

Öz

Giriş: Mezenkimal kök hücreler, bir çok dokuda da bulunan ve kıkırdak hasarının iyileştirilmesi de dahil bir çok hastalığın tedavisinde kullanılabilecek çok farklı hücrelere dönüşebilen kök hücrelerdir. Bu çalışmada, göbek kordonundan elde edilmiş ve geliştirici doku kültürü medyumunda zenginleştirilen kök hücrelerin sıçanlardaki hasarlanmış kıkırdak tamirindeki etkisi araştırıldı.

Gereçler ve Yöntemler: Yirmi dört adet 3 aylık erkek sıçan, 2,3 ve 4 aylık aralık ile değerlendirilen 3'ü kontrol 3'ü deney 6 gruba ayrıldı. Uygulanan işlem (tedavi) 5 kez tekrar edildi. Göbek kordonukök hücreleri 19 günlük hamile sıçandan elde edildi. Sıçanlarda, Kirschner teli kullanarak, el ile ve mekanik olarak, femur iç kondilinde bir boşluk oluşturuldu (D:-1mm; h:-1 mm). Tedavi gruplarında, kıkırdakta bir boşluk oluşturulduktan sonra sıçanlara doku medyumu içinde bekletilmiş kök hücreleri 1ml/kg (vücud ağırlığı) olacak şekilde haftada 5 kez olacak şekilde verildi. Kondrositlarin oluşumu ve fibröz doku, histopatolojik olarak Hemotoxylin-Eosin boyaması kullanılarak irdelendi. Veriler, O'Driscoll ölçütü kullanılarak ve SPSS 16.0 yazılımı ile Kruskal-Wallis testi yapılarak irdelendi.

Bulgular: Geliştirici doku sıvısının içinde gelişen mezenkim kök hücreleri, Wistar sıçanlarda oluşturulan kıkırdak hasarını onarmada daha yüksek doku oluşumu, yüzey düzenliliği, yapısal bütünlük, bitişik kıkırdağa bağlanma ve yeni kıkırdak oluşumunda kontrol grubuna göre anlamlı ölçüde etkili oldu. Kontrol grubuna göre çalışma grubunda komşu kıkırdak dokusuna bağlanma istatistiksel olarak anlamlı düzeyde daha yüksek bulundu ($p=0.028$). Ancak, oluşan doku skoru ($p=0.064$), yüzey düzenliliği ($p=0.064$), yapısal bütünlük ($p=0.075$) ve yeni oluşan doku ($p=0.088$) ölçümlerinde, iki grup arasında istatistiksel açıdan anlamlı fark saptanmadı. Dördüncü ayda yapılan incelemelerde, kollajen ifadesinin 2. ve 3. aylardaki irdelemelere göre istatistiksel olarak anlamlı derecede farklı olduğu saptandı.

Sonuç: Kültür ortamına mezenkimal kök hücrelerin eklenmesi hasarlanmış kıkırdak dokusunun tamirini kolaylaştırır.

Anahtar Kelimeler: Göbek kordonu kökenli mezenkimal kök hücreler, kıkırdakta boşluk, histoloji

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Turk J Immunol 2018; 6(2):57–64

Introduction

Lesions of articular cartilage can lead to potential crippling symptoms like swelling, pain, and mobility decrease. If it improperly treated, it will develop into osteoarthritis (OA).[1] Therefore, the ultimate goal in OA therapy is to restore the knee function by regeneration of hyaline cartilage in the defect.[2] Various procedures of treatment for the injuries were performed.

Mesenchymal stem cells (MSCs) are cell populations with infinite proliferation capabilities that can differentiate into mesenchymal strains.[3] Several studies have reported that the population of MSCs can differentiate into osteoblasts, chondrocytes, cardiomyocyte cells, muscle cells, and adipocytes. In certain culture conditions, MSCs are capable of trans-differentiation into nerve cells. MSCs can be isolated from various sources of adult tissues, including bone marrow, adipocyte cells, umbilical cord, and peripheral blood.[4]

Stem cells involve in bone remodeling as evidenced by RANKL expression in MSCs cultures or stromal cells and functionally contribute to osteoclastogenesis *in vitro*. MSCs populations also play an indirect role as a progenitor source of osteoblasts which is responsible for anabolic from homeostatic balance and regulate osteoclastogenesis through RANKL and OPG expression.[5-7] A preliminary study suggested that mesenchymal stem cells derived from the umbilical cord have good proliferation and differentiation capabilities.[8] This study also showed that the Umbilical Cord-derived Mesenchymal Stem Cells (UCMSCs) have the ability to differentiate into unipotent with chondrocytes' properties.[8]

The joint surface defect is an articular cartilage focal lesion. These injuries occur frequently that can be detected for approximately 20% in all arthroscopic procedures.[9] Clinical symptoms can lead to disability with pain accompanied by locking, resulting in continued cartilage loss and osteoarthritis (OA).[10] Articular cartilage damage remains one of the main concerns in orthopedic surgery.[11] One of the potential therapies is the use of stem cells. MSCs transplants have been introduced to minimize some of the side effects and complications of cartilage damage.[12] The crucial role of growth factor of the superfamily transforming growth factor (TGF), such as TGF-β1, TGF-β3, Bone Morphogenic Protein (BMP)-2, BMP-6 and BMP-9 will stimulate MSC to differentiate into cells similar to chondrocytes producing chondro-specific extracellular matrix.[13] Intracellular signals respond to chondrogenic differentiation.[14] Therefore, this study aimed to investigate the effect of conditioned medium of umbilical cord mesenchymal stem cell on histologic conditions of damaged cartilage in Wistar rats (*Rattus norvegicus*).

Methods

Research design

This study was conducted in Physiology Laboratory, Faculty of Animal Husbandry, Gadjah Mada University, Yogyakarta, Indonesia. The research design was true experimental with post-test only control group. This study has been approved by Ethical Clearance Commission of Gajah Mada University, Yogyakarta, in Mei 6th 2015 (Letter number: 263/KEC-LPPT/V/2015).

The animal models

There were thirty-six male Wistar rats aged 3-month, which were obtained from Gadjah Mada University, Yogyakarta, Indonesia. The homogeneity was confirmed through the balanced body weight. They were divided into 6 groups, including control , group receiving treatment for two months(K2), 3rd group receiving the treatment for 3 months(K3) and group 4 in which the rats having 4 months of treatment(K4). 3 months (K3), and 4 months (K4). Treatment in each group was repeated 5 times. Treatment groups were E2(2 months of treatment), E3(3 months of administration) and E4(treatment lasted 4 months). Rats in treatment groups were injected with 1 mL/kg body weight (BW) conditioned medium of UCMSCs 5 times with interval a week after creating the cartilage defect. Moreover, the cartilage defect was created by surgery on the medial condyle area of the right rear femur in the weight bearing area, followed by the destruction of cartilage through manual mechanical technique by drilling (750 rpm) using Kirschner Wire (D=1.0 mm; h=1.0 mm). This study was conducted in an accordance with appropriate animal ethics in Physiology Laboratory, Animal Husbandry Faculty, Gadjah Mada University, Yogyakarta, Indonesia.

Isolation and culture of umbilical cord-derived mesenchymal stem cells

The umbilical cord was obtained from 19 days-pregnant Wistar rats through caesarean section. In sterile

conditions, one centimeter of the umbilical cord was washed with 10% iodine solution and sterile physiological saline solution. The umbilical cord blood was transferred into a centrifugal tube containing Dulbecco's Eagle Modified Medium (DMEM) (Gibco, USA) with 200 µg/mL of penicillin, 200 µg/mL streptomycin, and 200 µg/mL fungizone. After being cut into 20 mm^3 pieces and cultured through enzymatic digestion procedures, the umbilical cord was dissolved in 0.25% Trypsin enzyme Ethylenediaminetetraacetic acid (EDTA) (Gibco, Canada) and incubated for 30 min at 37°C. A total of 2 mL complete medium (DMEM, 10% fetal bovine serum, 50 µg/mL streptomycin penicillin, and 2.5 µg/mL fungizone) was added into a tube containing umbilical cords that had been digested by enzyme, and then centrifuged at a rate of 3000 rpm for 10 min at 4°C. The supernatant was discarded, the cell suspension was mixed with the complete medium and incubated in incubator with temperature of 37°C and 5% CO_2. The media was changed every three days until the cell growth reached 80% confluence. The confirmation of stem cell formation was done through microscopic examination with the Haematoxylin Eosin, Giemsa and Sirius Red staining.

Production of conditioned media

Cell cultures of umbilical cord-derived mesenchymal stem cells (UCMSCs) that formed 80% confluence were harvested through warm tripsinization method. The cell suspension was centrifuged at a rate of 3000 rpm for 10 minutes right after trypsin removal. The supernatant was removed, while the cell deposit was washed with Phosphate-buffered saline (PBS) (Merck KGaA, Darmstadt, Germany) 3 times. The precipitate was further suspended with the new medium with concentration of 10.000 cells per mL. The stem cells were stimulated into embryoid bodies and were planted on culture plate with complete medium to form confluence between embryoid bodies. The conditioned medium was produced by washing the embryonic culture with sterile PBS and filling the embryonic culture plate with 10 mL complete medium without serum. After 48 hours, the conditioned medium was stored in -20°C.

Treatment with conditioned medium

Rats were anesthetized with ketamine: xylazine (9:1). Rats' condyle femur cartilages were destructed by performing manual mechanical destruction with Kirshner wire drilling (diameter: 1 mm, depth: 1 mm, 750 rpm). Following the destruction, rats were injected with conditioned media 5 times with interval a week and were observed after 6 weeks later without injection. Rats were euthanized with ketamine/xylazine combination. Furthermore, histopathologic examination of extracted cartilages was done after Hematoxylin Eosin staining.[15]

Haematoxylin eosin staining

The procedure of histopathologic preparation using haematoxylin eosin (HE) staining was done according to method published by Marquass.[16] Cartilage tissues were washed with physiological NaCl and were fixed in 4% formalin buffer for 18–24 hours. The tissues were then dehydrated with alcohol in gradual levels 30%, 50%, 70%, 80%, and 90%. Tissues were soaked in alcohol/xylol for 1 hour continued in pure xylol solution for 2x2 hours. Embedding process was conducted by soaking the tissues in liquid paraffin for 2x2 hours. Paraffin blocked-tissues were cross sectioned with microtome (4 microns). The slides were put on the object glasses that were previously smeared with polylisine followed by incubation to remove the paraffin residues. The slides were then incubated with Mayer's hematoxylin solution for 5 minutes to stain the nuclei in dark and 10 minutes in 0.5% Eosin solution to stain the fibers in red. The slides were mounted with 1–2 drops of Xylene-based mounting media and then were examined with microscope Olympus BX51 to analyze the cartilage repair in tissue level.

Data collection and O'driscoll scoring

The quality of cartilage repair in rats was assessed through O'driscoll scoring method covering five categories (nature of predominant tissue, surface regularity, structural integrity, the adjacent cartilage bonding, and level of the newly formed tissue). This assessment method was frequently used for cartilage analysis in animal studies (Table 1).[17-19]

The primary data were obtained from observation of cartilage repair histopathology with HE staining.

Data analysis

The descriptive data was displayed in the average ± SD or median while the frequency was displayed as percentage. The difference of the dependent variable between experimental and control group was analyzed through nonparametric statistical test Kruskal-Wallis. The difference was considered to be significant if the significance value $p<0.05$.

Turk J Immunol 2018; 6(2):57–64

Table 1. Assessment criteria of O'driscoll scoring for cartilage analysis used in the study

No.	Criteria	Score
1	Characteristics of the Predominant Tissue	(1) fibrous tissue, (2) poorly differentiated cartilage or mesenchymal cell, or (3) hyaline-like cartilage
2	Surface Regularity	(1) completely disrupted if there was severe fibrillation and fissuring, (2) partially disrupted if there were minor fissures or slight amounts of fibrillation, or (3) smooth and intact if there were no fissures or fibrillation
3	Structural Integrity	(1) completely disrupted if there were large horizontal clefts within the newly formed tissue or between it and the underlying subchondral bone, (2) partially disrupted if minor cleavage planes were observed or if fissures extended from the surface to the subchondral bone, or (3) intact
4	Adjacent Cartilage Bonding	(1) no tissue bonding if the tissue at the end of the defect was bonded to the adjacent cartilage, (2) partial bonding if the tissue at one end of the defect or part of the tissue at each end of the defect was bonded to the adjacent cartilage, or (3) completely bonded if the tissue at both ends of the defect were completely bonded to the adjacent cartilage.
5	Level of the Newly Formed Tissue	(1) elevated, (2) level, or (3) depressed

Results

The effect of conditioned media of mesenchymal stem cell in inducing cartilage repair was observed by comparing animals in experimental group with control group within 2, 3, and 4 months. The cartilage repair level was measured by giving scores to five criteria, i.e:

1. Nature of the predominant tissue
2. Surface regularity
3. Structural integrity
4. Adjacent cartilage bonding
5. Level of the newly formed tissue

1. Nature of the predominant tissue

As previously explained, this study involved two factors, namely experimental factor and observation time, which both were potentially affecting each other. Therefore, the Kruskal-Wallis test was done for three factors, i.e. experiment, observation time, and interaction.

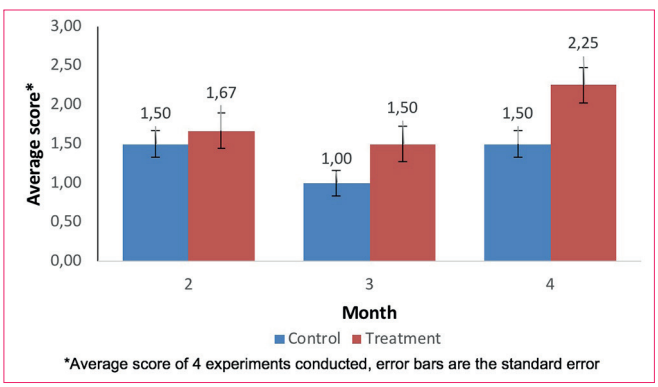

Figure 1. The average score of the patients at 2nd, 3rd and 4th months

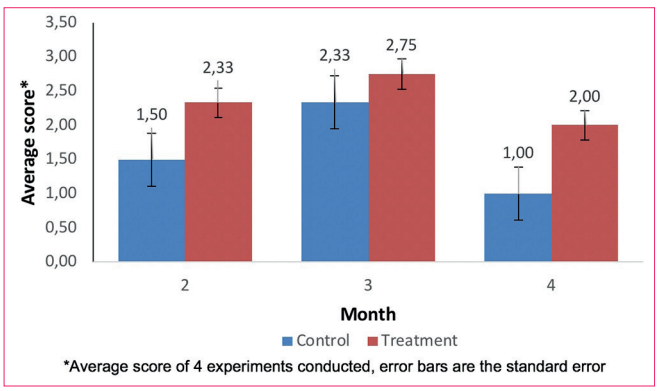

Figure 2. The average scores for surface regularity

It can be seen on Figure 1 that shows the scores. It decreased in the third month of observation, but it increased in the fourth month. The treatment groups (red) have higher score than control that of group (blue), It was found that cartilage was more rapidly healed by administering conditioned media of mesenchymal stem cells within observation time. Compared to the control group, administration of conditioned media of mesenchymal stem cells statistically significantly increased the tissue score at every month of experiment.

2. Surface regularity

The treatment groups had higher score than control group (Figure 2). It shows that the cartilage was repaired by administration of conditoned medium of mesenchymal stem cell. The differences were not statistically significant.

3. Structural integrity

Structural integrity increased with conditioned medium of mesenchymal stem cell (Figure 3). However, the

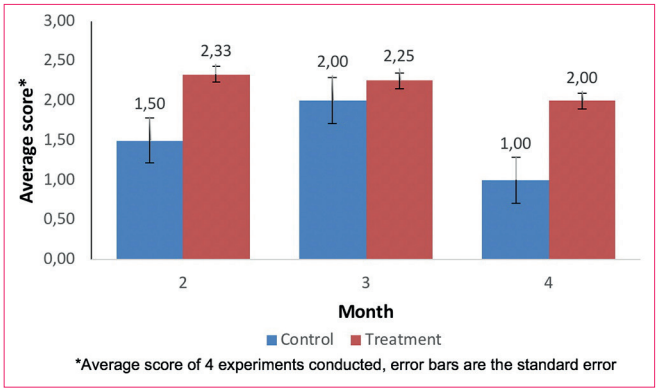

Figure 3. The average scores for structural integrity

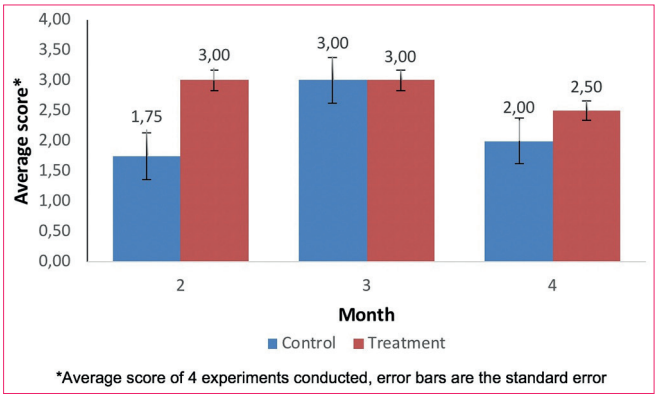

Figure 5. The average scores for level of the newly formed tissue

difference was not statistically significant (p=0.075). Scores decreased in the 4th month of observation.

4. Bonding to the adjacent cartilage

Cartilage bonding was found statistically significantly higher with conditioned medium of mesenchymal stem cell (Figure 4; p=0.028).

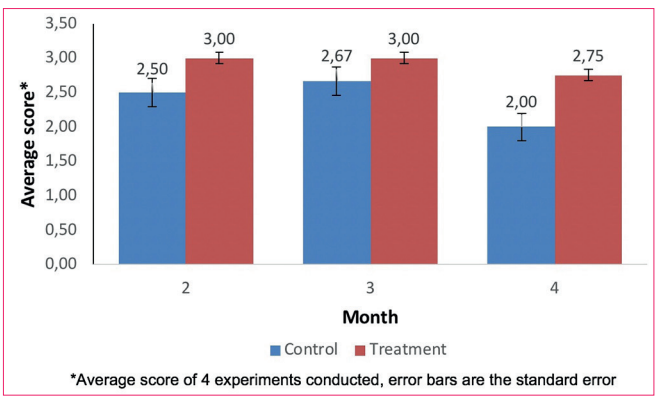

Figure 4. The average scores for bonding to the adjacent cartilage

5. Level of the newly formed tissue

Conditioned medium of mesenchymal stem cell administration statistically insignificantly increased newly formed tissue (p=0.088) (Figure 5).

In the second month of treatment, tissue score was 1.67, surface regularity and structural integrity score was 2.33, bonding to the adjacent cartilage score and level of the newly formed tissue score was 3.00 (Figure 6). In control group, those scores were 1.50, 1.50 and 2.50 (Figure 7).

In the third month of treatment, tissue score is 1.50 and 1.00, surface regularity scores were 2.75 and 2.33, structural integrity scores were 2.25 and 2.00, bonding to the adjacent cartilage scores were 3.00 and 2.00, newly formed tissue scores were 3.00 and 3.00 in treatment and control group respectively (Figure 8 and Figure 9).

In the fourth month of treatment, tissue scores were 2.25 and 1.50, surface regularity scores were 2.00 and 10.00, structural integrity scores were 2.00 and 1.00, bonding

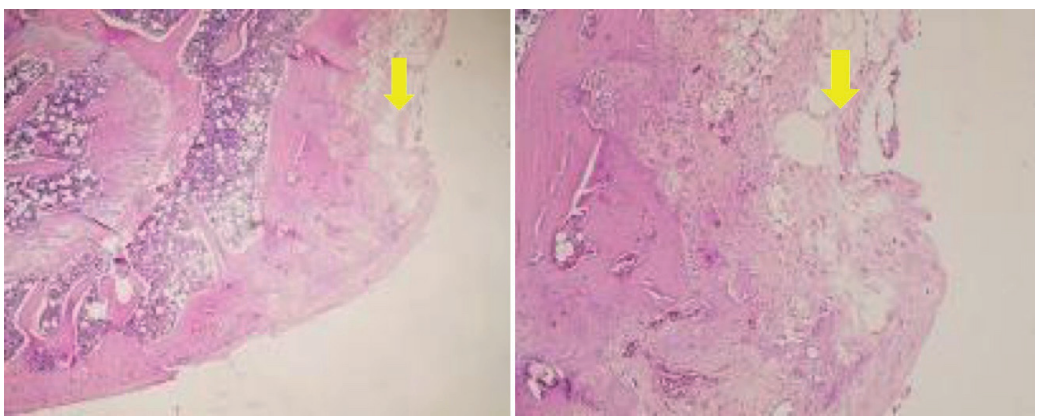

Figure 6. The cartilage healing after 2 months of treatment (MT) Hematoxylin Eosin staining under 4x (left) and 10x (right) magnification according to the O'driscoll score. The yellow arrows show cartilage defect sites.

Turk J Immunol 2018; 6(2):57–64

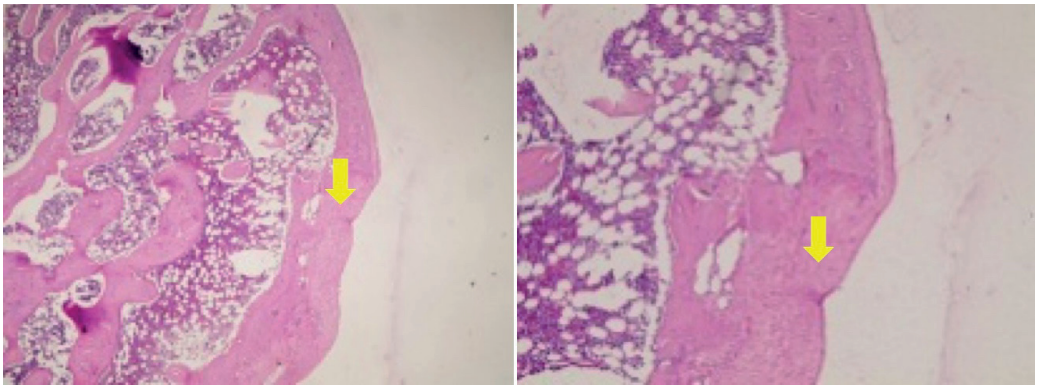

Figure 7. The histology of cartilages after 2 months in control group (MK) with Hematoxylin Eosin staining under 4x (left) and 10x (right) magnification according to the O'driscoll score. The yellow arrows show the cartilage defect sites.

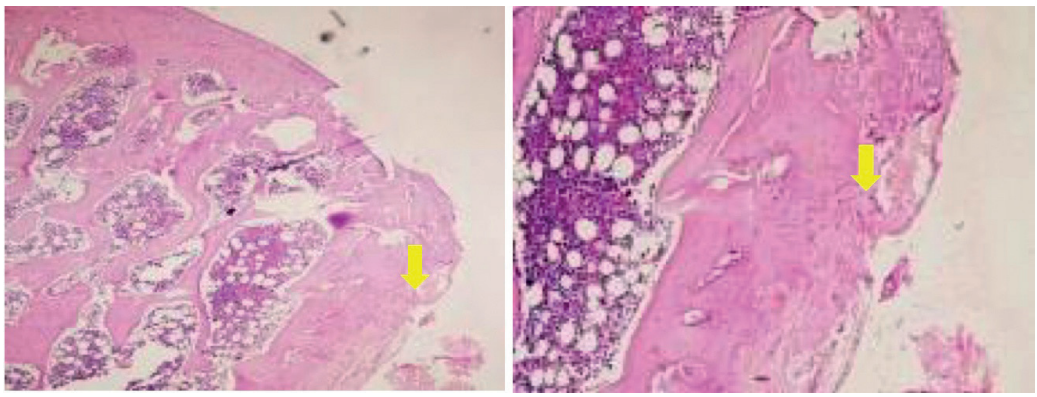

Figure 8. The cartilage condition after 3 months of treatment (MT) with Hematoxylin Eosin staining under 4x (left) and 10x (right) magnification according to the O'driscoll score. The yellow arrows show cartilage defect sites.

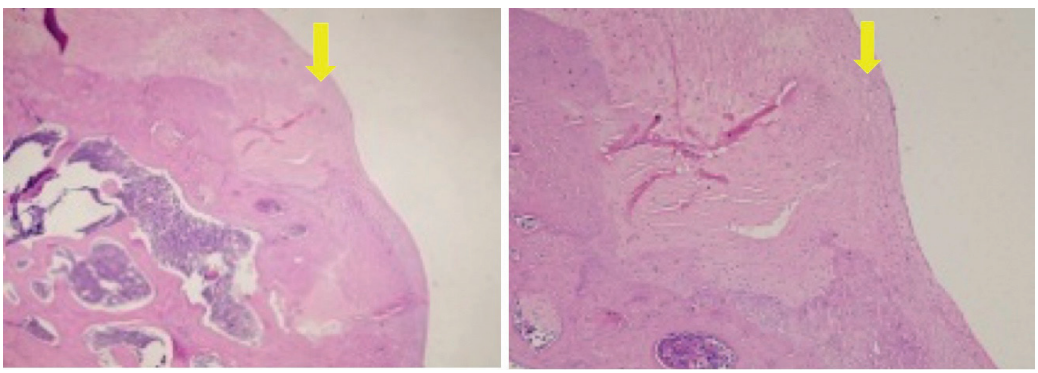

Figure 9. The histology of cartilage after 3 months in control group (MK) with Hematoxylin Eosin staining under 4x (left) and 10x (right) magnification according to the O'driscoll score. The yellow arrows show the cartilage defect sites.

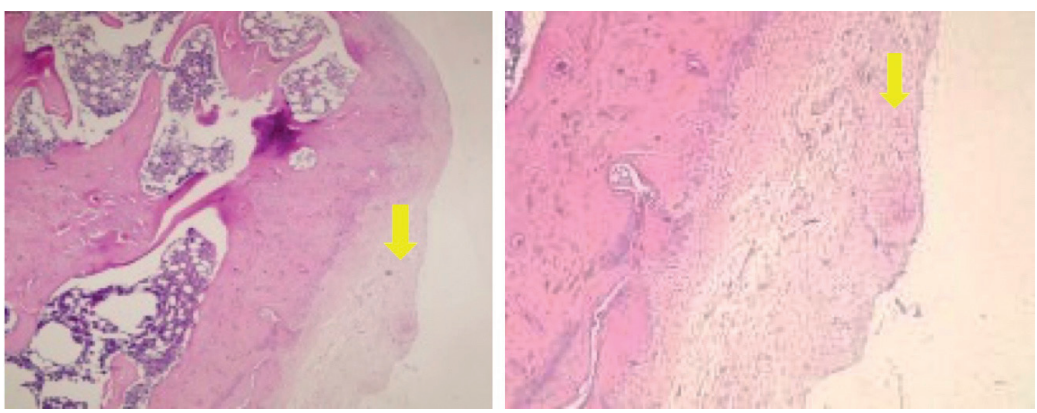

Figure 10. The cartilage condition after 4 months of treatment (MT) with Hematoxylin Eosin staining under 4x (left) and 10x (right) magnification according to the O'driscoll score. The yellow arrows show cartilage defect sites.

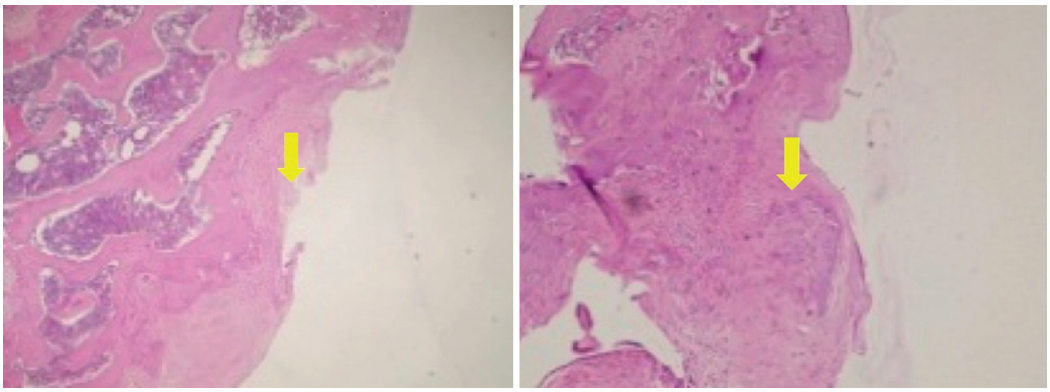

Figure 11. The histology of cartilage after 4 months in control group (MK) with Hematoxylin Eosin staining under 4x (left) and 10x (right) magnification according to the O'driscoll score. The yellow arrows show the cartilage defect sites.

to the adjacent cartilage scores were 2.75 and 2.00, of the newly formed tissue scores were 2.50 and 2.00 in treatment and control groups respectively (Figure 10 and Figure 11).

Discussion

In the study, we showed that conditioned medium of mesenchymal stem cell had healing effect in severed cartilages. However, the difference was not found statistically significant. Previous study on rabbit cartilages indicated better tissue scores in the treatment group that.

Another study examined the effect of injection of synovial MSC in cartilage defect of mice' knee.[20] There were five criteria: full-thickness defect of cell morphology, matrix staining, surface regularity, thickness of cartilage, and integration with native cartilage. It was found that the treatment group showed higher score in surface regularity than that of the control group, although the increase was not statistically significant. In addition, Marquass et al. reported that MSCs gel implantation in defected femoral condyle femur, showed better score in surface regularity than that of control group with statistical significance.[16] In our study, scores in the 2nd, 3rd and 4th month period of observations in the treatment group were higher than of those in the control group, where the highest score was obtained in the 3rd month without statisrtical significance. In control group, decrease scores should be considered to be part of equilibrium process to prevent the hypertrophic changes in articular cartilage.

Mehrabani et al. examined articular defects in twelve rabbits that were given MSCs.[21] It was reported that structural characteristics in the transplant group had higher scores than those of the control group. In accordance, Chung et al. reported that injected blood derived MSCs had positive effect in the repair of cartilage defects in mice.[22] It is found that the treatment group had better histological scores than that of the control group, which were characterized by collagen pattern and cellular arrangement as healthy cartilage articular characters. In our study, we found that treatment groups showed a higher score in structural integrity than that of the control group. However, the increase was not statistically significant.

The adjacent cartilage bonding of sheep that has been implanted with MSC gel shows significant differences compared to the control group without MSC implantation.[16] Similar to our study, there was found significant differences of bonding to the adjacent cartilage score which p-value was 0.028 ($p<0.05$) in treatment group than in control group based on Kruskal-Wallis test.

Giannini et al. conducted study in 48 patients with dome-bone marrow MSC transplant.[23] This study shows that cartilaginous tissue remodeling was marked by the formation of new tissue in all patients after the transplantation of bone marrow MSC. In our study, there was an increase of the newly formed tissue in in treatment group. However, the increase was not statistically significant.

In summary, the treatment with conditioned medium of umbilical cord mesenchymal stem cells has shown a potential for repairing damaged cartilage tissue in Wistar rats. This conclusion was proved by the higher scores of nature of predominant tissue, surface regularity, structural integrity, bonding with adjacent cartilage and the level of new tissue formed in the treatment group compared to control group. Furthermore, the duration of conditioned medium of umbilical cord mesenchymal stem cell should be prolonged to more than 4 months. The optimum doses of conditioned media and the optimum time needed in cartilage repair should be investigated in the further studies.

Ethics Committee Approval: This study has been approved by Ethical Clearance Commission of Gajah Mada University, Yogyakarta, in Mei 6th 2015 (Letter number: 263/KEC-LPPT/V/2015).

Peer-review: Externally peer-reviewed.

Conflict of Interest: No conflict of interest was declared by the authors.

Author Contributions: Concept: BS, MH, HS; Design: BS, MH, HS; Supervision: HS, YF; Resources: MH, HS; Materials: BS, MH; Data Collection and/or Processing: HS, YF; Analysis and/or Interpretation: BS, MH, HS; Literature Search: MH, YF; Writing Manuscript: BS, MH; Critical Review: HS, YF

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