

THE EFFECT OF INJECTION OF INTRA ARTICULAR ALLOGENIC BONE MARROW MESENCHYMAL STEM CELL-PLATELET CELL RICH PLASMA (BMSCs-PRP) ON FULL-THICKNESS ARTICULAR CARTILAGE DEFECT REGENERATION IN RABBIT

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ABSTRACT

The joint cartilage defect full thickness is still a problem today because its current treatment still has not delivered maximum results. Current treatment uses cartilage engineering using mesenchymal stem cells alone and or combining growth factor. The aim of this study was to investigate the effect of intra-articular injection of Allogenic bone marrow mesenchymal stem cell - Platelet rich plasma (BMSCs-PRP) on regeneration of cartilage defect full thickness in rabbits. The design of this study was a post-test only control group design using 36 New Zealand white rabbits divided into three groups. Each group were treated with PRP, BMSCs and BMSCs-PRP. Results were evaluated after 10 weeks. In the evaluation, macroscopic images showed the best healing in the BMSCs-PRP group. Histopathologic examination showed that in the MSCs-PRP group, there was a significant increase in the number of chondrocytes ($p = 0,000$), cartilage area ($p = 0,000$), as well as the number of Agc coprogenitor expectation cells ($p = 0,000$) and collagen type 2 ($p = 0,000$). BMSCs were able to differentiate into chondroblasts which then synthesize aggressive and collagen type 2. Platelet rich plasma (PRP) contains growth factor BMP, TGF, FGF and IGF which can accelerate the occurrence of MSCs differentiation. Intra-articular injections Allogenic bone marrow mesenchymal stem cell (BMSCs-PRP) is able to regenerate and cure full-thickness joint cartilage defects through differentiation of MSCs into chondroblasts.

Keyword: Allogenic, Bone marrow Mesenchymal stem cell, Cartilage, Platelet rich plasma, Full-thickness.

INTRODUCTION

Cartilage of joints is an important part of the joint component. Damage to joints often occurs in sports activities, where some studies show nearly 49% of joint damage are due to sports injuries (Aroen et al., 2004). Its high ability to hold and absorb weight is helpful in resisting the mechanical forces acting on joints during sports activities (Williams et al., 2007).

Cartilage joints are perfect for network engineering applications because of its properties.

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resisting the mechanical forces acting on joints during sports activities (Williams et al., 2007). Cartilage joints are particularly suitable for tissue engineering applications due to their avascular, aneural and alymphatic properties (Khan and Hardingham, 2007). The affected cartilage of the affected joints shows limited healing ability. Damage to joint cartilage that affects the subchondral bone shows signs of healing due to the release of bone marrow mesenchymal stem cells (BMSCs). This is the principle of microfracture (Punwar and Khan, 2011).

Generally mesenchymal stem cells implanted in joint cartilage defects are included in the three-dimensional scaffold. Some issues relating to the use of scaffolds are material selection, the ability to support cell viability and differentiation, retention and degradation problems at the site. Injection of intra-articular MSCs has been performed with satisfactory results on the regeneration of meniscus with osteoarthritis in the caprine model. Injection of MSCs in joints is focused by using carrier scaffold to localize MSCs at the site of the defect. Injection of MSCs with solvent fluid facilitates migration and attachment of MSCs to the site of the defect. Various materials are used as carriers such as hydrogel, fibrin matrix and hyaluronic acid (McIlwraith et al., 2011).

METHODS

This research was true experimental research with post test only control group design. In this study, there were no control treatments, no replication, and randomization.

Platelet rich plasma (PRP) has been used as a treatment for cartilage damage joints. In PRP found platelets with high concentrations. PRP is not immunogenic, can be absorbed by the body (bioabsorbable), sterile, easily prepared and applied to the patient. Platelets consist of β -secretory granules containing transforming growth factor (TGF) -1, insulin growth factor (IGF) and platelet derived growth factor (PDGF) that can stimulate joint cartilage regeneration (Chen et al., 2007).

Currently, there is no study that combined allogenic MSCs and PRPs in cartilage defects, whereas either MSCs or PRP itself has been shown to have a positive effect on cartilage regeneration. Giving PRP to MSCs cultures can increase in vitro proliferation and tendency to increase MSCs differentiation into chondroblasts and osteoblasts (Getgood, 2009). Allogenic MSCs can improve cartilage regeneration and do not cause autoimmune rejection reactions. MSCs have immunosuppressive syphles that allow them to be used allogenicly (Caplan AI and Dennis JE, 2006). In this study, we investigated the effect of intra-articular injection of allogenic bone marrow mesenchymal stem cell-rich plasma platelet (BMSCs - PRP) to the regeneration of full-thickness joint cartilage defect in rabbits.

The experimental unit was divided into three different treatment groups, then evaluated after 10 weeks. This research was conducted for 6 months from May 2014 - October 2014 at Airlangga University's Stem Cell Research and

Development Center Laboratory, Central Installation of Biomaterials and Tissue Bank of RSUD Dr. Sutomo, Integrated Electron Microscope Laboratory Medical Faculty of

Universitas Airlangga, Anatomical Pathology Laboratory, Integrated Diagnostic Center RSUD Dr. Soetomo, Surabaya.

RESULTS

Results of research on in vitro

Isolation, culture and expansion of Bone Marrow Mesenchymal stem cells (BMSCs). To obtain sufficient amount of BMSCs for

injection 2×10^7 was done culture for 2 weeks. This amount is reached on passage 3. The optimal number of BMSCs for mobilization to the defect in a rabbit knee is 1×10^7 per mL.

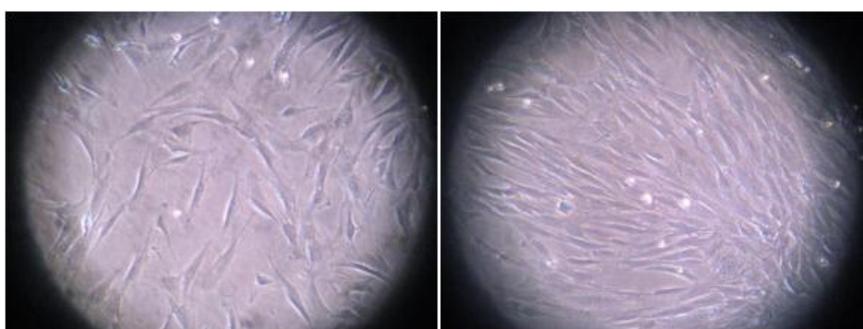


Figure 1. A. Bone marrow BMSCs has reached 40% confluent, neutral filter without staining, B. BMSCs has reached 70% confluent, netral without staining microscope filter

The results of Platelet Rich Plasma (PRP)

The making of platelet-rich plasma (PRP) was performed with two revolutions of 3000 rpm for 13 minutes at 1 and 3000 rpm for 15 minutes at stage 2. The final result of 20 ml of

peripheral blood taken then processed was 2 ml PRP. It was found that the number of RPP was 5 times the number of platelets in peripheral blood.

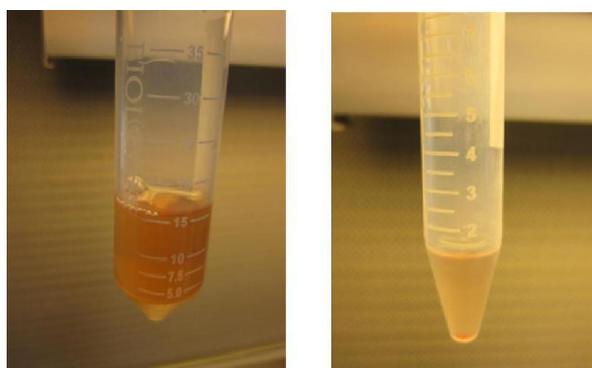


Figure 2. The process of making the PRP. A. Result of centrifuge stage 1. B. Result centrifuge stage 2.

The results of macroscopic examination of the knee joint of rabbits

After sacrifice at the 10th week in the experimental animal then took the knee joint that received the injection BMSCs-PRP. Macroscopically in the evaluation of defects in groups receiving the platelet-rich plasma (PRP) and Bone marrow mesenchymal stem cells (BMSCs). Visible healing defects in BMSCs, although not all closed. The defect had been filled with a whitish cartilage network where the boundary with healthy tissue was still visible. The healing surface of the healing tissue in the defect is also lower than the surrounding healthy tissue. In the knee joint group who received PRP the visible defect clearly. The defects were still

hole-shaped with a bit of healing tissue inside.

In the group receiving BMSCs-PRP, the defect was almost invisible. The defect area was filled with the same whitish cartilage tissue with the color of the surrounding cartilage. The boundary of the healing region with its surrounding was not clear with the same surface height as its surroundings. This shows the best healing in the BMSCs-PRP group. Comparison of microscopic display of each treatment.

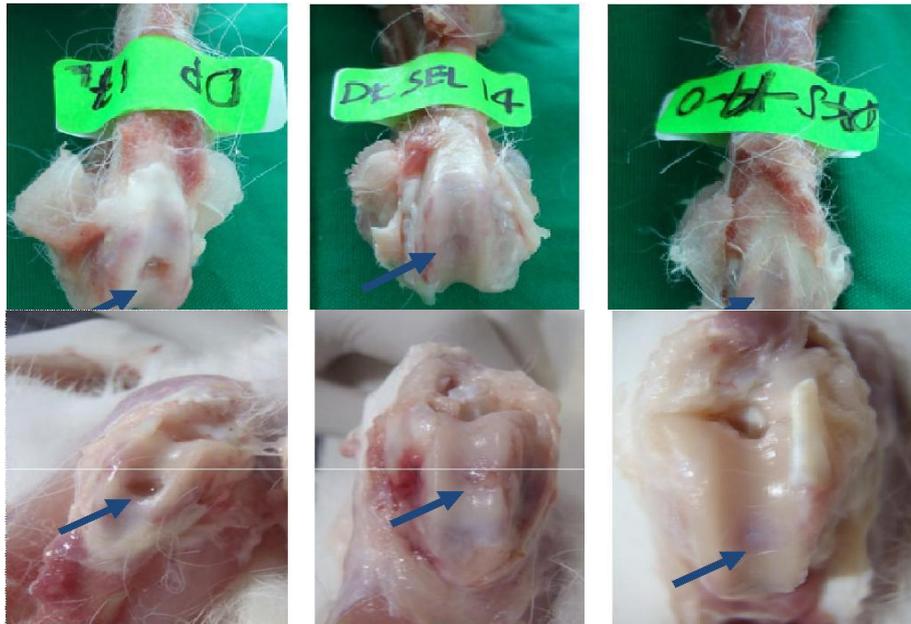


Figure 3. Overview macroscopic defects of the knee joint after 10 weeks. A. Injection of PRP. B. Injection with BMSCs. C. Injection of PRP BMSCs-PRP

Histological examination results

Total chondrocytes

Table 1 shows the difference in number of chondrocytes, highest in BMSCs-PRP group

and lowest in the PRP group. Average number of chondrocytes in the PRP group was 6.1×10^4 , whereas in BMSCs-PRP group was 2.5×10^5 .

Table 1 Number of Condroytes in treatment group PRP, BMSCs dan BMSCs-PRP

Group	n	Number of Condroytes				Anova one-way Statistic
		\bar{x}	sd	min	max	
PRP	10	60655,28 ^a	17058,28	34320,3	86435,3	Stat : 8,82 P= 0,000*
BMSCs	11	130870,44 ^b	26593,54	87645,5	174543,5	
BMSCs-PRP	11	247157,93 ^c	26834,91	198648,4	286842,4	

Note : Significant $\alpha= 0,05$

^{a,b,c}The different superscript show the difference between group (based on multiple comparison LSD)

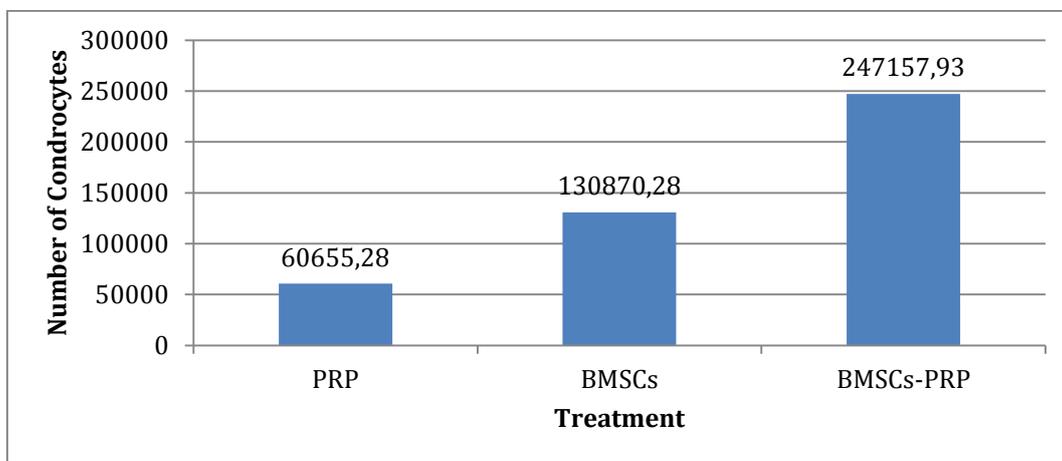


Figure 4. Comparison number of chondrocytes in the three treatment groups

Width Cartilage

Table 2 shows the difference in width cartilage, highest in BMSCs-PRP group and

lowest in the PRP group. Width cartilage in the BMSCs-PRP group was $2,4 \times 10^5$, whereas in PRP group was $8,2 \times 10^4$.

Table 2. Luas cartilage pada kelompok perlakuan PRP, BMSCs dan BMSCs-PRP

Groups	N	Width Cartilage (μm^2)				Brown-Forsythe Statistic
		\bar{x}	SD	Min	Maks	
PRP	10	82774,33 ^a	10834,22	45456,81	77348,74	Stat=3,755 p=0,000*
BMSCs	11	142955,27 ^b	26894,92	108826,5	186354,64	
BMSCs-PRP	11	244646,56 ^c	21100,28	213731,2	278654,36	

Description: * significant at $\alpha = 0.05$

^{a, b, c} of different superscript indicate a difference between groups (based on multiple comparisons Games-Howell)

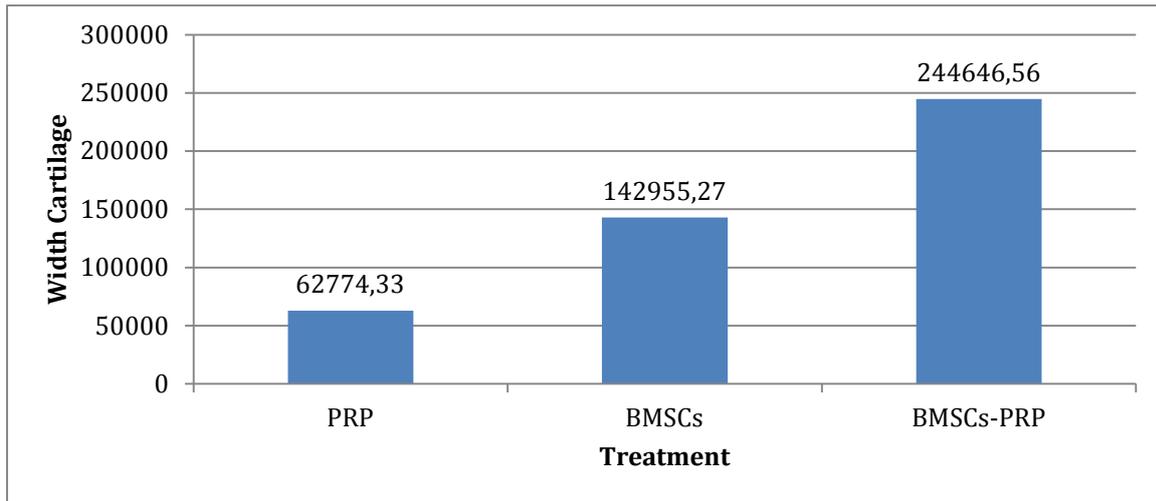


Figure 5. Comparison of the cartilage area in the three treatment groups

Immunohistochemical examination results

Number of agrecan expression cells

Table 3 shows the difference in the number of Agrecan expression cells, mostly in the

BMSCs-PRP group and slightly in the PRP group. The number of cells in the BMSCs-PRP group was 24.9%, whereas the PRP group was 2.7%.

Table.3. Percentage of Agrecan in the treatment group PRP, BMSCs and BMSCs-PRP

Group	N	Agrecan (%)				Brown-Forsythe Statistics
		\bar{x}	SD	Min	Max	
PRP	10	2,70 ^a	0,63	1,65	3,67	stat = 8.81, p = 0.000 *
BMSCs	11	9.77 ^b	1.14	8.64	12.31	
BMSCs-PRP	11	24.90 ^c	2.32	21.12	28.54	

Note: * significant at a = 0.05

^{a, b, c} of different superscript shows the difference between groups (based on multiple comparisons Games-Howell)

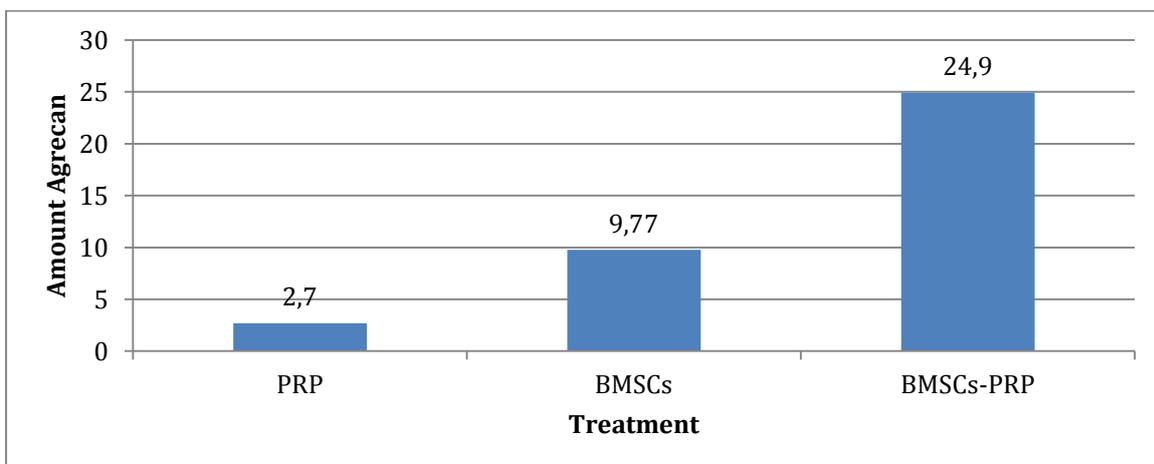


Figure 6. Comparison of the number Agrecan in the three treatment groups

Figure 6 shows the percentage of Agrecan in each treatment group. The results of analysis

by *Brown-Forsythe Statistic* p value = 0.000 which means there is a significant difference

between the number of cells expression Agrecan the PRP group, BMSCs and BMSCs-

PRP.

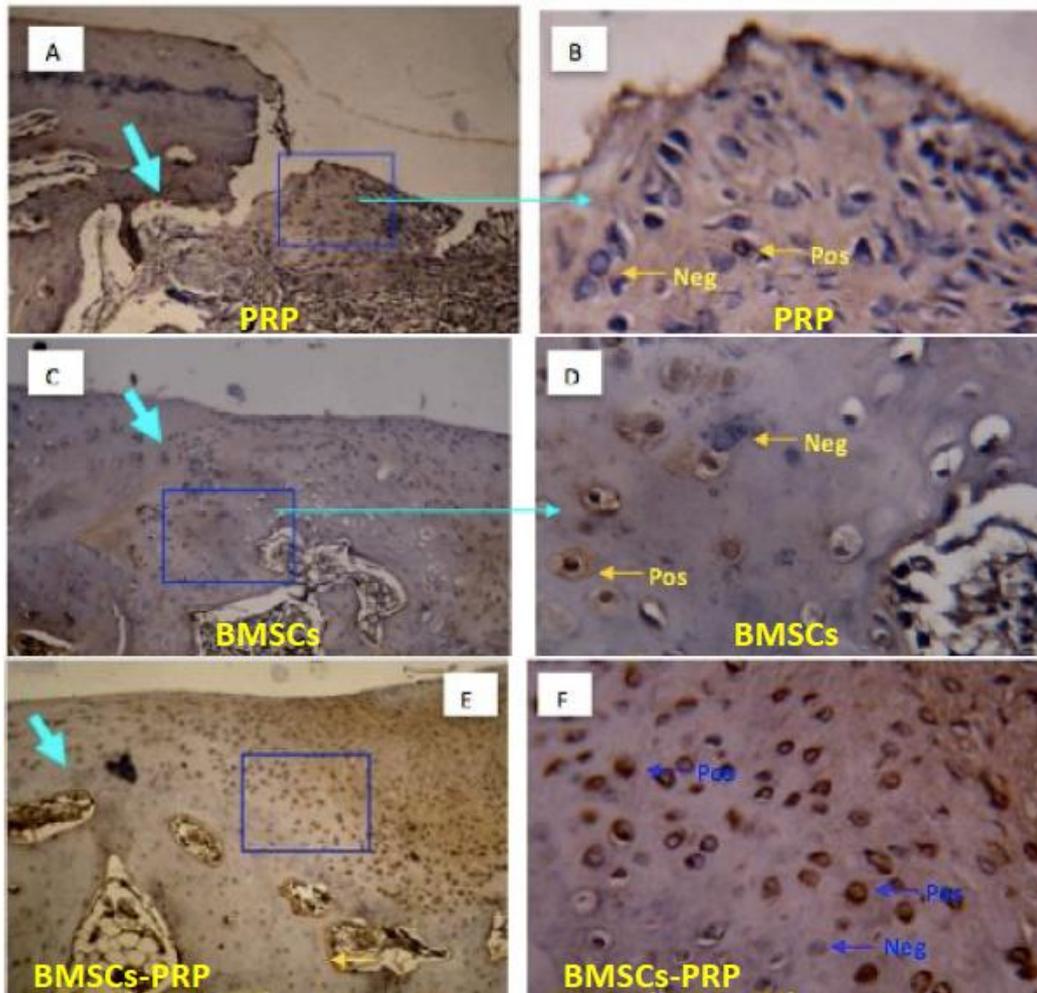


Figure 7 Total expression of Agrecan chondroprogenitor cell defect joints

A and B. Injection of PRP. Shown positive reaction to a brown color, not brown negative. arrows indicate cartilage growth boundary (kondrogenesis). (A, preparations no.8 and B. preparation no.5).

C and D. Injection BMSCs. Arrows indicate cartilage growth boundary (kondrogenesis) defect, which showed positive reaction to a brown color, notnegative.brown (Preparations no.12)

E and F. BMSCs-PRP injection. Arrows indicate cartilage growth boundary (kondrogenesis), which indicates a positive reaction in brown, brown negative.

The Agrecan immunohistochemical examination in the figure above shows the differences in the number and pattern of the Agrecan expression cell. The difference in numbers was seen in the agrecan expression of different cells between tissue matrices in each treatment group using x100 magnification. Based on the figure above, the number of

Agrecan expression cells is found more in the BMSCs-PRP group (Figure 5E) than in the PRP and BMSCs groups. Differences in the nuclear, nucleating, and nucleotide-aging cells of the Aggressive Aggressive expression of the nuclear enzyme in the X400 magnification. In the BMSCs-PRP group (Fig. 7F), the largest number of positive cells compared to the PRP

group (Figure 5B) and BMSCs (Figure 7D)

BMSCs-PRP group and at least in the PRP group. The number of cells in the BMSCs-PRP group was 18.58%, while the PRP group was 2.70%.

Number of collagen type 2 expression cells

Table 4 shows the difference in the number of type 2 collagen expression cells, most in the

Table 4 Percentage of collagen type 2 treatment group PRP, BMSCs and BMSCs-PRP

Group	N	Arecan(%)				Brown-Forsythe Statistics
		\bar{x}	SD	Min	Max	
PRP	10	2,70 ^a	0,52	1,89	3,64	Stat = 8.81 p = 0.000 *
BMSCs	11	8,07 ^b	1,41	5,64	10.02	
BMSCs	11	18.58 ^c	1.07	16.87	20.02	

Description: * significant at a = 0.05

^{a, b, c} different superscript indicate a difference between groups (based on multiple comparisons Games-Howell)

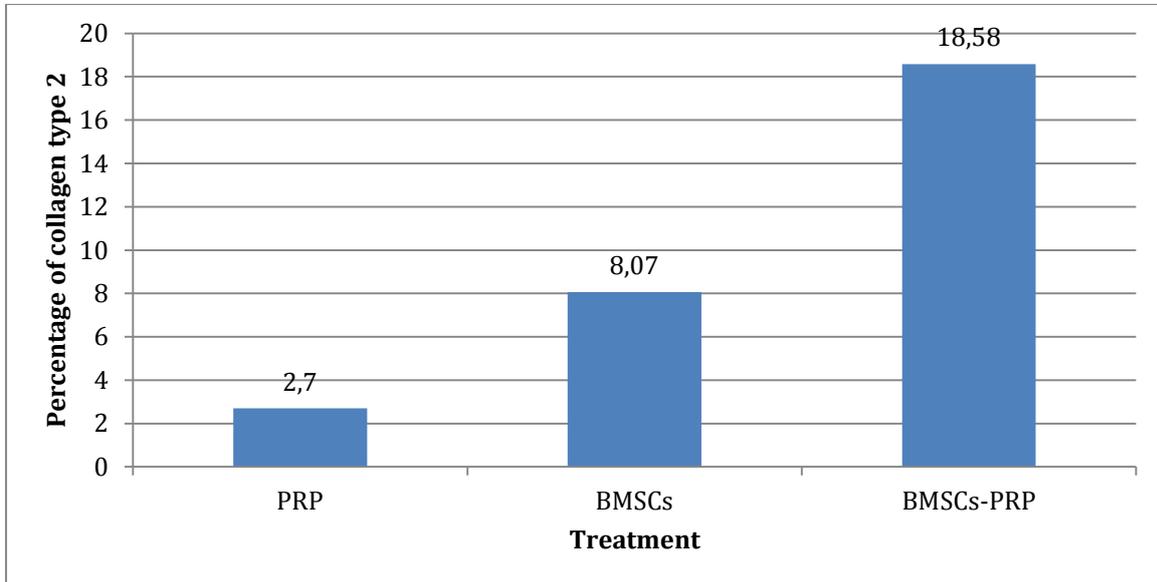


Figure 8 Comparison of type 2 collagen in the three treatment groups

Figure 8 presents the percentage of type 2 collagen in each treatment group. The results of analysis with Brown-Forsythe Statistic obtained p value = 0,000 which means there is

significant difference between the number of cells expression Collagen type 2 in the group PRP, BMSCs and BMSCs-PRP.

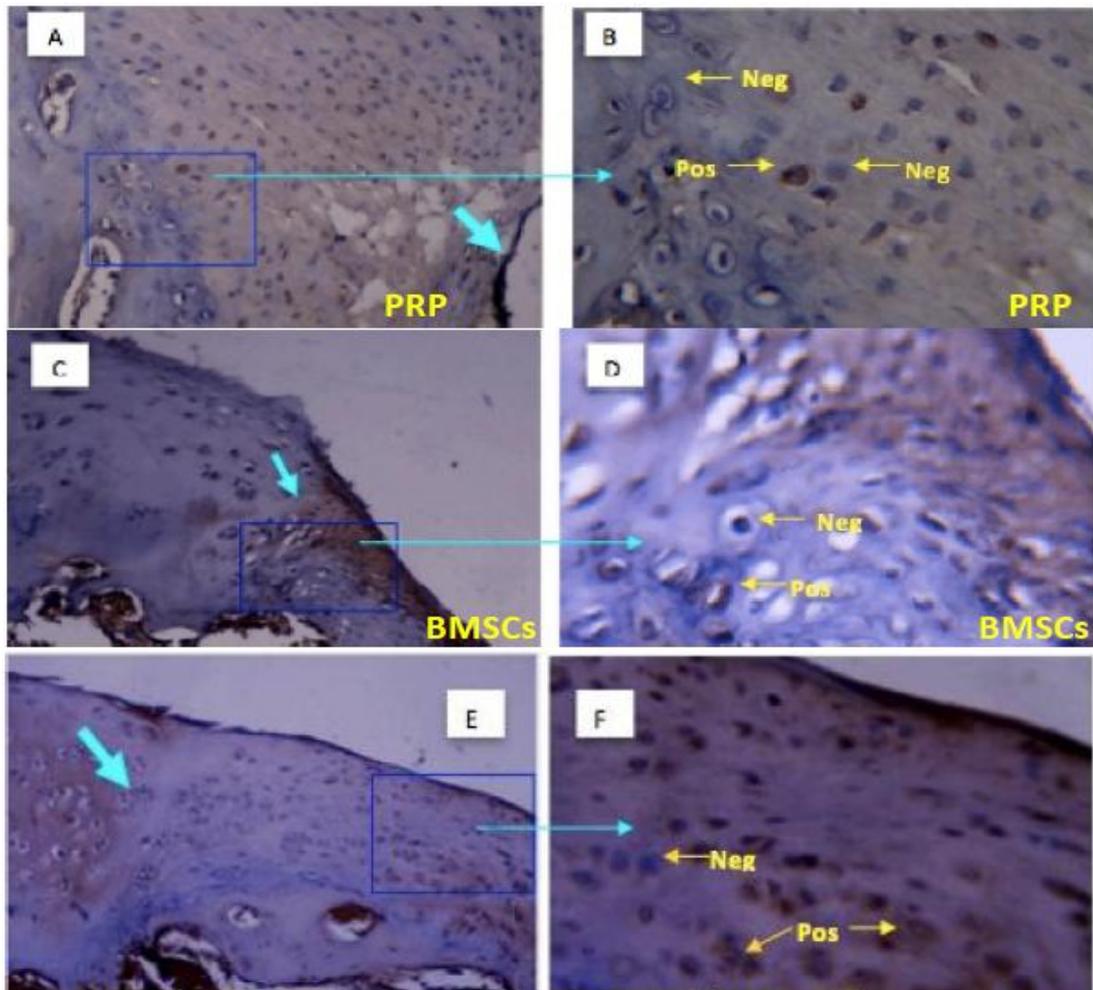


Figure 9 The number of cells condroprogenitorpengekspresi collagen type 2 defect joints

A and B. Injection of PRP. Shown positive reaction to brown, color less negative brown arrows indicate cartilage growth boundary (kondrogenesis). (Apreparation and B stocks no.8 no.5).

C and D. Injection BMSCs. Arrows indicate cartilage growth boundary (kondrogenesis) defect, which showed positive reaction to a brown color, notnegative. brown (Preparations no.12)

E and F. BMSCs-PRP injection. Arrows indicate cartilage growth boundary (kondrogenesis), which indicates a positive reaction in brown, brown negative.

Immunohistochemical examination Collagen type 2 in the figure above shows the difference in the number of collagen type 2 expression cells in each group. The amount difference can be seen in the network matrix in each treatment group using x100 magnification. The number

of collagen type 2 expression cells was found more in the BMSCs-PRP group (Figure 5.5E) than in the PRP group (Figure 9A) and BMSCs (Figure 9C). At x400 magnification, each treatment group expresses a condroprogenitor cell. (Fig. 9B, D and F).

DISCUSSION

Platelet Rich Plasma as a scaffold on cartilage repair

Platelet rich plasma (PRP) in this treatment serves as a scaffold because its 3-dimensional shape, it provide an environment suitable for BMSCs proliferation and differentiation. PRP application is now a rapidly growing method because it is able to provide growth factor easily, safely and effectively. This is evidenced in in vitro where PRP can increase the occurrence of proliferation and differentiation. PRP also plays an important role in the process of bone repair by stimulating migration, mitogenesis and differentiation into osteoblasts and angiogenesis. PRP acts as a vehicle for transport MSCs, so that it can be applied to the development of cartilage engineering techniques. In experiments with rabbits, it was found that subcutaneous PRP injection in seeding with chondrocytes could form tissues such as cartilage at in vivo. Minimal invasive PRP procedure can be applied to an irregular cartilage tissue by mixing the PRP fluid with the MSCs prior the attachment to the defect (Lee KB, 2007).

In PRP the resulting platelet concentration varies depending on the method of manufacture employed. The effect of these various methods of manufacture on the efficacy of PRP is not clearly known. Differences in PRP products (centrifugation, platelet concentration and percentage between leucocytes and erythrocytes) may also be the reason for the difference in outcomes of various clinical applications.

This study used the procedure

according to Rofi'i research (2013), where the process of centrifugation done 2 times with a speed of 3000 rpm for 13 minutes at 1st and 3000 rpm for 15 minutes in the second round. In another study, another commercial system was used in which leukocytes-PRP were classified according to DohanEhrenfest. In this system PRP produced with platelet concentration of about 220,000 platelets / μl . Preparation of PRP consists of a stage where the poor platelet plasma (PPP) was not disposed. The advantage of this process is to avoid excessive manipulation that can lead to platelet stress in the second centrifuge, and avoid removing growth factors that depend on PPP. Another advantage is the closed circuit system in its purification process which causes the procedure to be safer. In this process usually after centrifugation of 8 ml of peripheral blood, platelet recovery > 95% and recovery of leukocyte > 58% (mononuclear cell recovery 93%) in 4 ml PRP. There are also other techniques which not activating PRP prior to injection (no thrombin, CaCl_2) added, which may cause clot fibrin formation, since this activation will decrease the availability of platelets (Gobbi A, 2009).

Multiple growth factors either singly or in combination with cells have been applied to cartilage and demonstrated its ability to stimulate cell proliferation and the formation of matrix in tissue engineering. But there are some shortcomings such as short life span and expensive price. For that reason developed PRP application as the source of growth factor. Granul-á in PRP releases growth factors such as PDGF, TGF- β , VEGF and FGF. In addition,

PRP if compared to whole blood (whole blood) contains PDGF with a concentration of 5.3 times. Contains TGF-dengan at a rate of 7.9 times and contains VEGF at 14.6 times. PRP therefore offers various advantages to accelerate the healing of cartilage obtained from the growth factor levels contained in platelets (Alsousou J, et al., 2009).

Currently, it is not clear whether the PRP effect on cell proliferation and cell differentiation is dependent on the dose of PRP or associated with growth factor concentrations. Various studies of PRP applications on tissues used differences in centrifugation, blood count, number of PRP obtained and growth factor concentrations contained therein (Weibrich G, et al., 2005).

The platelet count levels in this study may provide optimal benefits. The concentration of platelets in PRP did not differ greatly with the Anitua study, which obtained platelet concentration results of about 2.5 times normal. Other studies have shown that an excessively high platelet count can decrease the expected effect (paradoxsal effect). The dose-response relationship between platelet concentration and the stimulated biological process remains unclear. Once the growth factor reaches the targeted receptor surface, the additional growth factor concentration has no effect. Growth factor will decrease the effect if the required upper (high) concentration limit is reached (Gobbi A and Bathan L, 2009).

Differentiation of BMSCs into chondroblasts on cartilage defects

Bone marrow mesenchymal stem cells (BMSCs) show some potential for regenerating tissues. Ito S, et al., Reported in 2012 the results of transplantation of MSCs and hydroxyapatite in rat tibia bones without co-culture. There was good bone formation and cell differentiation to osteoblast-like cells. This shows the osteogenic potential of MSCs. In 2005 Izuta, et al., Examined the ability of MSCs through cultures in meniscus defects of experimental animals, proliferation of cells was obtained and the formation of extracellular matrices were numerous. This shows that BMSCs are an effective method for tissue regeneration (Izuta, et al., 2005; Ito S, et al., 2012).

Several studies have shown MSCs to cure cartilage defects through scaffold as carriers to collect MSCs at the site of the defect. Scaffold is currently widely studied is the use of hydrogel and fibrin-glue. In 2000 Wakitani S, et al. transplantation of MSCs attached to collagen gel on cartilage defects of rabbit joints. Histologic and Immunohistochemical healing results are obtained. This proves that BMSCs can differentiate into various derived cells depending on where they are transplanted (Wakitani S, 2000).

In another study Agung, et al., In 2005 injected BMSCs on the knee joint where ACL, meniscus and cartilage were injured. It was found that BMSCs can move towards tissues that are injured and differentiate into meniscus and cartilage which then synthesize the

network matrix (Agung, et al., 2005).

Murphy, et al., In 2007 performed an injection of 1×10^7 MSCs cells in the animal model knee joints made by osteoarthritis by performing ACL and meniscus resection. Six weeks after the injection on observation found joint cartilage degeneration, remodeling osteophytes and subcondral sclerosis were milder than controls. These results are consistent with this study in terms of the contribution of BMSCs to injured tissue, although this study focuses more on acute trauma such as ACL injuries, meniscus and cartilage (Murphy, et al., 2003).

Healing in cartilage defects with implantation of BMSCs - PRP

This study, the best healing cartilage in MSCs injection group combined with PRP was obtained. Injection of BMSCs alone results in better cartilage cure than PRP ($p < 0.05$). This is seen from the amount of chondrocytes and cartilage area that formed after 10 weeks of treatment pemeberian. Immunohistochemical evaluation also showed that the formation of cartilagous tissue formed was clearly better in the BMSCs-PRP group.

The positive outcomes in this study differ from those of Wilke MM, et. all., 2007 using a combination of MSCs and fibrin matrices to correct defects in cartilage. In this study, MSCs in the fibrin matrix showed benefits obtained only in the initial phase. After 8 months evaluation there was no difference when compared with fibrin alone. In vitro studies found that there was a problem with migration and proliferation of MSCs

when embedded with fibrin. In dilution as in this study will facilitate the proliferation and migration of MSCs to the site of the defect. PRP acts as a diluent liquid to facilitate the spread of MSCs in joints (Wilke MM, et al., 2007).

This study is in line with Kisiday, et.al., 2011 using Intra-articular Injectable MSCs with Hyaluronin (HA) directly into pig joints with partial thickness defects. Histologic and morphological evaluation performed after weeks 6 and 12 resulted in better cartilage recovery than controls (saline and HA solution). In another study to determine the ability of MSCs to improve repair cartilage, intra-articular injection of MSCs was done after microfraktur with drilling defek with 4 mm drill bit. The results showed that MSCs mixed with HA resulted in the best histological improvement (Kisiday, et al., 2011).

Intra-articular injection study of BMSCs on osteoarthritis raised by bone debris and cartilage from osteochondral fragments, there was a significant decrease in postaglandin levels in the synovium fluid. This effect is not found when an injection of MSCs is sourced from fat. In this study there was an increase of tumor necrosis factor (TNF) levels in the synovium fluid. The effects of BMSCs on cartilage improvement are derived from inhibition of catabolic effects and stimulation of anabolic effects through cytokine mediators.

A recent study on horse experimental animals for 2 to 5 years found that BMSCs produce protein core and chondroitin sulfate with longer chain and some shorter protein molecules than chondrocytes. This shows that

BMSCs produce aggrecans with younger phenotype characteristics than those produced by chondrocytes. These results are in accordance with the results of this study where there is an increase in aggrecan content in defects given BMSCs (Kopesky PW, et al., 2010).

Allogenic BMSCs

Allogenic BMSCs are a new hope for healing cell-based cartilage. Allogenic BMSCs allow patients to be injected only once, no need for any 2 action for harvesting cells and implantation. This allogenic technique makes the treatment of defects in cartilage to be reliable, easy to apply and not invasive.

The concept of MSCs injection is based on the potential of MSCs to produce healing to cartilage defects with multiple pathways. MSCs are able to differentiate into target cells and populate new networks. In a previous study it was found that MSCs secrete various cytokines and growth factors that have autocrine and paracrine effects including local immune suppression, fibrosis inhibition and apoptosis in addition to mitotic stimulation and stem cell differentiation. The effect known as the trophic effect is different from the direct differentiation of MSCs into cartilage tissue. It is also demonstrated that mesenchymal stromal

cells present in the body can be reinforced by the paracrine effect of the MSCs themselves (Kaplan AI., 2006).

CONCLUSION

It is concluded that:

1. Intra-articular injections of allogenic bone marrow mesenchymal stem cells - platelet rich plasma (BMSCs-PRP) can increase the differentiation of BMSCs into condroblasts which produce aggrecan and collagen type II on joint cartilage defect fullthickness.
2. Intra-articular injection of allogenic bone marrow mesenchymal stem cell - rich plasma platelet (BMSCs - PRP) results in the formation of more aggressive and collagen type 2 expression cells than only injected BMSCs or PRP alone in fullthickness joint cartilage defects.
3. Intra-articular injection of allogenic bone marrow mesenchymal stem cells - platelet rich plasma (BMSCs - PRP) results in the formation of more chondrocytes and cartilage area than those injected with only BMSCs or PRP alone in fullthickness joint cartilage defects.
4. Giving PRP is able to form a suitable environment for MSCs so that MSCs proliferate and differentiate into more condroblasts.

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