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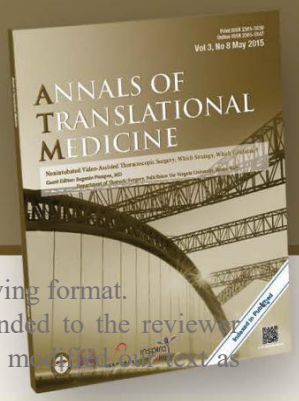


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Peer Review File

Article information: http://dx.doi.org/10.21037/atm-20-2215

Manuscript ID: ATM-20-2215

Title: Hemovac blood after total knee arthroplasty as a source of stem cells

Reviewer A

This study isolated and characterized a group of stem cells from the hemovac blood. These cells formed colonies, expressed MSC surface markers and showed trilineage-differentiation potential in vitro. However, some experiments could have been done to improve the paper quality.

1. Since the hemovac blood was collected from older patients, the biological behavior of the isolated cells could be affected. Therefore, the cell proliferation and migration ability should be assessed and compared with BMSCs from younger patients. The expression of senescence-related gene should also be evaluated.

◈ Reply1 :

We agree with your recommendation. Since the specimens obtained for this study are from older individuals there is a necessity to compare the proliferative and migratory capacity of the BMSCs with those of younger people. However, the patients' age of this study was relatively high as the total knee arthroplasty (TKA) is generally performed on older aged patients. Hence, to obtain BMSCs in younger patients, a new clinical study protocol approval would be needed from IRB. Therefore, to address your comment, we performed another experiment to compare

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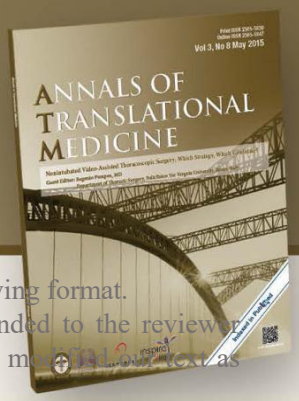


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cell proliferation and migration between HVBC and BMAC. As such, we have added the data in Figures 4B, 4C, 4D, as well as in the revised Methods and Results sections as follows:

◈ Change in the text :

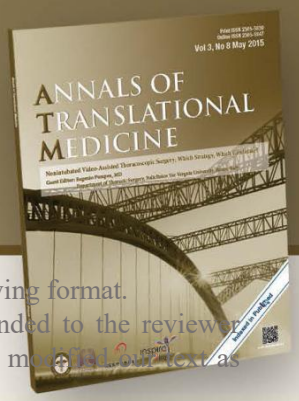
[Methods text]

"*Cell proliferation assay*

Cell proliferation was assessed using the cell counting kit (CCK-8) assay (Dojindo, Japan). Briefly, mesenchymal stem cells isolated from the HVBC and BMAC were seeded at 1 x $10^3$ cells/well in a 96-well plate. The assay was performed from 1 to 7 days after cell seeding. Mesenchymal stem cells (MSCs) from each time point were mixed with 10 uL of CCK-8 solution/well and incubated for 2 h at 37 °C. The cellular dehydrogenase activity of the cells was then measured at 450 nm using a microplate reader. The assay was performed in triplicate." (Page 8-9)

"*Transwell migration assay*

Migration assays were performed in Transwell plates (cat no. 3422, Corning Costar, Cambridge, MA, USA), 6.5 mm in diameter with 8 um pore filters. P2 MSCs (5 x $10^3$ cells) isolated from the HVBC and BMAC were added to the upper chamber in basal medium (αMEM without FBS). After overnight culture, 20% FBS αMEM was added to the bottom chamber. Basal medium served as a negative control. After 12 h incubation at 37 °C, with 5% $CO_2$, the upper chamber of the filters was carefully washed with cold PBS, and cells remaining on the upper side were removed with a cotton swab. Migrated cells through the chamber, or adhered to the lower membrane, were fix in 4% paraformaldehyde. After

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staining with 0.1% crystal violet, the images were observed microscopically. The absorbance of migrated cells were measured at 520 nm after being dissolved in 100% methanol. Each experiment was performed in triplicate." (Page 9)

[Results text]

*"Cell proliferation*

The proliferation of MSCs isolated from the HVBC and BMAC was observed. After day 1 to 7 of culturing, the optical density (OD) of cells increased from 0.087 ± 0.012 to 0.508 ± 0.028 in HVBC, and 0.083 ± 0.010 to 0.552 ± 0.003 in BMAC (average ± SD; Figure. 4B). There was no significant difference between the HVBC and BMAC in OD value from day 1 to 3 (Figure. 4B, $0.05 > P$). Meanwhile, the OD values representing proliferation during days 4 to 7 were significantly different between the HVBC and BMAC (Figure. 4B, $P < 0.05$, $P < 0.01$). The OD values were 0.383 ± 0.022 on day 4, 0.502 ± 0.011 on day 5, 0.519 ± 0.009 on day 6 in HVBC, and 0.451 ± 0.016 on day 4, 0.540 ± 0.016 on day 5, and 0.553 ± 0.004 on day 6 in BMAC. The pattern of increasing OD values for cell numbers between the HVBC and BMAC was similar." (Page 13-14)

*"Cell migration*

Next, the migration ability of MSCs isolated from the HVBC and BMAC was observed using crystal violet staining. The cells from the HVBC and BMAC were morphologically similar (Figure. 4c). The measurement of the OD value of stained cells represents the number of migrated cells. The OD value of 20% FBS αMEM was 1.182 ± 0.024, which was approximately 2.2-fold higher than the control (αMEM without FBS; 0.538 ± 0.045) in HVBC. Meanwhile, the

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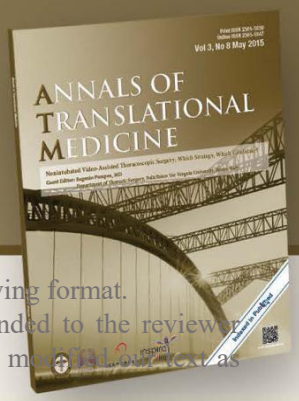


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OD value of 20% FBS αMEM was 1.18 ± 0.019, and approximately 2.3-fold higher than the control (αMEM without FBS; 0.515 ± 0.019) in BMAC. There was no significant difference between the HVBC and BMAC in OD values (Figure 4D, 0.05 > P)." (Page 14)

[Figure text]

"**Figure 4. …**B. Proliferation of cells from BMAC and HVBC incubated for 1 to 7 days and analyzed by CCK-8 assay. The proliferation rate of BMAC was higher than that of HVBC from day 4 to day 7 (# P > 0.05, * P < 0.05, ** P < 0.01). C. Representative images of migrated cells for the HVBC control, HVBC, BMAC control, and BMAC. D. Quantitative analyses of migrated cells in BMAC and HVBC (*** P < 0.001, # 0.05 > P)." (Page 31)

2. Please add the cell seeding density for CFU-F assay. In addition, semi-quantitative analysis should be done for colony numbers.

◈ Reply2 :

We plated 1 mL volume of HVB, HVBC, BMA, and BMAC for the CFU-F assay. We compared the number of colonies in the same volume of hemovac blood and bone marrow before and after concentration. The proliferative capacity of colonies was then compared. We added this data to Figure 2B and the corresponding results to the revised manuscript as follow:

◈ Change in the text :

[results text]

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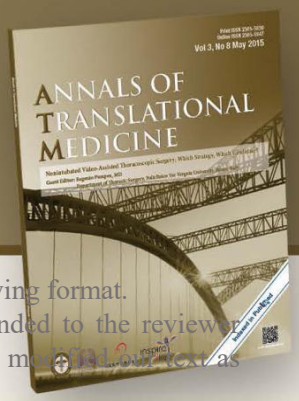


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"The number of colonies were 2.30 ± 1.87 (20 patients) in HVB, and 3.13 ± 1.73 (15 patients) in BMA, with no statistically significant difference (P = 0.365). Meanwhile, there were 14.30 ± 9.45 (20 patients) colonies in HVBC, and 17.07 ± 7.89 (15 patients) in BMAC, without statistically significant differences observed (P = 0.187). However, the number of colonies obtained before and after concentration were significantly different in each group (P < 0.05; Figure. 2B)." (Page 13)

[Figure text]
"**Figure 2.** …B. After 14 days of culture, colonies were counted. The number of colonies in HVB and BMA was not significantly different (P = 0.365). The number of colonies in HVBC and BMAC was not significantly different (P = 0.187). Hemovac n = 20, bone marrow n = 15; # P > 0.05. However, the number of colonies in HVB, BMA (before concentrate) and HVBC, BMAC (after concentrate) in each group showed a significant difference. * P < 0.05." (Page 28)

3. The discussion should be improved. The authors should focus on the translational potential of the HVB stem cells and their advantage or disadvantage compared with other sources of stem cell.

◈ Reply3 :

According to your suggestion, we have provided information on HVB stem cells and their advantages or disadvantage compared with other sources of stem cells as follows:

◈ Change in the text :

[Discussion section]

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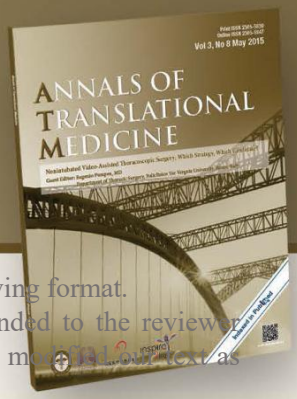


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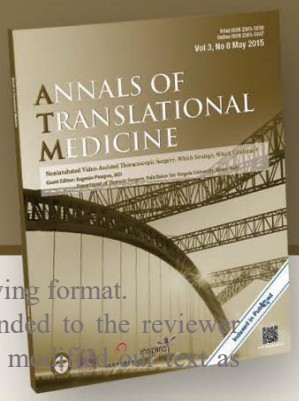
"Bone marrow mesenchymal stem cells (BMSCs) have served as the primary source of stem cells for many years. However, harvesting BMSCs is a painful procedure and they exhibit signs of senescence at an early stage of expansion compared with MSCs derived from other sources [31, 32]. Nevertheless, BMSCs require a relatively short culture period, [33, 34] and many clinical studies using this source are actively being conducted. Alternatively, adipose tissue derived MSCs can be readily isolated [34] with morphological and phenotypical characteristics similar to BMSCs, which are stable during the culture period [35]. Moreover, although umbilical cord blood derived MSCs (UCB-MSCs) have an associated long cultivation period, they exhibit high proliferation capacity [36, 37]. Meanwhile, peripheral blood derived MSCs are easily obtained, which is the reason for their application in many animal studies [37-44], however, they are present at low levels in mononuclear cells [37].

Compared to the other sources of stem cells, HVB can be readily obtained after various bone surgeries, including TKA, without the need for special procedures that can cause additional pain and be met with ethical issues. Although the amount of HVB may vary between patients, a sufficient number of MSCs can be obtained through concentration and cultivation of the cells. Nevertheless, the hemovac must be treated carefully and aseptically following TKA. Should the HVB become contaminated, the hemovac must be removed immediately from the patient to avoid the complications associated with TKA infection. Hence, to be used as a regular source of stem cells, an advanced care protocol for hemovac is required." (Page 16-17)

[Reference 31-44]

31.Berebichez-Fridman R, Gómez-García R, Granados-Montiel J, Berebichez-Fastlicht E, Olivos-Meza A, Granados J, Velasquillo C, Ibarra C: The holy grail of orthopedic surgery:

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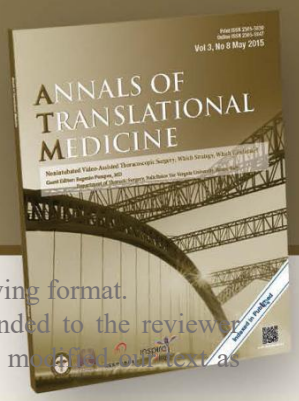


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44. Fu Q, Tang N-N, Zhang Q, Liu Y, Peng J-C, Fang N, Yu L-M, Liu J-W, Zhang T: Preclinical study of cell therapy for osteonecrosis of the femoral head with allogenic peripheral blood-derived mesenchymal stem cells. Yonsei Medical Journal 2016, 57:1006-1015

The discussion on stem cell general characteristics is redundant and is suggested to be removed.

➔ We have removed the text pertaining to the stem cell general characteristics in the Discussion

➔ ◈ Change in the text :

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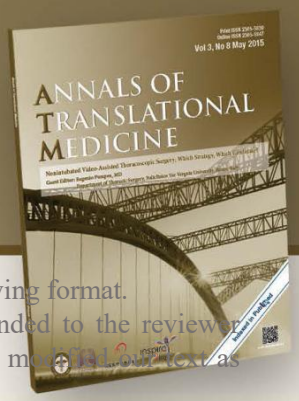


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~~The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has suggested a minimal standard to define human MSCs (31). MSCs have to adhere to a plate and be capable of multi-differentiation. Additionally, positive markers (CD105, CD90, etc.) of stem cells should be expressed with a lack of expression of hematopoietic surface markers (31).~~

~~MSCs have multi-differentiation ability in vitro [45]. MSCs have to adhere to a plate and be capable of multi-differentiation. Additionally, positive markers (CD105, CD90, etc.) of stem cells should be expressed with a lack of expression of hematopoietic surface markers [45]. The cells derived from hemovac blood adhered to the culture flask with fibroblast-like morphology showed multi-lineage differentiation and expressed stem cell markers.~~

~~CD marker analysis of HVB and HVBC showed over 96% expression of positive markers (CD29, CD44, CD90, CD105) of mesenchymal stem cells. These results were very similar to BMA and BMAC and showed no significant difference.~~

Reviewer B

The authors concluded that hemovac blood from TKA patients can be a source of stem cells, however, the HVBCs are mixed blood, and the dose of blood depended on the operator level. Secondly, the mixed blood is more susceptible to be infected due to TKA. Among of HVBCs maybe contains histiocytes, which misleads the results of the experiment. The HVBCs may not be a good source of stem cells.

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◈ Reply :

I agree with your concern regarding the care required to ensure hemovac blood is not contaminated. Accordingly, the following text has been added to the revised manuscript. We have also included a detailed colony count in the revised manuscript, which represent cells attached to the flask and are different from histiocytes. Taken together, these results demonstrate the potential of HVBC as a stem cell source.

◈ Change in the text :

[discussion section]

“Compared to the other sources of stem cells, HVB can be readily obtained after various bone surgeries, including TKA, without the need for special procedures that can cause additional pain and be met with ethical issues. Although the amount of HVB may vary between patients, a sufficient number of MSCs can be obtained through concentration and cultivation of the cells. Nevertheless, the hemovac must be treated carefully and aseptically following TKA. Should the HVB become contaminated, the hemovac must be removed immediately from the patient to avoid the complications associated with TKA infection. Hence, to be used as a regular source of stem cells, an advanced care protocol for hemovac is required.” (Page 16-17)