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# An efficient procedure of isolation, cultivation and identification of bone marrow mesenchymal stem cells

***Vitalie Cobzac,** MD, PhD Applicant; **Andrei Mostovei,** MD, PhD, Associate Professor; **Mariana Jian,** BioD; **Viorel Nacu,** MD, PhD, Professor

Tissue Engineering and Cells Cultures Laboratory
Nicolae Testemitsanu State University of Medicine and Pharmacy, Chisinau, the Republic of Moldova

*Corresponding author: vitaliecobzac@yahoo.com
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## Abstract

**Background:** Bone marrow mesenchymal stem cells (MSC) have a wide application in domain of Regenerative Medicine. Of a great importance is utilization of a suitable bone marrow extraction technique that can provide a sufficient number of MSC to perform laboratory tests without seriously affecting the health of the laboratory animal. At the same time, before using in researches and clinical application, the MSC needs to be identified.
**Material and methods:** The study was conducted in rabbits (n = 9), in which, from one iliac bone, by aspiration were taken 3.39 ± 1.27 ml of bone marrow. The nucleated bone marrow cells were separated through centrifugation using concentration gradient. The specific for stem cells culture medium was used, and MSC were multiplied during 2 passages. From the obtained MSC, $1x10^6$ cells were subject to differentiation by chondrocytes lineage for other 20 days. The obtained chondrocytes aggregates were morphologically examined by Hematoxylin–Eosin staining and specific cartilage staining with Safranin O and Toluidine blue/fast green.
**Results:** There was a strong correlation between the volume of collected bone marrow and the time required to achieve a 70-80% of MSC confluence (p=0.01). Also, the MSC isolated from bone marrow extracted from rabbit iliac bone were differentiated successful on chondrocyte line in all cases, confirmed through the specific cartilage staining with Safranin O and Toluidine blue/fast green (p<0,001).
**Conclusions:** The volume of 3.39 ± 1.27 ml of bone marrow, harvested from rabbit iliac bone is sufficient to obtain a large number of MSC for the laboratory tests *in vitro* and *in vivo.* As a standard method for MSC identification could be used just the capability of the cells to differentiate in the specialized cell, including chondrocytes.
**Key words:** mesenchymal stem cells, bone marrow, rabbits, cellular identification, iliac bone, autocells.

## Introduction

Mesenchymal stem cells (MSC) are multipotent cells that can differentiate into different cell lines depending on the micromedium in which they are stored [1, 2, 3, 4, 5]. These cells had a wide utilization in researches for regeneration of bone tissue, cartilaginous tissue, tendon, meniscus, degenerative lesions of the locomotor apparatus, nervous system and internal organs [6, 7, 8, 9, 10, 11, 12, 13]. Methods of bone marrow harvesting from laboratory animals for isolation and cultivation of MSC for *in vivo* and *in vitro* tests are diverse. Most common method of bone marrow extractions is slaughtering of the animals with further jet flushing of diaphyses and epiphyses of long tubular bones [14, 15]. However, this method allows to use obtained stem cells for *in vitro* tests and allogeneic transplantation for *in vivo* tests. Another way is the aspiration of bone marrow from the metaphyseal areas of the long tubular bones after perforating the bone with a drill bit [16, 17, 18]. It requires a deep anesthesia and a lot of time for the procedure. This method allows experiments on the animals with autocells, but the deep anesthesia, the surgery that certainly will not be the last and the postoperative recovery period may endanger the experiments success due to suffering or even death of the animal [19]. Another way is performing of a short-term superficial anesthesia with local potency and harvesting the bone marrow from the iliac bone [2, 3], a similar procedure performed in humans [20]. A similar method is nucleated cells separation from iliac crest after resection [21], since this method involves extraction and shredding of a bone piece, followed by trypsin treatment, isolation only of MSC is compromised. It is a great risk that the culture can be contaminated with a large number of osteoblasts and fibroblasts, also the time needed for the procedure is bigger [22].

## Material and methods

The study was performed on 9 house rabbits from 4 to 5 months old, 6 females and 3 males, with average weight of 3.78 ± 0.25 kg, in which, from one iliac bone were taken 3.39 ± 1.27 ml of bone marrow, followed by separation of nucleated cells with concentration gradient HiSep LSM 1077 (HiMedia, India). Then, the cells were cultured with mesenchymal stem cell expansion medium HiMesoXL (HiMedia, India) in incubator at 37°C, 5%$CO_2$ (SMART CELL, Heal Force) during the first 2 passages. In order to demonstrate the presence of MSC in culture, the MSC from the 2nd passage were differentiated into chondrocytes using the chondrocyte differentiation medium HiChondroXL (HiMedia, India). The differentiated chondrocyte aggregates were histologically examined by cartilage specific staining. The research on rabbits received a positive decision at the ethics committee meeting of 14.12.2016, No 31.

**Cell media preparation**

Culture medium for MSC was obtained by adding 11.4 ml of component B in 500 ml of component A of the medium for the MSC expansion HiMesoXL (HiMedia, India) and 5 ml of antimycotic antibiotic solution (HiMedia, India) [23].

The medium for chondrocyte differentiation is obtained by adding component B to 100 ml of component A of the chondrocyte differentiation medium HiChondroXL (HiMedia, India) and 1 ml of antimycotic antibiotic solution (HiMedia, India) [24].

The medium was prepared according to the manufacturer's instructions, with further sterilisation by filtration with 0.22 μm pores diameter PES filters (Sofra, China) and stored in the refrigerator at 4-8°C.

**Isolation and cultivation of MSC from bone marrow harvested from the iliac bone**

On the day of bone marrow harvesting, the animals were not fed, after weighing, they were anesthetized by intramuscular injection of 5 mg/kg xylazine and 2 mg/kg diazepam solutions. With a trimmer, the fur was removed from the dorsal part of the basin followed by aseptic processing with betadine and 70% alcohol solution. To potentiate the anesthesia, at the level of the iliac wing were injected 4 ml of 1% lidocaine. Was prepared a 5 ml syringe with 1250-2000 U heparin. A 18 G needle with trocar was used to perforate the first cortical of the iliac bone at the level of iliac wing. After bone perforation, the trocar was extracted and the bone marrow was aspirated with the heparinized syringe [2, 3] (fig. 1). The bone marrow went to laboratory for further processing and the animal was taken back to the vivarium.

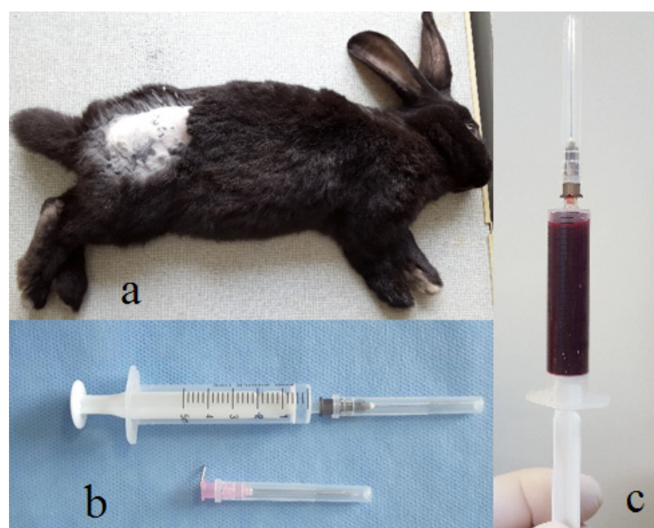


**Fig. 1. Bone marrow harvesting from a rabbit iliac bone. Preparing the animal for bone marrow harvest (a), preparation of necessary tools for the procedure (b) and the harvested bone marrow in 5 ml syringe (c).**

In the laboratory, the concentration gradient HiSep LSM 1077 (HiMedia, India) and PBS (Lonza, Belgium) preventively, were heated at 37$^0$C, in the water bath. In a 15 ml sterile tube the concentration gradient was poured in an equal volume with harvested bone marrow. The bone marrow was shifted in a 10 or 20 ml syringe containing the same volume of PBS (Lonza, Belgium). After homogenization, the PBS with bone marrow have been poured cautiously on the concentration gradient from the 15 ml tube without mixing. The tube was centrifuged at 400 x *g* for 15 minutes followed by removal of upper 2/3 of platelets and adipocytes layer, the mononuclear cells layer was collected in a separate tube along with 1/3 of the remaining overlying layer and the upper 1/3 of concentration gradient layer [25]. Then the tube was filled with PBS, followed by a careful pipetting and centrifuged at 170 x g for 10 minutes (fig. 2). The supernatant was removed and the centrifugation has been repeated after pipetting the cells with 10 ml of culture medium. After centrifugation the cells were resuspended in 5 ml culture medium, placed in a 25 cm$^2$ cell culture flask (Nunc, Denmark). The cells were cultivated in the incubator (SMART CELL, Heal Force) at 37°C with 5% $CO_2$, changing every 2-3 days of a half of the nutrition medium (fig. 3).

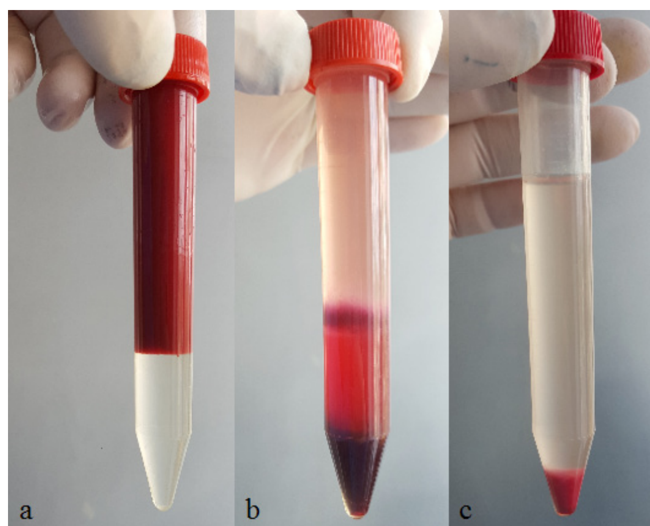


**Fig. 2. Processing of harvested bone marrow. The 15 ml tube with bone marrow mixed with PBS located upon the concentration gradient in a ratio of 1: 1: 1 (a), separation in layers after centrifugation (b) and the bone marrow nucleated cell layer separated in another tube (c).**

After a 70–80% confluence, the attached cells were washed twice with PBS , followed by addition of 2 ml trypsin–EDTA 0.25% solution into the flask. The flask was placed in the incubator for 3 to 5 minutes, after which 2–3 coups were applied to the flask, followed by visualisation under phase contrast microscope to evaluate the detachment of the cells. Trypsinization was stopped with 3 ml of soybean trypsin inhibitor (Lonza, Belgium). The cells suspension was centrifuged at 170 x *g* for 5 minutes. After supernatant decantation, 5 ml of culture medium were added and the cells gently pippeted. The cells were counted with haemocytometer and viability was assessed with 0.4% trypan blue (Sigma, UK). Then all cells were placed in 75 cm$^2$ cell culture flasks (Nunc, Denmark) at a density of 1x10$^4$ ± 1x10$^3$ cells/cm$^2$, with total culture medium change every 2

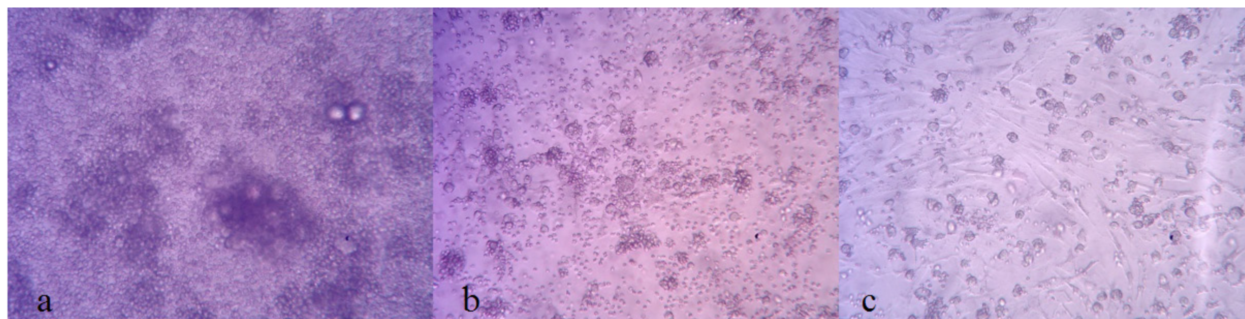

**Fig. 3. Isolation of MSC from bone marrow. x60. Concentrate of bone marrow nucleated cells (a), appearance of fusiform cells attached to the cell culture flask bottom after 2 days of cells culture (b) and the 70–80% confluence of the MSC after 5 days of culture (c).**

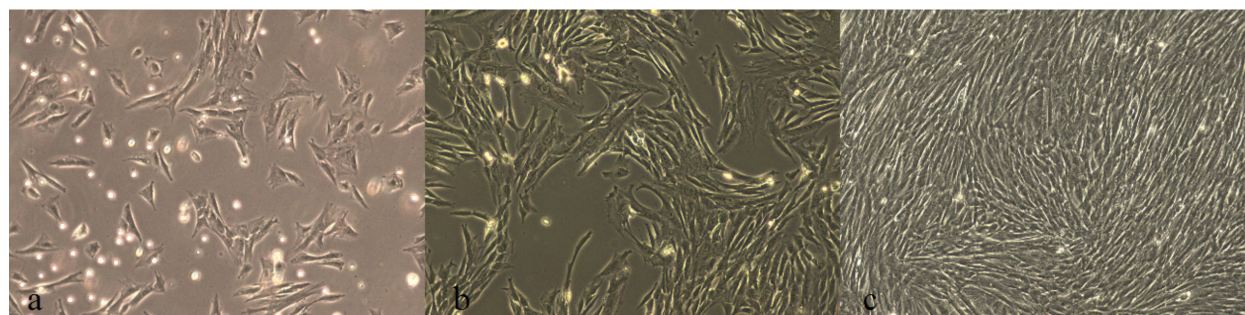

**Fig. 4. MSC culture in the 2nd passage. x60. MSC attached to the culture surface with traces of bone marrow cells after 24 hours of culture (a), CSM on the 3rd day of culture (b) and the 90% confluence of MSC at the 5th day of culture (c).**

days, until a 80-90% confluence (fig. 4). After trypsinization and cells counting with trypan blue (Sigma, UK), from each culture have been isolated $1x10^6$ cells and differentiated on chondrocytes lineage .The remained cells were frozen in concentration $5x10^5$ cells/ml with 10% DMSO (OriGen Biomedical, Germany) for future use.

**Chondrocyte differentiation from MSC**

The MSC differentiation potential is considered to be a functionally reliable criteria for their identification and their distinction from preadipocytes, preosteocytes or prechondrocytes [2, 23, 3]. Was used the chondrocytes line differentiation medium HiChondroXL (HiMedia, India).

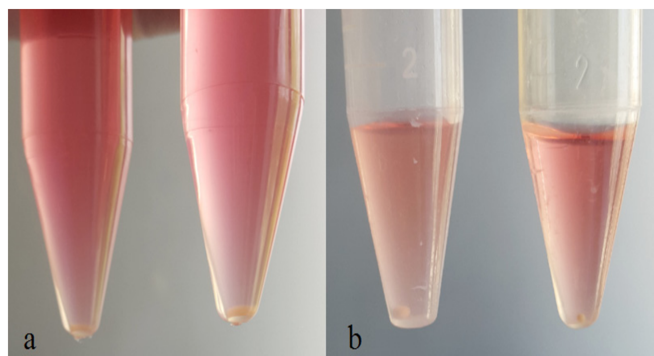

**Fig. 5. MSC differentiation on chondrocyte line in 15 ml polypropylene tubes. The aspect of $1x10^6$ MSC before the differentiation on chondrocyte lineage (a) and chondrocytes aggregates (b).**

In a 15 ml polypropylene tube were introduced $1x10^6$ cells/ml of MSC culture medium. The tube was centrifuged at 45 x *g* for 10 minutes, followed by supernatant elimination and addition of 1 ml of chondrocyte differentiation medium HiChondroXL (HiMedia, India). The cells were gently resuspended in medium and the tube was centrifuged again. The tube was placed into the incubator with a gently opened lid at 37°C, 5% CO2, without disturbing the cell pellets. The chondrocyte differentiation medium was changed every 48 hours for 20 days [17]. On the 5th–7th days of differentiation, at the bottom was observed formation of spherical or oval shape aggregates (fig. 5). After 20 days the aggregates were introduced into 10% buffered formaldehyde and stained with Hematoxylin–Eosin and specific staining for cartilage with Safranin O and Toluidine blue/fast green [26].

The statistical analysis of the obtained data was carried out using Excel and SPSS Statistics 17.0 programs.

## Results

The time needed for bone marrow harvesting from the beginning of anesthesia is 36±3 minutes. During the bone marrow harvesting and after that, complications in experimental animals were not recorded. Once the cells from the 1st passage reached a 70–80% confluence they were trypsinized, thus, the average duration of the first passage cultivation was 7±1 days with a strong correlation between the volume of harvested bone marrow and the number of days required to achieve a 70–80% cells confluency (p=0.01). In Table 1 are presented the results of the MSC obtained from rabbit bone marrow resulted from cultivation in the first 2 passages.

In all cases the 2nd passage was cultivated for 5 days, sufficient time to achieve a 80–90% cellular confluence in all cases. So the average duration of cells cultivation during the first 2 passages was 12 ± 1 days, with a surprisingly 100% cell viability in all cases.

In the process of chondrocyte differentiation, cells aggregates were of irregular spherical shape attached to the bottom of the tube, which later detached and floated freely in the medium. Also in the first days of differentiation, the cells could be easily dispersed by pipetting, but after that they became floating aggregates, the cells were no more dispersed by pipetting. Though, the cells aggregates consisted of the same number of cells, they could have different sizes, which vary between 1.5 and 3 mm in diameter (fig. 5).

**Table 1**

**MSC obtained from rabbit bone marrow resulted from cultivation in the first 2 passages**

| Rabbit body mass (kg) | Volume of harvested bone marrow (ml) | Procedure duration (min) | The 1st passage cultivation (days) | Number of cells from the 1st passage | Number of cells from the 2nd passage |
|---|---|---|---|---|---|
| 3.2 | 4.5 | 40 | 6 | 775000 | 4250000 |
| 3.8 | 2.5 | 40 | 7 | 675000 | 3500000 |
| 3.6 | 4.0 | 35 | 6 | 750000 | 4200000 |
| 3.9 | 2.0 | 30 | 8 | 850000 | 4900000 |
| 3.2 | 1.5 | 35 | 8 | 800000 | 4550000 |
| 3.8 | 4.5 | 35 | 6 | 800000 | 4300000 |
| 3.6 | 5.0 | 35 | 5 | 725000 | 3800000 |
| 3.8 | 2.5 | 35 | 8 | 725000 | 4050000 |
| 3.9 | 4.0 | 40 | 6 | 625000 | 3500000 |
| 3.64±0.27 | 3.39±1.27 | 36±3 | 7±1 | 747222 ±68970 | 4116667± 464354.4 |

At the histological examination with Hematoxylin–Eosin, a rich cellularity of aggregates was determined, highlighted by a high density of cells and extracellular matrix formation (fig. 6).

At Toluidine blue/fast green staining, the obtained structure was intensely colored in purple and blue. As the cells are arranged in conglomerates, their nuclei can not be distinguished due to the overlapping of a large number of them, at the same time the blue color represents the cartilage extracellular matrix synthesized by chondrocytes (fig. 7).

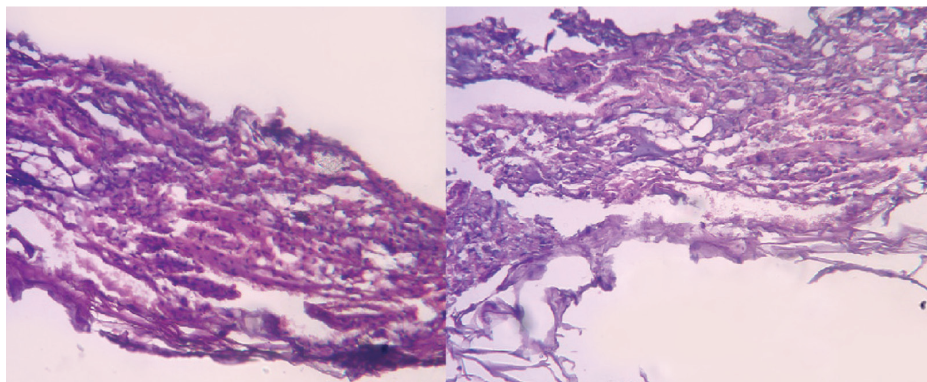

**Fig. 6. Hematoxylin-Eosin staining of the chondrocytes aggregates. x80.**

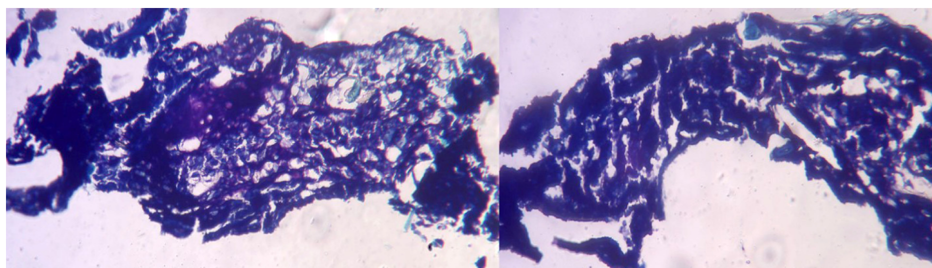

**Fig. 7. Toluidine blue staining and Fast Green of formed aggregates. x80.**

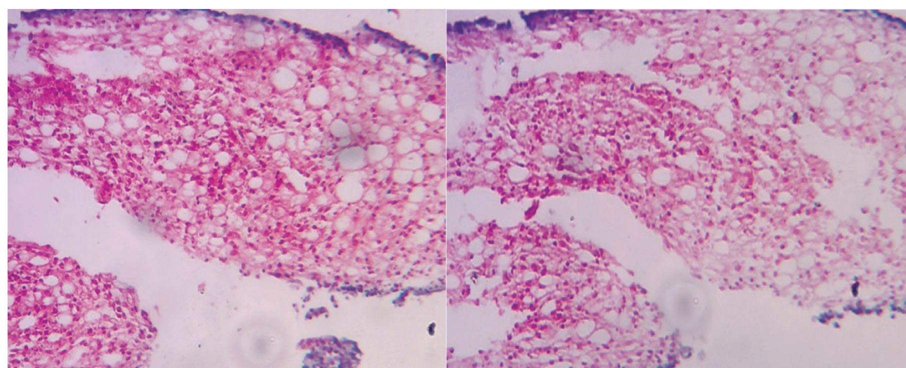

**Fig. 8. Safranin O staining of the cells agregates. x 80.**

After staining with Safranin O, a large number of darkened nuclei were determined and the extracellular matrix secreted by the cells was stained in red, this being specific to cartilaginous tissue (fig. 8).

The identification of obtained cells aggregates was positive at the specific staining for cartilaginous tissue in all cases (n=9) and is statistically significant ($p<0.001$).

## Discussion

Bone marrow harvesting from rabbit iliac bone serves as an effective way to isolate and cultivate mesenchymal stem cells. This method allows performing *in vivo* tests with rabbit own MSC, without subjecting the animal to great suffering which could adversely affect the results of the experiments [19]. Numerous cases of MSC isolation from long tubular bones after rabbit sacrifice are described in the literature [14, 8, 15, 12], or aspiration of bone marrow from metaphyseal areas of long tubular bones, like femur or even tibia [16, 17, 12, 18] and nucleated cells separation from iliac crest after resection [21].

The iliac bone is smaller than the femoral bone, respectively the volume of harvested bone marrow will be smaller. However, the thickness of the iliac bone at the perforation site, in an adult rabbit, ranges between 4.3 and 4.8 mm (fig. 9), respectively the iliac bone can be easily penetrated with a 18G needle and the volume of harvested bone marrow may reach 4.5–5 ml, just from one side (fig. 1), without harming the animal's health and exposing it to risks which can cost us time and money [19]. At the same time, it must be taken into account that the obtained volume of bone marrow is more than enough to obtain in a relatively short period of time, 12±1 days, a number of 4116667±464354.4 cells, which is quite important.

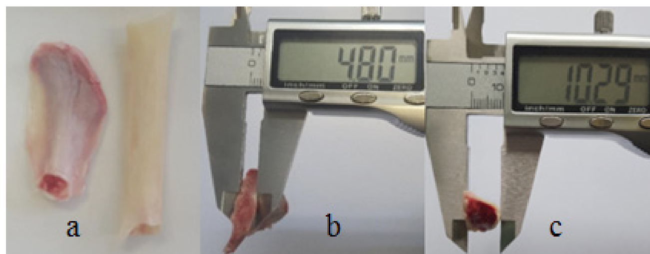


**Fig. 9. The comparative dimensions of the iliac and femoral bones. Rabbit femoral and iliac bones (a). Assessment of iliac bone (b) and femoral bone (c) thickness.**

According to literature data it is known that, in the bone marrow the number of MSC is very small, between 0.01 – 0.001% of total number of bone marrow nucleated cells [27], or 1/10000–1/100000 according to other sources [28, 29, 30]. MSC can be multiplied for 500 times during 50 generations, finally getting billions of cells [29, 30], also, from the 6th passage of *in vitro* culture, MSC lose their stem cells characteristics and the ability to differentiate [28, 30]. In other words, if we continue cultivating the obtained cells further in passages at a density of $8x10^3$ cells/cm$^2$, at the 5th passage we would have over 1 billion cells with differentiation potential. Therefore, even 1 ml of bone marrow taken from a single iliac bone in rabbits, represents a sufficient volume to get a large number of MSC capable to differentiate, for *in vitro* or *in vivo* tests in rabbits, which is groundless denied and ignored in the literature [15, 10, 4, 5, 16, 17, 12].

Stem cells identifying is an important step in working with them. At the moment, the most common ways to identify MSC are RT–PCR, cell differentiation by adipogenic, osteogenic and chondrogenic pathway [2, 3], flow cytometry [29, 3], immunofluorescence microscopy [28, 29]. In our research we identified bone marrow MSC only through chondrogenic differentiation pathway. Therefore, according to the criteria of the International Society of Cellular Therapy, MSC have not been fully identified, but in our opinion this is sufficient, because bone marrow MSC are multipotential cells with differentiation potential in specialized cells. However, the differentiation in chondrocytes line was confirmed by specific staining for cartilaginous tissue with Safranin O and Toluidine blue/fast green [31, 26].

## Conclusions

The volume of 3.39 ± 1.27 ml of bone marrow, harvested from rabbit iliac bone is sufficient to obtain a large number of MSC for the laboratory tests *in vitro* and *in vivo*. As a standard method for MSC identification could be also the capability of the cells to differentiate in the specialized cells, including hondrocytes.

## Disclaimer

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