THE EFFECT OF AUTOLOGOUS SERUM OBTAINED AFTER FRACTURE ON THE PROLIFERATION AND OSTEOGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELLS

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Abstract – The purpose of this study was to investigate the potential of Mesenchymal Stem Cells (MSCs) obtained from patients suffering from fractures to proliferate and differentiate towards osteogenic lineage with the use of autologous serum. In addition the effect of medium supplementation with the use of autologous serum obtained at different time points (patients' admission, first, third and seventh post-operative day) was investigated. In total eight patients suffering from lower limb long bone fractures with mean age of 39 (range 22- 68 years) were included in this study. MSCs were isolated and cultivated in 10% of either Fetal Calf Serum (FCS) or autologous serum. Cellular proliferation was examined by XTT assay and Vybrant assay. The osteogenic differentiation was assessed by total calcium production and alkaline phosphatase production. Cellular proliferation and osteogenic differentiation was significantly statistically higher in patients' serum obtained on admission than in FCS. A negative effect on proliferation was noted with serum obtained on the first postoperative day. Subsequently, both proliferation and differentiation were gradually increased with autologous serum collected during the 3ʳᵈ and 7ᵗʰ postoperatively days. Autologous serum obtained after fracture is superior in terms of proliferation and osteogenic differentiation to the currently used FCS. Surgery seems to have a negative effect on the quality of serum. These findings should be considered in cases where ***ex-vivo*** expansion of MSCs is needed. Recuperation of serum's quality takes place at a later time point within the first weeks after fracture.

Key words: Fracture, autologous serum, MSC biology.

INTRODUCTION

Mesenchymal Stem Cells are highly involved in bone healing and bone regeneration. They are non-hematopoetic, stromal cells that exhibit multilineage differentiation capacity being able to give rise to diverse cells like adipocytes, osteoblasts, chondrocytes, myocytes, tenocytes and possibly neural cells.[25]

Bone healing is a well orchestrated, complex cascade of events aiming at the restoration of the continuity of the fractured bone and finally the remodeling which will allow bone to return to its pre-fracture shape.[5] Signals for this process derive from several cytokines, cells and growth factors which are released during the healing process.[5,27] MSCs are the main recipients of these signals which force them initially to proliferate in order to increase their number and then to differentiate towards osteogenic or chondrogenic lineages.[5,6,27] A large number of animal models have described these cellular events but few data exist in humans concerning the production and release of growth factors in peripheral blood and their exact mechanism of action. Therefore, it is yet unclear whether bone healing represents a locally based biological process or there is also a systemic involvement.

Bone regeneration and tissue engineering with various techniques including scaffolds, slow releasing growth factors, isolation and ex-vivo expansion of MSCs represent modern and attractive ways of treatment.[9,10,14] Till now, various animal models together with the limited

clinical work support the beneficial role that these cells could play for the regeneration of various tissues.[9,25] A major limiting factor for the widespread use of MSCs is their rarity in tissues, especially in trabecular bone and bone marrow. Bone marrow aspirates contain MSCs as less as 0.01 % of the whole number of mononuclear cells.[4,15] In addition, MSCs isolated from other tissues including fat and muscle have been found less prompt to differentiate towards osteogenic and chondrogenic lineages, indicating that their use for bone regeneration is limited.[28]

In the clinical setting, MSCs could be used in two ways. Firstly, as bone marrow aspirates together with all other bone marrow cells [7,8,12,32] or secondly isolated and ex-vivo manipulated.[26,37,38] Both approaches seemed to have favorable outcomes.[7,8,12,26,32,37,38] Bone marrow aspirates with subsequent injection of autologous bone marrow in non-unions have been proven capable to initiate healing and repair of bone defects.[25,32] In femoral head osteonecrosis bone marrow injection plays a beneficial role promoting osteogenesis and angiogenesis.[7,8,12] In addition, in chronic skin wound, it has been shown that autologous bone marrow can accelerate the process of regeneration.[2,39]

MSCs isolated and ex-vivo manipulated is an alternative of bone marrow injections together with diverse applications with the use of scaffolds and growth factors. The healing of bone and cartilage defects as well as tissue engineered bone has been facilitated with this method. [26,37,38] Utilisation of these techniques results is a higher number of MSCs but the choice of serum contained in the expansion medium remains an important issue.

Media commonly used today contain heat inactivated fetal calf serum (FCS). MSCs themselves are low immunogenic and FCS heat inactivation destroys viral parts and micro-organisms. [23,36] Therefore, the risk for transmission of diseases seems to be relatively low but is not inevitable.[25] In addition, FCS cultured MSCs carry fetal calf proteins in high amounts and potential application may have immunogenic effects.[33] In fact, immune reactions have been reported in cells cultured in FCS. [30,37] Furthermore, life-threatening arrhythmias in clinical application of MSCs for cardiomyoplasty seems that have been avoided when human autologous serum was used instead of FCS.[3] These data have forced researchers to develop serum free media or to use autologous serum for ex-vivo expansion. Serum free media seems to be the most appropriate approach but it is not fully explored yet. We believe that autologous serum for ex-vivo expansion is currently the best choice with the lowest risk in the clinical setting. The aim of this study therefore was three fold. Firstly, to assess whether human autologous serum is as good material for proliferation and osteogenic differentiation as FCS. Secondly, to investigate whether there is an optimum timing for autologous serum isolation post-operatively (is late postoperative serum as good as the early post fracture one?) Thirdly, to indirectly evaluate whether there is an associated systemic response contributing to the localized fracture healing process enhancing the proliferation and differentiation of osteoprogenitor cells.

MATERIALS AND METHODS

Isolation and culture of MSCs

Trabecular bone pieces were isolated from 8 patients after written consent and approval from the local ethics committee. All patients had sustained lower limb long bone fractures (femoral or tibial). The average age was 39 years (range 22- 68 years). The bone pieces were weighted and washed thoroughly with PBS (Invitrogen, #14190-094). They were transferred in Eppendorf tubes and 1 ml of collagenase 0,04 % (StemCell Technologies, #07902) was added for 4 hours (37°C, 5% CO_2). Nucleated cells were filtered through a Cell Strainer (BD Biosciences, #352350) and the remaining bone pieces were discarded. The cells were normalized according to the initial bone weight (0,3 gr per flask) and plated in flasks in medium composed of DMEM (Invitrogen, #61965-026), 1% penicillin and streptomycin (Invitrogen, #15140-122) and 10 % of FCS (StemCell Technologies, #06750). When cells reached confluency they were trypsinised (Invitrogen, #15400-054) and splitted till passage 3 (P3). At P3 they were frozen in liquid nitrogen prior further use.

Isolation and preparation of Human Serum

From each donor approximately 15 ml of peripheral venous blood were collected on admission, first, third and seventh postoperative day. (Figure 1) The collection of blood from admission took place 24 hours before surgery in all patients whether the following blood samples were collected early in the morning before drug administration and morning meal. The blood was collected into 10 ml vacutainer tubes without anticoagulant and allowed to clot. Blood was processed within 2 hours of collection while was kept in ice. The clotted blood was then centrifuged at 1500 rpm for 10 minutes and serum was extracted. Serum was aliquoted in Eppendorf tubes (Alpha, #cp5514). Heat inactivation of human serum was not performed. Finally serum was stored at -80°C prior to further use.

Analysis of Proliferation

Cellular proliferation was assessed by two individual methods. Firstly by XTT assay (Roche, #1465015) in which metabolically active cells cleave the modified tetrazolium salt XTT to a water-soluble formazan, which is directly measured by an Elisa plate reader.

Secondly by the vibrant CFDA SE cell tracer kit, (Molecular Probes, #v12883) a fluorescent dye binding to intracellular phospholipids bilayer membranes of cells. This dye is not cytotoxic so is suitable for labeling and tracking cellular divisions.

The medium used to assess proliferation was DMEM containing 1% penicillin and streptomycin and 10 % of either FCS or autologous serum.

i) XTT. Cells was counted and two seeding densities of 4000 and 500 cells per well in triplicates (96 well plate) were used. After cellular attachment to the plastic, the medium was changed and 100 μl of new medium supplemented with either FCS or autologous serum was added. Cells were left to proliferate for three days. 50 μl of XTT dye was added according to manufacturers' instruction. After an incubation period of 4 hours (37°C, 5% CO_2) the plates were read at a plate Elisa reader at 450 nm. The optical densities were proportional to the number of viable cells.

ii) Vybrant cellular division tracking. Cells were plated in 24 well plates in a seeding density of 10 thousand cells per well. After adherence, cells were labeled by 1 μl/ml f vybrant dye over a period of 15 minutes in 37°C, 5% CO_2. The dye was removed and washed thoroughly (4 times) with PBS. The media containing the respective serum was added. During the 1st, 3rd and 5th day following initial plating, the cells were trypsinised and assayed. Propidium iodide (PI, Sigma,) was added to exclude fluorescent dead cells and flow cytometric analysis was performed.

Analysis of Osteogenic Differentiation

Osteogenic induction medium was added in the P3 cells. The medium contained 100 nM Dexamethasone (Sigma, #D1756), 100 μM L-ascorbic acid 2-phosphate (Sigma, #A8960) and 10 mM β-glycerol phosphate (Sigma, #G6251-10G). The serum content of osteogenic medium was 10% of either FCS or autologous serum.

i) Total Ca production assay
Ten thousand cells per well in triplicates (96 well plate) were plated. The osteogenic medium containing the respective serum was added and cells stayed in culture for 21 days. Cells were fed twice per week with fresh medium. For calcium extraction the cells were washed with PBS (no $Ca2^+$, no $Mg2^+$) and 50 μl of 0.1 N HCl was added for 10 minutes. The surface of each plate was scraped and the content was transferred in Eppendorf tubes and mixed in 4 °C for 4 hours. For the quantification of calcium the Cresolphthalein Complexone method (Thermo, #TR29321) was used and values were obtained using an Elisa plate reader.

ii) Alkaline Phosphatase assay
Alkaline phosphatase activity was measured after 10 days of osteoblast differentiation. Cells were lysed and the supernatant was collected. 20 μl of cell supernatant was added to 90 μl of p-nitrophenyl phoshatase (Sigma, #N-7653) and incubated for 30 minutes at 37 °C. The absorption was read with the Elisa reader at 405 nm. Protein content was determined (Protein assay, BioRad, #500-0114) and normalization of ALP was performed.

iii) Prostaglandin E2 assay
The total PGE-2 production of MSCs undergoing osteogenic differentiation was measured. During feeding, the discharged medium was assayed revealing the amount of PGE-2 present (R&D systems, #DE0100). The initial medium content on PGE-2 was considered together with any addition during feeding.

Statistical Analysis
All calculations were done on a personal computer using the SPSS software standard version 13.0 for Windows. Assumption of normality was tested with a one-sample Kolmogorov-Smirnov test. Data are expressed as means (standard error) or median (range) as appropriate. Parametric and nonparametric data were compared using the Student's t test and Mann-Whitney U test, respectively. The cut-off value for significance was p = 0.05.
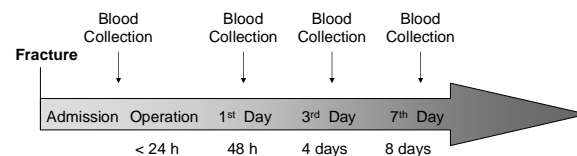

Figure 1. Time points of peripheral blood collection. (In the arrow: time after surgery, Under the arrow: time after injury)

RESULTS

Medium supplementation for MSCs' cultivation with autologous serum isolated from all postoperative days was capable to preserve their biological activity in culture. In order to study the serum's effect on cellular proliferation two different seeding densities were used which we have previously reported that respond differently during proliferation[24]. In high seeding density (4000 cells per well) it was obvious that autologous serum from admission upregulated proliferation by 12%, followed by a drop in the 1st post-operative day where no statistical significant difference was observed compared to FCS. Thereafter, a gradual increase of proliferation was noted during the 3rd and 7th postoperative days (Figure 2). The highest effect on proliferation was obtained at day 7 which was increased by ~21% compared to FCS.
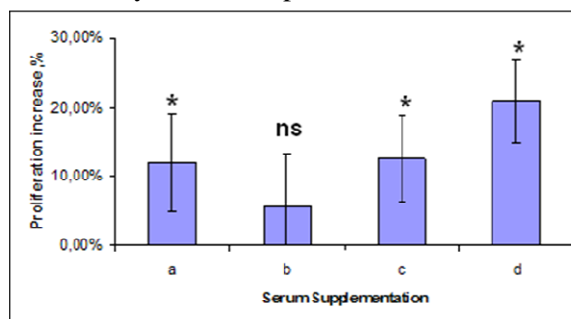

Figure 2. Percentage of enhancement of cellar proliferation in high seeding density compared to FCS (at 4000 cells per well). a. Serum of admission, b. Serum of first postoperative day, c. serum of third postoperative day, d. serum from seventh postoperative day. (*P <0,05, ns Not statistically significant difference)

As far as low seeding density is concerned we could depict higher differences. In particular, serum obtained on the day of admission, enhanced proliferation by 33 % followed by a drop during the first post-operative day. Then, a gradual increase was apparent during the 3rd and 7th postoperative days by 38 % and 48 % respectively (Figure 3). It's of note that the trend was preserved for both high and low seeding densities. Vybrant assay showed the kinetics of MSCs proliferation (Figure 4). It validated the results of XTT revealing therefore that autologous serum resulted in a more even and homogenous proliferation.
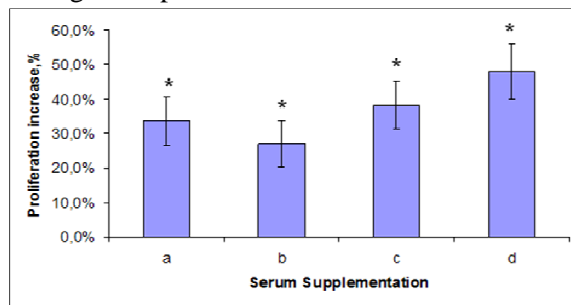

Figure 3. Percentage of enhancement of cellar proliferation in low seeding density compared to FCS (500 cells per well). a. Serum of admission, b. Serum of first postoperative day, c. serum of third postoperative day, d. serum from seventh postoperative day. (*P <0,05)
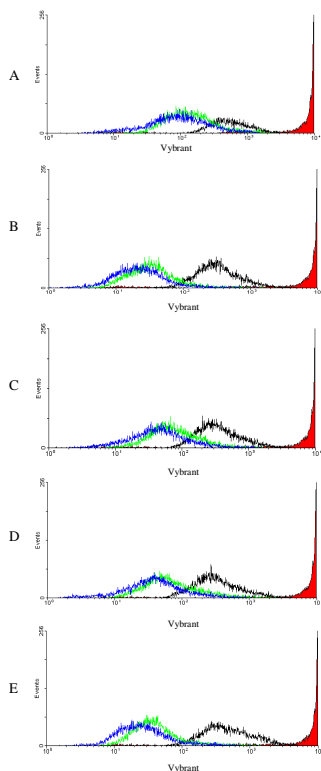

Figure 4. Vybrant assay: Cellular proliferation Kinetics. A. FCS cultured cells. B Serum of admission, C. Serum of first postoperative day, D. serum of third postoperative day, E. serum from seventh postoperative day. Red: day 0, Black: day 1, Green: day 3, Blue: day 5.

Osteogenic differentiation

For the determination of serum's effect on osteogenic differentiation two well validated methods being calcium production and alkaline phosphatase activity were used. Calcium production was found to be increased by 29 % when the serum from the admission day was used. Serum from the first post-operative day had a slightly negative effect on calcium production. Subsequently, an upward trend during the 3rd and 7th post-operative day was noted (Figure 5). The alkaline phosphatase production followed the same trend. It was increased by 45 % with the use of the serum from the admission day followed by a drop during 1st postoperative day and increasing thereafter from 40% to 51% between 3rd and 7th post-operative days (Figure 6).
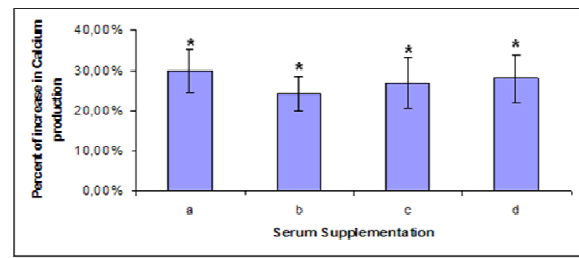

Figure 5. Percentage of increase of calcium content of autologous serum cultures compared to FCS. a. Serum of admission, b. Serum of first postoperative day, c. serum of third postoperative day, d. serum from seventh postoperative day. (*P <0,05)

Figure 6. Percentage of increase of Alkaline Phosphatase activity of autologous serum cultures compared to FCS. a. Serum of admission, b. Serum of first postoperative day, c. serum of third postoperative day, d. serum from seventh postoperative day. (* P <0,05)
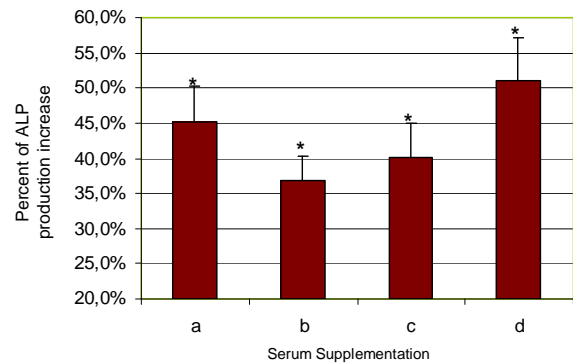
PGE-2 production of MSCs was found statistically significant higher only in cells supplemented with serum from the 3rd and 7th post-operative days reaching values of 130% and 142% respectively as compared to the fetal one

(Figure 7). However, admission and 1st postoperative day serum supplementation slightly increased PGE-2 production without reaching significant difference.
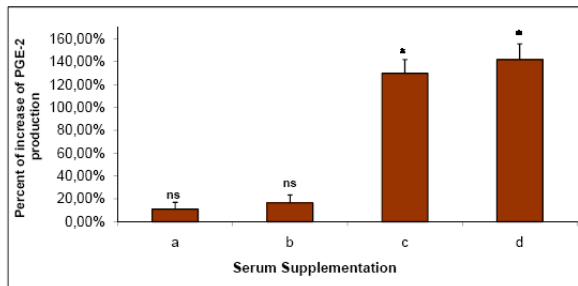

Figure 7. Increase of Prostaglandin E2 production from MSCs during osteogenic proliferation after respective serum use compared to FCS. a. Serum of admission, b. Serum of first postoperative day, c. serum of third postoperative day, d. serum from seventh postoperative day. (*P <0,05 , ** Not statistically significant difference)

DISCUSSION

The need of a high numbers of MSCs for tissue engineering together with their rarity in tissues underscores the need of their *ex-vivo* expansion. Serum supplementation is unavoidable as it is essential for cellular viability. In addition, research on the development of serum-free media is on its way; however, their use is not yet feasible.

A dilemma exists on the most appropriate choice of serum for medium supplementation. On one hand, fetal sera are available and their use is approved for clinical use but they carry risk for immune reactions and transmission of diseases[13]. On the other hand, human autologous serum is the best option but its isolation is difficult.

Cellular proliferation is highly affected by the choice of serum. Several studies have been conducted comparing autologous serum with fetal animal sera. Their results were divergent. Koller et al found that autologous and allogenic serum is equivalent to human plasma, but inferior to fetal sera[19]. Similarly, a number of studies suggested that autologous serum is equivalent or slightly inferior to animal sera but can be used for the expansion of MSCs [20,29,34,41]. On the other hand, some studies suggested that autologous serum is superior to fetal animal sera [11,18,21,31]. Hankey et al presented a 134% increase of MSCs proliferation with the use of autologous human serum[11]. Likewise, Shahdadfar et al found that MSCs proliferated faster in autologous serum compared to fetal bovine serum but the use of allogenic human serum resulted growth arrest and death of MSCs[31]. Our results suggest that autologous serum, isolated from patients suffering from fracture and within the first week from fracture occurrence, has an up-modulatory effect on cellular proliferation. This effect is higher when low initial seeding density is used. We speculate that this effect is due to the presence of circulating growth factors in the serum that have been released in the systemic circulation as a result of the trauma sustained. Zimmermann et al studied the levels of TGF-β1, BMP-2 and BMP-4 after fracture[42]. They observed increase of TGF-β1 serum levels during the first two weeks after fracture, but they were unable to detect BMP-2 and BMP-4. The authors further suggested that serum levels of TGF-β1 can serve as a marker for delayed union[42]. In another study it was reported that circulating levels of IGF-I gradually increase after fracture[35]. It is of note however, that a negative effect in cellular proliferation was observed when media were supplemented with serum of the 1st postoperative day.

Osteogenic differentiation was found to be upregulated with the use of human autologous serum obtained from patients who had sustained lower limb fractures. Our functional assays revealed higher calcium and alkaline phosphatase production being indicative of a higher degree of osteogenic differentiation. Again a negative effect on both assays was detected after supplementation of osteogenic medium with serum of the 1st postoperative day followed by an increase with the use of sera of 3rd and 7th postoperative day. PGE-2 production was higher when MSCs were supplemented with serum of the 3rd and 7th day suggesting a higher osteogenic activity and potentially higher BMP-2 and BMP-7 expression as suggested by recent studies[1,35]. Available studies present again convergent results. Shahdadfar et al presented that although medium supplementation with autologous serum increased proliferation of MSCs compared to fetal sera, it failed to enhance osteogenic differentiation[31]. On the other hand, Stute et al suggested that autologous serum supplementation resulted superior osteogenic differentiation compared to fetal sera.[34]

The finding that the serum obtained on the first post-operative day has the poorest effect on cellular proliferation and osteogenic differentiation is open to a number of interpretations. Firstly, due to the administration of fluids (crystalloids) there may well be a

dilution effect of the serum circulating growth factors which constitute a normal physiological environment for the proliferation and differentiation of MSCs. Secondly, administration of blood products (transfusion) as a result of the blood loss could affect the serum's quality in terms of neutralisation of growth factors secondary to antibody reactions. Thirdly, surgery exerts an immunologic burden and affects host defence mechanisms which could affect the quality of the serum. Finally, administration of agents (drugs) from anaesthesia remaining in the circulation for a period of time after surgery prior to their excretion via the liver or the kidney could be responsible for this finding. It is possible however that all of the above factors could be responsible.

The gradual increase of proliferation and osteogenic differentiation with the serum obtained during the 3rd and 7th postoperative day indicates that there is a systemic recovery from the effect of surgery. In fact, comparing the effect of serum from admission to other postoperative sera, we could see that full recovery takes place in-between the 3rd and 7th day.

In conclusion, media supplemented with autologous serum obtained from patients suffering from fracture are superior to media containing FCS. Addressing the suitable time point for serum isolation it seems that serum obtained after the 3rd postoperative day has better potential for *ex-vivo* expansion of MSCs. In addition, this study supports indirectly the findings of other authors that during fracture healing a systemic response, possibly due to the circulation of growth factors or other stimulatory molecules, takes place contributing to the overall healing process.

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