Supercharging allografts with mesenchymal stem cells in operating room during hip revision

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Background: bone marrow derived mesenchymal stem cells (BM-MSCs) have been proposed to improve allografts used during hip revision. However no study has reported the number of MSCs that could be associated to the allograft and the best technique to load MSCs in allografts. The optimal loading technique should combine methods to increase the initial cell density and create an appropriate environment to accelerate the efficiency of the cell-allograft constructs into clinically applicable grafts. We designed a study to evaluate the number of MSCs in an autograft femoral head considered as the gold standard and to determine the best operating room procedure for loading in allograft with MSCs to approach the same number as in an autograft femoral head.

Methods: For femoral head autograft, we determined with bone marrow aspiration and bone fragmentation the number of MSCs in the living femoral head of patients who had hip arthroplasty for osteoarthritis and compared this number to the number present in the iliac crest of the same patients. Bone marrow for injection in allografts was obtained from residual marrow from patients undergoing surgical procedure with concentrated bone marrow. With this bone marrow (with and without concentration) we tested different techniques (injection and soaking) to load stem cells in allografts of different sizes: bulk allografts, pieces or blocks (8 or 1 cm^3 blocks) and morselized fragments (from 125 to 8 mm^3) or particules (1 mm^3). We also evaluated the release of MSCs from fragments of autografts and allografts loaded with MSCs in cultured medium.

Results: The femoral head autografts contained a lower concentration of MSCs than the iliac crests of the same patient. However in absence of concentration, with bone marrow aspirated from the iliac crest, we were not able to load in the femoral head allograft the same number of MSCs as the number present in an autograft. The loaded volume of bone marrow (and the corresponding number of MSCs) depended of the technique (injecting, soaking), and on the volume, and shape of the allografts. The seeding efficiency of loading MSCs in allografts increased with the concentration of MSCs in the bone marrow. With concentrated bone marrow, supercharging the allograft with MSCs (as compared with an autograft) was possible in the operating room, and the number of MSCs supercharged in allografts was predictable.

Conclusions: The loaded volume of bone marrow depended of the technique (injecting, soaking), and on the volume, and shape of the allografts. With concentrated bone marrow, the allograft could be charged with a similar or higher number of MSCs than the number present in a femoral head autograft.

Supercharging allografts with mesenchymal stem cells in operating room during hip revision

Despite years of ongoing research, reconstruction of large bone defects remains a challenging clinical problem. Classic therapeutic approaches rely on autologous bone graft, which today is considered the 'gold standard' treatment used (for example using the femoral head as autograft for acetabular reconstruction in THA with developmental dysplasia of the hip). However, the use of autograft, which often results in a favorable clinical outcome, is limited in quantity and is associated with complications at the harvesting site. For hip revision allograft femoral head is commonly used. The procedure of bone allografting associated with a reinforcement device has been widely used for the reconstruction of major osteolytic lesions of the acetabulum during revision total hip arthroplasty (THA). However, as many authors have reported, these lesions can present reconstructive challenges and in absence of biologic fixation of the allograft, failure of the reconstruction will occur |11].

Adult mesenchymal stromal cells (MSCs) can be isolated from bone marrow [3,7,15]. These cells are further characterized by their multi-potential capacity for differentiation into osteoblasts [4]. Thus, the homing ability of MSCs to injury sites, their paracrine secretions enhancing cell migration, differentiation, or angiogenesis as well as their immunomodulatory properties make them an ideal cell type to be associated with an allograft to mimic a bone autograft which contains all of the key components required for bone repair, an osteoconductive scaffold, cells with osteogenic potential, and growth factors for osteoinduction and vascularization [3,7,15].

Bone marrow derived mesenchymal stem cell (BM-MSCs) have been proposed with encouraging results to improve union |8,9] and with allografts used during hip revision [2,14]. However no study has reported the number of MSCs that could be associated to the allograft and it has not been evaluated if the association of MSCs and structural allograft could be equivalent to a femoral head autograft in hip surgery, at least according to the number of MSCs that can be loaded in the allograft. Furthermore to our knowledge, no report has described the best solution to load MSCs in an allograft. The cell loading technique in the allograft (as in a bone substitute) may affect the osteogenic activity of MSCs loaded bone grafts because they determine the initial density and spatial distribution of cells in the graft as well as their subsequent behaviors (e.g. proliferation, differentiation, migration).

In animal's experimentation, it has been demonstrated that cells may be loaded by static or dynamic methods. In the static method, a suspension containing cells (soaking technique) is dispensed on a graft or the graft is soaked in the suspension, followed by a period of rest to allow the cells to adhere to the surface of the graft. With this method, the cell density (the number of cells which attached without culturing) in the graft can be increased by increasing the cell concentration of the suspension within a certain range, though at the expense of seeding efficiency (i.e. the percentage of cells that adhere or entered the graft), but probably cannot be further increased beyond a plateau level. In comparison, in the dynamic method, cells are allowed to adhere to the allograft in a dynamic fluid flow created for example by an injection technique. With this method of injection in the graft, the cell agglomeration should accelerate with the cell density in the suspension, but also with the number of injections, thus facilitating the increasing density of cell, and improving the spatial distribution of cells inside the graft. However no such an evaluation has been performed for human allograft, and particularly there is no indication for the surgeon in the operating room.

The optimal loading technique should combine methods to increase the initial cell density and create an appropriate environment to accelerate the efficiency of the cell-allograft constructs into clinically applicable grafts. We designed a study 1) to evaluate the number of MSCs

present in a femoral head autograft as compared with the number present in the iliac crest of the same patient; 2) to determine whether there is a simple readily available operating room procedure for loading with bone marrow aspirate from the iliac crest of the patient a femoral head allograft to approach the same number of MSCs as present in an autograft: We investigated different loading techniques and compared also these techniques on three different allografts sizes (bulk, fragment, morcelized; 3) and in final we also evaluated whether concentration of the bone marrow aspirated from the iliac crest allowed supercharging the allograft with MSCs.

MATERIAL AND METHODS

MATERIAL

1) *femoral heads autografts:* To determine the number of MSCs present in an autograft, we used the bulk femoral head autograft of 12 patients who had THA for osteoarthritis and gave their informed consent to have (at the time of hip arthroplasty) aspiration of bone marrow from the femoral head and from the iliac crest during surgery under general anaesthesia; and gave there informed consent to give the the autograft that was not used during THA as "res nullus" for research. The rationale for the investigation and the accompanying risks were discussed with each patient, and an informed consent form approved by the University Hospital was signed. Bone marrow was collected from the femoral head in at the beginning of THA before cutting the femoral neck and before incision of the skin. At the same time bone marrow aspiration was obtained from the iliac crest of the patient to act as control and to allow comparison with the bone marrow injected in allografts.

2) *Femoral head allografts* were obtained and prepared by a bone bank approved by the national health agency. Twenty-five femoral allografts were obtained from the National Blood and Tissue Services. They were retrieved and packed under sterile conditions, fresh frozen, and stored at -80 degrees C packaged frozen and had undergone sterilization with irradiation using 25KGy. These allografts complied with the European Union Directive 2004/23/EC.32 at the time of revision surgery. The femoral head allograft was thawed in warm saline before being loaded with MSCs.There was no significant difference in terms of age, BMI, and size of the femoral head among autografts and allografts.

3) *Bone marrow* for injection in allografts was obtained from residual marrow from 30 patients undergoing surgical procedure with concentrated bone marrow. BM was aspirated under general anesthesia from iliac crests of patients. After concentration patients received from 30 to 20 cc of concentrated bone marrow according to the indication and the residual that was not used (usually from 10 to 30 cc) was available for this study (and loaded in allografts). Patients gave their informed consent to use this residual for research. Bone marrow used for loading allografts was experimented with concentration and without concentration to evaluate a seeding effect in relation with the concentration of stem cells. Concentration of 5 times (Gambo technology) and 10 times (Celling biosciences technology, Austin, USA) was obtained.

METHODS

1) Evaluation of the number of MSCs in autografts

a) -*With bone marrow aspiration* from the *Femoral head autograft:* Bone marrow was collected from the femoral head of patients at the beginning of Total hip arthroplasty, just

before skin incision through a trochanteric approach under general anaesthesia, the needle was rinsed with a heparin solution, introduced by hand through the femoral head in three different parts of the half proximal part of the femoral head (Figure 1) and 1 mL were aspirated to get the higher number of cells as previously reported .

b) -*With bone fragmentation*

The bone fragments were obtained from the same half distal part (Figure 1) of the femoral head. Fragments were cut in cube of 8 cm3, 1 cm3, 125 mm3, 27 mm3, 8 mm3 and pilled in 1 mm3 particles, and powder. Fragments and particules were plunged in a solution of cultured at 37°C in 95% air and 5% CO2 in alpha-minimum essential medium (a-MEM) with 10 μg/ mL ciprofloxacin (Ciflox 400 mg/200 mL; Bayer Pharma, Leverkusen, Germany) and 10% pre-screened FCS (FCS medium) or 5% PL (PL medium) complemented with 2 UI/mL heparin to avoid platelet gel formation. After 3 days, mononuclear cells were isolated from the supernatant (non-adherent and adherent cells) and viable cells counted to have another evaluation of the number of mononuclear cells and MSCs in an autograft or in allograft. Two femoral heads were also prepared as a cube of 64 cm3 to allow comparison with a bulk femoral allograft head.

c) -*iliac crest:* At the same time bone marrow aspiration was obtained from the iliac crest of the same patient to act as control and to allow comparison with the other group of patients. With use of an established technique that has been validated in previous studies bone marrow cells were aspirated directly into 10.0-mL syringes that had been preloaded with heparinized saline solution (1000 units of sodium heparin in 1.0 mL of saline solution). A smear was made at the time of each aspiration to confirm the presence of nucleated cells and the adequacy of the aspirate. 1-mL samples of marrow were collected at 3 sites of 2 cm distance from each other along the IC.

d)- *Determination of the number of mononuclear cells and MSCs:* Two parameters were measured directly or calculated from the results of cell culture: (1) the bone marrow nucleated-cell count (the number of bone marrow nucleated cells per 1 cc of marrow aspirate); to assess the cell recovery in each syringe, the number of total mononucleated cells was determined by counting marrow smears on a hemocytometer; the prevalence of MSCs per 10^6 nucleated cells estimated by counting the number of colony forming units (CFUs). 2X10^6 cells from the bone marrow aspirate were plated into 25 cm2 tissue culture flasks and the number of colonies containing at least 50 cells following 10 days under standard growth conditions as previously described was evaluated. The concentration of MSCs was calculated for each sample as the product of the nucleated cell count and the prevalence of MSCs.

2) ***Evaluation of loading techniques MSCs in allografts with bone marrow***

This phase of the study was performed to quantify the volume of concentrate bone marrow and the quantity of MSCs that could be loaded with different techniques on different size human fragments of femoral head allografts

a) For injection technique, two injection techniques were tested *for the entire bulk* femoral head: injection through the cartilage (Group A) and through the neck (group B).

- In group A, 10 mL of bone marrow was injected into a fresh frozen femoral head through the cartilage several times at different points (figure 2). After testing different techniques, the maximum of bone marrow that could be injected was obtained with a 10 cc syringe injecting bone marrow from different entry points through the cartilage. Injected bone marrow that flawed outside the femoral head through the neck was kept in a recipient, then re-aspirated and re-injected. The total volume that remained inside the femoral head was calculated as the difference between the volume that was injected and the volume that remained in the recipient. After a plateau was obtained for the volume that could be loaded, the cells in the supernatant were separately counted to evaluate loading by injection until the maximum volume was

obtained. The loaded charge was analyzed as the difference between the number of cells present in the supernatant before and after injecting the allografts. The cells in the supernatant after injecting were separately counted by hemocytometer and recorded as 'remaining cell number'. Then injection was continued with the same marrow and with the same volume of injection (if the plateau was 10 mL, then 10 mL were injected again. The cells in the supernatant after this second volume injection were separately counted to evaluate if the number of loaded cells could be increased by injecting several times the same volume of bone marrow.

- In the group B, (Figure 3) the needle was inserted from osteotomied femoral neck side to proximal. The tip of needle was positioned at subchondral bone area and at the center of the femoral head. 10cc of bone marrow was injected several times, then the leaked volume of liquid was measured and the volume charged into the femoral head was estimated when the plateau was obtained.
- We not able to inject bone marrow in small fragments of the femoral head;

b) the soaking technique was tested on the bulk femoral head, on pieces of femoral head and on morcelized fragments or particles.

- for the bulk femoral heads (Group W) the cartilage was removed and the femoral head shaped as a cube of 4cm square (64 cm3), was soaked and mixed with 20 cc of bone marrow. The volume of absorbed bone marrow by the bulk allograft bone was estimated by the method of displacement of fluid. The loading efficiency was analyzed after contact during 15 mn, 30 mn, and 60 mn.

-For the pieces of femoral head (Group C), the femoral head without cartilage was sliced into quarters of 8 cm3 (Group C1). This quarter of the femoral head then divided into 1 cm^3 blocks (Group C2). The blocked allograft bones were soaked and mixed with the MSCs solution. The volume of absorbed C-BM into the blocked allograft bone was estimated after contact during 15 mn, 30 mn, and 60 mn by the method of displacement of fluid.

- For the morselized femoral head (Group D), the femoral head was morselized into 125 mm3 (Group D1), 27 mm3 (Group D2), 8 mm3 (Group D3) fragments or pilled in 1 mm^3 (Group D4) size using bone mill. These morselized allograft bones were soaked and mixed with bone marrow. The loading efficiency was analyzed after contact during 15 mn, 30 mn, and 60 mn.

3) Evaluation of the loading efficiency with concentrated bone marrow

a) - bone marrow concentrate ((5 or 10 times) was used to evaluate whether there was an initial cell density effect on the quantity of cells that could be loaded in the allograft. The seeding efficiency was calculated as the number of MSCs loaded per cubic centimeter of allograft. The volume of the allograft pieces was evaluated by displacement of fluid measurement and size of pieces by caliper. The loading charge of cells in the allograft was analyzed by comparison of the number of cells remaining in the allograft to the number of cells present in the liquid before and after soaking or injecting the allograft. For the soaking technique the loading efficiency was also analyzed after contact during 15 mn, 30 mn, and 60 mn in the cell suspension. The spatial distribution of cells into the graft (Figure 2C) or at the surface of the graft was evaluated by methylene blue to color the bone marrow.

b)- Evaluation of release of MSCs from allografts loaded with MSCs; Blocks , fragments or particles obtained from allografts loaded with MSCs were plunged in the same culture medium as autografts, and mononuclear cells and MSCs were obtained and counted after 3 days like for autografts.

STATISTICAL ANALYSIS

Each experiment was performed in triplicate. Data are reported as mean $\pm$ 1 standard deviation and the significance level was set at a probability value of less than 0.05. Analysis

was conducted to evaluate the relationship between the set of variables of the different groups. The non-parametric Mann-Whitney U test was used to identify the significance of the differences between groups. The chi square test was used to identify trends within groups with categorical variables.

RESULTS

1) The femoral head autografts contained a lower concentration of MSCs than the iliac crest of the same patient

- *With bone marrow aspiration:* The mean concentration of nucleated cells in the processed bone marrow aspirate samples from the iliac crest was 27.3 ± 14.6 x 10^6 cells/mL (range, 12 to 54 x 10^6 cells/mL). The concentration of nucleated cells in the samples from the femoral head was 13.7 ± 9.8 ·106 cells/mL (range, 5 to 32 x 10^6 cells/mL). The mean prevalence of osteogenic progenitor cells per million cells in the samples from the iliac crest was 40.4 ± 21.9 (range, 29 to 64). The prevalence per million cells in the samples from the femoral head was 25.9± 10.2 (range, 0 to 130). Therefore in the femoral head autograft the concentration of MSCs per 1.0 mL of bone marrow aspirates was average 354±116 MSCs/mL and significantly ($p < 0.05$) lower than in the paired iliac crest (average 1102± 324 MSCs/mL). There was a relationship ($p < 0.05$) between the number of MSCs obtained by bone marrow aspiration in the iliac crest and in the femoral head.

- *There is a relationship between the number of MSCs obtained by bone marrow aspiration and the number of MSCs released by bone fragmentation:* Considering several bone marrow aspirations at the same site in the femoral head, 90% of the total of MSCs that could be obtained after 9 bone marrow aspirations of 1 cc with a 10 cc seringe at the same site were obtained after 3 bone marrow aspiration (figure 4). For MSCs released from pieces of different sizes of autograft the, their number - related to the same volume of autograft bone (1cm3) - increased with fragmentation to reach a maximum number (plateau) for fragments of 2 and 1 mm size (figure 5). The curve obtained suggest than MSCs are released from the superficial part of the bone with a relationship between the increased number and the increased surface when the fragments decrease in volume ($p <0.05$). Considering the fact that increasing the number of aspiration more than 3 times or increasing the fragmentation more than 2 mm did not change the number of MSCs (figure 5), one can consider (from a schematic point of view) than the total number of MSCs present in one cubic centimeter of an autograft is average 700±264 MSCs per cm³, and is 2 times the number obtained by the first aspiration of 2 cc of bone marrow in the femoral head , and also similar to 2/3 of the number obtained by the first 2cc of bone marrow aspiration from the iliac crest. *This number was considered as the target number to load in an allograft*.

2) In absence of concentration, a femoral head allograft could not be charged with the same number of MSCs as the number present in an autograft

The statistical analysis demonstrated no statistically significant difference in connective tissue progenitor concentrations in the iliac crest of patients who had THA with those who had their bone marrow used to load allografts. Bone marrow aspirated from iliac crest of patients who had their bone marrow used to load allografts contained average 30.1±14.2 total nucleated cells/mL (range 14-51) and average 39.1±13.5 MSCs /million cells (range 26-61). Therefore the concentration of MSCs per 1.0 mL of aspirates was average 1173 ±525 MSCs/mL before any concentration.

- *The loaded volume of bone marrow depended of the technique (injecting, soaking), and on the volume, and shape of the allografts*

The mean volume of the femoral allografts was 62.1 ±14.2 mL. The maximum volume of bone marrow charged by injection into bulk femoral heads (20 points of injection) was 6.3±1.47 mL in the group A and 2.5±1.32 mL in the group B. The maximum volume of bone marrow loaded by soaking was obtained in 1 hour. The seeding effect was small in absence of concentration and there was not a significant difference when the loading efficiency was analyzed after contact during 15 mn, 30 mn, and 60 mn. The maximum volume of loaded bone marrow was 2.3±1.1 mL for the whole femoral head (Group W); for the block bone of group C respectively 5.4± 1.5 mL for C1, 9.4±1.8 mL for C2; and for the morselized bone of group D respectively 15.2±1.9 mL for D1, 25.3±2.3 mL for D2, 23.4±2.2 mL for D3 and 21.7 ±2.5 mL for D4. The volume of absorbed bone marrow per mL (cm^3) of allograft was therefore 0.1 (6.3/62.1) mL per 1 cm^3 of bone for group A, 0.04 for group B, 0.15 for group C2, 0.41 for group D2, and 0.04 for group W. The spatial distribution of bone marrow in the graft with the injection technique corresponded to a cone inside the graft from each point of injection. The spatial distribution was at the surface of the graft for fragment or morcelized allografts (soaking techniques): the thickness of the film of bone marrow fixed at the surface of the bone was average 0.3 mm (range 0.4-0.2) and appeared similar whatever the size of the fragment, without penetration in bone (<0.1). The best ratio of bone marrow (that could be loaded) to the volume of bone was 0.41, and was obtained for particles of 3 mm (group D2). Considering the nature of the paste that was obtained, the size of 3mm fragment or particle appeared to give a better consistence than particles of 2 mm or 1 mm where the final composite appeared to be more liquid than with particles of 3 mm.

- *For bulk allografts* therefore, with unconcentrated bone marrow (1173±525 CFU F/mL) the average number of MSCs loaded in the allografts of group A was therefore 117± 53 MSCs per cm^3 of allograft (figure 6) ; 43±21 MSCs per cm^3 of allograft for group B, and was therefore signifiacantly lower than the number that could be aspirated per ml of bone marrow in an autograft.

- *Fragmentation and morcelization of the allograft increased the concentration of MSCs that could be loaded with the soaking technique as compared with bulk allografts*, but this number, even for the best result (479±230 MSCs per cm^3 of allograft for group D2), remained lower (figure 7) than the number present in an autograft (evaluated at 700±264 MSCs per per cm^3 of autograft.

3) With concentrated bone marrow, the allograft could be charged with a similar or higher number of MSCs than the number present in a femoral head autograft

- *The comparison between autograft and allograft* demonstrated that with concentrated bone marrow, a similar (or higher) number of MSCs could be charged in an allograft as compared with an autograft. For example, when the bone marrow was concentrated 2 times the number of MSCs loaded by soaking became greater (figure 7) than the number of MSCs present in an autograft for morcelized fragments of 3mm size or less.

- *The seeding efficiency of loading MScs in allografts increased with the concentration of MSCs in the bone marrow*

There was a seeding effect when the concentration of the bone marrow increased more than 5 times: the cells in the recipient (counted by hemocytometer and number of colony forming units (CFUs) were at a higher concentration before loading than after loading. With the

injecting technique the seeding effect was increased when the femoral head continued to be loaded with concentrated bone marrow after the plateau value of volume was reached. With the soaking technique (figure 7), it increased with the time of contact of the graft with the suspension: the plateau was usually reached after 60 mn of contact, with 90% of the load obtained at 30mn and 70% at 15 mn.

- *When the bone marrow was concentrated 5 or 10 times*, the average number of MSCs loaded in the allografts with the seeding effect (20 injections) was respectively therefore 720 and 1350 MSCs per cm^3 of allograft for group A (figure 7); 875 and 1750 MSCs per cm^3 of allograft for group C; 5700 MSCs per cm^3 of allograft for group D could be loaded after 1 hour of contact.

- *Association of injecting and soaking with concentrate bone marrow allowed supercharging the allograft with MSCs*
When injecting and soaking methods were associated, the number of MSCs that was loaded in the allograft was still increased. For example a surgeon using a femoral head allograft for hip revision can first load the allograft the allograft with the injecting technique. If he needs a fragment and morcelized pieces for the reconstruction, after cutting the allograft, the different pieces should be soaked in the bone marrow to continue to load the allograft. Using figures 6 and 7, the number of MSCs that can be loaded in different situations can be determined. For example when the allograft was injected with a 10 times concentrate bone marrow, and cut in 8 cubes of 2 cm that were soaked during 15 minutes, each cubic centimeter of allograft was loaded with 2166 (1350+816) MSCs. When the allograft was cut in fragments of 27 mm3, 1cm3 of fragments (37 fragments) was loaded with 5310 (1350+3960) MSCs, as compared with 700 MSCs for fragments of the same size coming from an autograft.

DISCUSSION

In recent years, the search for a substitute to cancellous bone autograft in large bone defects has focused on composite materials. Several parameters such as the nature of the conductive material, differentiation state of implanted cells, and the local supplementation in growth factors have been shown to be crucial in bone repair. Owing to the number of parameters to be tested, their relative importance and interactions still require further basic research. The geometry and macrostructural properties of biomaterials have been shown to play important roles: to supply nutrients and oxygen, to allow infiltration of cells and tissue, and to provide pores, channels, concavities, or spaces in which processes leading to heterotopic bone formation can occur. At this moment none of these biomaterials replicate exactly an allograft. Developments in allograft technology have primarily addressed issues pertaining to graft sterility, storage, and longevity. Enhancing incorporation of these grafts with the host bone, and improving the ongoing repair and remodeling of these grafts in vivo remain important challenges. By sterilizing and preparation process, allograft bone acts mainly as an osteocondutive matrix. There is no progenitor cells, and few osteoinductive properties. Many authors reported low incorporation rate of grafted allogenic bone. However, as the bone marrow can contain the osteoprogenitor, osteoinductive properties, it seems quite logical that the composite allograft-autologous bone marrow can improve allograft properties. Several studies have tested with success the possibility to culture and to get expansion of MSCs on allograft, but to our knowledge, none has tried to evaluate the number of MSCs present in an autograft and none has tested the possibility to load with the same number an allograft in the operating room.

We compared the seeding efficiency of loading an allograft with the technique of injection or soaking, or with association of the two techniques; 3 different types of allografts were tested according to their size: bulk, fragmented or morcelized. We also tested seeding efficiency of the initial cell density used to load the allograft (changed by bone marrow concentration (concentrated 2, 5, and 10 times), and observed increased seeding efficiency with increased concentration; this phenomenon may be explain by adherence of cells between themselves. Regardless of the initial cell densities, the MSCs-allograft constructs showed continued increase in cell density when the time of soaking increased from 10 minutes to one hour, with approximately 70% of the cells loaded in 15 minutes, this time being acceptable for the surgeon. In this study, regardless of the initial cell densities, we found that when comparing soaking and injecting techniques, the MSCs-allograft constructs achieved lower plateau values of stem cells with soaking technique on bulk allograft, but higher values on morcelized allografts with the same technique. The association of soaking and injecting increased the cell density in MSCs-allograft constructs, achieving a higher cell density than each technique alone and therefore when possible is the optimal one among the three methods studied here. However injecting cells in the femoral allograft is only efficient when it is performed on the whole femoral head with cartilage, and the cell suspension is injected through the cartilage.

With the hypothesis that the gold standard is the autograft, we found that that the femoral head contain less MSCs than the iliac crest of the same patient, when evaluated by bone marrow aspiration, or when bone marrow mononuclear cells (BMMNCs) were isolated from the supernatant of spongious bone fragments. We are not aware of any prior studies evaluating osteogenic progenitor cells from the iliac crest and femoral head of the same patient. This study has confirmed that the quantity of osteogenic progenitor cells is greatest at the iliac crest compared with the femoral head. Although the number of MSCs is higher in the iliac crest, it seems difficult without bone marrow concentration to load a femoral head allograft with a similar number as in an autograft. However we were able to demonstrate that in the operating room it is possible to supercharge an allograft with the same concentration of MSCs that are present in a femoral head autograft, as soon as the surgeon uses the correct technique of loading according to the size and shape of fragment allografts, and as soon the bone marrow is concentrated. In many circumstances the concentration may be not only similar but higher than in the femoral head autograft.

The favorable effect of injecting MSCs in bone before cutting fragments in the femoral head may be attributed to three factors. First, the injection generated fluid flow in the construct, which enhanced cells transfer and improved the cell distribution: our analysis suggested that sufficient flow fluid (with several injections) can be generated in bone tissue allograft to load stem cells. Second, the hydrodynamic condition due to injection might promote physical cell-matrix interaction, with some cells being stopped by the porosity of the system. Third at the difference of the soaking technique that could only load cells at the surface of the MSCs-allograft constructs, the injecting technique is able to load cells deep in the bone. The injection through the cartilage is more efficient than through the femoral neck cut. This can be explained by several reasons; the difference of bone density [6] in a femoral head with a low distal density at the neck side than in the subchondral zone and therefore more leakage from femoral neck side when the trocard is inserted in the neck; when the needle is inserted in the cartilage, the elasticity around the needle works as a barrier; we observed that no liquid leaked from the cartilage, and that the leakage was always at the femoral neck side.

Considering morselized or fragmented bones, only the surface of the bone was loaded with stem cells (no penetration deeper than 0.1 mm). A larger volume of bone marrow was loaded in the group of morselized bone as compared with the fragmented bone group. This is explained by the difference of bone surface area and the mechanism of the wetting[13]. The volume of wetting is fixed by the properties of adhesivity and cohesivity. When the bone

block is divided into smaller pieces, the bone surface is more extended and the volume of wetting proportionally increased.

This paper has several limits. We do not know how many cells can survive in the center of the graft due to the absence of vascularisation and hypoxia, but it is the same problem for a bulk autograft; we have not checked in this study how many MSCs can survive at the surface of the allograft due to the risk of an immunologic response, but we have previously reported the results of another study where human MSCs [5,13] were loaded on bone allografts of animals demonstrating viability, osteoblastic differentiation and bone formation. These results are confirmed by other reports.

Despite these limits, the results obtained in this study can help the surgeon to have an idea of the number of MSCs than can be loaded into an allograft femoral head in the operating room as compared with the gold standard represented by the femoral head autograft. This number will depend of the concentration of the bone marrow, of the technique used to load the cells, and will be related the size of the allograft pieces. The efficiency of the MSCs-allograft constructs in the long term as compared with autograft remains to be determined by other studies.

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Legends of figure

Figure 1: the arrows indicate the bone marrow aspiration from the proximal part of the femoral head; the cones in yellow the bone marrow aspiration; the cubes in yellow the part of the femoral head used for bone fragmentation

Figure 2:
A: one entry point for injection
B: several entry points for injection
C: Injected bone marrow that flawed outside the femoral head through the neck was kept in a recipient, then re-aspirated and re-injected.
D: Injection of 1cc of the methylene blue showing diffusion as a cone into cancellous bone.
E; Injection of bone marrow showing a diffusion as a cone into cancellous bone.

Figure 3:
A: the needle was inserted from osteotomied femoral neck side to proximal.
B; The tip of needle was positioned at subchondral bone area and at the center of the femoral head.

Figure 4: number of MSCs obtained after each bone marrow aspiration performed at the same point (9 successive bone marrow aspirations); the total number was 700 MSCs; This number was assumed to be the total number of MSCs present in 1 mL of bone marrow of the femoral head autograft since the extremity of the needle remained at the same site and only one 1mL was aspirated each time; 90% were obtained after 3 bone marrow aspiration.

Figure 5:
Number of MSCs released per 1 cm3 of autograft according to the size of the fragments

Figure 6:
with unconcentrated bone marrow the average number of MSCs loaded in the allografts of group A with the injecting technique was 117± 53 MSCs per cm^3 of allograft. With concentration there was a seeding effect between 10 and 20 injections to reach with a 10 times concentration a load of 1350±150 MSCs per cm^3 of allograft

Figure 7:
MSCs loaded with the soaking technique, according to the time of contact and according to the concentration of the bone marrow

Figure 1

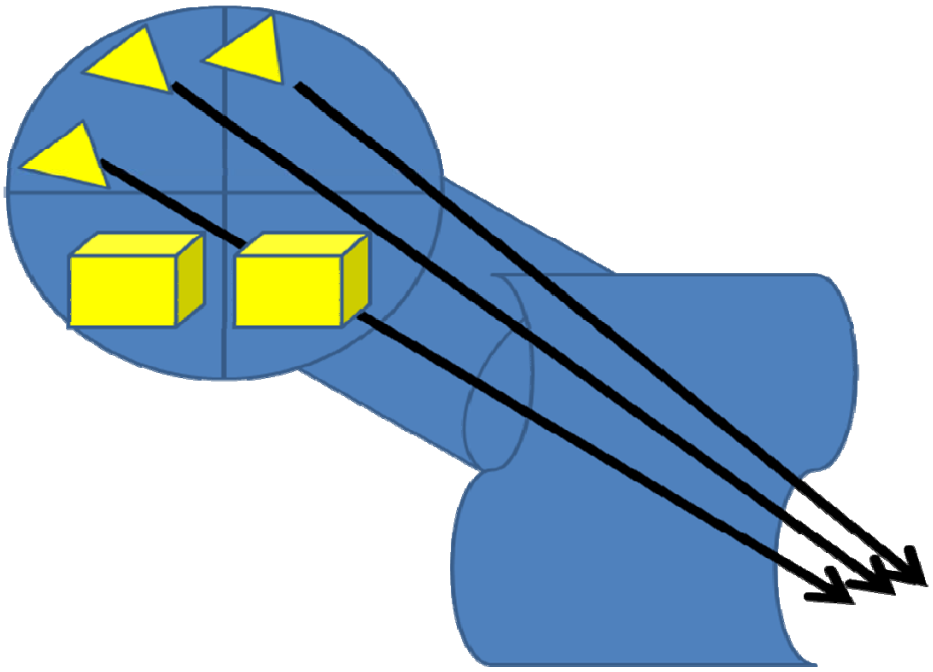
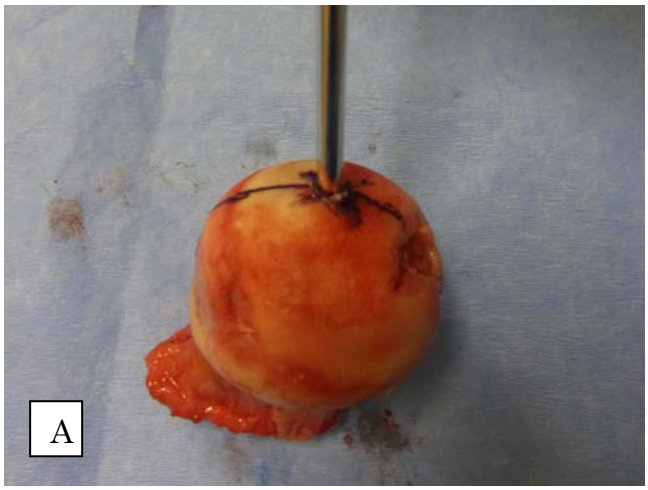
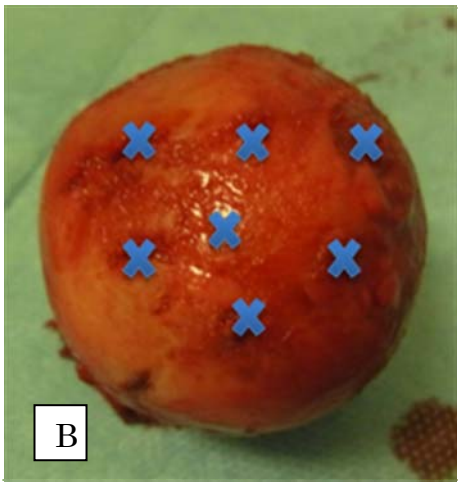
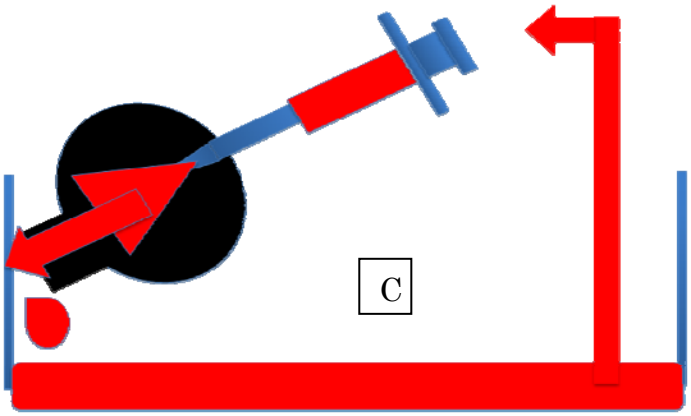
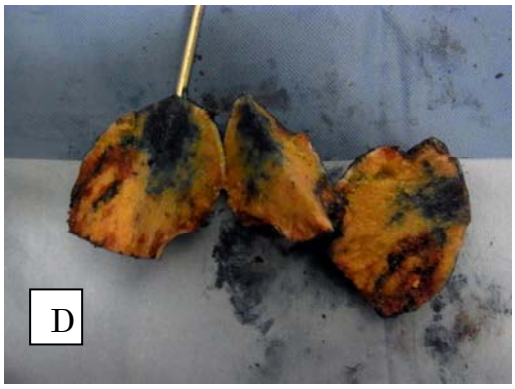


Figure 2









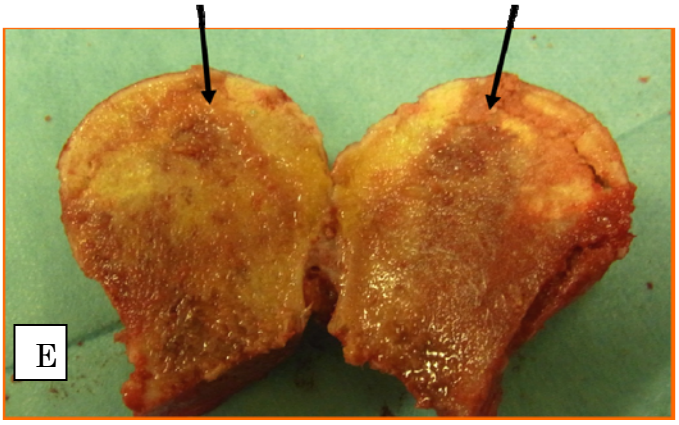
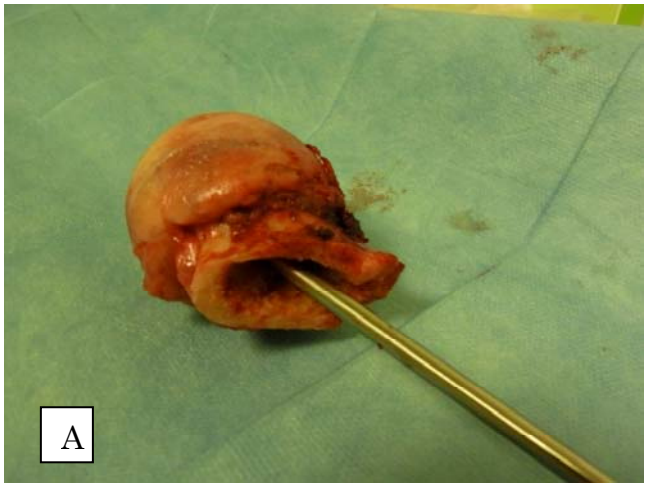


Figure 3



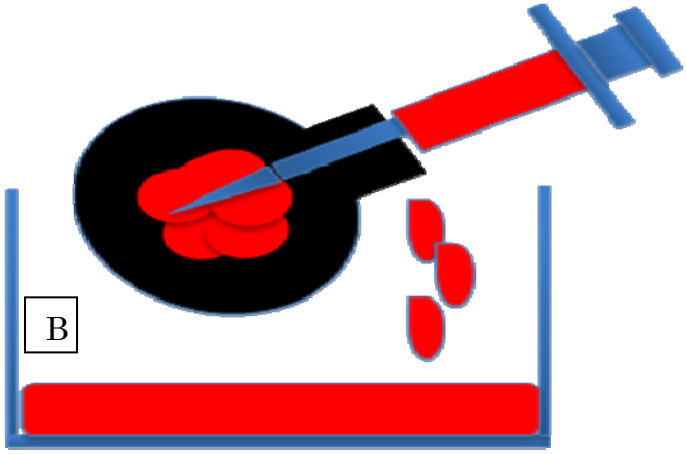


Figure 4

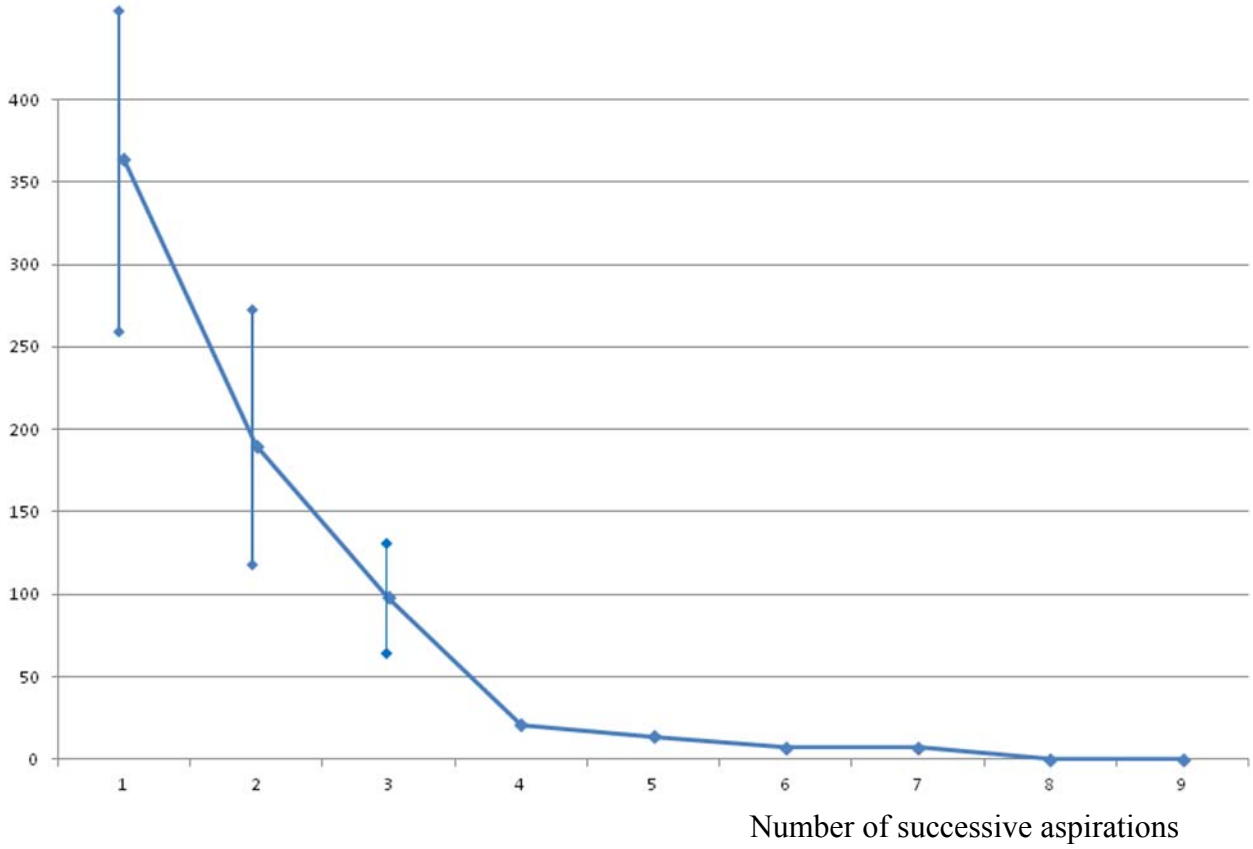

Number of MSCs per 1cc aspiration
400
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
Number of successive aspirations

Figure 5

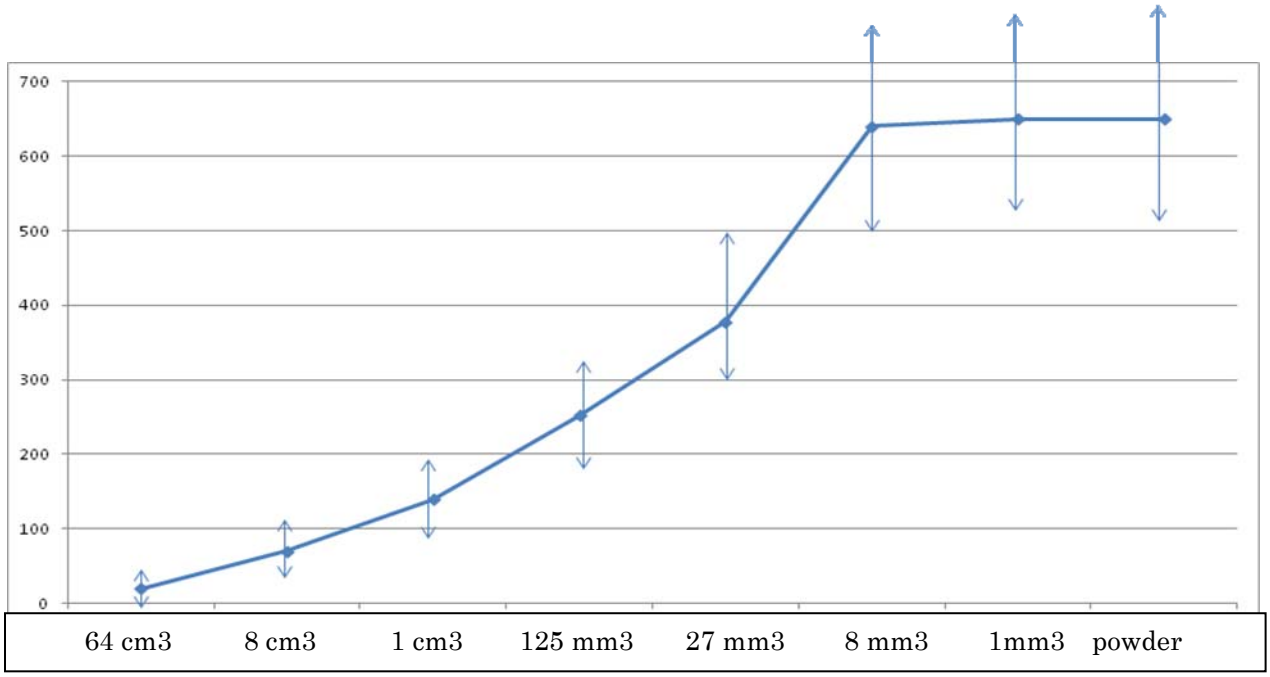
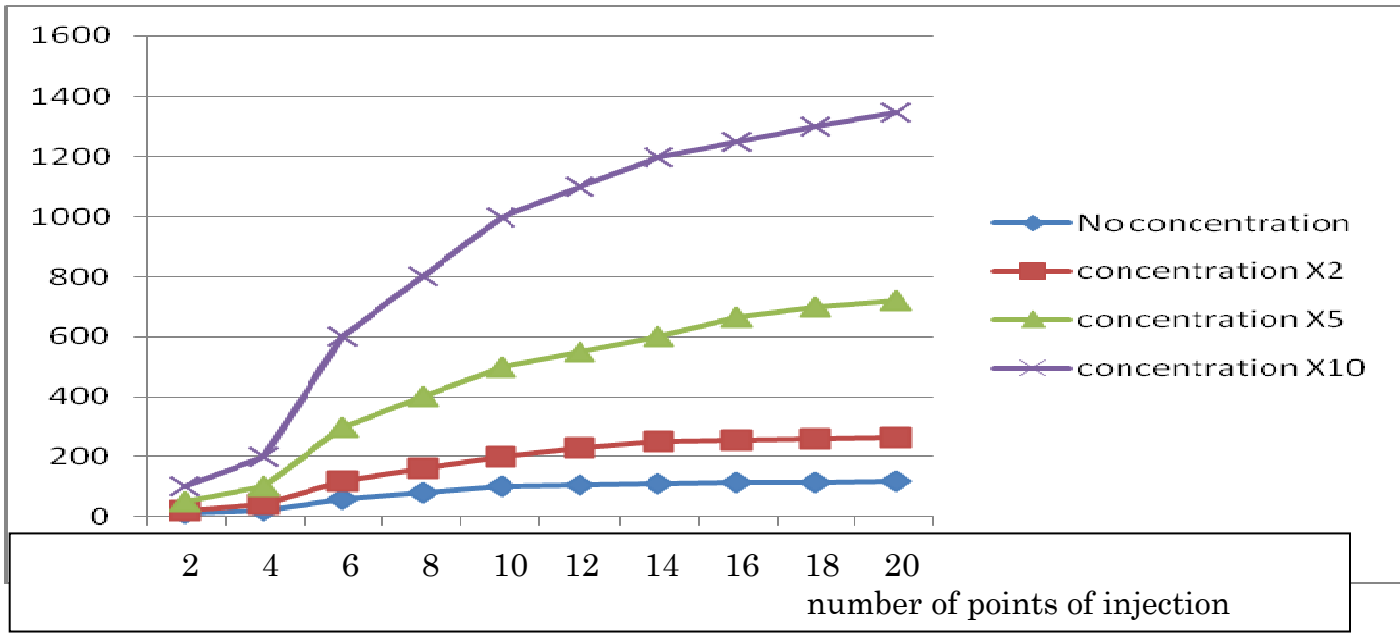

Number of MSCs released
per 1 cm3 of bone autograft
700
600
500
400
300
200
100
0
64 cm3
8 cm3
1 cm3
125 mm3
27 mm3
8 mm3
1mm3
powder

Figure 6

Number of MSCs injected
per 1 cm3 of bone allograft
in a bulk allograft



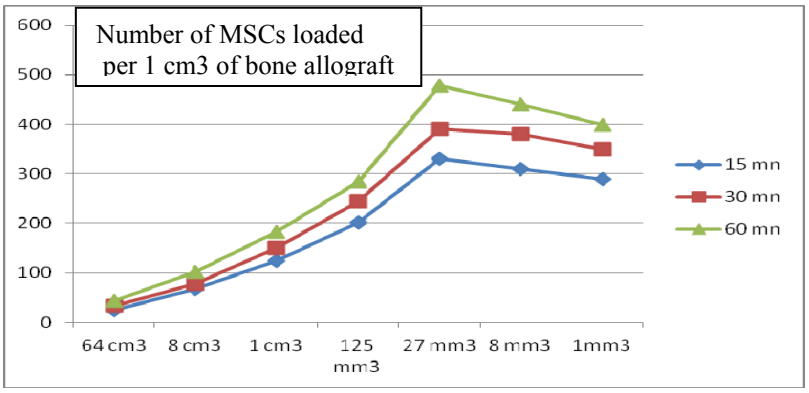
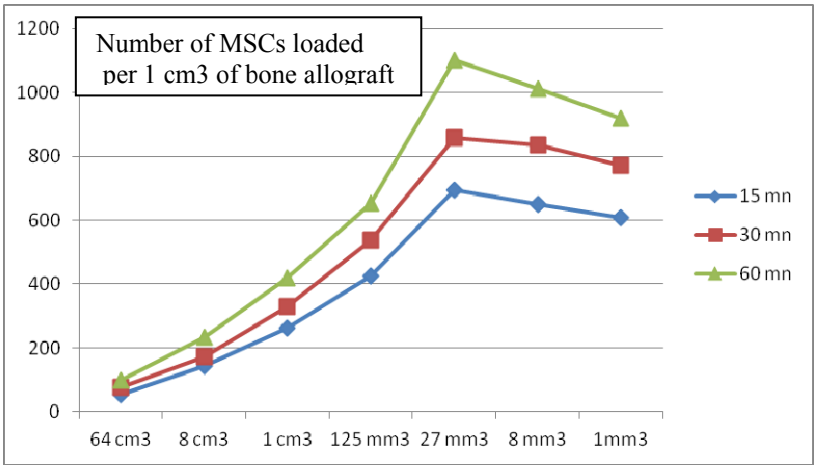
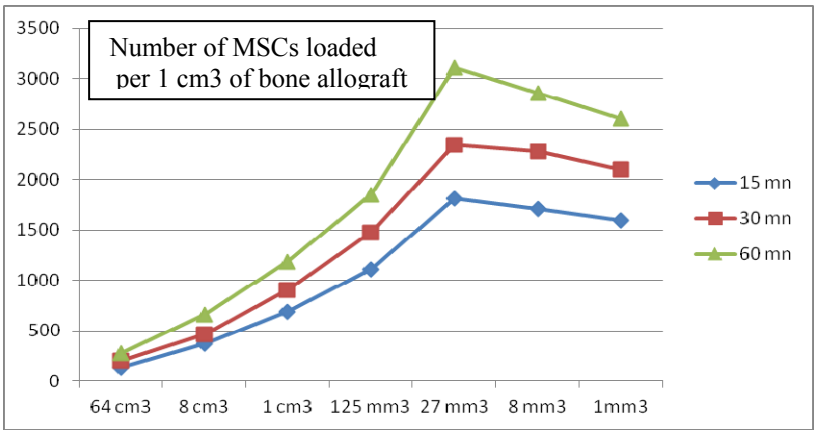


Figure 7

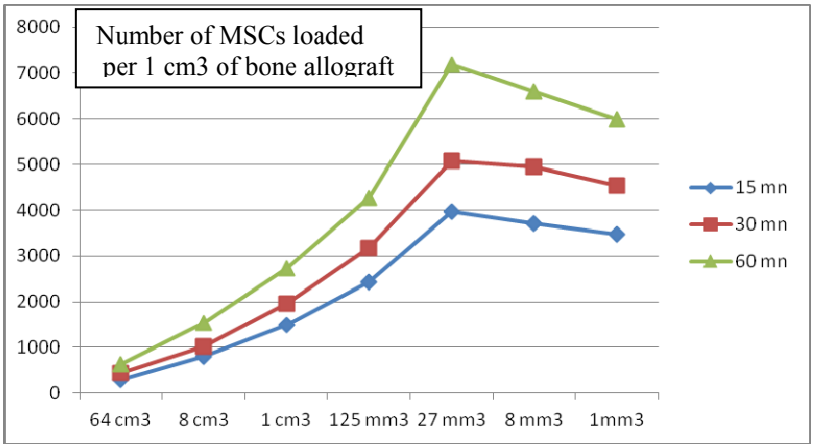
No concentration



Concentration X 2



Concentration X 5



Concentration X 10