A CLOSED, SAFE AND COST EFFECTIVE ALTERNATIVE ALGORITHM OF ISOLATION OF BONE MARROW DERIVED MONONUCLEAR CELLS.

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ABSTRACT

The new concept of use of stem/progenitor cells of desired lineage for the treatment of various diseases are under trial in several animal and human studies with promising results. In adults the mesenchymal tissue-preferentially the BM and adipose tissue are used to derive MNCs having several types of stem/progenitor cells. Instead of a standard protocol, the teams of past and ongoing trials have their own methods of aspiration, isolation and infiltration of BM for the treatment of various diseases either alone or as add on to conventional treatment. Although the process of BM aspiration from different sites of the body is almost standardized, optative improvements in the method of collection, transportation and purification have been done by us. Use of single closed aseptic quadruple blood bag tends to minimal the chance of infection to BM sample. Moreover, we use simple differential centrifugation for MNCs (Buffy coat) separation i.e. devoid of any reagent or solution except a clinical grade anticoagulant; hence does not require any cell retrieval process. Following BM aspiration and purification, the desired amount of MNCs was infused at the site of non union. Our alternative algorithm provided the more aseptic, pure and concentrated form of MNCs and added the use of stem cell property of BM-MNCs optimally in the better regeneration of defective sites.

KEYWORDS: BM- Bone marrow, CPDA- Citrate phosphate dextrose adenine, MNCs-Mononuclear cells, MSCs- Mesenchymal stem cells, CD- Cluster of differentiation, FACS-fluorescence activated cell sorting, TNC- Total nucleated cells.

INTRODUCTION

Scope: This study has been conducted to move one step ahead in the management of patients with certain seldom cured diseases. And to achieve this goal we had done work on two very beneficial purposes that are -

- Standardization of an alternative algorithm of BM aspiration and collection, better MNCs fraction isolation and its infusion at the site of interest.
- Comparison of CD 34^{+} cells concentration in aspirated and processed bone marrow samples.
- Finally the purposes provided us full basic platform to acquire the better concentrated and calculated MNCs for infusion purpose.

Background:

Naturally the adult stem cells may be uni, oligo and multipotent in nature. The best example of multipotent stem cell in adults is bone marrow that has natural ability to make various types of blood and bone tissues. It is found that the MNC fraction of BM contains MSCs that also have fate to develop some other types of tissues such as neural cells, muscular cells [10], cardiac cells etc either in vitro or in vivo [3]. Within the body, the infusion of autologous adult stem cell to any niche other than its origin have shown that the fate of stem cell change according to new niche microenvironment that fosters the growth of resident stem cells. Studies on different lower and higher animals certified that the behavior of stem cells that are govern due to the collective effect of many layers of regulation in response to local, systemic, and environmental factors present in their surroundings [4, 9]. By using these lineage-tracing studies it has been proved not only stem cell activity level but also stem cell behavior changes according to the needs of the surrounding tissue. The process of autologous BM aspiration and infiltration is now well established in the treatment of numbers of cancerous as well as non-cancerous conditions affecting blood. Now it is under trial in the management of various disorders related to delayed and non union of bone, spinal cord injury, cartilage and ligament development, neural cell, cardiac cell and myogenic cell development. But even after several researches a definitive treatment protocol could not be established and nothing much could be done in their better management. The teams of several past and ongoing trials have their own methods of aspiration, isolation and infiltration of BM for the treatment of various diseases either alone or as add on to conventional treatment. So to resolve this problem our team of translational research planned to standardize an alternative algorithm for the better use of BM in various diseases which required the infusion of BM resources.

Literatures have shown that the concentration of TNCs per ml of BM aspirate (crude BM) has power of 10^7. TNCs fraction contains WBCs, erythroblasts and progenitor cells [1, 11]. The

WBC count of per ml crude bone marrow normally contributes 4×10^6 to 1×10^7 WBCs [8]. Different reagent based BM MNCs separations (like Lymphoprep, Ficoll-Paque, Percoll separation etc) have shown that the near about 20-30 % of total WBCs were recovered as MNCs in buffy coat fraction of whole concentrate occupying in 10-15 % of total volume. It has lymphocytes, monocytes, macrophages and rare progenitor cells leaving granulocytes, RBCs, platelets etc in different other layers. Most of the literatures have been shown that the BM MNCs concentration has power range of 10^6-10^7 in per ml of BM concentrate [5, 7, 12]. According to different literatures the percentage of CD 34^+ cells varies between 2-4% of total MNCs in any normal bone marrow sample and they have properties of stem/progenitor cells. Different literatures have shown that the CD 34^+ cell count of per ml of BM concentrate has power in the range $10^5 – 10^6$ [5, 12].

Our achievement of BM MNCs and $CD34^+$ count is almost same among the all related studies that have shown the best count of MNCs and $CD34^+$ cells in purified BM.

Material & Methods:

Patients:

For the standardization of this alternative algorithm of BM aspiration and collection, its MNCs fraction isolation and infusion we have done study on 20 cases of established diaphyseal non union of tibia between January 2013 to June 2014. Patients more than 18 years of age of either gender were selected and enrolled for the study. Informed consent was taken from all the enrolled patients. The parts of research like patient's enrollment, BM aspiration and BM-MNCs infusion were done in the Department of Orthopaedic Surgery, King George's Medical University, Lucknow (UP), India. Rest of the research parts like BM-MNCs separation and their CD 34^+ count were done in the Department of Transfusion Medicine of same institution.

Our research work is ethically approved by the Institutional Stem Cell Ethics Committee of KGMU- UP, Lucknow- India as topic entitled "*Enumeration and correlation of osteo-progenitor cell concentration and activity of autologous bone marrow and bone marrow derived mononuclear cells in the recovery of non union tibia*" in October 2012.

Materials:

- Quadruple CPDA blood bag (Mitra Industries)
- Differential centrifugation machine (Thermo Scientific™ Heraeus™ Cryofuge™ 6000i)

- Compo Safe™ Intelligent Expressor E 250
- PE (Phycoerythrin) conjugated CD34 antibody (BD Pharmingen)
- FACS Calibur (Becton Dickinson)
- Bone marrow aspiration needle – 18 gauge (as Sternal puncture needle by Medifit Healthcare Product)
- Abgel®©™ brand medical absorbable gelatin sponge as gel foam by Shri Gopal Krishna Labs Pvt. Ltd etc along with complete OT and molecular biology laboratory setups.

Methods:

Since various studies on animals and human have already stated the positive impact of use of autologous bone marrow in various diseases. This study was conduct only to standardize a much significant alternative algorithm for the use of bone marrow or any of its products in better way, along with the increment of stem/progenitor cells concentration in the infusion sample. The study does not include the correlation of BM MNCs count with the final outcome in the patient, as that will be our future prospect. This alternative algorithm includes three consecutive stages and forth one as their cumulative impact over the count of $CD34^+$.

1. Aspiration and collection of bone marrow from iliac crest:

Since the patients were adult so we used the most common BM aspiration site i.e. posterior iliac crest (in prone or lateral decubitus position). The double layer packed sterile *Quadruple CPDA-1 Blood Bag system* (includes one primary bag with anticoagulant CPDA-1 solution USP and three satellite bags) was open and kept over an aseptic place in OT just before the aspiration procedure. According to standard norm there is 49 ml CPDA for collecting 350 ml blood or BM. So before the collection of aspirated blood into CPDA bag the CPDA of bag is reduced to about 15ml (30-35%) of it's initial to collect average 100 ml of BM. About 100 – 150 ml bone marrow was aspirated from patient using 16 gauge BM aspiration needle and 10 ml sterile needleless syringes under local anesthesia or general anesthesia (for non surgical and surgical cases respectively) under strict aseptic conditions in OT. X-ray navigation was used for the proper identification of needle puncture site. The aspirated blood was collected slowly in the required amount of CPDA of primary bag through its afferent tube. Now after sealing the afferent tube using tube sealer about 2-3 ml of crude BM was taken back into the sampling bag. The sampling bag was cut and sealed and kept for MNCs and CD 34^+ count of crude bone marrow. The aspirate was kept hanging for 10

minutes for proper settling down of blood cells according to their weight under the gravitational force.

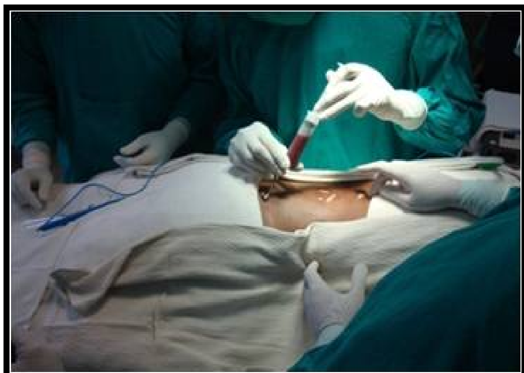

Fig.1- Aspiration of bone marrow from posterior iliac

Fig.2- Collection of bone marrow in CPDA blood bag

2. Differential Centrifugation and concentration:

It was performed in high volume cryocentrifuge based on the simple differential centrifugation of blood components according to their density. It involves following steps:

- The first round centrifugation was performed at 1200 rpm for 10 min at 10° C.
- Supernatant was collected (plasma with nucleated cells) in one of the three satellite bags of quadrupled set using manual plasma expresser. The primary bag with left RBCs, platelets along with some plasma components was separated from set.
- Plasma with nucleated cells was centrifuged again at 2500 rpm x10 min, 10° C.
- Again using plasma expresser the supernatant was separated out to second satellite bag leaving precipitate (about 10-15 ml) of mononuclear stem cells (MNCs) below in the first satellite bag called buffy coat. The buffy coat contains most of the mononuclear cell fraction (about 70 %) of bone marrow.
- Approximately 4-5 ml of precipitate of BM MNCs was collected, cut and sealed in efferent tube of second satellite bag for MNCs and $CD34^+$ count of purified sample.
- Left of the 6-10 ml precipitate left in first satellite bag was used as inoculum for infusion.

Note- The separation of components using manual plasma expresser must be done very carefully so that no RBC or its any hemolyzed product could mix with real sample because it leads to lowering of number of real MNCs in a definite desired volume of infusion. It is also to be too careful during the demarcation of desired volume of MNCs precipitate from the rest of the supernatant at the final step of separation.

Note- If there was any coagulated RBC component left with real MNC containing inoculum in the second satellite bag then the inoculum must be separated to third satellite bag carefully leaving coagulated RBC component left in the second satellite bag.

Fig.3- Differential centrifugation of bone marrow

Fig.4- BM-MNCs separation using plasma expresser

3. Infusion of bone marrow mononuclear cells:

BM MNCs infusion at the site of interest was added as a synergetic aide to conservative treatment.

- Immediately after isolation the sample was carried to OT table for infusion at the end of surgery.
- The 10 ml sterile syringe without needle was used to pull out the sample from efferent tube end of sample satellite bag.
- The infusion of BM MNCs sample was done under X-ray navigation. The infusion was done at the bone fracture site of interest already covered by the gel-foam paste to stabilize the sample over fracture site for its long term action.
- The infusion was done very slowly (about 1ml/5sec). So that the sample could not discharge out any way. If there was any such problem then we managed that by using hydrophobic antiseptic band(s).
- If at any point the infusion was interrupted then we slightly changed the direction as well as up or down movement of syringe for ease of infusion.

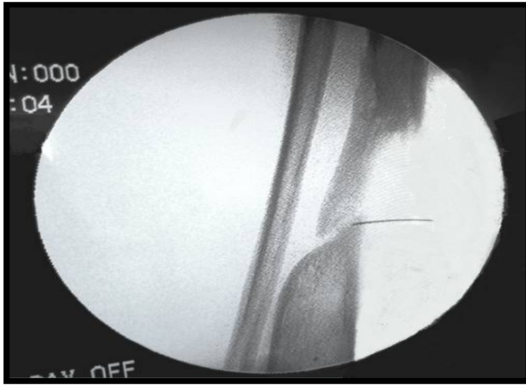

Fig.5- X-ray navigation of infusion site of non union

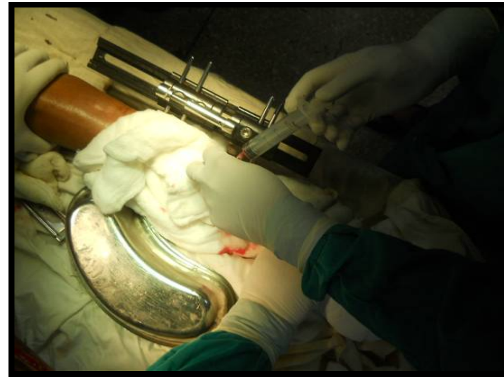

Fig.6- BM-MNCs infusion at the site of non union

4. CD 34^{+} counts by flowcytometry (FACS):

It was done for CD 34 positivity count of samples taken before and after differential centrifugation as crude BM (aspirate) and purified BM MNCs fraction (infusion sample) respectively to check how many folds the concentration we achieved. CD34 positive cells in bone marrow samples was assessed by using PE (Phycoerythrin) conjugated CD34 antibody (BD Pharmingen) and fluorescence of 10,000 cells was acquired on FACS Calibur (Becton Dickinson) and subsequently analyzed using Cell Quest programme.

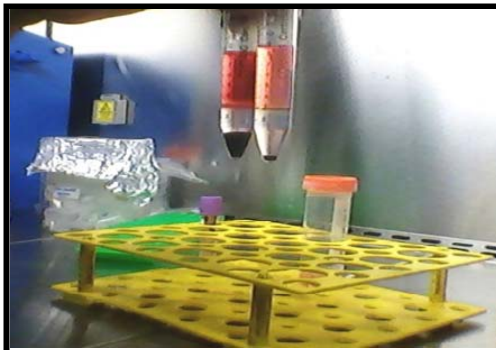

Fig.7- Crude BM and buffy coat processing for CD34+ count

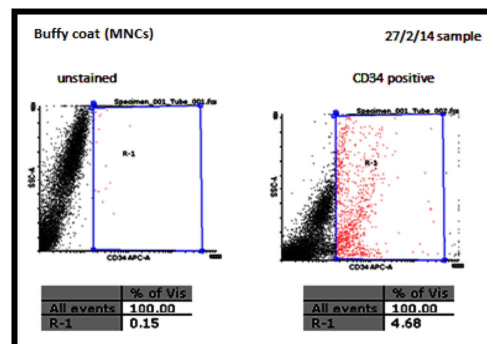

Fig.8- CD34+ count of a buffy coat sample by FACS analysis

Results:

1) Standardization of aspiration and collection protocol:

- Reduction to 30-35% of initial volume of CPDA was done to maintain the maximum possible real concentration of cells in aspirate.
- We used 10ml syringes at place of large volume syringes because:
  - In Large volume syringes the aspirate remains longer in the syringes that may result to coagulation.

- It provided desired range of 'pressure of pull' for maximum concentration of desired cells in aspirate.

- Once the aspiration started the syringe was not detached until the required volume was collected into the syringe. It avoided coagulation due to any aeration.
- Due to any reason if the aspiration became slower or ceased then the current volume of aspirate was instantly transferred to bag without any time loss. The needle direction or depth (but not the site) was changed for further aspiration.
- The transfer of blood from syringe to blood bag through the attached tube was done slowly i.e. about 1ml/ sec to avoid hemolysis of blood.

2) Standardization of differential centrifugation protocol:

In general the component separation of donated blood is done by single time differential centrifugation at 1000 rpm. Since the BM is denser than peripheral blood so it requires spin at greater rpm for component separation. But it should not be much more because it leads to hemolysis of RBCs. So keeping this safety in mind we retraced that the1200 rpm was most accurate to separate highest density bearing components of BM i.e. RBCs followed by granulocytes, that were settled down in the bag leaving rest of the components in primary supernatant. Since the cellular component with lesser density have tendency to suffer greater rpm without cell rupture, the primary supernatant (having plasma, platelets and MNCs fractions etc) was spin at favorable retraced rpm of 2500 at which next highest density bearing component i.e. MNCs fraction settled down in the bag leaving rest of the components as secondary supernatant. The un-useful secondary supernatant was separated out in second satellite bag leaving 6-10 ml MNCs precipitate (buffy coat) at the bottom of first satellite bag as final product for infusion.

3) Standardization of infusion protocol:

To avoid the any wastage of infusion sample:

- The infusion was done carefully after skin closer under X-ray navigation.
- 10 ml syringe were used because it has moderate pressure of push and required needle size for effective reach up to the exact site of non union gap.
- The infusion was done very slowly (about 1 ml/5 sec) to avoid any force full discharge from any minute pore of stitches or pricks etc.

4) Concentration measures of MNCs in crude BM sample and centrifuged sample:

- The MNCs of crude BM and isolated buffy coat (purified BM concentrate) samples were manually counted using 3% acetic acid followed by trypan blue staining of nucleus under an optical microscope using hemocytometer.
- The samples has shown a mean ± SD of $2.34 \pm 0.81\times10^6$ and $36.2 \pm 13.8\times10^6$ MNCs per ml of crude BM and buffy coat samples respectively with the p-value < 0.0001 using 'unpaired T test' when compared independently with mean of some previous papers [2, 7, 12]. Further by flowcytometry we have found a mean percentage of 1.51±0.65 % and 3.83±1.26 % of $CD34^+$ stems/progenitor cells in crude BM and buffy coat samples respectively with the p-value < 0.0002 using 'unpaired T test' when compared independently with mean of corresponding papers [2, 6,7, 12].
- FACS analysis have shown that the CD 34^+ cells were 2 to 4 fold more in purified BM sample (buffy coat) as compared to crude (aspirated) BM sample. The calculated mean of $CD34^+$ count was found equal to $3.99\pm2.91\times10^4$ and $153\pm105\times10^4$ per ml of crude BM and buffy coat samples respectively. The $CD34^+$ and BM MNCs count of our study is almost same to those studies showing the best result of MNCs and $CD34^+$count in purified BM using most widely used MNCs purification method i.e. Ficoll-paque density gradient separation method. However our purification method becomes much more significant over others because it includes a cheap and time saving protocol performed in closed aseptic setting and does not require any retrieval process.

Discussion:

- In our study we had chosen the iliac crest as a site of aspiration because the iliac crest has almost no weight bearing, no mobility, more surface area and volume so lead to better recovery and more aspiration volume respectively.
- It is better to use purified sample of BM as compared to crude BM because crude BM has RBCs, platelets, coagulating factors and other plasma components that have several factors indicating the niche of origin and so produces those unfavorable conditions which cause the hindrance in proper functioning of stem cells of BM concentrate.
- The use of quadruple CPDA-1 blood bag system for the collection and processing of bone marrow is one of the most aseptic and systematic methods among all other methods of buffy coat separation. If needed, more amount of sample can be collected in the blood bag as compare to generally used centrifuge tubes. Since at each pull greater the volume of aspirate to be taken greater will be the pressure generated by the syringe inside the

aspiration site. Since the cells have more density than the liquid component of blood or BM, so if we use less pressure to pull the aspirate then it will lead to an aspirate with more liquid component and less number of essential cells. Opposite to this if we will use more pressure of pull then the chance of mixing of other mature cells or tissues that are loosely attached to nearby surrounding surfaces become increases. This will lead to unnecessary increase of nonessential mature cells and decrease of real concentration of stem /progenitor cells in the bone marrow aspirate. So for assuring the required volume of aspirate and a moderate pressure of pull we were used 10 ml sterile syringes at place of 5ml or 20 ml syringes that require either very less or much more pressure leading to above specified unfortunates.

- The use of two step simple differential centrifugation of BM for MNCs separation has a special feature that it does not require any reagent, solution or filter (as required by other methods using one step centrifugation) except an anticoagulant CPDA that is accepted worldwide for the purpose of blood donation. Due to the non external add the concentration of desired cells in the concentrate becomes maximize and hence the cells retrieval process from centrifuged MNCs fraction (Buffy coat) is not required. Since the final product is an autologous product without any external impurity hence it has been provided the results as none of the infusion was showing any type of allergic reaction.
- The best thing of overall process is that the infusion sample is prepared within an hour since the operation started and does not face storage problem and much better than the methods required storage resulting as decrease in active stem cells.
- Since average 100 ml of aspirated crude BM sample was concentrated as approx- 10 ml of final infusion sample, so assumed to be near about 10 fold purification. But it is not exactly as assumed because the loss of MN cells during different steps of separation of plasma and buffy coat is not well demarcated.

Conclusion:

This alternative standardized algorithm provides us a complete procedure of use of BM and its derivatives as regenerative medicine for synergetic aide to various incurable diseases. The aspiration and MNCs isolation procedures of the algorithm have been standardized keeping all the aspects assuring the safest and most efficient use of BM for any clinical purpose. While our third step of algorithm i.e. infusion although used here for cases

of non union tibia but it provides a comparative knowledge for infusion in other sites of interest for other diseases.

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