Xenotransplantation of Caprine Mesenchymal Stem Cells into Rabbits for Full Thickness Skin Wound Healing

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ABSTRACT:
Mesenchymal stem cells (MSCs) are currently thought to be a source of cells for regenerative medicine having the capacity to differentiate into cells with mesodermal, ectodermal, and endodermal characteristics. The previous studies suggest that MSCs might be immune-privileged cells. The surface characteristics of MSCs are believed to allow them to avoid rejection. So in this study, we examined the benefits of Caprine bone-marrow derived mesenchymal stem cells (cBM-MSCs) in full thickness skin wound healing in rabbit models. Four wounds of 2cm×2 cm, two on the left side and two right side of the midline were created on the back of each rabbit under xylazine-ketamine anesthesia. The injection of cBM-MSCs, of passage P12 labeled with immunofluorescent dye PKH-26, was given into wound margins and wound bed (Treatment) of right side, whereas only PBS was injected in left side wounds (Control). Wounds were observed for 22 days for wound contraction and time to complete healing. On 22nd day of transplantation, the tissue was taken from around the wound and histopathological evaluation was done. It was observed that within 22 days post surgery, there was complete healing of wound in control as compared to treatment, where very small areas were still left for healing but the difference was statistically non significant (P<0.05). However, histopathological finding showed that, in control, although, there was complete epithelialization of the wound but the stroma had moderate amount of well aligned fibrocytes with less quantity of thick collagen. On the contrary, in the treatment group, apart from complete epithelialization, the underline stroma had more fibroplasia with higher amount of slightly irregular thick collagen than in control. The study indicated that the Caprine MSC, treatment could be used for proper skin wound healing.

KEY WORDS: Caprine, Bone marrow-mesenchymal stem cells, Xenotransplantation, Skin wound healing, Rabbit

INTRODUCTION:
Wound healing is a highly complex biological process which requires a well-orchestrated integration of the complex biological and molecular events of cell migration and proliferation and extracellular matrix (ECM) deposition, angiogenesis, and remodeling (Martin P., 1997; Falanga V., 2005; Singer and Clark, 1999). However, this orderly progression of the healing process is impaired in many chronic diseases, including diabetes (Falanga V., 2005). About 15% people with diabetes worldwide suffer from foot ulcerations, which often become nonhealing chronic wounds (Boulton et al., 2005). The best available treatment for chronic wounds achieves only a 50% healing rate that is often temporary. The coverage of skin defects remains a major concern when the defective area is widespread, severely contaminated by microorganisms, or poorly vascularized, as can be the case with irradiation defects, congenital skin disorders, or extensive burns. Extensive research has been carried out on the use of topical medication in healing, but many of the products
investigated in domestic animals do not affect wound healing and some inhibit it (Swaim and Henderson, 1990). In recent years, great success has been achieved in accelerating the capacity for wound healing. However, the prognosis of extensive and deep burn is not entirely satisfactory because of scar formation and the loss of normal function and skin appendages, which severely affects the quality of life after survival. Therefore, reducing the formation of scar and reestablishing the normal anatomy and function of the skin and its appendages have become the aim of regenerative medicine research. Fortunately, with research into and better understanding of stem cell biology, experiments have shown that mesenchymal stem cells (MSCs) can participate in tissue and organ regeneration in almost all lesions.

The embryonic stem cells have a great capacity for self-renewal and plasticity, but their use is limited by scientific, political, and ethical considerations. The use of adult stem cells, especially mesenchymal stem cells (MSCs), is not burdened by many of these problems. MSCs can be isolated from bone marrow and other tissues such as adipose tissue, nerve tissue, umbilical cord blood and dermis (Kim et al., 2007; Dai et al. 2007; Perg et al. 2006, Shih et al. 2005; Jones et al. 2002). Although several studies have found that MSCs can contribute to skin reconstitution in cutaneous wound (Yashikawa et al. 2008; Sasaki et al. 2008; Lataillade et al. 2007). Kim et al. (2009) found that there was no significant difference being observed in wound healing between autologous and allogeneic MSCs transplantation.

To heal the skin defects, the MSCs have the capability of self renewal and multilineage differentiation into adipocytes, chondrocytes, and osteoblasts under appropriate environmental cues (Caplan and Bruder, 2001). MSCs are also immune-modulatory as they can inhibit the maturation and functions of various immune cells (e.g., dendritic cells, natural killer, T, and B cells; Uccelli et al. 2008). The results of previous studies suggest that MSCs might be immune-privileged cells (Bartholomew et al. 2002; Diniola et al. 2002). The surface characteristics of MSCs are believed to allow them to avoid rejection. Although several reports are available about effective role of autologous as well as allogeneic MSCs in skin wound healing. However, to date, no study has been performed to elucidate the effects of xenogeneic ruminants MSCs transplantation in skin wound healing. Although MSCs are immunosuppressive and can be tolerated after auto and allo-transplantation, Whether MSCs are suitable for Xenotransplantation remains to be established. For instance, Human mesenchymal stem cells (hMSCs) were depleted rapidly after direct injection into the myocardium of rats (Grinnemo et al. 2004; Terrovitis et al. 2008) while xenotransplantation of rat MSCs into the quadriceps of immunocompetent mice led to the engraftment and differentiation of cells into human hepatocytes with the aid of immunosupression (Sato et al. 2005). Xenotransplantation of hMSCs into the rat spinal cord led to long-term survival of cells under, but not without, appropriate immunosupression (Ronsyn et al. 2007). Strikingly, intravenous injection of hMSCs into the rats led to the survival and migration of hMSCs to the spinal cord injury site and improved the functional recovery even without immunosupression (Cizkova et al. 2006). The long term survival and chondrogenic differentiation of hMSCs after xenotransplantation into the rat intervertebral discs without immune-suppression (Wei et al., 2009). Mansilla et al. (2005) created a full-thickness skin defect in mice and used human MSCs to accelerate the wound healing; all the skin wounds healed without a scar or retraction at a median time of 14 days after wounding. The bone morphogenetic protein 2 expressing Baculo virus (Bac-CB) engineered hMSCs promoted the cell differentiation and new bone formation in the immunocompetent rats (Chuang et al. 2010).

The present study was designed to evaluate the wound healing potential of Xenotransplantation of Caprine bone marrow derived mesenchymal stem cells (cMSCs) in full-thickness excisional skin wounds in rabbits without any suppressive drugs and without dressing of wounds.

MATERIALS AND METHODS:
Animals
Six clinically healthy New Zealand white rabbits of both sexes weighing between 2kg to 2.5kg and of between six months to one year of age were used for skin wound creation where as 6month to 2 years old black
Bengal goats of either sex were used for the bone marrow aspiration. The institute Animal Ethics Committee of the Indian Veterinary Research Institute (IVRI), Izatnagar, India, approved the study. All the rabbits were obtained from the Laboratory animal research section of the Animal Genetics Division at the Indian Veterinary Research Institute. All the goats were obtained from Animal farm shed of the Veterinary physiology and Climatology Division at the IVRI. Animals were housed individually and provided with healthy diet and water ad libitum. They were maintained under uniform conditions and were acclimatized to people approaching and handling them for a period of 10 to 15 days before the start of the study.

Animal Preparation:
Wound creation and bone marrow aspiration were carried out aseptically under general anaesthesia using xylazine administered intramuscularly, followed 10 minutes later by ketamine intramuscularly. Anaesthesia was maintained by additional doses of intravenous ketamine if required. In rabbits, enrofloxacin (5mg/kg intramuscularly) and meloxicam (0.2mg/kg intramuscularly) were administered preoperatively. The animals were restrained in lateral recumbency for collection for collection of bone marrow aspirate and in sternal recumbency for the creation of experimental wounds.

Bone marrow aspiration and isolation of Mesenchymal stem cells from Caprine:
Aspiration of bone marrow was done using the method described by Crow and Walshaw (1997), with some modifications. The bone marrow aspiration were carried out aseptically from the 6 month to 2 years old black Bengal goats of either sex under general anesthesia using 0.3 mg/kg body weight xylazine administered intramuscularly, followed 10 minutes later by 30 mg/kg body weight, ketamine intramuscularly. Enrofloxacin (5 mg/kg) and meloxicam (0.2mg/kg) were administered intramuscularly preoperatively. An 18-gauge bone marrow biopsy needle was inserted with little force through the skin and muscle of the iliac crest (external angle of the ilium) of the hip joint. Once the needle (with the stylet in place) had contacted with the bone, it was advanced deeper by rotating it slowly with steady pressure until the cortical bone had been penetrated and the needle had entered the marrow cavity. A sudden change to the penetration force of the needle was usually felt at this point, which indicated that the needle was in the marrow cavity. The stylet of the biopsy needle was then removed and the needle was connected to a 20 ml syringe containing 5000 IU of heparin. A negative pressure was applied by forcefully pulling back the plunger and approximately 5 ml of bone marrow aspirate was collected from each side. The mononuclear fraction of the bone marrow was isolated with a Ficoll-paque density gradient, using the volume reduction centrifuge “Buffy coat” protocol (Kasten et al.2007). The bone marrow aspirate was transferred into a sterile tube and centrifuge at 2000 rpm for 30 minutes. The supernatant plasma was discarded and the buffy coat was aspirated using a micropipette. While collecting the buffy coat, it was ensured that no trace of buffy coat was left in the tube by aspirating a small quantity of plasma with red blood cells. It was re-centrifuged for 5 minutes at 1500 rpm to further reduce the volume. Finally, concentrated nucleated cells were mixed with 5 ml of DMEM low glucose (catalog no.SH30021.01, Hyclone® ) with 10% FBS (catalog no.16000-044), 100 U/ml penicillin, 100 μg/ml streptomycin (penstrep, catalog no-15140-122), 1ml/100ml glutamine (100mM,catalog no.25030), 25mg/ml amphotericin B, gentamicin (Sigma); all supplied by GIBCO® Invitrogen Corporation) and were cultured in T-25 tissue culture flasks. The flask was incubated at 37°C in 5% CO₂ for 3 days and after 72 h the non-adherent cells were removed while replacing the culture medium. BM-MSCs were first selected by their adherent property preferentially attaching to surface of tissue culture flasks. The culture media was changed every third days till confluency. After 12 days, the culture reached to confluence stage and the monolayer cells were washed twice with 2 ml of Phosphate Buffered Saline (PBS) (pH-7.2). The cultures were digested with 4 ml of 0.20 % Trypsin-0.02 % Ethylenediamine tetra acetic Acid (EDTA, Sigma,USA) and distributed on the surface of the layer for 5 minutes while checking the cells under the microscope till the regained their round shape and lifting up of the cells. Then Dulbecco’s modified eagle’s medium containing 10% FBS was added to the medium and then gently tapped to detach cells for next three subcultures. Passage 12 cells were used for the experiments.

Flow cytometry analysis:
The cells were detached with Trypsin-EDTA and counted. The trypsinized cells were prepared at a

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concentration of $1 \times 10^5$ cells/ml in DMEM low glucose with 10% FBS, then incubated for 15 min at 4°C with a 1:9 dilution of normal goat serum in PBS to block nonspecific binding of the primary antibodies. The cells were resuspended in 400 μl of PBS and pelleted by centrifugation for 5 min at 400 x g. Then cells were labeled with primary antibodies against CD-45 (catalog no.SC-25590), CD-105 (Endoglin,catalog no.SC-19793), CD-73 (catalog no.SC-14682) and FITC conjugated CD-90 (Thy-1, catalog no.SC-6071) for overnight and then counter stained with FITC- conjugated respective secondary antibodies. The cells stained with FITC labeled respective secondary antibodies only were used as negative control. All primary and secondary antibodies were supplied by Santa Cruz Biotechnology. The cells were washed with 1x PBS. The cells were acquired using FACS Calibur (B D= Becton Dickinson,USA) and analyzed using WinMDI cell Quest software (BD Biosciences, USA).

**Labeling and preparation of cells for transplantation to the wound:**

For tracking the transplanted cells in the skin wounds, the cells were labeled with PKH-26 red fluorescent Cell Linker kit for general cell membrane labeling (catalog no- P9691,Sigma) before the transplantation was carried out according to the manufactures instructions. The PKH-26 linker kit labeled the cell membrane with a fluorescent dye which incorporates in long aliphatic tails into lipid regions of the cell membrane. The labeled cBM-MSCs were used in vivo studies.

**Differentiation assay:**

The cells were cultured to confluency in complete medium and then differentiated by appropriate inducing media for 21 days.

For osteogenesis, the cultured cells were incubated in osteogenic medium containing DMEM low glucose, 10% FBS supplemented with 10 mM β-glycerol phosphate (catalog no-G9422-50G,Sigma), 0.3mM L-ascorbic acid (catalog no-A4403-100MG,Sigma), and 10 mM dexamethasone (catalog no-D2915-100MG,Sigma). The cultured cells were incubated at 37°C and 5% CO₂ for 3 wks. The culture media was changed three times a week for 3 wks. The cells were fixed with methanol for 10 min at room temperature and stained with Alizarin Red S, pH-4 (catalog no.A5533-25G, Sigma ) for 30 min at room temperature. For further confirmation of differentiation, untreated cells were subjected to RNA extraction and reverse transcriptase PCR (RT-PCR) analysis.

For adipogenesis, the cultured cells were incubated in adipogenic medium containing DMEM low glucose, 10% FBS supplemented with 50 g/ml Indomethacin (catalog no- I7378-5G,Sigma), $10^{-7}$ M dexamethasone (catalog no-D2915-100MG,Sigma) ,10 g/ml Insulin(catalog no.-I- 4011,Sigma). The cultured cells were incubated at 37°C and 5% CO₂ for 3 wks. The culture media was changed three times a week for 3 wks. The differentiated cell were analyzed by Oil Red-O staining. The cells were fixed for 60 minutes in 4% formaldehyde at room temperature and washed with 70% ethanol . Then stained for 10-15 minutes in a fresh working solution consisting of three parts Oil Red-O stock solution(0.5% Oil Red-O in 99% isopropyl alcohol) and two parts distilled water, which were mixed for at least 5 minutes and filtered before use. The excess stain was removed by washing with 70% ethanol followed by several changes of distilled water prior to observation. For further confirmation of differentiation, untreated cells were subjected to RNA extraction and RT PCR analysis.

**Immunofluorescence analysis:**

Cells were grown on the cover slip kept in a 6-well tissue culture plate until 50% confluence was reached. Specimens were rinsed with PBS and fixed with 4% Para formaldehyde (PFA) for 20 min at room temperature. After rinsing with 1x PBS(3 times), cells were blocked with 10% normal goat serum in 1x PBS for 40 min in humified chamber at room temperature and wash with 1x PBS, and permeabilized with 4% BSA, 0.3 % Triton x-100 in PBS for 20 min. Specimens were subsequently incubated with primary antibodies CD-105 (Endoglin, catalog no.SC-19793), CD-73 (catalog no.SC-14682) and FITC conjugated CD-90 (Thy-1, catalog no.SC-6071) in dilution 1: 50 in PBS BSA 3% for overnight at + 4°C. A negative control with no primary antibodies was also included. Cells were rinsed three times in 1x PBS and then incubated with counter stained with FITC- conjugated respective secondary antibodies (in dilution 1:50) in the same buffer used for primary antibodies in the dark for 1 hours at room temperature. Specimens were rinsed with1x PBS.
three times and counterstained with DAPI diluted in 1x PBS. Then stained specimens were rinsed with 1x PBS three times, mounted with Prolong® Gold antifade reagent (catalog no.-P36930, Invitrogen™) and analysed under the microscope (Zeiss) and image overplay was performed using Axiovision software.

RNA extraction and reverse transcriptase PCR analysis of gene expression:
Cells of each group were harvested, and washed with PBS 1x and pellets in 100 1 of PBS 1x were frozen in -80°C immediately. The total RNA was extracted from cells using Quick-RNA™ reagent (Catalog No. R1050, MicroPrep, Zymo Research). The O.D. of RNA was maintained at 900 ng/µl. The cDNA was synthesized using iScript select cDNA Synthesis kit (catalog no. 170-8897) and used for real time PCR study. Relative quantification was performed using a real time polymerase chain reaction method. Briefly, a Biorad CFXManager™ Software and EvaGreen supermix (catalog no.172-5200), as a double stranded DNA-specific fluorescent dye were used to determine the mtDNA copy number. For Amplification of genes, primers were designed in beacon software. No template control (NTC) containing all the first strand synthesis reagent but lacking RNA template, was also prepared. Samples were tested as triplicates, and only variations of less than 1.5 thresholds cycles were tolerated. Thresholds cycles after cycle 45 were defined as invalid. The real time PCR thermocycling conditions were: an initial denaturation step at 95°C for 30 min followed by 40 cycles of 95°C for 5 sec, annealing for 15 sec. Reverse transcriptase (RT) was performed by 30 min incubation at 50°C, followed by 2 min at 94°C to inactivate the RT. The reaction products were resolved by electrophoresis on a 1% agarose gel and visualized with ethidium bromide. Transcript level of all genes were quantified using the relative quantification method based on comparative threshold cycles values (Ct). The abundance of gene was determined relative to the abundance of the housekeeping gene GAPDH. Data evaluation was performed using the ▲▲C_T method.

Primers for real time PCR were as follows:
Thy-1 (CD-90); fwd 5´CCT CCT GCT AAC AGT CTT AC 3´ rev 5´ATC CTT GGT GGT GAA GTT G3´; (Annealing temperature = 60°C, Product size = 271 bp).
Endoglin (CD-105); fwd 5´AGC GAT GGC ATG ACT CTG 3´ rev 5´AGG CTG TCC GTG TTG ATG 3´; (Annealing temperature = 65°C, Product size = 251 bp).
Osteocalcin; fwd 5´GAC CAT CTT TCT GCT CAC TCT G 3´ rev 5´GTG ATA CCA TAG ATG CGT TTG TAG3´; (Annealing temperature = 60°C, Product size = 276 bp).
Adipsin; fwd 5´ATG GTA TGA TGT GCA GAG TGT AG3´ rev 5´CAC ACA TCA TGT TAA TGG TGA C3´; (Annealing temperature = 60°C, Product size = 276 bp).
Lipoprotein lipase (LPL); fwd 5´GAG GAC ACT TGT GCA GAG TGT AG3´ rev 5´CCT TCT TAT TGG TCA GAC TTC C3´; (Annealing temperature = 55°C, Product size = 376 bp).
GAPDH ; fwd 5´GGA GAA ACC TGC CAA GT ATG3´ rev 5´TGA GTG TCG CTG TTG AA GTC3´; (Annealing temperature = 65°C, Product size = 126 bp).

Wound creation, treatment and postoperative care:
Using a clean transparency sheet template and a permanent marker, the vertices of the wounds, two on the right side and two on the left, were outlined in six clinically healthy New Zealand white rabbits. Four full-thickness skin wounds, including subcutaneous tissue measuring 2 x 2 cm were excised with # 21 Bard-Parker blades in each rabbits. A gap of 2 cm was kept between the two wounds on both sides and wounds were created 2 cm away from the midline on either side of the dorsum. The wounds were created at the same location on the trunk of each animal regardless of the differences in the body size. Hemorrhage, if any, was controlled by applying pressure with sterile cotton gauze. The injection of 1.5×10⁶ Caprine bone marrow derived mesenchymal stem cell (cBM-MSCs), of passage P12 labeled with immuno-fluorescent dye PKH-26, was given into wound margins and wound bed (Treatment group) of right side, whereas only 0.5 ml of PBS was injected in left side wounds (Control). For the wounds of treatment group, 1.5×10⁶ cBM-MSCs diluted with 0.5 ml of PBS were loaded in a 1 ml sterile insulin syringe and the contents of the syringe were then injected subcutaneously around the wound margins and wound bed. The antibiotic enrofloxacin and the anti-inflammatory analgesic meloxicam were administered in all the animals for three consecutive days after surgery. But the dressing of the wounds with antiseptics after surgery was not done.
Wound contraction: Wound contraction was measured at day 0, 5, 10, 12, 14, 18 and 22nd days after surgery as a per cent reduction in the wounds. A progressive decrease in the wound area was monitored periodically by tracing the wound margin on tracing paper and measuring the area using graph paper. Wound contraction (WC) was calculated as a percentage change in the initial wound size by following formula (Surekha kamath, et al.; 2006).

WC% = \frac{Initial\ wound\ size - specific\ day\ wound\ size}{Initial\ wound\ size} \times 100

Time to complete healing:
This was recorded as the day on which the wound healed completely. Healing was considered to be complete when the epithelium covered the entire wound and the area of the remaining granulation tissue was zero (Bigbie and others 1991).

Gross and histopathology of post healing skin wound:
The samples of full-thickness skin tissue from healing wounds in each treatment and control group were collected after complete healing of the wound (day 22) and fixed in 10 per cent neutral buffered formalin. After fixation of the tissue, thin pieces (2-3mm) were cut involving entire thickness of skin. Thin tissue pieces were processed for paraffin embedding and making of paraffin tissue blocks as per standard method (Luna---). Tissue paraffin blocks were cut with the help of microtome to obtain 5 thick paraffin sections on glass slides. The sections on slides were staining with routine Haematoxylin and Eosin (H&E) method. Masson’s trichrome staining was also done for demonstration of collagen in healing tissue.

Data analysis:
The means of parametric observations were compared by analysis of variance (ANOVA) as described by Snedecor and Cochran (1989) using SPSS software. For each comparison, differences between groups were considered significant at P < 0.05.

RESULTS:
Characterization of Caprine BM-MSCs:
Fluorescence-activated cell sorting (FACS) analysis of our BM-MSCs showed that they were positive for typical surface antigens such as CD73,CD-105 and negative for CD-45 (Fig 2,3, & 4). The immunocytochemistry results showed that they were positive for cell surface markers like CD-73,CD-105 and CD-90 (THY-1)(Fig 5,6 & 7). These cells were also positiveive for Endoglin (CD-105) and Thy-1 (CD-90) on molecular characterization. When cultured in adipogenic and osteogenic medium, they differentiated into adipocytes (Fig, 8) and osteoblasts (Fig,9).

Wound contraction
Although the original wound created was 2x2 cm, some of the wounds expanded to various extents, possibly due to the loose nature of the skin of the rabbit dorsum. Thus, after the surgery, some wounds had an area greater than 400mm². However, immediately after surgery, there was no significant difference in the mean wound area.

By day 5,10, 12,14,18 and day 22,The mean percentage wound contraction were insignificantly higher (P<0.05) in control group (22.375,45.500,66.812,72.625,95.125 and 100.00 percent, respectively) than cMSCs treated wounds (19.085,41.700,53.377,70.905,92.500 and 99.147 percent). It was observed that within 22 days post surgery, there was complete healing of wound in control as compared to treatment, where very small areas were still left for healing but the difference in percent wound contraction was statistically non significant.
At day 22, the wound contractions were almost similar in control and treated group (table 1, Fig. 10 A to C).

**Table 1.**

<table>
<thead>
<tr>
<th>Number of days after surgery</th>
<th>Day5</th>
<th>day 10</th>
<th>day12</th>
<th>Day14</th>
<th>day18</th>
<th>day22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(WC%)</td>
<td>22.375a</td>
<td>45.500b</td>
<td>66.812c</td>
<td>72.625c</td>
<td>95.125d</td>
<td>100.00d</td>
</tr>
<tr>
<td></td>
<td>(0.21)</td>
<td>(0.82)</td>
<td>(0.60)</td>
<td>(0.02)</td>
<td>(0.82)</td>
<td>(0.01)</td>
</tr>
<tr>
<td>Treatment(WC%)</td>
<td>19.085a</td>
<td>41.700b</td>
<td>53.377bc</td>
<td>70.905c</td>
<td>92.500d</td>
<td>99.147d</td>
</tr>
<tr>
<td></td>
<td>(0.92)</td>
<td>(0.54)</td>
<td>(0.91)</td>
<td>(0.53)</td>
<td>(0.11)</td>
<td>(0.29)</td>
</tr>
</tbody>
</table>

*Means bearing common superscript are not differ significantly (P<0.05).

**Gross and histopathology of post healing skin wound:**

It was observed that within 22 days post surgery, there was complete healing of wound in control as compared to treatment, where very small areas were still left for healing but the difference was statistically non significant (P<0.05). However, histopathological finding showed that, in control, although, there was complete epithelialization of the wound but the stroma had moderate amount of well aligned fibrocytes with less quantity of thick collagen. On the contrary, in the treatment group, apart from complete epithelialization, the underline stroma had more fibroplasia with higher amount of slightly irregular thick collagen than in control (Fig.11). This was indicated the better healing of cMSCs treated wound than control.

**Survival and Regeneration ability of transplanted xenogenic Caprine BM-MSCs into rabbit skin:**

The PKH-26 labeled Caprine bone marrow MSCs into PBS (2.0 x 10^6 cells) were injected into treated wounds of rabbit. After 22 day of wound healing, the tissue around healed area was taken. The survival and regeneration ability of transplanted Caprine MSCs were identified by presence of PKH-26 labeled cells (Fig12,a,b,&c).
Endoglin (CD-105) - 11.41%

CD-45

0.07%

CD-73
Endoglin (CD-105)

Thy-1 (CD-90)

In vitro differentiated adipoblast cells (10X) (Oil O staining)
In vitro differentiated osteoblast cells (10X) (Alizarin Red S positive cells).

A. 13th day wound

B. 18th day wound
Fig. Skin wound healing after artificially created wound in New Zealand White rabbits. 2X2 cm² size of cutaneous wounds, two on either side of the mid dorsal line, were created. The right side of wounds were injected with 2x10⁶ goat mesenchymal stem cells of passage P12 in 150 µl of PBS on margins as well as in the centre of the wound bed. In left side wounds, only 150 µl of PBS was injected. A. 13 th day wound B. 18 th day wound and C. 22nd day wound.

Fig. Skin sections taken from artificially created wound site after the healing from New Zealand White rabbit. 2X2 cm² size of cutaneous wounds, two on either side of the mid dorsal line, were created. The right side of wounds were injected with 2x10⁶ goat mesenchymal stem cells of passage P12 in 150 µl of PBS on margins as well as in the centre of the wound bed. In left side wounds, only 150 µl of PBS was injected as above. HE: Haematoxylin and Eosin staining, MTS: Masson's trichrome staining. X100.
PKH26 labeled Caprine Mesenchymal stem cells before transplantation (10X)

Fig a

PKH26 labeled Caprine Mesenchymal stem cells transplanted skin section (Treatment group) (10X).

Fig b

PBS injected skin section (control group) (10X)

Figure c

Fig: Skin sections taken from artificially created wound site after the healing from New Zealand White rabbit. 2x2 cm² size of cutaneous wounds, two on either side of the mid dorsal line, were created. The right side of wounds were injected with pkh-26 labeled 2×10⁶ goat mesenchymal stem cells of passage P12 in 150 μl of PBS on margins as well as in the centre of the wound bed. In left side wounds, only 150 μl of PBS was injected as above.
DISCUSSION:

It has been reported that BMSCs may be immune-privileged cells that do not elicit immune responses due to an absence of immunologically relevant cell surface markers. In addition, BMSCs have immunomodulatory function (Bartholomew et al. 2002; Le Blanc et al. 2003; Ringe et al. 2002; Rasmusson, 2006; Wang et al. 2006). Such immunological characteristics of BMSCs theoretically can make them impervious to immunorejection following xenogenic transplantation, irrespective of the use of immune suppression. Previous studies have reported conflicting results following xenogenic BMSCs transplantation into non-immunosuppressed hosts, ranging from no survival to differentiation into destination cells (Grinnemo et al. 2004; MacDonald et al. 2005; Saito et al. 2002; Tashiro et al. 1995). There are limited studies about Xenogenic Human mesenchymal stem cells transplantation into animal models like mice, sheep, and rabbits. However, these animals have not close relationship in origin from human. In our literature review, we were unable to find any study that had investigated the results of xenogenic ruminants BMSCs transplantation in skin wound healing model. The authors therefore performed the current study to investigate the hypothesis that xenogenic caprine BMSCs transplanted into the full thickness skin excisional wound healing in rabbit model without immune suppression and dressing of wounds.

The bone marrow mesenchymal stem cells that secrete a large number of growth factors and cytokines, which are critical in the repair of damaged tissues (Mcfarlin et al. 2006; Wu et al. 2007). Wu et al. (2007) opined that bone marrow cells enhance neovascularisation and promote wound healing through differentiation and the release of proangiogenic factors.

There has been evidence that adult bone marrow cells transplanted into the skin wounds of mice differentiate into epidermal keratinocytes, sebaceous gland cells, follicular epithelial cells, dendritic cells and endothelial cells (Kataoka et al. 2003), and fully differentiated skin (Krause et al. 2001, Badiavas et al. 2003, Kataoka et al. 2003). In vivo experiments by Yamaguchi et al. (2005) demonstrated that bone marrow cells differentiate into wound myofibroblasts when they enter the micro-environment of a wound. Opalenik and Davidson (2005) showed that adult bone marrow derived cells participate in wound repair by differentiating into wound fibroblasts.

In the present study, we tested the therapeutic value of mesenchymal stem cell therapy to enhance skin wound healing. We used Caprine bone marrow mesenchymal stem cells transplanted into a rabbit xenogeneic host model of excisional skin wound lesions, based on the accumulating evidence. The Caprine bone marrow MSCs were injected into the wound margins and wound bed. It was observed that within 22 days post surgery, there was complete healing of wound in control as compared to treatment, where very small areas were still left for healing but the difference was statistically non significant (P<0.05). However, histopathological finding showed that, in control, although, there was complete epithelialization of the wound but the stroma had moderate amount of well aligned fibrocytes with less quantity of thick collagen. On the contrary, in the treatment group, apart from complete epithelialization, the underline stroma had more fibroplasia with higher amount of slightly irregular thick collagen than in control. The histo-pathological results showed the better wound healing observed in the treatment group compared with the control groups. The survival and regeneration ability of transplanted Caprine MSCs were identified by presence of PKH-26 labeled cells. Caprine MSCs were differentiated into myofibroblasts and fibroblasts.

It has been suggested that Histomorphological assessment of healing in open wounds allow a more precision than clinical examination (Abramo et al. 2004). Liu et al. (2006) reported that, in response to the wound microenvironment, bone marrow stem cells augment wound healing through the responsive secretion of growth factors that enhance angiogenesis and promote wound repair.
Diabetic ulcers and other chronic wounds are difficult to heal, and little improvement has been shown in preventing the associated morbidity and disability in the past few decades (Boulton et al. 2005). The best available treatment for chronic wounds achieves only a 50% healing rate that is often temporary. Innovative treatments to enhance wound healing and regeneration are needed.

An optimal scar is one that most resembles the collagen structure, architecture of normal skin and tensile strength. Thus, the biological status of the repaired wound depends on the proper chemical reorganization of the collagen fibers during the remodeling stage, which affects the tensile strength.

The concentrated conditioned medium from MSCs can modulate wound repair without MSCs being present in the wound. The therapeutic effects of MSCs might be attributable to their ability to differentiate and transdifferentiate into tissue-specific cells, to fuse with the resident cells, to secrete a wide array of paracrine factors in order to stimulate the survival and functional recovery of the resident cells, or to regulate the local microenvironment or niche and immune response. These mechanisms are probably independent but not mutually exclusive. In many circumstances, a combination of these protective mechanisms might work together to affect skin wound healing (HaiHong Li and Xiaobing Fu 2012). Although the specific mechanisms in each type of cutaneous or skin wound are still unclear and controversial.

In conclusion, our results suggest that xenogenic BMSCs may provide an alternative to autologous as well as allogenic BMSCs in situations where the host has limited availability of autologous and allogenic BMSCs due to old age, metabolic and inherited diseases. This suggests the intriguing possibility of utilizing genetically engineered and specifically bred animals that are free of transmissible diseases as a source of BMSCs. The further studies are needed to investigate the appropriate dose of xenogenic BMSCs. The study indicated that the Caprine MSC treatment could be used for proper skin wound healing.

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