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COMPARATIVE EVALUATION OF ALLOGENIC AND XENOGENIC STEM CELLS IN WOUND HEALING

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Abstract: The present study was conducted to compare wound healing potential of allogenic and xenogenic MSCs in G.pig model. Bone marrow derived MSCs of different species viz. canine, caprine and guinea pig were isolated, *in vitro* expanded, phenotypic characterization and trilineage differentiation potential was done and stem cells was collected at third passage of BM-MSCs. Stem cells were applied towards dermal healing following their topical application in full thickness ($2\times2cm^2$) skin wound in a guinea pig model. Bone marrow sample was collected from the bone marrow and mononuclear cells were separated by density gradient centrifugation and were seeded in growth medium. Morphologically these spindle-shaped cells resembled typical fibroblasts; and reached 70% confluence in 8–10 and 10–12 days, respectively but species of guinea pig derived BM-MSCs were reached 70% confluence in 6-8 days. Positive surface markers for MSCs along with a negative marker were localized by immunofluorescence, flow cytometric analysis. MSCs cell surface antigens (CD73, CD90 and CD105 were localized in BM-MSCs (canine, caprine and guinea pig) monolayers by positive immunofluorescence staining and were negative for the CD34. All the three cell lines of BM-MSCs were having potential of tri lineage differentiation. It was found that allogenic stem cells healed significantly faster than xenogenic stem cells and quality of healed wound was also better. The score of neovascularization was significantly ($P\leq0.05$) lower with advanced stages of vascularisation in all the stem cells treated groups than the control groups and lowest score was for G. pig followed by caprine and canine stem cells. Similarly, collagen deposition score was significantly ($p<0.05$) high in control than stem cells treated group and it was lowest for G.pig stem cell treated group. It can be concluded that allogenic stem cells is better than xenogenic stem cells in excision wound healing.
Keywords: Guinea pig, Allogenic, Xenogenic. Mesenchymal Stem cells, Excisional wound

Introduction

Stem cells are quiescent, unspecialized or undifferentiated cells, which undergo asymmetric cell division and produce daughter cells that maintain the stem cell pool and a progenitor cell which undergoes rapid proliferation followed by differentiation (Verfaillie, 2002). The ability of stem cells to differentiate into multiple cell lineages has proposed in exciting possibilities for stem cell based therapies, which help to regenerate and repair damaged tissues and organs. Stem cells may be isolated from the embryos, fetal (umbilical cord and placenta) and adult tissues (Ilancheran et al., 2007). MSCs comprise a rare population of multipotent progenitor cells which are capable of supporting hematopoiesis.

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Regenerative therapy, a relatively new field based on the use of stem cells to generate biological substitutes and improve tissue functions, restoring damaged tissue with high proliferability and differentiability (Toda et al., 2007). Hence it is necessary to understand the full spectrum of stem cell actions and preclinical evidence for safety and therapeutic efficacy. The role of animal models for gaining this information has increased substantially, larger animals, such as dogs, pigs, goats, sheep, and non-human primates, are often better models than that of mice for this purpose as they have a longer life span and many physiological parameters (for example, immune system properties are much closer to humans than are those of rodents). Large animals also have significant advantages regarding the number and types of stem cells that can be reproducibly extracted from a single animal and manipulated, in vitro in sufficient quantity for analysis and for various applications. These species also provide significant advantages for modeling specific human disease conditions and testing stem cell therapies, hence, provide better model. Studies are required in variety of model animals as source of stem cells and host for allogeneic and xenogeneic tissue grafts. It will help to establish proof-of- concept and test the safety of potential therapies (Harding et al., 2013). Till date no information is available regarding comparative efficacy of allogenic and xenogenic stem cell in wound healing.

Materials and methods

The present study was conducted in Reproductive Physiology Laboratory,
Physiology and Climatology Division, ICAR-Indian Veterinary Research Institute,
Izatnagar, Bareilly,India.

1. Chemicals and reagents

All the chemicals and media used for the culture were procured from the Sigma (St.Louis, MO, USA) were culture grade and endotoxin tested. The description of chemicals is given in appendices and text, wherever required. The chemical composition of all the media, buffers and reagents used in the present study are listed in the Appendix-I, II. Stock solutions were used within 30 days of preparation, whereas, the working solutions were prepared, filtered through membrane filters (0.22 μm) purchased from Millipore (Molsheim, France) and pre-equilibrated with 5% CO2 at 37^0C for 12 hrs prior to use.

2. Plastic wares and glass wares

All the plastic wares used for the culture viz., culture dishes, multi well plates, centrifuge tubes and culture flask etc., were purchased from established firms such as Nunc (Roskilde, Denmark), Axygen Scientific Private limited (USA) and Greiner (Austria). The plastic wares

were either certified to be free from DNAse, RNAse activity or treated with DEPC and autoclaved to render them DNAse and RNAse free. All the glass wares used for RNA isolation and RT-PCR work in the present study were procured from Schott Duran (Germany). The glass wares were cleaned as per standard laboratory procedures and autoclaved at 120^0C and 15 psi for 15 min after overnight exposure with 0.01% DEPC treated water to render them DNAse and RNAse free. All the plastic wares and glass wares were UV treated for 30 min before use.

3. Equipments and Instruments

All the standard equipments and instruments available in Reproductive Physiology Lab of Physiology and Climatology, Division and Central Instrument Facility (CIF) of Pathology, Indian Veterinary Research Institute, were used for desired purposes. Specification of the instruments/equipments is given at appropriate places in the text wherever necessary.

4. Molecular biology reagents, kits and enzymes

All the molecular biology reagents, kits and enzymes used in the present study were obtained from established firms such as Sigma, Thermo scientific, Fermentas, Invitrogen, Ambion, Bangalore-Genei, and Merck. Specifications of the chemicals, molecular reagents and bio chemicals are given at the appropriate places in the text, wherever necessary.

The present study was conducted to compare isolation, in vitro expansion; phenotypic characterization and tri lineage differentiation potential of bone marrow derived MSCs of different species viz canine, caprine and guinea pig and collection of stem cells at third passage of BM-MSCs of different species. Stem cells of third passage was applied towards dermal healing following their topical application in full thickness (2×2cm) skin wound in a guinea pig model. In order to achieve our objective, the study was divided into 3 major experiments. The results of each experiment have been described separately.

Results and discussion

1. Evaluation of allogenic and xenogenic stem cells in wound healing

This experiment was conducted for comparative evaluation of therapeutic potential of allogenic and xenogenic stem cells towards dermal healing following delivery to full thickness (2×2cm) excision skin wounds in the guinea pig model. Full thickness excisional wounds of 2×2cm were created on the dorsal surface of guinea pig, however after recovery from anesthesia all the wounds expanded to an area ranging to 4.5-5.5cm to possibly due to the loose nature of the guinea pig dorsum skin (Fig 1 and Table 1).

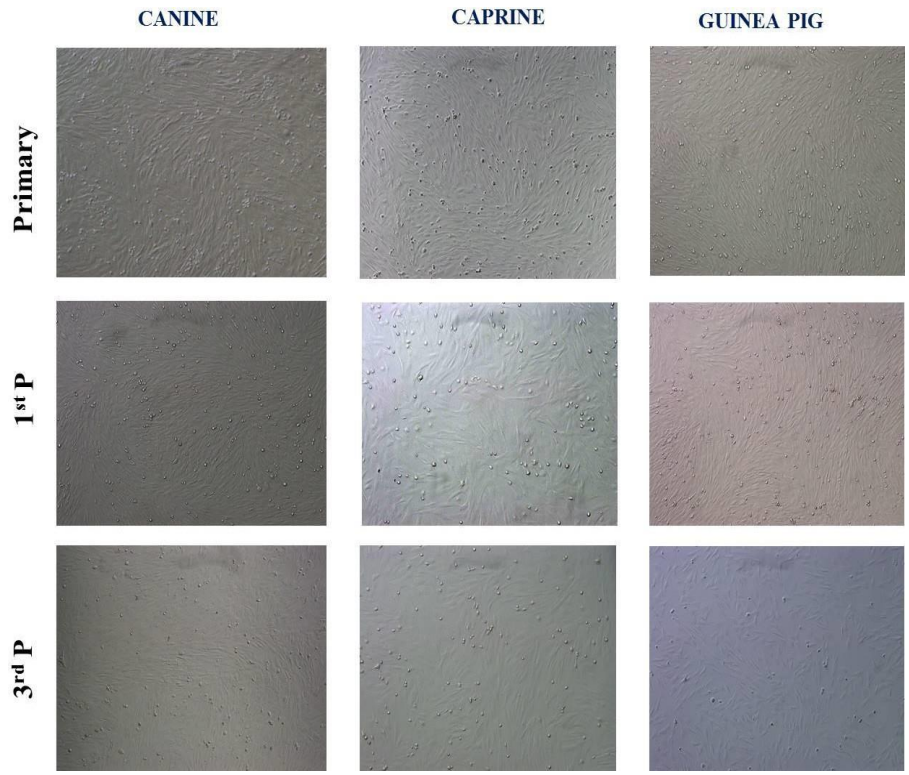

Fig .1 Culture and *in vitro* expanded of BM-MSCs of canine, caprine and guineapig observed under bright field microscope (Primary, 1st and 3rd Passage)

Macroscopic evaluation of the wounds

Digital photographs showing the gross reduction in wound area on 7 th, 14 th, 21 th, 28 th days after application of stem cells therapy are represented in Fig 2. On comparing the wound area (Fig.2), a significant (p< 0.05) change was observed on day 14 th between treatment groups. All the different species of stem cells treated groups registered more than 50% contraction of the wound, but it was only 35.10% in the control laminin group. A significant ($p \leq 0.05$) increase in % wound contraction (60%) was seen at day 14 th in different stem cells of guinea pig, canine and caprine bone marrow derived mesenchymal stem cells. But the differences were not significant (p> 0.05) when compared with allogenic and xenogenic stem cells. The percent wound contraction on 21st and 28 th days post surgery was significantly (p< 0.05) better in allogenic stem cells group than other groups and caprine stem cells group did not significantly (p>0.05) better than canine and control group (fig 2 and table 2).

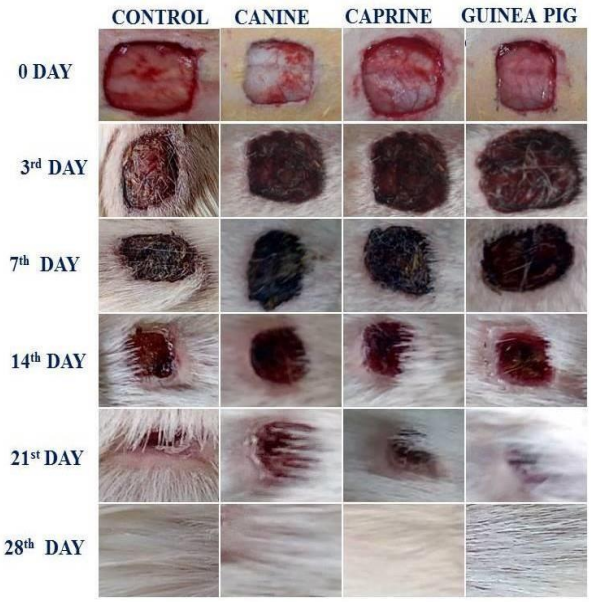

Fig.2: Digital photographs (representative) showing gross reduction of wound area after treatment with stem cells therapy on oth , 3rd , 7th, 14th, 21st, and 28thday post-incision in different treatment groups.

Table 1 Wound area (2x2cm) of the excisional wounds in different treatment groups at different time intervals

Days	Laminin gel	Canine stem cells at 3rd passage	Caprine stem cells at 3rd passage	Guinea pig stem cells at 3rd passage
0day	5.07±0.15	5.01±0.18	4.90±0.12	5.30±0.11
3 rd day	4.78±0.17	4.63±0.06	4.56±0.10	4.99±0.16
7 th day	4.42±0.19	4.28±0.05	4.33±0.04	4.63±0.17
14 th day	3.29±0.16 a	1.74±0.12 b	1.49±0.06 bc	1.23±0.24 c
21 th day	1.23±0.16 a	1.06±0.07 a	0.90±0.19 b	0.37±0.17 c
28 th day	0.07±0.06 a	0 b	0 b	0 b

Values (means±SE) in a row bearing different superscripts in the row differ significantly ($p \leq 0.05$) in different groups.

Table.2 Percentage wound contraction of excisional wounds in different treatment groups at different time intervals.

Days	Laminin gel	Canine stem cells at 3rd passage	Caprine stem cells at 3rd passage	Guinea pig stem cells at 3rd passage
3 rd day	5.78 a	6.95 a	7.45 a	5.56 a
7 th day	12.86 a	13.07 a	14.32 a	14.56 a
14 th day	47.36 a	67.34 b	69.24 b	72.52 b
21 th day	76.66 a	78.76 a	81.44 ab	94.26 b
28 th day	89.63 a	99.59 b	99.93 b	100 b

Values (Means) in a row bearing different superscripts in the row differ significantly ($p \leq 0.05$) in between different groups

3. Histomorphological observation

Skin biopsy samples were taken on day 28th post treatment and tissue samples were evaluated for epithelization, neovascularization, collagen deposition and its arrangement as shown in Fig 3 and table 3. It was found that the surface epithelium of allogenic and xenogenic stem cells treated groups was restored with better scores and appeared more closer to normal skin section compared to the control group. The score of neovascularization was significantly (P≤0.05) lower with advanced stages of vascularisation in all the stem cells treated groups than the control groups and lowest score was for G. pig followed by caprine and canine stem cells. Similarly, collagen deposition score was significantly (p< 0.05) high in control than Laminin gel treated group and it was lowest for G.pig stem cells treated group. A significantly (p≤0.05) more dense, thick and well arranged collagen fibres (fig.4.11) were seen in G.pig stem cells group than other groups and caprine stem cells group also did significantly better than canine and control group.

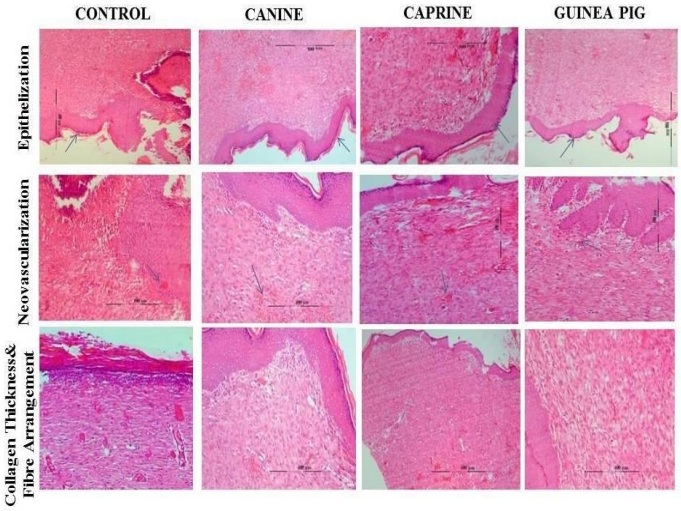

Fig. 3: Histomorphological evaluation of epithelization, neovascularization and collagen thickness and collagen fibre arrangement in the tissue section ofdifferent groups on 28th days post incision stained with hamatoxylin and eosin.a) laminin gel b) Canine stem cells c) Caprine stem cells d) Guinea pig stem cells.

Table 3. Histomorphological evaluation of wound in different groups.

Parameters	Laminin gel	Canine stem cells at 3rd passage	Caprine stem cells at 3rd passage	Guinea pig stem cells at 3rd passage
Epithilization	3.14±0.33 a	1.42±0.28 bc	1.57±0.33 b	1.26±0.28 c
Neovascularization	2.36±0.17 a	1.96±0.57 b	1.27±0.33 c	1.09±0.04 c
Collagen thickness	2.64±0.19 a	1.67±0.067 b	1.64±0.17 b	1.35±0.28 c
Collagen fibre arrangement	2.96±0.23 a	2.18±0.33 b	1.06±0.06 c	1.06±0 c

Values (Means±SE) in a row bearing different superscripts differ significantly (p≤0.05).

BM-MSC conditioned medium, which showed high levels of VEGF and SDF-1, known potent chemo attractants to endothelial cells and endothelial progenitor cells (Urbich et al., 2004; Lamalice, 2007). BM-MSC-derived secreted mitogens may stimulate the proliferation of dermal fibroblasts, keratinocytes and endothelial cells in vitro (Javazon et al., 2007; Lee 2009; Smith 2010; Kim2007) and possibly in vivo as well. Wound healing mechanism mainly involves 3 different phases, inflammatory, proliferative and remodeling phases. These events are overlapped without any clear demarcation for their time periods. Immediately following cutaneous injury, blood elements and vasoactive amines extravasate from locally damaged blood vessels within dermis to start the inflammatory response. Vascular permeability is temporarily increased to allow neutrophils (PMNs), platelets and plasma proteins to infiltrate the wound. Vasoconstriction follows, in response to factors released by these cells. Coagulation then occurs as platelets aggregate with fibrin, which is deposited in the wound following its conversion from fibrinogen. Platelets release several factors, including platelet derived growth factors (PDGF) and transforming growth factors β (TGFβ), which attracts PMNs to the wound, signaling the beginning of inflammation. After 48hr macrophage replace PMNs as the principal inflammatory cell. Together, PMNs and macrophages removes debris from the wound, release growth factors, and begin to reorganize the extracellular matrix, the prolifreration phase begins at about 72hr as fibroblast, recruited to the wound by growth factors released by inflammatory cells, begin to syntheize collagen. Although the rate of collagen synthesis slows down after about three weeks, collagen cross linking and reorganization occur for months after injury in the remodeling phase of repair (Diegelmann et al., 2004).

Conclusion

Better wound healing in allogenic stem cells than xenogenic stem cells may be due to the fact that some of the growth factors may not be identical in structural/chemical nature and thus not acted properly in xenogenic model. Apart from this fact, MSC secreted several other molecules like micro RNAs in micro vesicles which may be differ in stem cells of different species, which needs to be investigated further.

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