Repair of Bone Gap Defect Using Human Wharton Jelly Derived Stem Cells in Canine Model: Radiological and Histopathological Study

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Abstract:
This study was conducted to evaluate the ability of human Wharton jelly derived stem cells (WJSCs) to induce bone formation when implanted in canine tibial gap defect. As well as to insure absence of immune rejection against stem cells when implanted xenogenically. It was carried out on nine apparently healthy males of mongrel dogs weighing 20 to 25 kg. The animals were randomly divided into three equal groups (1, 3 and 6 months) according to the observation periods. Two 10 mm diameter holes were surgically created at the proximal third of the tibia. The first hole was filed at the time of the operation with WJSCs suspension while, the second one was left empty as control negative. Radiological and histopathological studies revealed that holes filed with WJSCs showed new bone formation which was faster and better compared to control one. As well as, no immune reaction was detected against WJSCs when implanted xenogenically. Our results support the hypothesis that mesenchymal stem cells can be used for bone grafting between different species without the fear of immune rejection.

Key words: Human Wharton jelly derived stem cells, Gap defect, Xenogenic, Dog, Histopathology
INTRODUCTION
Bone is the most transplanted human and animal’s tissue. When bone injury occurred, it retains a good capacity to repair itself (Schmidt-Bleek et al., 2014). Spontaneous healing usually not allowed in some cases of bone affections. In such cases, surgical intervention and bone regeneration strategies are required to induce bone healing (Jafarian et al., 2008; Moghaddam, et al., 2015; Westhauser et al., 2015; Stanovici et al., 2016). Autologous bone grafts still considered as the gold standard for treatment of bone defects (Salgado et al., 2004). Although it is superior to allografts and xenografts (Finkemeier, 2002), its application in orthopedic felids is usually limited by a considerable donor site morbidity and the limited amount that can be supplied by the donor (Spitzer et al., 2002; Kim et al., 2009). Allografts and xenografts are the second widely used bone grafting materials. It possesses many advantages over autograft bone in terms of; it reduces the donor morbidity, it can be supplied with large number and different shapes as well as, it constitutes a major source of osteogenic cells to the implantation site (Cho and Lee, 2006, Stievano et al., 2008; Heo, et al., 2011; Emara et al., 2013a&b). Their clinical application is usually hindered by the risk of immune rejection and pathogen transmission to the implantation site (Shegarfi & Reikeras, 2009; Nandi et al., 2010; Herberths et al., 2011).

Several biomaterials have been used as bone substitutes. It well accepted and tolerated by living body. It also, possesses the advantages of an unlimited availability, good osteoconductivity and high biocompatibility (Hak, 2007; Ghosh et al., 2008; Nandi et al., 2008; Rahaman et al., 2011; Emara et al., 2013b). On the other hand, most of the known bone substitutes lacks osteoinductive properties which made them unable to heal large bone defects when used alone (Ajeesh, 2010; Emara et al., 2013b).

Recently tissue-engineering in the form of cells capable of osteogenic activity was introduced into orthopedic fields an alternative to bone grafting materials for inducing bone regeneration (Jang et al., 2008; Jafarian et al., 2008; Chen et al., 2009; Udehiya et al., 2013). This approach has several advantages over traditional methods of bone grafting; including ease of handling and manipulation of the cells, good quality repair, low morbidity at the donor site and low risk of immunorejection and pathogen transmission (Pountos et al., 2007).

Human and animal bodies houses several types of uncommitted progenitor stem cells capable of giving rise to daughter cells. It can grow and differentiate into one or more types (Schwartz & Reyes, 2002; Smith & Webbon, 2005; Pountos et al., 2006; Jung et al., 2008; Yamachika & Iida, 2013). They were isolated from different location in the body (Tuli et al., 2003; Pountos et al., 2006). Among adult stem
cells, mesenchymal stem cells (MSCs) are the most commonly used type suitable for bone tissue engineering. It represents an ideal stem cell source for cell therapies due to their ease of isolation, expansion and their ability to differentiate into different tissues under certain stimuli (Chao et al., 2007; Patel et al., 2008; Udehiya et al., 2013; Brennan et al., 2014).

Autologous and allogenic MSCs were used alone or seeded with synthetic or natural osteoconducting matrix for bone regeneration (Planka et al., 2008; Jafarian et al., 2008; Pagni et al., 2012; Yamachika & Iida, 2013). The cells may be implanted directly after harvesting (Centeno et al., 2006; Le Nail et al., 2014; Scaglione et al., 2014; Thua et al., 2015; Ajiboye et al., 2015), implanted after being isolated, expanded and directed to form specific tissues using specific stimuli (Dong et al., 2013; Brennan 2014).

The use of autogenic MSCs has its limitations; in which to obtain the proposed number of the cells, a large quantities of bone marrow are required to be aspirated which subjects the patient to more than one operation (Hernigou et al., 2005; Kim et al., 2007). In addition, several weeks are required for expansion of the cells before implantation (Watson et al., 2014). The availability of both allogenic and Xenogenic MSCs and its ability to overcome host immune rejection have made these cells an attractive alternative to autogenic MSCs for reconstructive surgery (Nishio et al., 2006; Kim et al., 2007; Jung et al., 2009; Stanovici et al., 2016; Westhauser et al., 2016). The aim of this study is to investigate the ability of human Wharton jelly derived stem cells (WJSCs) to induce new bone formation when used to reconstruct an artificially induced critical size bone gap defect in canine tibia. As well as to

MATERIALS AND METHODS

1. Experimental design

The study protocol followed the guidelines of faculty of veterinary medicine, University of Sadat city, Egypt for the use and care of animals. Nine males apparently healthy, Mongrel dogs weighing 20 to 25 kg. were used in this study. The animals were randomly divided into three equal groups 1, 3 and 6 months according to the observation periods. The dogs were used as recipient for WJSCs

2. Isolation, Characterization and processing of Human Wharton jelly derived stem cells (WJSCs)

2.1. Isolation and culture of Wharton jelly derived stem cells (WJSCs)

WJSCs were isolated from donated umbilical cord (UC) tissue samples (n=15). Umbilical cord samples were collected from normal, full-term deliveries following cesarean section. Informed consent from all others was taken. Umbilical cords were transported to the laboratory in normal saline and cell isolation was carried out within 24 h. from tissue collection. After removal of
arteries and veins, Wharton jelly was cut into 0.5- to 1-cm³ pieces and suspended in fresh complete nutrients medium which includes: Dulbecco modified Eagle low-glucose media (DMEM-LG) with L-glutamine, 10% FBS, Penicillin-streptomycin (10,000 U/ml and 10,000 µg/ml), Fungizone (250 µg/ml), all from Lonza. The flasks were incubated in a 37°C humidified incubator with 5% CO₂ to allow cells to migrate from the explants. The first change of the media was at day 7 then the media was changed twice weekly until reaching 70% to 90% confluence by inverted microscope (Huang et al., 2010).

2.2. Characterization of MSCs by flow cytometry
The harvested MSCs were characterized by flow cytometric analysis for PE CD34 (Imunostep), PE CD44 (BD Pharminogen) and FITC Oct3/4 (BD Pharminogen). 100 ul of cell suspension was incubated with 10 µl monoclonal antibody incubated in the dark at 4°C for 20 minutes. The tubes were washed with 2 ml phosphate buffered saline (PBS) at 1800 rpm for 5 minutes. Analysis was performed using the BECTON DICKINSON software (BD Biosciences).

2.3. WJSCs processing for application:
After 80% confluence was reached, and at least 3-4 hours before the operation the adherent cells were harvested by trypsin-EDTA 0.25% solution. Viability was assessed by trypan blue 0. 4% (Sigma). Cells was counted by hemocytometer and 20 × 10⁶ cell suspension in DMEM-LG were stored in CO₂ till the operation time.

2.4. Surgical procedures
2.4.1. Anaesthesia and Preoperative animal preparation technique:
All animals were pre-mediated with I/V injection of mixture of atropine sulfate 0.05 mg/kg (Atropine sulfate®: 1mg/ml Med. Co., ARE) and diazepam 1 mg/kg (Neuril®: 0.5% sol. Memphis Co. for Pharm. &Animal Ind. Cairo A.R.E). Anaesthesia was induced immediately through I/V injection of a mixture of Ketamine 10 mg/kg (Ketalar®: 5% sol. Amoun Co. A.R.E.), and Xylazine 1 mg/kg (Xylaject®: 2% sol. ADWIA Co., A.R.E). The anaesthetic depth was maintained with 2.5% thiopenal sodium (Thiopenal®: EPICO Co., ARE), administrated by I/V rout (Schmidt et al., 1995). The lower region of the hind limb (tibia) was prepared for aseptic surgery followed by routine orthopedic operative draping and gowning procedures. A prophylactic course of Cefotaxime sodium (Cefotax®: EPICO, ARE) at dose of 4.5 mg/kg/bw was administered intravenously before the operation and repeated every 8 hours for five successive days post operation.

2.4.2. Surgical procedure (Fig. 1 A & B)
A 10-cm skin incision was made at the proximal third of the medial surface of the right tibia. The incision includes skin and periosteum to expose
the bone (Fig. 1A) (MacNeill et al., 1999). Two 10 mm diameter holes were created at the proximal third of the tibia. They were 1 cm apart from each other. Each defect extended through only one cortex. The drilled holes were packed using sterile gauze to control hemorrhage from the medullary cavity. The previously prepared WJSCs suspension was added to the first hole at the time of the operation using micropipette while, the second one was left empty as control negative (Fig. 1B). The surgical wound was closed using polyglactin 910 (Vicryl®).

2.5. Post-operative follow-up evaluations

2.5.1. Clinical evaluation
All operated animals were subjected to daily clinical examination, including appetite, wound drainage, local reaction, regional lymph node size and the wound status.

2.5.2. Laboratory assessment of inflammation
Blood samples were collected directly before the operation and at time points 1, 2, 3, 4 weeks post operation to evaluate post-operative inflammation. Inflammatory markers including total leukocytic count (TLC), erythrocyte sedimentation rate (ESR) and quantitative measurement of C-reactive protein (CRP) were measured. TLC and ESR were evaluated using the classical Westergren method (Westergren, 1957), while, CRP was evaluated using immunoturbidimetric method (Peltola, 1982).

2.5.3. Radiological evaluation
Medio-lateral views were taken on a standard 30×40 cm film at 50-52 kVp, 85 FFD and 10 mAs by using X-ray apparatus (Semens 300). First radiographs were taken immediately post-operation and once every 2 weeks until the end of the study (six months). Radiographs were evaluated for radiographic density of the implanted and the control holes.

2.5.4. Morphological studies
At the end of each observation period 1st, 3rd and 6th months. The dogs of each group were euthanised using large dose of thiopental sodium. The operated tibiae were harvested and examined grossly. Each hole was inspected for complete or partial filling in relation to the adjacent bone.

2.5.5. Histopathological Examination
Bone samples were collected from the operated tibiae. Each sample contain the defect hole with its surrounding healthy tissue was immediately fixed in 10% formalin for one week. The samples were decalcified using 10% EDTA di-sodium solution (P.93®: El Nasr pharmaceutical chemical, Egypt) for one month (Shibata et al., 2000). Decalcified samples were routinely prepared and impeded in paraffin wax. A 3-5-micron sections were mounted on glass slides, deparaffinized, rehydrated and stained with hematoxylin and eosin for histopathological examination.
RESULTS

Isolation and Immunophenotypic characterization of WJSCs:
Nine from fifteen samples were successfully isolated with viability 96-100%. It showed adherence to plastic surface and fibroblastoid morphology reaching 70%-80% confluence at day 15-21 of culture (Fig. 2). Using flowcytometry, the cells were positive for CD Oct3/4, Cd44 and negative or low expression to CD34 (Fig. 3 & Table 1).

Clinical assessment:
There is no evidence of infection or seromal reaction in the operated animals as, assessed by visual and palpable monitoring the degree of swelling and temperature of the operated limbs, regional lymph node size as well as, absence of wound drainage.

Laboratory results of inflammatory markers
TLC, ESR and CRP were increased from basal level before the operation to postoperative periods 1st and 2nd weeks and begin to decrease at the 3rd week then returned to its normal levels by the end of the 4th week (Table 2)

Radiographic results:
On examination of the radiographs taken for the operated tibia directly after the operation and throughout the observation period, it was noticed that; both control hole and hole implanted with WJSCs appeared radiolucent immediately post-operation (Fig. 4A). By the end of the 2nd week post operation, a slight radiopaque zone at the hole’s margin implanted with WJSCs was noticed, while the control one showed no detectable changes (Fig. 4B). By the end of the fourth weak post operation; the holes implanted with WJSCs showed irregularity at its margins with slight reduction in its width compared with previous period. At this period, the control hole showed a slight increase of radiopacity at its margin (Fig. 4C). At 12th weeks post operation, the holes implanted with WJSCs showed marked reduction
in its diameter compared to the control ones (Fig. 4D). At 24th week post operation the holes implanted with WJSCs was difficult to be detected radiographically, while the control one could be easily detected with a marked radiolucent zone at its center (Fig. 4E).

**Histopathologic results**

Microscopical examination of bone samples collected from control group euthanized after one month and three months’ post-operation showed that, the entire bone holes were filled with collagen bundles (Fig. 5A & B). After six months’ post-operation, control bone-samples begins to form immature bone cells mixed with collagen bundles (Fig. 5C). In the other hand, bone holes treated with WJSCs showed that the entire bone holes were filled with organized fibrous connective tissue with newly formed capillaries at one-month post-operation (Fig 5D). At three months’ post-operation immature bone cells mixed with collagen bundles were observed (Fig. 5E). Well-developed mature bone matrix mixed with remnant of immature bone matrix were detected at 6th month post-operation (Fig, 5F).

**Figure 2:** Wharton jelly derived MSCs showed fibroblastoid adherent cells at 15 day (a) with 80% confluence appearance at 21 day (b).
Figure 3: Mesenchymal stem cell markers by flowcytometry showing positive expression for CD44 and oct 3/4 and negative for CD34

Table 1: Percentage expression of Wharton jelly MSCs markers by flowcytometry

<table>
<thead>
<tr>
<th>The studied group</th>
<th>Okt3/4</th>
<th>CD44</th>
<th>CD34</th>
</tr>
</thead>
<tbody>
<tr>
<td>N= 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range (%)</td>
<td>53.5-68.7</td>
<td>43.5-63.0</td>
<td>1.4-4.7</td>
</tr>
<tr>
<td>$\overline{X} \pm SD$</td>
<td>61.57±5.6</td>
<td>54.68± 6.32</td>
<td>3.4± 1.15</td>
</tr>
<tr>
<td>Median</td>
<td>59</td>
<td>57</td>
<td>3.7</td>
</tr>
</tbody>
</table>
Table 2: Inflammatory markers of the studied groups basal level and postoperative at different time points

<table>
<thead>
<tr>
<th>The studied group</th>
<th>Total leukocytic count</th>
<th>CRP</th>
<th>ESR</th>
</tr>
</thead>
<tbody>
<tr>
<td>N= 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>4.2-13.8</td>
<td>2.0-5.0</td>
<td>10.0- 20.0</td>
</tr>
<tr>
<td>X ±SD</td>
<td>7.54±2.93</td>
<td>3.88± 1.0</td>
<td>14.4 ± 3.39</td>
</tr>
<tr>
<td>1st week</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>14.5- 24.8</td>
<td>24- 36</td>
<td>40- 60</td>
</tr>
<tr>
<td>X ±SD</td>
<td>20.22± 3.25</td>
<td>29.1± 4.8</td>
<td>50.33± 4.8</td>
</tr>
<tr>
<td>2nd week</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>7.8- 11.4</td>
<td>12- 20</td>
<td>28- 45</td>
</tr>
<tr>
<td>X ±SD</td>
<td>9.84 ±1.37</td>
<td>14.55± 3.57</td>
<td>34.22± 5.51</td>
</tr>
<tr>
<td>3rd week</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>7.6- 11.1</td>
<td>6-15</td>
<td>10-20</td>
</tr>
<tr>
<td>X ±SD</td>
<td>9.73 ±1.13</td>
<td>11.0± 2.8</td>
<td>16.33±3.2</td>
</tr>
<tr>
<td>4th week</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>4.8- 9.8</td>
<td>3-8</td>
<td>3-15</td>
</tr>
<tr>
<td>X ±SD</td>
<td>7.53 ±1.59</td>
<td>4.88± 1.61</td>
<td>9.0 ±4.0</td>
</tr>
</tbody>
</table>

DISCUSSION

Several techniques of bone reconstruction have been used to treat different bone affections, especially that resulted in bone loss (Torroni, 2009; Kim et al., 2009; Nandi et al., 2010; Heo et al., 2011; Emara et al., 2013a&b). The current techniques are directed toward enhancement of bone ability to regenerate itself. This was achieved by using cells capable of bone formation when delivered to a skeletal defect, thus avoiding the need for harvesting bone (Pountos et al., 2007; Jang et al., 2008; Jafarian et al., 2008 Chen et al., 2009; Udehiya et al., 2013). Although the use of autogenic mesenchymal stem cells provides high quality repair without the risk of immune rejection (Udehiya et al., 2013), the increased rate of donor site morbidity and the prolonged time required for their expansion before implantation are the most common limitation of their wide use (Kim et al., 2007; Watson et al., 2014).

In the present study, we have focused on characterization of MSC populations originating from the Wharton’s jelly matrix of human fetal umbilical cord (UC) termed WJSC and evaluate their ability to repair critical size gap defect in canine model. As well as to proof that stem cells has the ability to overcome host immune system especially when they implanted xenogenically. We isolate nine from fifteen samples, six samples failed to be completed because of contamination. The isolated cells have fibroblastoid morphology and express the immunophenotypic markers consistent with MSCs. This MSC source has many advantages over other stem cell sources in terms of: this source has abundant cell
availability, relative ease of access for stem cell isolation with minimal or no tissue morbidity, as well as, the isolated cells has an immunogenic profile favorable for cellular transplantation (Puissant et al., 2005; Weiss et al., 2008).

In this study the used experimental animals (dogs) may give us an idea about the nature of the repair process in human because of similarity of the biological repair process between dogs and human (Burchardt, 1987).

**Fig. 4:** Showing sequential radiography of the operated tibia: A) at day zero. B) two weeks’ post-operation. C) 1 month post-operation. D) 3 months post-operation. E) 6 months post-operation. The hole number (1) is implanted with stem cells while hole number (2) left empty as control one.
Fig. 5: Dog, tibial bone. A, B & C representing control group at 1, 3 & 6 months’ post operation, respectively. A & B) showing collagen bundles filled the entire bone holes (asterisks). C) showing that the defected area begins to form immature bone cells (arrow) mixed with collagen bundles (asterisk). D, E & F representing WJSCs treated group at 1, 3 & 6 months’ post operation, respectively. D) showing that the entire hole filled with organized fibrous connective tissue (asterisk) with newly formed capillaries (arrows). CB, compact bone. E) showing that the defected area filled with immature bone cells (thin arrow), collagen bundles (asterisk) and blood vessels (thick arrow). F) showing well-formed mature bone (arrow) in between remnant of immature bone. H &E stain, X 10.
In our study, we choose the end of the observation period (six months) depending on the radiographic evaluation results. By the end of the observation period the WJSCs implanted hole completely disappeared and couldn’t be detected radiographically compared to the control one, which indicated new bone formation regardless the quality of the newly formed bone (Arinzeh et al, 2003). The histological findings complimented the radiological results and indicated new bone formation in the hole implanted with WJSCs by the end of the observation period as compared to control holes. The increased osteogenesis may be attributable to the conversion of stem cells into osteoblasts then into osteocytes that responsible for tissue formation and mineralization (Jang et al., 2008; Jiang et al., 2010; Udehiya et al., 2013), as well as, their ability to secrete several growth factors which might have accelerated healing (Chen et al., 2009). Concerning the quality of the newly formed bone, the hole implanted with WJSCs showed mature bone formation interspersed with small amounts of immature bone remnants. According to the authors opinion, the quality of the newly formed bone can be improved by prolongation of the observation period or by using a scaffolding material to enhance the development and migration of the stem cells, but this wasn’t proofed here and require further investigation.

The measured inflammatory markers here showed marked increase directly after the operation and during the post-operative period till the end of the 3rd week and returned to its normal level by the end of the 4th week. This may be attributed to traumatic injury during the operation. According to our results there is no any immune response provoked against WJSCs when they were implanted xenogenically in dogs, this result found in accordance with previous reports published by (Guo et al., 2009; Udehiya et al., 2013) whom stated that mesenchymal stem cells have little or no immunogenicity when they used in bone healing. It seems that mesenchymal stem cells overcome immune rejection by three broad mechanisms including: (1) absence of major histocompatibility complex II (MHC II) on their cell surface, which responsible for the antigenicity of the cells, (2) direct and indirect prevention of T cell responses and (3) induction of suppressive local microenvironment (Di Nicola et al., 2002; Ryan et al., 2005; Uccelli et al., 2006; Nauta and Fibbe 2007; Planka et al., 2008; Guo et al.,2009; Jung et al., 2009). In conclusion; this study supported the hypothesis that mesenchymal stem cells have no any detectable immune reaction and can be used xenogenically in animals to enhance and accelerate bone healing.

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