Regeneration of Salivary Gland Defects of Diabetic Wistar Rats Post Human Dental Pulp Stem Cells Intraglandular Transplantation on Acinar Cell Vacuolization and Interleukin-10 Serum Level

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Abstract

Objective: To investigate the regeneration of rat’s salivary gland diabetic defect after intraglandular transplantation of Human Dental Pulp Stem Cells (HDPSs) on acinar cell vacuolization and Interleukin-10 (IL-10). Material and Methods: HDPSs isolated from the dental pulp of first premolars #34. HDPSs from the 3rd passage was characterized by immunocytochemistry of CD73, CD90, CD105 and CD45. Twenty-four male Wistar rats, 3-month-old, 250-300 grams induced with Streptozotocin 30 mg/kg body weight to create diabetes mellitus (DM) divided into 4 groups (n=6); positive control group on Day-7; positive control group on Day-14; treatment group Day-7 (DM+5.10⁵ HDPSs); treatment group on Day-14. On Day-7 and Day-14, rats were sacrificed. Histopathological examination performed to analyze acinar cells vacuolization while Enzyme-linked Immunoabsorbent Assay to measure IL-10 serum level. Data obtained were analyzed statistically using multiple comparisons Bonferroni test, Kruskal Wallis, Shapiro-Wilk and Levene’s test result. Results: The highest acinar cell vacuolization found in control group Day 14 (0.239 ± 0.132), meanwhile the lowest acinar cell vacuolization found in treatment group Day 7 (0.019 ± 0.035) with significant difference (p<0.003). The highest IL-10 serum level found in treatment group Day 14 (175.583 ± 120.073) with significant difference (p=0.001). Conclusion: Transplantation of HDPS was able to regenerate submandibular salivary gland defects in diabetic rats by decreasing acinar cell vacuolization and slightly increase IL-10 serum level.

Keywords: Diabetes Mellitus; Totipotent Stem Cells; Interleukin-10; Acinar Cells.
Introduction

Diabetes Mellitus (DM) is a progressive and chronic disease characterized by an increase in blood glucose levels. The prevalence of DM patients in the world was 8.8% and in Indonesia it was 6.2% [1]. Salivary gland defect is one of the complications of DM in the oral cavity. Based on a recent meta-analysis study, the global prevalence of xerostomia in DM patients is 42.4%. Salivary gland defects can reduce the patient's quality of life because it causes difficulty in swallowing, speaking, chewing, dysgeusia, caries and an increase in plaque accumulation [2]. Clinically, salivary gland defects are characterized by decreased salivary secretion volume and histologically characterized by acinar cell vacuolization, which is an early sign of cell degeneration [3,4]. Nowadays, salivary gland defects are treated by performing salivary stimulation either through oral receptors using ascorbic acid, stimulation of mastication by chewing gum, and by pharmacological stimulation using pilocarpine. However, all of these therapies cause various complications and are only temporary relief; therefore, long-term definitive therapy is still needed [5-7].

Bone Marrow Mesenchymal Stem Cells (BMMSC) which are considered to be a solution to problems in various tissue damage related to the tissue regeneration capacity by differentiating into various cell lines, having a paracrine effect and having immunomodulation capacity. However, the isolation of BMMSC is very invasive. Human Dental Pulp Stem Cells (HDPSC) are considered as a better source of MSC because their extraction is less invasive than BMMSC, possess the ability of self-renewal, higher plasticity, and proliferation compared to BMMSC, but has a similar pattern of gene expression, fenotip and protein in vitro with BMMSC [8-11].

DPSC allegedly has a good salivary gland regeneration capacity by being a component of epithelial cells (acinar) itself or by being a salivary gland mesenchymal component to induce and support the regeneration process of the salivary glands. Therefore, stem cell-based regenerative therapy is expected to produce replacement tissue that can mimic the natural structure and function of the salivary glands and provide long-term benefits for salivary gland defects [12-15].

Material and Methods

Ethical Clearance

This study received ethical clearance for the use of animal subjects, number 047/HRECC.FODM/V/2018. Ethical permission was obtained in accordance with the guidelines of the Board for Animal Experiments at the Faculty of Dental Medicine, Airlangga University, which related to the Guidelines on the Care and Use of Animals for Scientific Purposes [16].

HDPSC Isolation and Culture

HDPSC was isolated from the healthy premolar tooth pulp #34 patients undergoing orthodontic therapy. The extracted teeth were immediately included in the Dulbecco’s Modified Eagle Medium (DMEM) (D5796, Sigma Aldrich Corp., St. Louis, MO, USA) and it was sent to the laboratory. The teeth were washed with Phosphate-Buffered Saline (PBS) solution. The teeth were
washed and cut to separate the crown and roots; then the pulp tissue is taken. The pulp tissue was digested in a solution of 3 mg / ml collagenase type 1 (C9891, Sigma Aldrich Corp., St. Louis, MO, USA) and 4 mg / ml dispase (D4818, Sigma Aldrich Corp., St. Louis, MO, USA) for 30-60 minutes at 37°C. HDPSC was obtained by filtering the digested tissue with 70 μm cell filters. 1 cell suspension (1x10^6 cells / flask) planted in α-minimum essential medium (α-MEM) (M8292, Sigma Aldrich Corp., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μM L-ascorbic acid-2-phosphate, 100 U / mL penicillin-G, 100 μg / mL streptomycin and 0.25 μg / mL fungizone (A9528, Sigma Aldrich Corp., St. Louis, MO, USA). Cells were conditioned at a temperature of 37°C in 5% CO2 medium was replaced every 2-3 days. The cell culture was passaged three times to get the desired cell number [17-19].

Immunocytochemistry Analysis

HDPSC in the 3rd passages was then examined for cluster differentiation markers to ensure positive MSC markers, namely CD73, CD90, CD105 and negative CD45 markers. Cells were coated with coverslips, and after incubation at 37°C for 1-2 hours, fixation was done with 10% formaldehyde (F8775, Sigma Aldrich Corp., St. Louis, MO, USA) for 15 minutes. Coverslips were rinsed four times. Monoclonal antibodies labeled FITC (Santa Cruz Biotechnology, Dallas, Texas, USA) CD105 (anti CD 105 sc-71042) positive, CD73 (anti CD73 sc-18849) and CD90 (anti CD90 sc-53116) positive and CD45 (anti CD45 sc-53665) negative [20]. Monoclonal antibodies were applied to cells and incubated for 60 minutes then rinsed with PBS twice and the cells were analyzed using the Olympus FSX100TM fluorescence microscope (Olympus Corporation of the Americas, Center Valley, PA, USA) with 200x magnification in 5 fields of view by two observers.

Diabetic Rats Model

All animals (Wistar Rats) were housed in polycarbonate cages, subjected to a 12-hour light-dark cycle at the constant temperature of 23°C, and fed a standard pellet diet (expanded pellets; Stepfield, Witham, Essex, UK) with tap water ad libitum at a temperature of 22°C ± 2°C [21]. Mice are fastened for approximately 12 hours before induction to empty the stomach and accelerate the occurrence of DM conditions. Diabetic rats model was induced by STZ (Bioworld Merchandising Inc., Irving, TX, USA) with a dose of 30 mg / kg which was dissolved in citrate buffer (CV. Gamma Scientific Biolab, Malang, Indonesia) 30 mg/mL (pH 4.5) injected intraperitoneally in the area beside the midline between two nipples or in right / left mouse umbilication. Induction was done once; the mouse was held and the part to be injected is rubbed with 70% alcohol. The needle was injected perpendicular to the right / left umbilical to the peritoneal cavity; then the ingredients are injected slowly. Mice were given 10% sucrose solution or 10% dextrose (PT Otsuka, Jakarta, Indonesia) during the first night after induction to avoid sudden hypoglycemic post [22]. Mice were declared diabetic if on the 7th day after induction of blood sugar levels ≥ 200 mg / dL checked using Accu check (0197, EasyTouch GCU, Bioptik Technology Inc., Taiwan) [22,23].
HDPSC in Vivo Injection

HDPSC was injected in rats in the treatment group with a single dose of $5 \times 10^5$ cells / 250 gr BW in 0.2 mL intraglandular PBS solution in the submandibular gland. The control group mouse has injected 0.2 mL of PBS intraglandularly in the submandibular gland. The mice were sacrificed on the 7th and 14th day using rodent anesthesia (Ketamine 70 mg / kg BW and Xylazine 5 mL). The sub-mandibular gland and blood tissue were extracted for further analysis [19].

Paraﬃn Block Preparations

Preparations were obtained from salivary gland incisions, which were ﬁxed with 10% neutral buffered formalin (NBF) (HT501128, Sigma Aldrich Corp., St. Louis, MO, USA) with solution volume of 10 times the size of the specimen. After ﬁxation, dehydration was done through the extraction of water from the tissue with alcohol (30%, 50%, 70%, 80%, 96% and absolute) for 60 minutes each. Clearing was then carried out, which was to clear the tissue to be transparent by inserting the tissue into xylol solution 2 times each for 60 minutes. Impregnation was done consequently by inﬁltration with soft paraﬃn for 60 minutes at 48°C and went through block process in hard paraﬃn in the mold and let set for a day. Paraﬃn blocks were attached to the holder and were cut using a microtome rotary for 4-6 µm.

Staining and Interpretation of Hematoxylin and Eosin

Deparaﬃnization was done by submerging the sample in xylol for 5 minutes. The sample was re-hydrated using alcohol from high to lower concentrations (96%, 80%, 70%) for 2 minutes and at the end using water for 10 minutes. Staining was done with Mayer's Hematoxylin (MHS1, Sigma Aldrich Corp., St. Louis, MO, USA) for 15 minutes and washed with running water until samples appeared blue for 5 minutes or less. The samples were submersed in 1% acidic alcohol (1% HCL in 70% alcohol) for 5-10 seconds. Rinsing with water until appeared blue again for 10-15 minutes or submerge in alkaline solution (ammonia water) followed by washing for 5 minutes. The sample was stained with 1% eosin Y for 10 minutes. Wash it with water for 1-5 minutes. The sample was dehydrated with alcohol 70%, 80% and 96% for 2 minutes of each. Clearing was performed by submerging the sample in xylol for 5 minutes and mounting with EZ Mount. Results showed a nucleus with a blue / black stain and cytoplasm with a pink stain. The examination was carried out by two observers in 5 different visual ﬁelds and using Nikon H600L light microscope (Nikon Imaging Japan Inc., Tokyo, Japan) at 400x magniﬁcation with a 300 megapixels Fi2 DS digital camera and image processing software Nikon Image System. Vacuolization observed by the number of acinar cells that showed vacuolization compared to the total acinar cells in 1 ﬁeld of view by two observers.

IL-10 ELISA Examination

IL-10 level examination used an ELISA kit (Cat. No E0108Ra, Bioassay Technology Laboratory, Birmingham, UK) according to the manufacturer's instructions. Serum was left to form
clots first for 10-20 minutes at room temperature before use, then centrifuged in 2000-3000 RPM for 20 minutes. All reagents, standard solutions and samples were prepared at room temperature before use. The process was carried out at room temperature and began by pouring 50μl standard in standard well, 40 μl samples and 10 μl anti-IL-10 antibodies into the sample wells, and 50 μl streptavidin-HRP (Horseradish Peroxidase) into the sample wells and standard wells and mixed them carefully. The plates were covered with a sealer and incubated for 60 minutes at 37°C. The sealer was removed and plates were rinsed 5 times with a wash buffer. Submersion was done using at least 0.35 ml wash buffer for 30 seconds to 1 minute for every washing. The plates were dried with paper towels. The substrate solution was added 50 μl and 50 μl of substrate solution B to each well. The plates were covered with a sealer and incubated for 10 minutes at 37°C in the dark. Stop solution was added to each well, to make the color changed from blue to yellow. Optical density (OD) readings were performed using a microplate reader with a 450nm wavelength 10 minutes after stop solution was added.

Statistical Analysis

Data obtained were analyzed statistically using Kruskal Wallis (p<0.05) based on a Shapiro-Wilk normality test and Levene's variance Homogeneity test (p>0.05) and then a multiple comparisons Bonferroni test (p<0.05) was done with IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA).

Results

After isolation and culture, DPSC was the passage for 3 times to obtain desired cell numbers (Figure 1). In 3rd passage, immunocytochemistry characterization was done on DPSC to determine the marker MSC expression - CD73, CD90, CD105(+) and not expressing HSC marker - CD45(-) (Figure 2).

Figure 1. The morphology of HDPSCs (yellow arrow). A) HDPSC first isolation. B) HDPSC first passage did not show a fibroblast-like appearance. C) HDPSC second passage showed a fibroblast-like appearance. D) HDPSC third passage showed more fibroblast-like structures. 200x magnification.
Acinar cell vacuolization appears in HE staining as rounded-white colored with defined border acinar cells (Figure 3).

The acinar cells vacuolization data obtained were not homogenous and not normally distributed (p<0.05). The highest acinar cell vacuolization found in control group Day 14 (0.239 ± 0.132), meanwhile the lowest acinar cell vacuolization found in treatment group Day 7 (0.019 ± 0.035) with significant difference (p=0.003, p<0.05) (Table 1).

Figure 2. HDPSCs expressed positively MSCs marker CD73(+), CD90(+), CD105(+), while HDPSCs did not express HSCs marker CD45(-) (yellow arrow) with ICC examination with FITC using fluorescence microscope. 200x magnification. The left column showed a non-contrast appearance and the right column showed contrast appearance. A1) Non-contrast appearance from ICC CD45 examination. A2) Contrast appearance from ICC examination which indicated negative CD45. B1) Non-contrast appearance from ICC CD73 examination. B2) Contrast appearance from ICC examination, which indicated positive CD73. C1) Non-contrast appearance from ICC CD90 examination. C2) Contrast appearance from ICC examination, which indicated positive CD90. D1) Non-contrast appearance from ICC CD105 examination. D2) Contrast appearance from ICC examination, which indicated positive CD105.

Figure 3. Acinar cells vacuolization (yellow arrow) in histopathological examination using HE staining. 400x magnification. Vacuolizations were clearly observed in control groups. A) Histopathological appearance of acinar cells in control group on 7th day. B) Histopathological appearance of acinar cells in the control group on 14th day. C) Histopathological appearance of acinar cells in treatment group on 7th day. D) Histopathological appearance of acinar cells in treatment group on 14th day.
Table 1. The analysis of acinar cell vacuolization number between groups.

| Groups | Mean ± SD     | Acinar Cell Vacuolization Number |
|--------|--------------|----------------------------------|
|        |              | Normality Test | Homogeneity Test | Kruskal Wallis Test |
| K+7    | 0.376 ± 0.024| 0.206            | 0.13             | 0.003*            |
| K+14   | 0.239 ± 0.132| 0.665            |                  |                  |
| P+7    | 0.192 ± 0.035| 0.002            |                  |                  |
| P+14   | 0.081 ± 0.078| 0.007            |                  |                  |

*Statistically Significant.

There were significant differences between K+14 and K+7, P+7 and K+14, P+14 and K+14 group (p<0.05) (Table 2). The IL-10 serum level data obtained were not homogenous and not normally distributed (p<0.05) with a significant difference (p=0.001, p<0.05).

Table 2. Multiple Comparisons result of acinar cells vacuolization between groups.

| Groups | K+7 | K+14 | P+7 | P+14 |
|--------|-----|------|-----|------|
| K+7    | −   | −    | −   | −    |
| K+14   | 0.002* | −    | −   | −    |
| P+7    | 1   | 0.001* | −  | −    |
| P+14   | 1   | 0.015* | 1  | −    |

Bonferroni test; *Statistically Significant.

The highest level of IL-10 serum was found in the treatment group on the 14th day (175.583 ± 120.075), meanwhile the lowest level of IL-10 serum found in the control group on the 7th day (24.071 ± 9.316) (Table 3).

Table 3. The analysis of IL-10 serum level between groups.

| Groups | Mean ± SD     | IL-10 Serum Level |
|--------|--------------|-------------------|
|        |              | Shapiro-Wilk Normality Test | Levene’s Homogeneity Test | Kruskal-Wallis Test |
| K+7    | 24.071 ± 9.316| 0.153*            | 0.0001*          | 0.001*            |
| K+14   | 136.491 ± 65.525| 0.049            |                  |                  |
| P+7    | 36.656 ± 17.323| 0.350*            |                  |                  |
| P+14   | 175.583 ± 120.075| 0.086*           |                  |                  |

*Statistically Significant.

There were significant differences between groups (p=0.001, p<0.05). There were significant differences between K+7 and K+14, K+7 and P+14, P+7 and P+14 groups (p<0.05) (Table 4).

Table 4. Multiple Comparisons Bonferroni test result IL-10 serum.

| Groups | K+7 | K+14 | P+7 | P+14 |
|--------|-----|------|-----|------|
| K+7    | −   | −    | −   | −    |
| K+14   | 0.048* | −    | −   | −    |
| P+7    | 0.989 | 0.090 | −  | −    |
| P+14   | 0.006* | 0.762 | 0.011* | −    |

*Statistically Significant.

Discussion

In this present study found that acinar cell vacuolization between the control and treatment groups Day 7 were not significantly different. This is indicated that the tissue regeneration process
by DPSC on the 7th day had not occurred well. This is supported by previous research that on the 7th day of the HDPSC injection post there were no significant results on the microvascular structure, which is one of the important factors in the regeneration process [24].

Acinar cells vacuolization between the control and treatment groups Day 14 were significantly decreased. It can occur because the acinar cell vacuolization value was observed by the number of acinar cells that showed vacuolization compared to the total acinar cells in 1 field of view, so a decrease on acinar cell vacuolization can occur because acinar cells vacuolization actually decreased or the number of normal acinar cells increased. The decrease in vacuolization value indicated that there was an improvement in the conditions of the treatment group compared to the control group in the 2nd week. This is consistent with the experiment conducted previously, which revealed that in the 2nd week of post-transplantation DPSC in co-transplanted in salivary glands showed that there was a significant increase in Alpha-Amylase-1 markers (AMY-1) marker of differentiation of acinar cells and FGF7 which are mesenchymal markers. This showed that the salivary gland regeneration by DPSCs occurs very well in the 2nd week and it is able to increase the differentiation of the salivary glands in vivo [25].

Acinar cells vacuolization tends to increase in both groups Day 7 and Day 14 due to blood glucose did not controlled; therefore, the progressive diabetic complication worsens each day. However, the acinar cells vacuolization increased significantly in the control group than the treatment group. This indicated that HDPSC only able to decrease and inhibit the progression of acinar cells vacuolization.

The literature shows that IL-10 inhibits macrophage activation [26]. Meanwhile, MSC will increase IL-10 expression through macrophages, dendritic cells, and peripheral blood mononuclear cells [27]. It means that the administration of MSC will increase IL-10 levels comparable to levels of macrophages in serum.

The elevated IL-10 levels in control group will reduce the levels of existing macrophages, so that the administration of MSC increases in IL-10 levels did not appear to be too significant. This can explain why in this experiment, there was no significant increase in the treatment group when compared with the control group both on day 7 and 14. This result did not in accordance with the previous study, which said that DPSC was able to significantly increase IL-10 production when DPSC was conditioned with Peripheral Blood Mononuclear Cells (PBMSC) [28].

It has been shown that DPSC transplanted in the sciatic nerve was able to decrease IL-10 levels in the sciatic nerve sample [29]. When compared between this study and others [28,29], the results of the study showed that there were no differences between the control group and treatment groups could occur due to differences in the injection location of DPSC and the samples used for IL-10 levels, namely DPSC injected intraglandularly in the submandibular gland while the IL-10 examination sample originated from peripheral blood serum.

However, the effect may be seen systemically if the dose from DPSC is increased as in an experiment carried out previously [30], which demonstrated that expression of IL-10 serum levels
would recover in repeated transplants from DPSC at a dose of 1x10^6 cells intra-muscular. In this experiment, only single-dose DPSC was used (5x10^5 cells intraglandularly). Hence, the absence of significant differences in this study could be due to the dose being too low and not being given repeatedly dose of DPSC, which caused intraglandular administration could not provide a systemic effect on serum levels of IL-10.

**Conclusion**

Transplantation of HDPSC was able to regenerate submandibular salivary gland defects in diabetic rats by decreasing acinar cell vacuolization and slightly increase IL-10 serum level.

**Authors’ Contributions:** IBN, VL, APN, DSE, SW, CP, FAR designed the study, performed the data collection, data analysis and interpretation, wrote the manuscript and reviewed the manuscript. AD, HS, EH, ISI performed the data collection, data analysis and interpretation, and wrote the manuscript. All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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**Conflict of Interest:** The authors declare no conflicts of interest.

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