Impact of fetal brain tissue derived mesenchymal stem cell and fibrin glue on facial nerve crash injury

Background/aim: To evaluate the clinical and histopathological effects of fetal brain tissue derived mesenchymal stem cells (FBTMSC) and fibrin glue (FG) on the facial nerve (FN) regeneration in rats with traumatic FN injury.

Materials and methods: 28 Sprague Dawley rats were included in the study and divided into 4 groups. Traumatic FN injury (FP) was created by a surgical clamp compression to the main trunk of left FN in all groups. In the control group (Group 1) no treatment was applied, in Group 2 (FBTMSC group) 2x10^6 FBTMSC was injected, in Group 3 (FG group) only FG was applied, in group 4 (FBTMSC & FG group) both FBTMSC and FG was applied to the injured section of the nerve. The FN functions were evaluated clinically, immediately after the procedure and at 3rd-5th-8th weeks postoperatively. The FNs of all subjects were excised after the 8th week; then the rats were sacrificed. The presence of stem cells in the injured zone was assessed by using BrdU (Bromo-deoxyuridine), and apoptosis was determined by TUNEL method.

Results: After the damage, total FP was observed in all subjects. Statistically significant functional improvement was observed in Group 4 compared to all other groups (p<0.005). TUNEL positive cell count was statistically significantly higher in the control group than the other groups (p<0.001). TUNEL positive cell count was statistically significantly lower in Group 4 than the other groups. The proportion of BrdU-stained cells in Group 4 (5%) was higher than Group 2 (2%).
Conclusion: Clinically and histopathologically FBTMSC applied with FG may play a promising role as a regenerative treatment in posttraumatic FP.

Level of Evidence: N/A

Keywords: Facial nerve, facial nerve palsy, trauma, stem cell implantation, fetal brain tissue derived mesenchymal stem cells.

1. Introduction

The facial nerve (FN), with its multiple functions, is one of the most sensitive cranial nerves against traumas due to its complex and unique anatomical structure [1]. The paralysis of the facial nerve (FP) causes worsening of the quality of life and consequent social and psychological disorders, owing to loss of many functions and aesthetic appearance in face. Therefore, full recovery of FP is very important.

The most common cause is Bell’s palsy. Traumatic FP that develops due to blunt and penetrating traumas, and iatrogenic causes is the second most common. Infections and tumors are rare causes of FP [1]. Recovery may not be satisfactory after complete axonal injury of FN [2]. In addition, Wallerian degeneration after axonal damage starts to develop within days [3].

Although many methods have been used so far in the rehabilitation of FP, there have been no excellent effective results in functional recovery [4,5]. These methods include observation with or without medical treatment, physical therapy, surgical FN decompression, neurorrhaphy, autologous nerve grafting, and nerve transposition [6,7]. Although the most
succesfull surgical procedures are neurorrhaphy and autologous nerve grafting, these procedures have some limitations including limited donor nerves, non-aesthetic scarring, infection, pain, long surgical times, and inadequate regeneration [2]. If sufficient regeneration does not develop, facial reanimation techniques such as muscle transpositions and static suspension procedures are other solutions. However, despite all these techniques, the healing process may result in sequelaes [8]. New treatment modalities are still being developed to prevent these results and improve the quality of life of patients.

Stem cell transplantation is a very successful treatment modality using tissue engineering in many different diseases. The last two decades of studies have shown the positive effects of various stem cell therapies related to nerve regeneration, in both the central and peripheral nervous system [2,9,10]. It has been shown that the stem cell is differentiated into Schwann cell phenotype and accelerates axonal regeneration and provides better remyelination [11,12]. It has also been shown that; stem cells increase growth factor secretion in peripheral nerve damage and thus augment regeneration with anti-inflammatory action, and replace damaged Schwann cells and motor neurons [2,9,13].

There are studies using adult, embryo and fetal stem cells for the regeneration of nerve damage. Many subtypes of these stem cells, such as bone marrow, mesenchymal and neural stem cells have been used for regeneration in peripheral nerve damage [2,9,13,14]. In previous years, there are animal studies carried out using many kind of stem cells in the FP that reported good results [15-22]. There is no research in the literature about the effects of fetal brain tissue-oriented mesenchymal stem cells (FBTMSC) on facial nerve damage. It has been shown that some bio-degradable materials and pharmacological agents may contribute to nerve regeneration. These materials provide a protein rich environment, increase stem cell adhesion and promote axonal growth [2,23]. For this purpose, many different
materials and pharmacological agents have been used [9,15,17-20, 22,24,25]. Fibrin Glue (FG) application alone has been shown in the studies that accelerate nerve regeneration [26,27].

In this study, we aimed to investigate the effects of local administration of rat FBTMSC together with FG, on functional and histological recovery of crush injury of FP in rats.

2. MATERIALS AND METHODS

2.1 Study population and design

A total of 28 adult female Sprague Dawley rats weighing between 250-300 g were included in the study. Subjects were hosted in the Experimental Animals Laboratory during the study, within an environment of 12 hours light/12 hours dark, temperature of 24 - 26 °C with 60-70% moisture, and fed ad libitum. The study was carried out with the approval of Animal Ethics Board of our hospital (2015/24).

The animals were anesthetized and then left facial nerves were identified surgically in all groups. The left facial nerves of all animals were damaged by a direct surgical mosquito compression. Fifteen minutes after the iatrogenic injury to the nerve, different treatment modalities were applied for each group:

1. **Control group**: No treatment was applied (Group 1, n=7).

2. **FBTMSC group**: 2x10^6 FBTMSC was injected into the damaged zone (Group 2, n=7).

3. **FG group**: Only fibrin glue (FG) (Tisseel VH; Baxter, Vienna, Austria) was applied over the damaged zone (Group 3, n=7).
4. **FBTMSC & FG group**: The damaged part was first injected $2 \times 10^6$ FBTOMSC and then covered with FG (Group 4, n=7).

Facial functions of the subjects were evaluated immediately after recovery from anesthesia, and then 3, 5 and 8 weeks after surgery. The damaged section of the FN of all subjects was excised at the 8th week for histopathological examinations (TUNEL) and evaluation of stem cell implantation (BrdU). All subjects were then sacrificed.

2.2. **Detection, Identification and Marking of Mesenchymal Stem Cells from Fetal Brain Tissue**

The brain tissue was removed from the fetus taken from an 18-days pregnant rat under deep anesthesia with xylazine and Ketamine in sterile conditions. It was divided into 1cm$^3$ pieces and seeded in culture dishes with the explant method. Passage of the mesenchymal stem cell cultures in 5% CO2 incubator (20% Fetal bovine serum (Lonza, Belgium), 2% L-Glutamine (Lonza, Belgium), 1% Penicillin, Streptomycin, Amphotericin (Biological Industries, Israel) and 77% Dulbecco's Modified Eagle Medium (DMEM-LG) (Lonza, Belgium)) was done when the cells filled 80% of the culture dishes by changing the medium once every three days. After the second passage, the cells were identified in flow cytometry (FACSaria III, USA) according to CD11b / c (BD, USA), CD45 (BD, USA), CD90 (BD, USA), CD44 (BD, USA) surface markers (Figure 1). Cells after the second passage were then differentiated into adipocytes, osteocytes, and chondrocytes by the following differentiation methods.

MSCs obtained from the second passage were placed in the culture dishes after the passage and when they covered more than 40% of the container and they were taken to the adipocyte medium (Adipocyte Differential Basal Medium and supplement (Gibco, USA)). Replacing 5
the medium on a three-day basis, dyeing was performed according to the principle that lipid
droplets appear red within the cell with Oil Red (Diagnostics BioSystem, USA) at the end of
the third week (Figure 2A).

For differentiation of osteocytes, Osteocyte Differentiation Basal Medium and supplement
(Gibco, USA) were used. MSCs from the second passage were planted in the culture dishes
after the passage and after covering 40% of the dishes, the medium was changed with
osteocyte medium every three days. At the end of the third week, staining was performed
according to the principle of black staining of calcium deposits with Von Kossa (Diagnostics
BioSystem, USA) (Figure 2B).

MSCs from the second passage were re-passaged, and when they covered 40% of the culture
dishes, Chondrocyte medium (Chondrocyte Differential Basal Medium and supplementation
(Gibco, USA)) was added for Chondrocyte differentiation every three days. At the end of the
third week, Alcian Blue (Diagnostics BioSystem, USA) was used to dye hyaluronic acid
based on the blue / purple staining principle (Figure 2C).

Before transplantation, the number and viability of the cells were evaluated with the
Countess® Automated Cell Counter (Invitrogen, USA) and the cells stained with 10 μL / ml
BrdU (Bromo-deoxyuridine,1 mM BrdU in 1 × Dulbecco's Phosphate Buffered Saline, BD
Pharimngen,US) that were prepared at a concentration of 2 × 10^6 cells / mL. The solution was
carefully added and incubated for 2 hours. Cells labeled with BrdU were given in a 1cc
insulin injector (1cc Dulbecco 1s PBS + 2 × 10^6 cells (FBTMSC)) to be used in the
experiment.
4-µm (micrometer) sections were obtained from formalin-fixed, paraffin-embedded tissues and then the tissues were treated with sequential alcohol and deparaffinized with xylen. Immunohistochemical staining was performed with BrdU Detection Kit (BD-Pharningen), after treatment with 3% H$_2$O$_2$ followed by washing with PBS. The ratio of BrdU(+) was obtained by counting 100 cells, 3 times in 20 (20X10) areas, from the specimens that included facial nerve segments from the rats treated with FBTMSC.

2.3. Surgical procedure

After anesthesia with xylazine hydrochloride (3 mg/kg) and ketamine hydrochloride (100 mg/kg), the left infraauricular region was shaved and sterilized to create FN damage. A horizontal incision extending from the postauricular region to the mandibular side was made and the skin and subcutaneous tissues were passed, and the parotid gland was reached. The parotid gland was excised and the FN main trunk was identified at the anterior aspect of the posterior digastric muscle. The nerve damage was created just proximal to the nerve trifurcation using microforceps compression for 30 seconds. The damage was repeated for another 30 seconds. The same researcher performed the compression to all subjects. Stem cells were injected into the damaged area, and FG was applied to the same area as well. The surgical area was then properly closed. The subjects were anesthetized 8 weeks after the initial procedure as mentioned above. The FN was re-exposed with the same incision, the damaged area was found and the injured nerve segment was resected. Then, FN specimen was prepared and fixated in 4% para-formaldehyde solution. The rats were then sacrificed.

2.4. Physical examination evaluation method
Immediately after the initial surgical procedure and at the 3rd, 5th and 8th weeks postoperatively, the FN motor function examinations of all rats were done according to the standardized scale with observation of vibrissae movement and the standardized scale with observation of blink reflex and eye closure [28].

2.5. **Histochemical Method**

The FN tissue specimens obtained from all subjects were first fixated in 10% formaldehyde solution for at least 72 hours for light microscopic examination. After fixation, tissue samples were placed in cassettes and washed under stream for 24 hours. Tissues were removed from the increased alcohol series (50%, 70%, 80%, 90%, 100%) for removal of water. Afterwards, the tissues were passed through xylene to be transparent and then embedded in paraffin. Four µm thick sections were cut from prepared paraffin blocks.

2.6. **TUNEL assay**

TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) method was used to determine apoptosis developed by DNA fragmentation. Millipore Apoptag Plus Peroxidase in Situ Apoptosis Detection kit (Millipore, Cat no: S7101) was used for this method. Cross sections were incubated at 61 °C. After deparaffinization, the tissues were incubated with 20 µg/ml proteinase K (Millipore, Cat no. 21627,Germany) at 37°C for 25 minutes. Endogenous peroxidase activity was blocked in tissues with 3% hydrogen peroxide for 15 minutes. The cross sections were incubated with the equilibration buffer for 5 min at room temperature. Sections were incubated with Working Strength TdT Enzyme solution in a humid environment at 37 °C for 60 min. Then, tissues were incubated for 10 min in stop/ wash
buffer solution and then incubated with antidigoxin peroxidase solution for 30 min in a humid environment. Subsequent staining with diaminobenzidine was used to identify TUNEL-positive cells. Mayer's Hematoxylin was used as a background stain. Cross sections were evaluated under a light microscope using a computer-supported imaging system to take photographs using the Leica Q Vin 3 program. TUNEL positive cells were counted at 400x magnification in random areas containing the nerve bundles in the tissue sections of the subjects.

2.7. Statistical analysis:

The normal distribution of the variables was analyzed graphically and by the Shapiro-Wilk test. Descriptive data were presented with Mean ± standard deviation and median (25th-75th percentiles) values. Kruskal-Wallis nonparametric analysis of variance was used to compare the skewed data that did not provide parametric test assumptions. Within time differences were analyzed by Friedman test. In order to determine the different group, Connover-Dunn pairwise comparison test was performed. Statistical analysis and calculations were performed using IBM SPSS Statistics Version 22.0 (IBM Corp., NA, USA) program. In statistical decisions p ≤ 0.05 was accepted as an indicator of significant difference.

3. RESULTS

3.1. Physical examination evaluation

Left total FP was observed in all rats immediately after the procedure, and at the 3rd, 5th and 8th weeks blink reflex, eye closure and vibrissae position were compared. According to intra-group comparisons; in all groups, the 5th and 8th week values were found to be statistically
significantly better than the postoperative results. According to the comparisons between
groups; in all weeks, blink reflex and eye closure degree in Group 4 were found to be
statistically better than the other groups (Table 1).

The vibrissae monitoring score was found to be the highest in Group 4 at the 3\textsuperscript{rd} week
evaluation, and there was a difference between Group 4 and Group 3 only at the 5\textsuperscript{th} week. The
results of Group 1 and Group 3 at 8\textsuperscript{th} week were significantly lower than that of Group 2 and
Group 4. When compared to the immediate post-injury evaluations; in all groups 5\textsuperscript{th} and 8\textsuperscript{th}
week results were better (Table 2).

3.2. TUNEL

TUNEL-positive cell values in all groups and were obtained as 31.42 ± 10.84; 12.06 ± 5.44;
8.28 ± 3.04 and 5.33 ± 2.44 respectively. The TUNEL positive cell count of group 1 was
significantly higher than the treatment groups (Figure 3,4a). The number of TUNEL positive
cells decreased in all treatment groups compared to the control group (p<0.001) (Figure 3).
The difference between group 2 (Figure 4b) and group 4 was found to be statistically
significant (p<0.001). Similarly, there was a statistically difference between group 3 (Figure
4c) and group 4 (Figure 4d) (p<0.041). However, there was no significant difference
between the groups 2 and 3 (p=0.167) (Figure 3,4).

3.3. BrdU Staining Results: BrdU staining results of BrdU Detection Kit (BD-
Pharmingen) showed the percentage of BrdU(+) (Prepared by BrdU and obtained by counting
100 cells 3 times in 20X10) in 20 (20X10) area of the facial nerve traction obtained from rats.
No staining was observed in Group 1 (Figure 5a) whereas 2% found in Group 2 (Figure 5b) as well as 5% in Group 4 (Figure 5c).

4. DISCUSSION

Although peripheral nerves are capable of self-healing after traumas; regeneration and functional improvements after severe damages may not be satisfactory [18]. Any new method or treatment modality to accelerate regeneration and provide full functional recovery in traumatic nerve paralysis, is invaluable. Recent advances in tissue engineering and stem cell transplantations with different cell differentiation capacities give a new perspective to this field. It has been shown in several studies that, stem cells, can accelerate the regeneration of the damaged nerves by producing adhesion molecules, neurotrophins and growth factors or by replacing Schwann cells or even by replacing the neurons [2,9,13,14]. It is reported that Schwann cell plays a major role in peripheral nerve regeneration and can be used in nerve injuries since the differentiation of the stem cell to the Schwann cell was determined [11]. Mesenchymal stem / stromal cells have been shown to have anti-inflammatory effects; by expressing IL-1 receptor antagonists and also have negative feedback effects on proinflammatory cytokines [29]. This is another feature that will explain the positive effects of stem cell therapy on nerve regeneration. Similarly, another study has shown that mesenchymal stem cell accelerates axonal growth by neurotrophic and pro-angiogenic activity [30].

The effectiveness of stem cell technology in different peripheral neural damages has been shown in studies. In one study, local mesenchymal stem cell injection in the damaged area has been shown to accelerate recovery in recurrent laryngeal nerve injury [31]. In their study,
Lerner et al. found no significant difference with the control group in terms of functional recovery in the recurrent laryngeal nerve injury with intravenous mesenchymal stem cell therapy; but the control group of this study also gained normal functions in less time than expected [32]. In experimental animal models, stem cells have been shown to provide nerve regeneration in sciatic nerve injury through anti-inflammatory effects, growth factor secretion, Schwann cell or motor neuron differentiation [13]. Matthes et al. showed that intravenous transplantation of mesenchymal stromal cells to rats with sciatic nerve injury contributed to peripheral nerve regeneration clinically and histologically with footprint analysis [33]. In this study, 21 days after intravenous transplantation of mesenchymal stromal cells, histological examination of the damaged nerve showed that these cells were present in the area where nerve damage was present and functional recovery was superior as well. The study of Yamamoto et al. is based on the potential neurotrophic and neuro-differentiated feature of mesenchymal stem cells. Dental pulp stem cell effect was compared with autograft and collagen scaffolds on the damaged sciatic nerve in rats and regeneration in the stem cell transected group was superior to other groups [34]. Considering the high likelihood of sequelae in severe traumatic FP and medical treatment for sequelae may not be sufficient, it is mandatory to develop a new treatment alternative. Unlike other skeletal muscles, the atrophy of the mimic muscles develops many years after nerve paralysis, but even without atrophy, FP causes a high level of psychological stress. There are some experimental stem cell studies in the literature for facial nerve injury.

In post-traumatic FP in humans, facial decompression was performed and a positive result was obtained in a study, in which bone marrow mononuclear stem cells were also applied to the damaged area [7]. Wang et al. compared the bone marrow mesenchymal stem cell application with Schwann-like mesenchymal stem cells in rabbits with facial nerve damage,
and concluded that the Schwann-like mesenchymal stem cell gave more positive results in axon regeneration and remyelination [15]. Batıoğlu-Karaaltın et al. showed the efficacy of human olfactory stem cell in nerve regeneration in facial nerve damage they formed in rats [16]. Zhang et al. in a model of facial nerve damage they created in rabbits, used neural stem cells supplemented with hyaluronic acid, collagen and neurotrophin-3 and determined that this might be an alternative modality in the management of the treatment of peripheral nerve defect [17]. Cho et al. in a model of facial nerve injury that they formed in guinea pigs, used neural mesenchymal stem cells together with platelet-rich plasma. In this study, the authors concluded that only stem cell administration is not effective but it promotes nerve regeneration of the stem cell used with platelet-rich plasma [18]. Ma et al. used neural stem cells supplemented with fibroblast growth factor on collagen scaffold in facial nerve injury and observed significant neural proliferation and functional recovery [19]. Sasaki et al. applied poly-DL-lactide-co-glycole tubes carrying dental pulp cells on damaged rat facial nerves and observed remyelination and regeneration in nerves [20]. Satar et al. in their study, created damage to facial nerve branches in rats and applied mesenchymal stem cells [21]. They stated that mesenchymal stem cell had positive effects on histopathological damage on facial nerve branches. Shi et al. applied neural stem cells composed of glia-derived neurotrophic factor, exfoliated polyglycolic acid on damaged rat facial nerves; and observed increased physiological, histochemical and histopathological regeneration [22].

Strategically, the first important point in stem cell therapy is to define which cells can be used in what kind of pathologies. The second important point is the preparation of such cells in a manner which will not harm the patient, and the third important point is the proper placement of these cells. There are various opinions about the superiority of local or systemic administrations in stem cell transplantation [15,35]. Akiyama et al. investigated the effects of
intravenous bone marrow cells on demyelinated spinal cord [36]. However, the desired effect in this study was less than expected probably due to the systemic distribution of the given cells. In the studies, stem cells for peripheral nerve injuries have been generally applied locally. For this reason, in our study local injection was performed because of our expectation to have more local effect which was also believed to be augmented with FG.

FG, with its fibrin-like effect that plays a role in coagulation, have been shown to be as effective as microsurgery on peripheral nerve injury [26]. In an in vivo rat model, it has been shown that regeneration is accelerated by FG in sciatic nerve injury [27]. In another study, growth factor added to FG was shown to increase the expression of p75NTR in peripheral nerve regeneration in Schwann cells [28]. It was stated that FG formed a 3-D matrix for nerve regeneration and its application was easy especially with stem cells. In our study, FG was used to provide stabilization of stem cell in the damaged area, and this 3-D environment was considered to be suitable for neurogenesis [37]. According to the results of our study, both functional and TUNEL outcomes were better in the FG group compared to the control group. The group 4, in which FG was administered with stem cells; yielded the best functional and TUNEL results compared to the other groups.

In the TUNEL test, the highest rate of apoptosis occurred in control group. The second highest rate was observed in group 2. It was considered to be relevant to the fact that FBTMSCs were destroyed very quickly due to high cytotoxic cytokine release in the damaged area. The low incidence of apoptotic cells and the high rate of BrdU(+) in the stem cells group where FBTMSCs were given with fibrin glue, could be interpreted as FG protected the stem cells from the cytokine release and contributed to tissue reparation by keeping these cells in the damaged area.
In our study, we determined that FBTMSC applied with FG provided the most appropriate microenvironment for nerve regeneration. Our clinical observation results showed that the group reaching the highest functional results was Group 4. Although, only stem cell and only FG groups also had better results compared with the control group, the expected clinical and histopathological effects were not as fast. At the end of the 8th week, significant clinical improvement was observed in all groups compared to immediate results after the surgical injury. This result could be attributed to the fact that the damage was a crush type rather than a full nerve cut.

In conclusion, in this study, it has been shown clinically and histopathologically that FBTMSC combined with FG local application might play a promising role as and adjunctive regenerative therapy in traumatic facial paralysis. In this context, it is thought that these results will provide a significant contribution to the literature, as well as satisfactory results for patients when they enter clinical practice. However, these results need to be confirmed in a larger population and by human studies. We believe that our study will provide a basis for human stem cell studies to provide functional clinical improvement in traumatic FP.

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Conflict of Interest: No conflict of interest was declared by the authors.

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Figures:

Figure 1: Characterization of FBTMSCs in flow cytometer according to their surface markers (FACSria III, USA) with: CD11b/c (-), CD45 (-), CD90 (+), CD44 (+).
Figure 2: Histochemical staining of adipocyte, osteocyte and chondrocyte transformation representing the transformation of three different germ-layers structures for the characterization of FBTMSCs: a: Demonstration of adipocyte differentiation (Oil Red (Diagnosis BioSystem, USA) with the appearance of lipid droplets in the cell to appear red-Leica D50, 10x40). b: Demonstration of osteocyte differentiation (Von Kossa (Diagnostic BioSystem, USA) with staining of calcium deposits according to the principle of black
staining - Leica D50, 10x10). c: Demonstration of chondrocyte differentiation (Alica Blue (Diagnostics BioSystem, USA) and hyaluronic acid staining based on blue / purple staining principle - Leica D50, 10x20).

Figure 3: Quantitative summary of TUNEL positive cell count for all groups.
Figure 4: Photomicrographs representing TUNEL-positive staining on the nerve tissues of all the groups (DAB, Hematoxylin) a: The highest number of positive cells in the control group; b: reduced number of positive cells in the FBTMSC group; c: reduced number of positive cells in the FG group; d: minimum number of positive cells in the FBTMSC & FG group.

Figure 5: Demonstration of BrR1-labeled MSCs in tissues by immunohistochemical staining (brown nucleated cells). a: Control group / Group 1 (BrdU (-)). b: FBTMSC group / Group 2 (BrdU (+)). c: FBTMSC & FG group / Group 4 (BrdU (+)) Leica DM 4000 (Germany)).
Table 1: Statistical evaluation of blink reflex and eye closure results.

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$p^1$: Kruskal Wallis test, $p^2$: Friedman test. *Statistically significant difference was occurred after surgery with others. (pctl:percentile)
Table 2: Statistical evaluation of Vibrissae clinical observation.

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p^1: Kruskal Wallis test, p^2: Friedman test. *: Statistical difference was occurred between after surgery and 5th-8th weeks.