Am J Stem Cells 2019;8(1):19-27
www.AJSC.us /ISSN:2160-4150/AJSC0077143

Original Article

Histopathological evaluation of mesenchymal stem cells in the healing of anastomosed carotid arteries

Adnan Altun[1], Cengiz Çokluk[2], Ercan Yarar[3], Enis Kuruoğlu[2], Keramettin Aydın[2], Eyüp Genç[4], Sezgin Gunes[5], Levent Yıldız[6], Abdullah Hilmi Marangoz[2]

[1]Neurosurgery, Medicana Private Hospital, Samsun, Turkey; [2]Department of Neurosurgery, Ondokuz Mayis University, Samsun, Turkey; [3]Department of Neurosurgery, Bafra State Hospital, Sinop, Turkey; [4]Department of Neurosurgery, Turhal State Hospital, Tokat, Turkey; [5]Department of Medical Biology, Ondokuz Mayis University, Samsun, Turkey; [6]Department of Pathology, Ondokuz Mayis University, Samsun, Turkey

Received November 22, 2015; Accepted March 20, 2019; Epub April 15, 2019; Published April 30, 2019

Abstract: The objective of this study was to evaluate the influence of mesenchymal stem cells on the healing of experimental carotid artery anastomoses histopathologically. Twenty-four female Sprague-Dawley rats were used in this study. After random separation of the subjects into two groups, in both groups carotid arteries were transected and anastomosed in end-to-end fashion. Anastomoses were locally treated with 1 ml 0.09% NaCl, and 1 ml mesenchymal stem cell suspension (1×10^6 cells) in control and trial groups, respectively. Anastomoses were wrapped with an 8 mm sheet of surgicel and soaked with BioGlue in order to sequestrate the stem cells. After patencies were confirmed via Doppler USG, surgical site was closed with 2/0 silk sutures. Histopathological evaluation was carried out after 4 weeks. In respect to endothelial continuity, vessel patency (along with presence or absence of restenosis), integrities of internal and external elastic laminae, muscularis and adventitia; no statistically significant differences were present between the trial and control groups. In Trial and Control Groups, luminal thrombus was present in 8 (66.6%) and 3 (25%) of the 12 subjects, respectively. The difference was statistically significant ($P < 0.05$). Recanalization was present in 6 subjects in trial group; 1 subjects in Control Group, respectively. Our results suggest that local administration of mesenchyme stem cell does not have a positive influence on success of an anastomosis.

Keywords: End to end carotid arteries anastomosis, local application of mesenchyme stem cells, histopathological evaluation, thrombosis

Introduction

Recent studies on mesenchyme stem cells (MSC) have yielded encouraging results (25-27). Experimentally, they are known to have enhanced proliferative capacity and ability to differentiate into the parenchymal cells of a damaged tissue or organ [1]. However, according to our literature review, their potential in enhancing the function of a micro vascular anastomosis has not been investigated, and their influence in other vascular surgery models are controversial [2-5]. In an angioplasty study using rats, Chen *et al*. found an increase in aortic restenosis in mesenchyme stem cell test group [2]. Whereas in a wire-induced vascular injury model, Wang *et al*. found that mesenchyme stem cells contributed to intimal hyperplasia and tissue regeneration after vascular injury [3]. And again, Wang *et al*. showed that cell-to-cell contact induces mesenchymal stem cells to differentiate into cardiomyocyte and smooth muscle cell [4]. Hashi *et al*. used nanofibrous vascular grafts integrated with mesenchyme stem cells and observed a very long-term vascular patency [5].

Materials and methods

This experimental animal study was approved by the Ondokuz Mayis University Ethics Committee for Animal Experiments. All experiments were carried out in accordance with international guidelines and local requirements at the Experimental Animal Implementation and Research Center of Ondokuz Mayis University.

Mesenchymal stem cell acquisition

The genetics division of our medical biology department provided MSC used in test subjects, from 4-week-old green fluorescent protein (GFP) transgenic Sprague Dawley rats. After the animals were deeply anesthetized, the femurs and tibias were dissected and the bone marrow was plated on Petri dishes containing Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and Primocin. Cells were allowed to adhere for 48 hours and nonadherent cells were removed by replacing the medium. Adherent cells were cultivated at 37°C in a humidified atmosphere containing 5% CO_2, and the medium was changed twice a week. After reaching near-confluency, the cells were harvested by a Trypsin/EDTA solution. After two to three passages, the cells were labeled with SPION and transplanted into animals. The cells were characterized as MSC by their spindle-shaped morphology and adherence to plastic, while their multipotency was confirmed by their differentiation into adipocytes, osteoblasts, and chondroblasts according to a standard differentiation protocol [6].

MSC identification with flow cytometry

The flow cytometry studies were undertaken according to the criteria defined by Dominici *et al*. [6]. Mononuclear cells harvested in interphase were placed in 3 ml vials and 6 vials were used for each mononuclear cell line. Tubes were labeled respectively as surface markers and control group. And then 100 µl cell suspensions containing 1×10^6 cells were added to each tube. 10 µL surface markers were added to the tubes number 1 to 5. None of the surface markers were added to the tube number 6. All of the tubes were vortexed and incubated at 4°C in dark environment for 15 minutes. Afterwards 1 mL washing solution was added and tubes were centrifuged at 1400 rpm for 5 minutes. Supernatant was removed before adding 0.5 ml washing solution to the residual cell sediment and using flow cytometers did cell identification. The procedure was repeated for all other cell samples. During this procedure as a result of HSC (hematopoietic stem cell) and MSC (mesenchyme stem cell) markers (CD11b/c, CD44, CD45, CD90, CD106) usage, respectively CD11b/c [97% (+)], CD44 [1% (-)], CD45 [99% (+)], CD90 [34% (+)] and CD106 [11% (-)] were demonstrated.

Same procedures were repeated for cell samples harvested by using tripsin-EDTA-C solution from passaged cultivated petri dishes. As a result of same markers usage CD11b/c [5% (-)], CD44 [97% (+)], CD45 [1% (-)], CD90 [97% (+)] and CD106 [30% (+)] were identified.

Surgical technique

In this study, 24 female Sprague - Dawley rats weighing between 200-300 g were used. This model has been chosen due to its consistent anatomy and relatively higher anastomotic patency rate [7]. Rats were divided into trial and control groups, with 12 subjects in each. Surgical prophylaxis was achieved via intramuscular administration of 10 mg/kg cefazolin sodium (Cefozin® Bilim pharmaceuticals). Test subjects were sedated by intraperitoneal administration of Xylazine (Rompun® Bayer) 5-10 mg/kg Ketamin HCL (Ketalar® Parke Davis) 50-150 mg/kg [7]. Afterwards, surgical sites were prepped with Povidone iodine (10%) (Betadin® Kansuk) and draped.

End to end anastomoses

In all subjects, carotid arteries were exposed and transected, followed by end-to-end anastomoses via back wall first simple interrupted technique, under 20× surgical magnification using 10/0 prolene suture (Prolen® ETHICON).

Control group: After the anastomosis, the vessels were wrapped with a 8 mm long surgicel sheet (surgicel® ETHICON) soaked with 1 ml physiological saline solution and kept in place using a tissue adhesive (BioGlue® CryoLife).

Test group: After the anastomosis, the vessels were wrapped with a 8 mm long surgicel sheet (surgicel® ETHICON) soaked with 1 ml MSC suspension (10^6 cell/ml) and kept in place using the same tissue adhesive

Patencies of all vessels were confirmed using Doppler USG. 150 U/kg intravenous (i.v.) heparin (Nevparin® Mustafa Nevzat) was administered to each subjects via their jugular veins.

Following surgery, the subjects were housed in separate cases with access to food and water ad libitum. After 4 weeks, the subjects were euthanised by ether and carotid vessels were harvested for histopathological analyses.

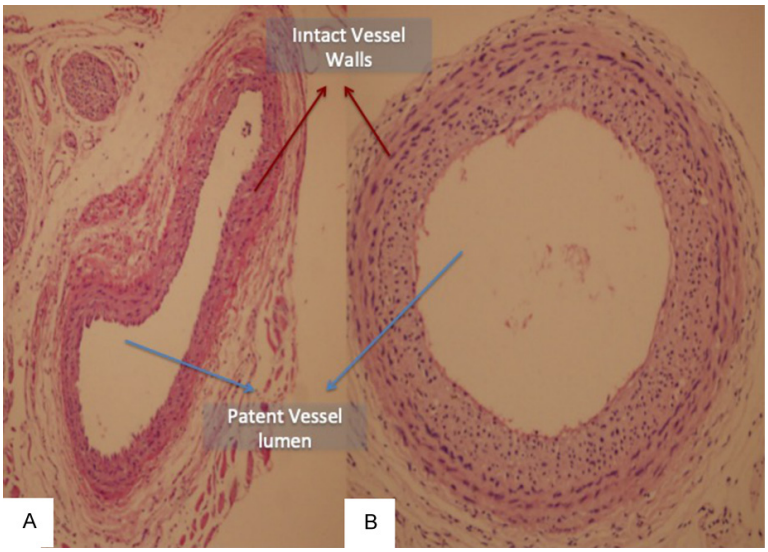


Figure 1. A. Patent vessel lumen in control group artery anastomosis. (×100 HE), B. Patent vessel lumen in MSC test group artery anastomosis. (×200 HE). In some cases there is no difference at vessel lumen patency between control and MSC test group. Also it shows the succession of anastomosis surgery.

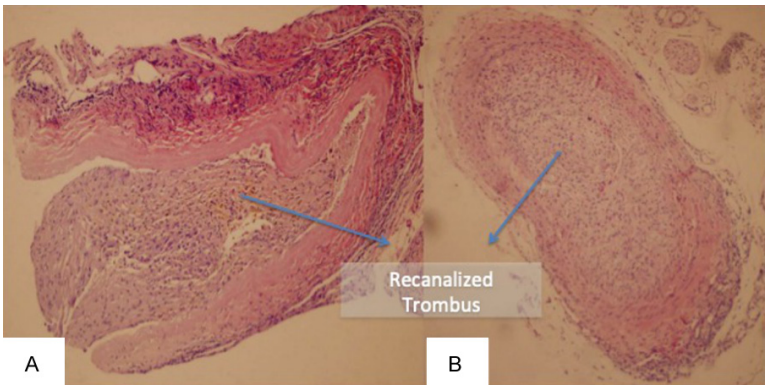


Figure 2. A. MSC test group artery anastomosis, blood vessel wall intact, recanalized thrombus (×100 HE), B. MSC test group artery anastomosis, blood vessel wall intact, recanalized thrombus (×100 HE). In 2 different microscopically sections, we can see the intact blood vessel wall but lumen is occluded by thrombus. Both vessel walls are intact so it demonstrates that thrombus formation is due to stem cell usage; not caused of surgical method.

We used BioGlue as it is frequently and safely used in tissue glueing, anastomosis and providing hemostasis [8]. İn a study Karl *et al*. reported that by using BioGlue in rat artery anastomosis, 100% vascular patency could be provided [9]. İn many microsurgery studies, it has been shown that anastomosis done with bioglue reduces sutures and shortens the surgery time [10, 11]. Especially in artery anastomosis less than 1 mm in diameter, bioglue is used frequently and if applied properly there would be no extra pressure onto artery [9, 12]. In our study bioglue is applied at anastomosis line and used for disengagement of anastomosis line from surrounding tissues. We particularly pay attention for not causing extra pressure onto anastomotic artery.

Histopathological studies

Following Formalin fixation for 24 hours, all specimens were embedded in paraffin blocks and 4-6 µm sections through the anastomosis line were stained with hematoxylin and eosin (HE) for light microscopy. (**Figures 1-6**) By using the evaluation of Control and test group specimens under the light microscope we constituted a table (**Table 2**) and scoring were done on it.

SRY amplification

Genomic DNA was isolated from the Spraque - Dawley rats by a PureLink™ Genomic DNA Kits (Invitrogen, Carlsbad, USA). *SRY* (sexdeterminingregion Y) amplification was carried out by a multiplex PCR reaction [Ise, 2004]. *β-actin* gene was used as an internal positive control. PCR was performed using the *SRY* primers (F: 5'-CAGAGATCAGCAAGCATCTGG-3'; R: 5'-TCTGGTTCTTGGAGGACTGG-3') and β-actin primers (F: 5'-AGAGAAGCTGTGCTATGTTGC-3'; R: 5'-GTACTCCTGCTTGCTGA-TCC-3'). The multiplex PCR amplification was performed in a volume of 25 µl containing 1×PCR buffer, 0.5 µM of SRY primer and 0.3 µM of *β-aktin* primers, 3.0 mM $MgCl_2$, 200 mM of each dNTPs (MBI, Fermentas, Lithuania), 5% DMSO and 2.0 U *Taq polymerase* (Promega, Maddison, WI, USA). After initial denaturation at 95°C for 5 minutes, 37 PCR cycles with denaturation at 94°C for 30 second, annealing at 57°C for 30 second, and extension at 72°C for 60 second were conduct-

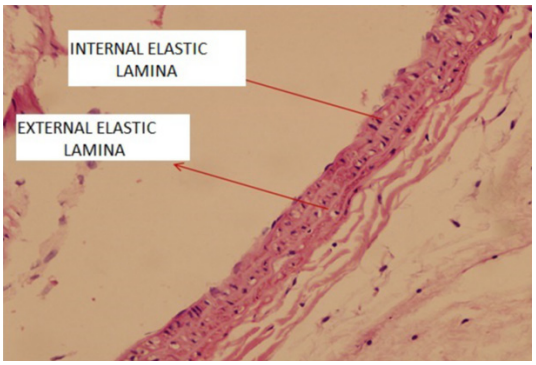


Figure 3. Control group internal elastic lamina, external elastic lamina and endothelium continuity were clearly evaluated (×400 HE). Successful surgical method and clear vessel structure are shown.

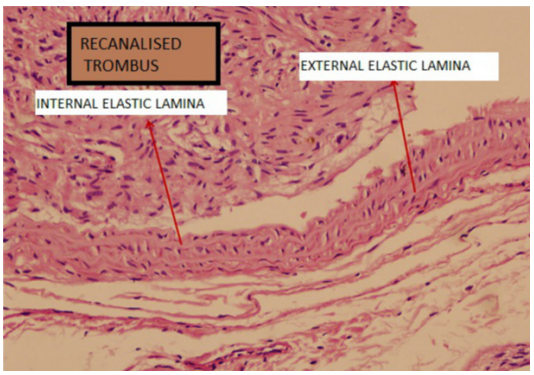


Figure 4. MSC test group anastomosis line, blood vessel wall intact, recanalized thrombus in lumen (×400 HE). Vessel walls are intact so it demonstrates that thrombus formation is due to stem cell usage; not caused of surgical method.

ed [23]. All of the PCR products were resolved on a 2.5% agarose. *SRY* and *β-aktin* gene products were identified by 400 bp and 288 bp respectively (**Figure 7**). This method demonstrates the existence of stem cell in MSC anastomosis line.

Results

Computer software chi-square test was used for statistical analysis (Statistical Package for Social Sciences (SPSS) version 16.0; SPSS, Inc., Chicago, IL). *P* values lesser than 0.05 were considered significant. Histopathological evaluations of anastomoses in both groups are shown in **Table 2**.

In this study, we aimed to see the effect of local application of MSC's on microsurgical anastomotic patency (**Figure 1B**) versus lumen patency in control group (**Figure 1A**) and also see MSC's thrombotic effects in microsurgical anastomoses (two different sections by **Figure 2A** and **2B**). We have investigated the effect of MSC's in patency and quality of rat carotid artery anastomoses. Anastomotic patency's and structural integrities of intimal, medial and adventitial layers around the anastomoses were histologically evaluated (**Figure 3**). Examinations were handled separately for each layer. Also we used the SRY (sex determining region Y) gene in order to prove MSC presence at the anastomosis site in MSC test group.

There were no significant difference in anastomosis line vascular layer healing ratio between control group and MSC test group (**Table 2**;

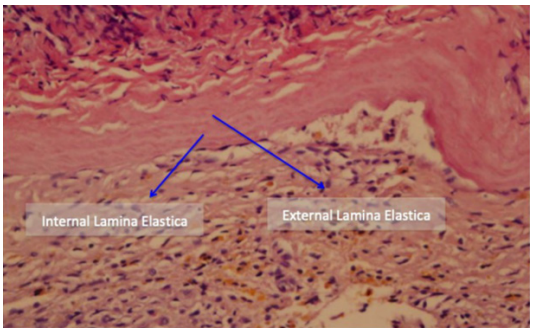


Figure 5. MSC test group anastomosis line, internal and external elastic laminas were clearly evaluated, endothelium continuity partially preserved (×400 HE).

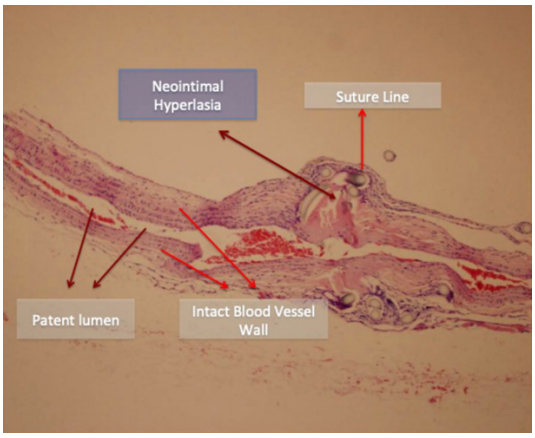
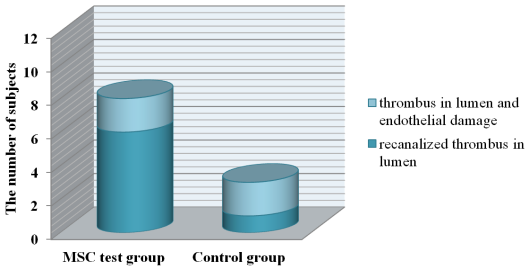


Figure 6. MSC test group anastomosis line longitudinal cut, patent lumen and intact blood vessel wall, neointimal hyperplasia in anastomosis line (×100 HE). Neointimal hyperplasia can be the cause of thrombus formation in MSC test group versus control group.

Table 1. Ratio of thrombus formation in control and MSC test groups



The comparisons of two groups are shown in **Table 1**. Thrombus was formed in 8 of 12 subjects (66.6%) and 3 of 12 subjects (25%) in trial and control groups, respectively. Thrombus formation probabilities in MSC test group were found statistically significant [x^2 = 4, 19, sd = 1, P < 0.05, (OR = 0.167), (0.028 < OR < 0.983)].

Figure 4). There were significant differences subjected to thrombus formed in vessel lumen in MSC test group anastomosis. We found that the endothelium continuity partially preserved in MSC test group anastomosis line (**Figure 5**) and neointimal hyperplasia occurred in MSC test group anastomosis line (**Figure 6**).

Our histopathological analyzes yielded no statistically significant difference between endothelium continuities, vascular lumen patency's, and healing capacities of internal and external elastic lamina, muscular layers and adventias of both groups (**Table 2**). As shown in **Table 2**; in all MSC test group, we detected MSC.

In first variable, in 2 of 12 (16%) subjects, we didn't determine endothelium cell in anastomosis line and ratio was same in control group; in 4 of 12 (33%) subjects we determined endothelium cells but we didn't detected continuity of endothelium in anastomosis line and ratio was 2 of 12 (16%) in control group; in 6 of 12 (50%) subjects, we determined continuity of endothelium in anastomosis line in MSC group and the ratio was 8 of 12 (66%) in control group. As shown, MSC application in anastomosis line decreases endothelium continuity.

In second variable, in 2 of 12 (16%) subjects, we determined total occlusion of vessel lumen in anastomosis line in MSC test group and the ratio was same in control group; in 6 of 12 (50%) subjects, we determined partial occlusion of vessel lumen in MSC test group in anastomosis line and this ratio was 1 of 12 (8%) in control group; in 4 of 12 (33%) subjects we didn't detected thrombus and lumen was totally patent; in control group this number was 9 of 12 (75%). This shows us that MSC application increases vessel lumen occlusion by thrombosis.

In third variable, in 2 of 12 (16%) subjects, we determined no neointimal hyperplasia and detected total occlusion of vessel lumen in anastomosis line in MSC test group and the ratio was same in control group; in 1 of 12 (8%) subjects, we determined neointimal hyperplasia in MSC test group in anastomosis line and this ratio was same in control group; in 9 of 12 (75%) subjects we didn't detected neointimal hyperplasia in MSC test group and same in control group This shows us that MSC application doesn't effect neointimal hyperplasia ratio.

The comparisons of two groups are shown in **Table 1**. Thrombi were formed in 8 of 12 subjects (66.6%) and 3 of 12 subjects (25%) in trial and control groups, respectively. Thrombus formation probabilities in MSC test group were found statistically significant. [x^2 = 4, 19, sd = 1, P < 0.05, (OR = 0.167), (0.028 < OR < 0.983)].

Discussion

Success of a micro vascular anastomosis depends mainly on the execution of the surgical technique. The major cause of pathological occlusion after the destruction of blood vessel integrity is neointimal hyperplasia [13, 14]. Hyperplasia is also the major cause of late-term occlusion. Even though the development of new treatment strategies, restenosis following anastomosis is still a clinically significant problem [15].

Disruption of the endothelial integrity plays a significant role in stenosis. Endothelial cell damage leads to decreased secretion of vaso protective mediators like nitric oxide (NO) and prostaglandins, which result in a more pronounced inflammatory response [16]. Also, adventitial damage inflicted by poor surgical technique further contributes to the progression of [17].

Recent studies have shown that circulatory MSC's originating from bone marrow contribute

Table 2. Endothelium continuity, vessel lumen patency subjected to thrombus, and neointimal hyperplasia scorings*

Rat No	MSC	Endothelium continuity at anastomosis line	Vessel lumen patency subjected to thrombus	Vessel lumen patency subjected to neointimal hyperplasia
1	1	2	1	2
2	1	2	2	2
3	1	1	1	1
4	1	2	2	2
5	1	1	1	2
6	1	1	1	2
7	1	1	1	2
8	1	2	2	2
9	1	2	2	2
10	1	2	1	2
11	1	0	0	0
12	1	0	0	0
13	0	1	2	2
14	0	2	2	2
15	0	1	1	1
16	0	2	2	2
17	0	2	2	2
18	0	2	2	2
19	0	0	0	0
20	0	0	0	0
21	0	2	2	2
22	0	2	2	2
23	0	2	2	2
24	0	2	2	2

*For **Table 2**: MSC presence 1 = yes, 0 = no; Endothelium continuity at anastomosis line; 0= no endothelium, 1= endothelium (+), endothelium continuity interrupted, 2 = endothelium (+), endothelium continuity (+); Vessel lumen patency subjected to thrombus; 0 = thrombus (+), lumen totally obstructed, 1 = recanalized thrombus (+), lumen partially patent, 2 = no thrombus, lumen totally patent. Vessel lumen patency subjected to neointimal hyperplasia; 0 = lumen totally obstructed, 1 = mild neointimal hyperplasia, 2 = no neointimal hyperplasia.

to the remodeling of blood vessels. MSC's could be reproduced following minimal bone marrow aspiration. Experiments involving MSC allotransplantation are increasing due to their minimal immunogenity that doesn't necessitate immunosuppression [18]. Undifferentiated MSC does not recognized by immune system and, thereby, cause an immune reaction; therefore MSC transplantations can be effectively used in treatment of several diseases [24, 25]. Zarbakhsh et al. investigated individual and synergistic effects of transplantation with Schwann and bone marrow stromal cells in axonal regeneration in experimental rat model of peripheral nerve injury, and showed the effectiveness of both approaches in functional recovery in 12 weeks [26, 27]. The body of literature investigating the effects of MSC's on vascular patency yields contradictory results. Chen *et al*. have reported that MSC's increase the risk of restenosis in an experimental aortic angioplasty model [2]. In another experimental study, Wu et al. shows that MSC's may inhibit smooth muscle cell proliferation of damaged blood vessels and may decrease restenosis [19]. In yet another in which vascular grafts incorporating MSC'S are investigated, it's reported that long term vessel patency is promising [5]. However, according to our literature review, there are no studies investigating the effect of MSC's on patency of micro vascular anastomoses.

I.V. administration of MSC's has shown to be safe in test subjects and humans [20]. In order to compare MSC's efficiency, we used criteria's defined by Dominici et al. in our experiment (The International Society for Cellular Therapy position statement) [6]. We applied MSC's topically and no pathological reactions such as allergy were observed.

In their study, Forte et al. showed that mRNa levels which were used as an indicator of MSC presence, have been increased not only at anastomotic vessel but also at nonanastomic vessels [21]. Amalia et al. used marked MSC's in a carotid artery anastomosis model and detected MSC presence at the damaged artery wall three days after IV MSC administration [16]. Likewise, WANG et al. have shown MSC presence in rat femoral artery seven days after IV MSC application [3]. In our study we didn't detect MSC's anywhere other than the vessel we locally administered it on.

During healing process of a damaged artery wall, endothelial healing will be complete by the

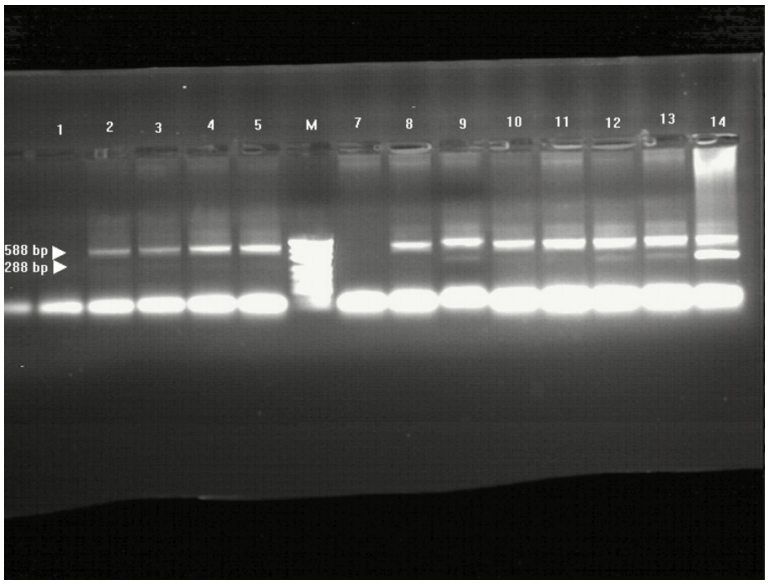


Figure 7. *SRY* amplification; *SRY* and *β-aktin* gene products were identified by 400 bp and 288 bp respectively.

end of the first week; however significant hyperplasia of the perianastomotic intimal layer will ensue during a course of four weeks [3, 5, 12, 22]. MSC presence can be shown at those sites following their systemic administration at this period [3, 16]. We sacrificed our subjects after four weeks so that we can better evaluate their presence and possible influence.

Craig et al. have showed that combining MSC's with nano fibrous vascular grafts improved their influence [5]. Shinichiro et al. showed that acelluar vascular grafts without MSC's lead to intimal thickening and thus restenosis while vascular grafts combined with MSC's had promising results [22]. It appears that combining MSC's with vascular grafts yields a more physiological endothelial and smooth muscle organization and prevents restenosis [5]. Craig et al. have pointed out the antiadhesive properties of MSCs in combination with vascular grafts, which prevent platelet aggregation [5]. In the light of all of these data we can say that MSC's have antithrombogenic effect.

Our histopathological analyzes yielded no statistically significant difference between endothelium continuities, vascular lumen patency's, and healing capacities of internal and external elastic lamina, muscular layers and adventias of both groups. However when we analyzed the presence of vascular lumen thrombosis; we found that thrombus were formed in 8 of 12 subjects (66.6%) in MSC test group while in 3 of 12 subjects (25%) in control group. Recanalization developed in 6 of 8 thrombotic vascular lumen in MSC test group. Whereas in control group only 1 of 3 thrombotic vascular lumen have been developed recanalization. These data suggests that MSC administration significantly increases the propensity of thrombus formation in microsurgical anastomoses [x^2 = 4, 19, sd = 1, P < 0.05, (OR = 0.167), (0.028 < OR < 0.983)]. These data appears to contradict with the literature, which suggests antithrombotic effects of MSC's [21].

Conclusions

• This study shows that local application of MSC's may yield different results when compared with their systemic administration.

• Local application of MSC's seemed to have no positive influence on microsurgical anastomotic patency.

• Local application of MSC's ensures their presence to be confined to the target vicinity.

MSC's have thrombotic effects in microsurgical anastomoses.

Disclosure of conflict of interest

None.

Address correspondence to: Adnan Altun, Neurosurgery, Medicana Private Hospital, Yeni Mahalle, Şehit Mesut Birinci Street No. 85, Canik/Samsun, Samsun, Turkey. E-mail: draaltun@hotmail.com

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