The effects of mesenchymal stem cells on the structure and contractile force of the carotid artery in a rat aneurysm model

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Background/aim: An aneurysm is a pathological enlargement of an artery characterized by the thinning of the elastic fiber layer in the tunica media. Because the aneuritic artery wall is weakened, these vessels can rupture and cause serious bleeding. Surgical methods are often used for the treatment of aneurysms. However, cell-based therapies are less invasive and potentially safer alternatives. In this study, the therapeutic efficacy of rat adipose tissue-derived mesenchymal stem cells (MSCs) was investigated in a new carotid artery aneurysm model.

Materials and methods: Arteries were pretreated with elastase to create aneurysms. Gelatin matrices containing MSCs were applied to the outer surface of the elastase-treated carotid artery sections.

Results: Healing of the aneuritic arteries for which MSC applications were performed was significantly better than in the aneuritic group. The histological structure of the vessels was largely reconstituted, and the contractile force of the MSC-treated group was similar to the untreated healthy group.

Conclusions: Application of MSCs facilitates the healing of aneurysms. Hereby, MSC application could be a promising approach for clinical applications after further validation processes are concluded.

Key words: Mesenchymal stem cells, carotid artery, aneurysm, elastase, animal model

1. Introduction

Peripheral artery diseases (PADs) are particularly common diseases. It is reported that more than 200 million people have PADs worldwide (1,2). Smoking, poor diet, diabetes, and atherosclerosis are the principle causes of PADs. There are many types of PADs, such as acute and chronic limb ischemia; abdominal aortic, iliac, and peripheral artery aneurysms; and stenosis (3,4).

Peripheral artery aneurysms, which have gradually increasing incidence, are the pathological enlargement of the artery wall (5,6). Although the process of aneurysm formation is not well understood, it is typically characterized by the degeneration of the elastin layer and thinning of the tunica media (7,8). The wall of an artery consists of three layers. The innermost layer, the tunica intima, is lined by a simple squamous epithelium surrounded by a connective tissue basement membrane with elastic fibers. The middle layer, the tunica media, is primarily made up of smooth muscle and is usually the thickest layer. The outermost layer, which attaches the vessel to the surrounding tissue, is the tunica externa or tunica adventitia. This layer is composed of connective tissue with varying amounts of elastic and collagenous fibers (9). Hematoxylin and eosin staining of different arteries has shown structural and thickness differences among these layers (10). The carotid artery is an elastic artery, like the abdominal aorta. However, the elastic layer of the carotid artery is comparatively thin and resembles other small arteries in the body. Therefore, the carotid artery is a good model for simulating small and midsized arteries of the body.

The capacity of mesenchymal stem cells (MSCs) to differentiate into multiple cell types was discovered 40 years ago. MSCs have attracted great attention because of their potential applications in regenerative medicine (11). Adipose tissue is a new and promising alternative source of MSCs, owing to the ease of collection and the increased numbers of MSCs in this tissue.

In the present study, MSCs were isolated from rat adipose tissue and evaluated in a new aneurysm model. The results reveal that MSCs can facilitate repair of an aneuritic carotid artery. Thus, MSC transplantation can be a promising therapeutic approach as an alternative to invasive treatment methods.

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2. Materials and methods

2.1. Cell isolation and characterization

All animal procedures were performed in accordance with the ethical standards of the Hacettepe University Ethics Committee (Protocol No. 2013 - 04 - 04). MSCs were isolated from the subcutaneous flank adipose tissue of male albino Wistar rats (250-300 g) with the primary explant culture technique and were characterized as shown in our previous studies (12,13). Two rats were used for cell isolation. The MSCs were passaged four times in 25-cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) (Biochrom AG, Berlin, Germany) containing 20% fetal bovine serum (FBS) and 0.2% penicillin/streptomycin (Biochrom AG). Approximately 650,000 cells were used for each carotid artery treatment.

2.2. In vivo experiments

Female albino Wistar rats (250-300 g) were anesthetized by application of xylazine (Richter Pharma AG, Wels, Austria) (10 mg/kg) and ketamine (Richter Pharma AG) (50 mg/kg). The ventral region of the neck was shaved and cleaned with povidone-iodine. To expose the carotid artery as it traverses behind the trachea, the skin was cut and the muscles were carefully separated using forceps. A fusiform-type aneurysm model was developed in the rats. To create a carotid artery aneurysm, a 0.5-cm portion of the carotid between the main branch and the end was encased in a UV-sterilized plastic tube and treated with type I elastase from porcine pancreas (9.1 U/mg, Sigma Aldrich Chemical Co., St Louis, MO, USA) (Figure 1). The optimal dose and application time for elastase for rat carotid arteries were determined according to previous studies (14,15). An elastase solution was freshly prepared at 0.18 mg (1.638 units) in 100 mL of phosphate-buffered saline (PBS) (Biochrom AG), just before application. The

elastase solution was applied to the carotid arteries for 20 min, after which the plastic tubes were removed and the carotid arteries were washed with PBS. The muscles were placed back in their original positions and the skin was sutured. The rats were then allowed to recover in their home cages for 5 days to ensure aneurysm development.

Rats were randomly divided into three groups. In the aneuritic group, the carotid arteries of all rats were damaged by elastase application and left to heal without any treatment. In the MSC-treated aneuritic group, the carotid arteries of all rats were damaged by elastase application and MSC-absorbed gelatin matrices were applied to the damaged area. In the control group, only the carotid arteries were exposed, and then the muscle and skin were sutured (healthy carotids).

2.3. MSC application

Before MSC application, the cells were centrifuged, the cell pellet was transferred to 100 μL of PBS, and the cells were absorbed by small pieces of gelatin matrices (0.5 × 0.5 cm, GELITA-SPON, Amsterdam, the Netherlands) (13,16). The gelatin matrices, containing approximately 650,000 MSCs, were then applied to the aneuritic region before the wound was sutured (Figure 2).

2.4. Histological experiments

For microscopic evaluation, paraffin-embedded carotid arteries were stained with hematoxylin and eosin & Verhoeff stain (H & E & V). The thickness of the elastic fiber layer was measured and compared among groups. Results were evaluated with the Mann–Whitney U test.

2.5. Recording of smooth muscle contractions

One week after surgery, each animal was anesthetized as described previously (12,13). The carotid arteries of all rats were quickly isolated and cut into 3-5-mm transverse rings without damaging the luminal surfaces. The rings were vertically fixed between two stainless steel wire hooks,
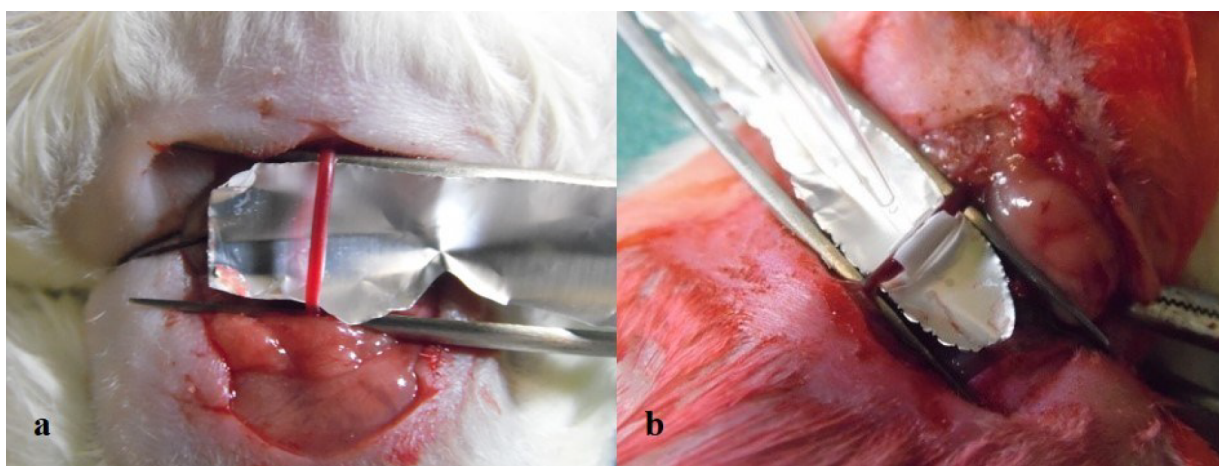

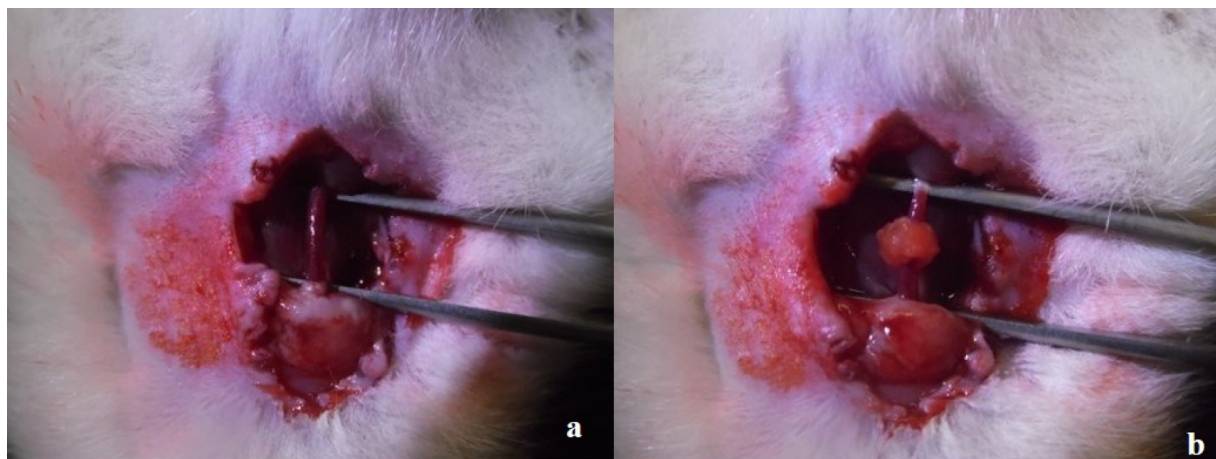**Figure 1.** a) Placement of the carotid artery, b) external application of the elastase.

Figure 2. Implantation of the MSCs into the aneuritic region: a) aneuritic carotid artery, b) application of the MSCs in gelatin matrix.

passing through the lumen of the vessels, and suspended under 0.5 g of resting tension in a chamber containing Tyrode solution (pH 7.4) at 37 °C, continuously aerated with 95% O_2 and 5% CO_2 (Figure 3).

Prior to the experiments, tissues were equilibrated for at least 10 min in the Tyrode solution and then the isometric muscle contractions of the vessel rings were recorded using a force displacement transducer (Model FT-03C; Grass Inst. Co., Quincy, MA, USA). The signals from the force displacement transducer were recorded and digitalized with a BioPac MP35 data acquisition system (Biopac Systems Inc., Goleta, CA, USA) at a sample rate of 500 kHz and were analyzed with Biopac Student Lab Pro 3.6.6 (Biopac Systems Inc.). The contraction and relaxation of the vessels were assessed by observing the contraction

of the rings after epinephrine administration (Galen Drug Industry Co., Craigavon, UK) at concentrations of 5 µM, 10 µM, 20 µM, and 40 µM and consequent relaxation was recorded when challenged with 5 nM, 10 nM, 20 nM, and 40 nM concentrations of acetylcholine (Sigma, St. Louis, MO, USA), a selective endothelium-dependent dilator. The contraction/relaxation patterns of the vessels were recorded for at least 2 min for each dilution. Between each measurement interval, the organ bath was replenished with 20 mL of fresh Tyrode solution.

3. Results

3.1. Cell isolation and characterization

MSCs were isolated from rat adipose tissue using the primary explant culture technique described in our
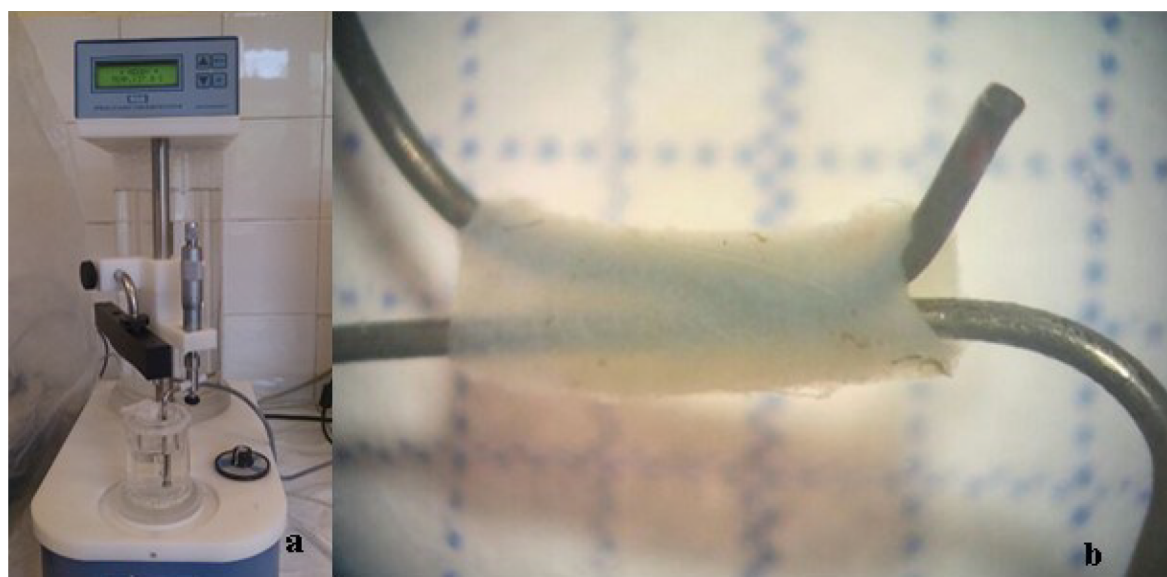

Figure 3. a) Carotid artery ring in the chamber, b) fixed ring between two stainless steel wire hooks.

previous study (12). The cells were passaged four times and phenotyped by the surface expression of CD13 and CD29 by immunofluorescence staining. Flow cytometric analyses showed that these MSCs were positive for CD29, CD90, CD54, and class I MHC and were negative for CD45, CD106, and class II MHC (12).

3.2. In vivo experiments and histological examinations

Elastic fibers are stained black by H & E & V staining. H & E staining showed that the tunica media was significantly thinner in the aneuritic group than in the control group. Additionally, the elastic fiber layer was significantly thinner in the aneuritic group than in the control group (Figure 4; Table 1).

The healthy, untreated carotid arteries had normal morphology and all layers were clearly observed by H & E staining. Elastic fibers, which were surrounded by smooth muscle cells in the tunica media, appeared black in H & E & V stained sections.

In contrast, the integrity of the carotid arteries was completely lost in some individuals in the aneuritic group due to connective tissue damage. Intravenous folds and pockets were also formed in some cases. Alternatively, carotid arteries in the MSC-treated aneuritic group had maintained their normal histological structures, as all layers were visible. Only the outer surface differed, as it was surrounded by thick connective tissue. H & E & V stainings showed the formation of elastic fibers in the MSC-treated aneuritic group. Since the MSCs were applied to the outer surfaces of the carotid arteries, we suggest that the MSCs contributed to cell proliferation in this layer (Figure 5).

3.3. Recording of smooth muscle contractions

The contractile force was compared among the three groups (aneuritic, MSC-treated aneuritic, and control) both before and after administration of the epinephrine and acetylcholine dilutions.

The comparison of contractile force among groups, administration of the highest dilution of epinephrine (40 μM), and administration of the highest dilution of acetylcholine (40 nM) (Figure 6) are shown in Table 2.

The contractile force results confirm the histological observations. When the number of elastic fibers and smooth muscle cells increased in the MSC-treated aneuritic group, contractile force of the carotid arteries were approaching that of the control group. As expected, epinephrine and acetylcholine administration led to dose-dependent contractions in the carotid arteries of the control group (Figure 7). The increased contractile force with increasing doses of epinephrine and the decreased contractile force with increasing doses of acetylcholine were significant ($P < 0.05$, Kruskal–Wallis test). However, contractile force was unsteady in the aneuritic group. Deterioration of the elastin layer and decreased numbers of smooth muscle cells or epinephrine and acetylcholine receptors may have resulted in the unsteady contractile force.

The differences between the MSC-treated aneuritic and control groups were not significant. This is possibly due to the low contraction capacity of the aneuritic group. In accordance with the literature, the contractile force in this study was also reduced after acetylcholine application. As a result, we observed the healing of the carotid artery by physiological parameters.

4. Discussion

The first aneurysm model was established using elastase perfusion in rats in 1990 (14). Elastase-perfused aortas contained aneurysms and had a total loss of elastic tissue. In another model, the infrarenal abdominal aorta was dissected free of the inferior vena cava through a laparotomic incision and the inferior mesenteric artery and all branches of the aorta below the left renal vein
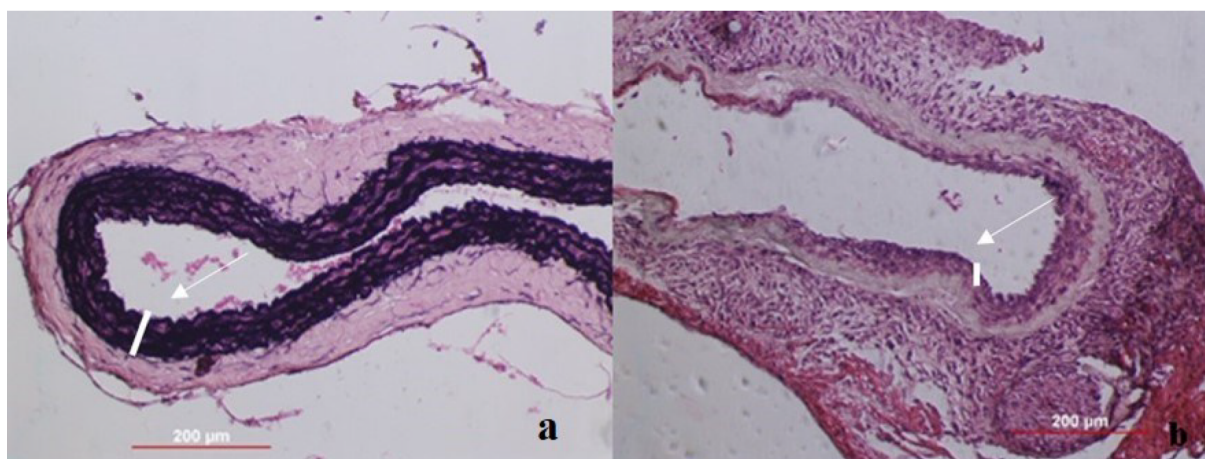

Figure 4. Representative images of the carotid arteries in a) the control and b) aneuritic groups with H & E & V staining (400×) (white arrow indicates the elastic fibers).

Table 1. Thickness of the elastin layer in MSC-treated aneuritic and control groups.

	Control group (μm)	MSC-treated aneuritic group (μm)
n_1	51.70	35.00
n_2	52.70	42.15
n_3	57.60	61.94
n_4	57.70	38.09
n_5	56.67	50.06
n_6	58.96	60.30
N_{avg}	55.87	47.92

were ligated. A polyethylene catheter was inserted into the femoral artery and advanced until its tip was positioned in the distal abdominal aorta. An atraumatic clamp was placed on the aorta at the level of the left renal vein and a ligature was secured around the distal aorta to encompass

the catheter just above the level of the aortic bifurcation. Over a 2-h period, the aorta was perfused with 2 mL of saline solution containing pancreatic elastase (17). In many studies, abdominal aortic aneurysm models were created by intraaortic elastase application (18–20). For instance, Zhang et al. created an experimental abdominal aortic aneurysm model in rats by using an intraaortic elastase application (21). In these studies, elastase was usually applied to the carotid arteries for 2 h.

In the present study, elastase was applied between the bifurcation and proximal carotid arteries using a newly improved method. Elastase was applied to the surface of the artery wall for only 20 min. Because the carotid artery is a relatively large vessel compared to the other peripheral arteries, it can be a promising model. The aneurysm occurred more efficiently and faster in this model than in previously described models (21) in the literature, with rapid disruption of the elastic structure of the carotid artery. Fibroblasts located in the tunica adventitia and the smooth muscle cells in the tunica media
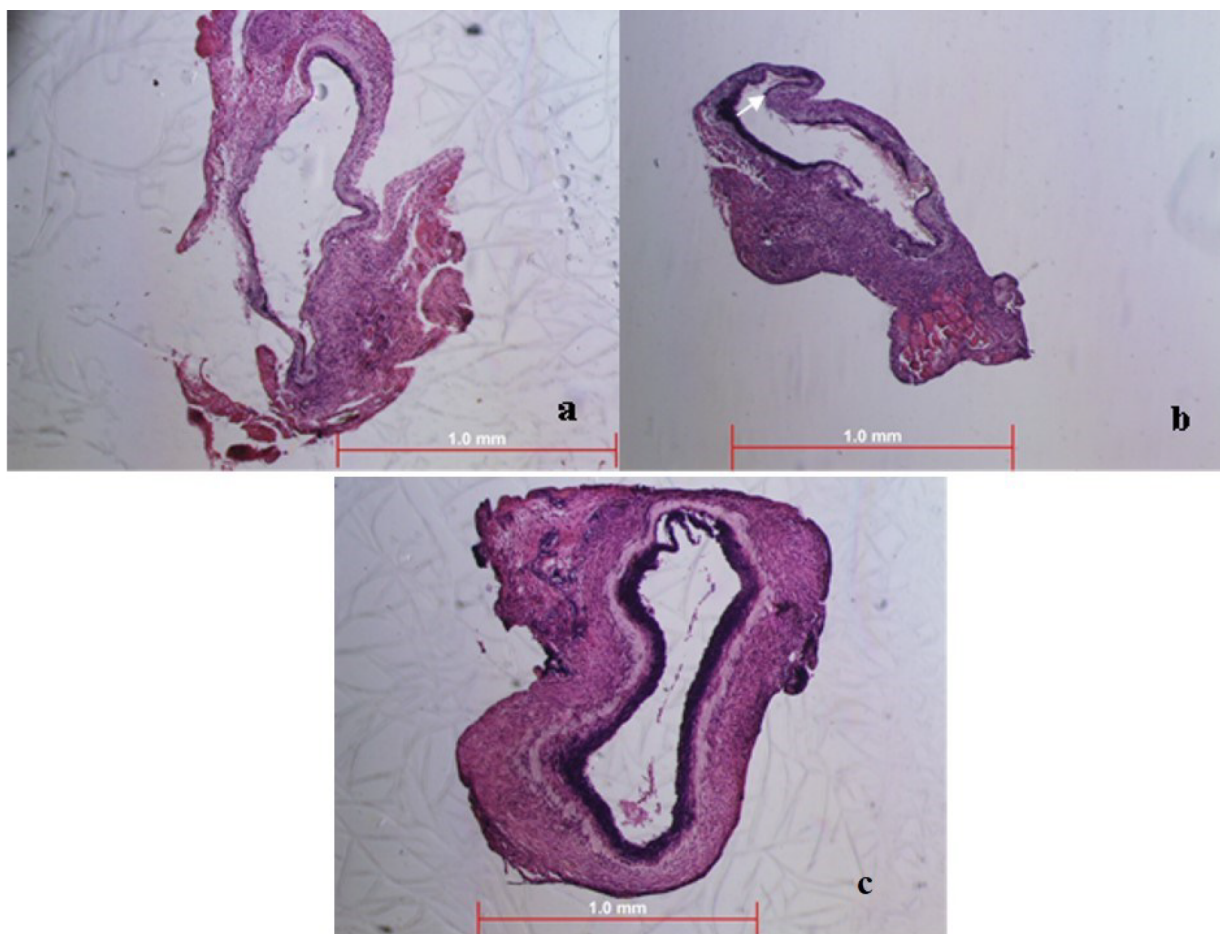

Figure 5. Microscopic images of the damaged carotid arteries in a) aneuritic group with H & E & V staining, b) intravenous folds and pockets (white arrow), c) MSC-treated aneuritic group (400×).
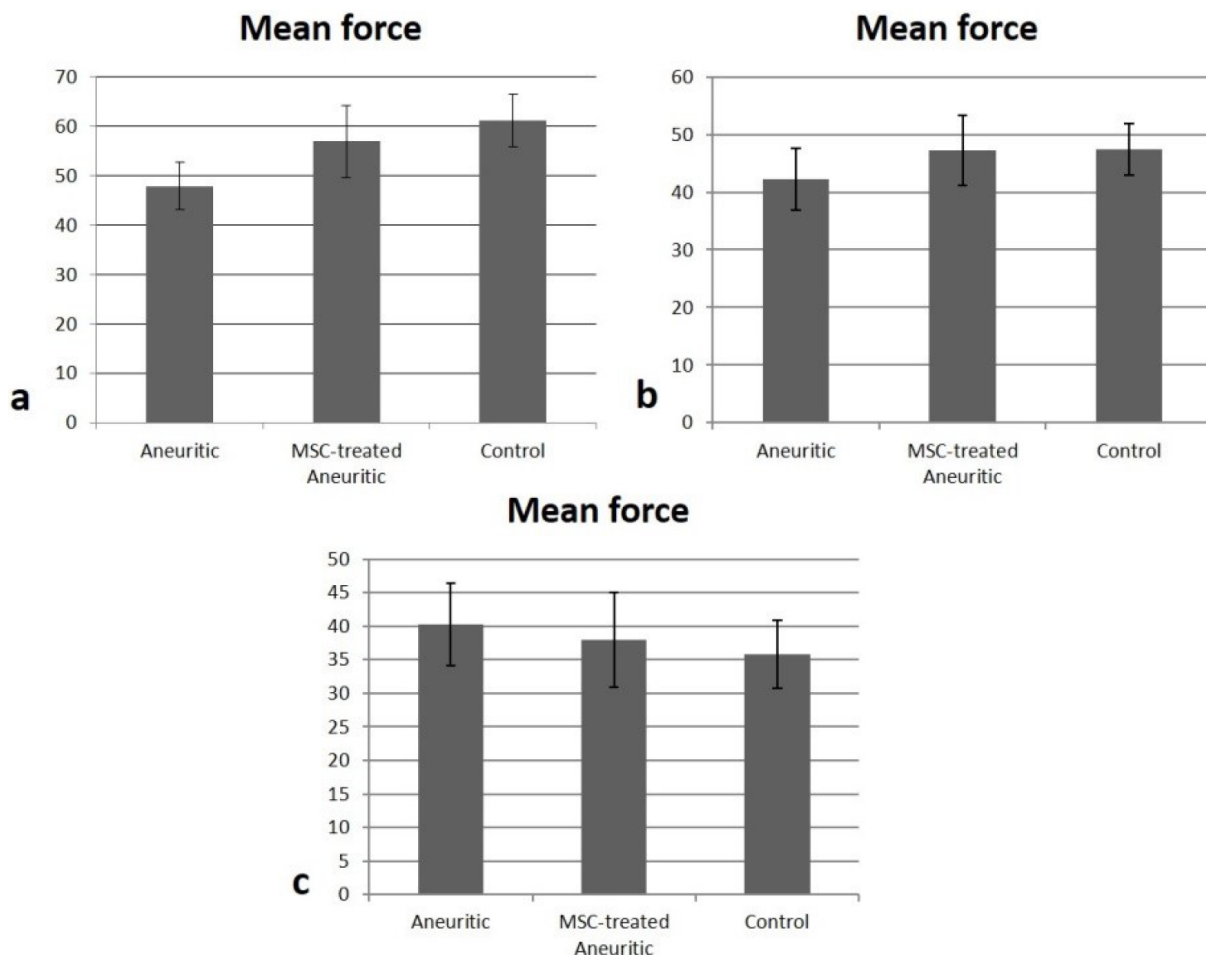
Mean force

Mean force

Mean force

Figure 6. Comparison of contractile force between groups, without epinephrine and acetylcholine administration.

Table 2. Effects of epinephrine and acetylcholine administration on contractile force for all groups.

	Groups		
	MSC-treated aneuritic to control	Aneuritic to control	MSC-treated aneuritic to aneuritic
No administration	Not significant	*	*
40 µM epinephrine administration	Not significant	*	*
40 nM acetylcholine administration	Not significant	*	Not significant

*: P < 0.05, Mann–Whitney U test.

are responsible for the synthesis of elastic fibers. Smooth muscle cells also play a primary role in the synthesis of the extracellular matrix, which is required for vessel stability. In our aneurysm model, the tunica adventitia and tunica media deteriorated, the layer of elastin-containing elastic fibers was lost, and the integrity of the carotid artery was disrupted. In the literature, aneurysm models are usually studied in the aorta. In the present study, unlike in the literature, the carotid artery was used to create an aneurysm model. The carotid artery is an elastic-type artery, like the aorta. However, the elastic layer of the carotid artery is thinner than that of the aorta. As a result of this feature, the carotid artery can be used as a good model for other smaller arteries.
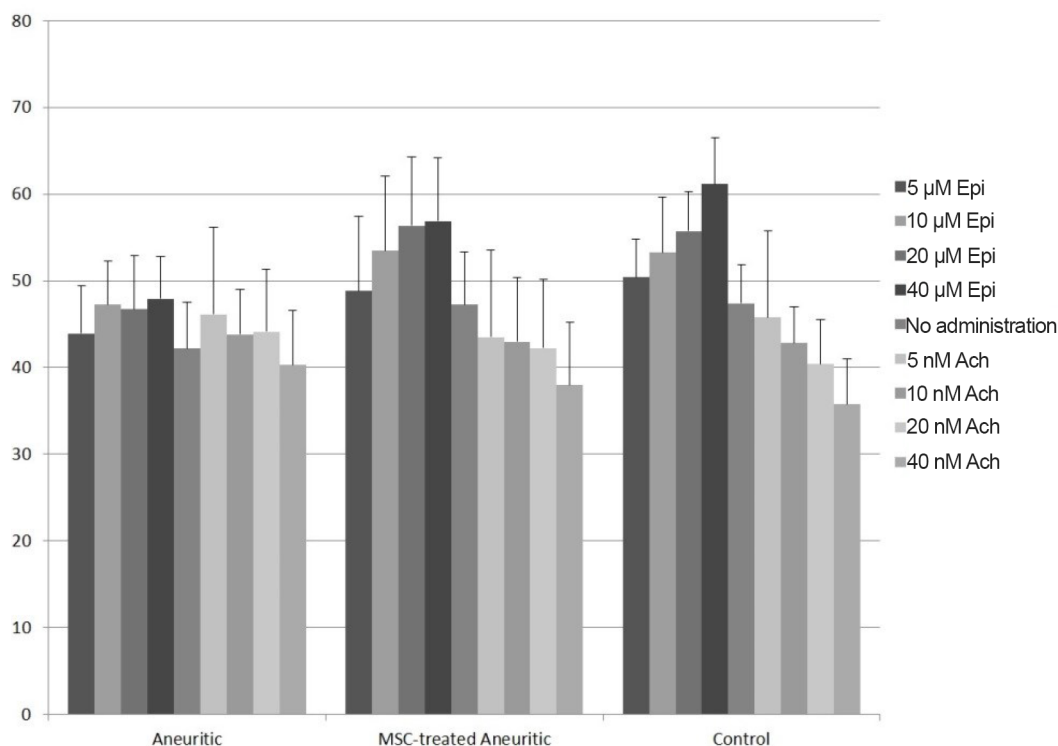
Mean Force

Figure 7. Epinephrine and acetylcholine administration led to dose-dependent contractions on the carotid arteries of the control group.

The pathogenesis of aneurysm formation is complex and the principal initiating factors are still unknown (22). However, elastin degeneration and weakening of the tunica media are typical features of the aneurysmal process (23,24). Our model recapitulates these events of aneurysm formation. Moreover, this model can be established rapidly, reducing the invasive period. Once the connective tissue containing the fibroblasts that produce elastic fibers is damaged, the aneurysm is irreversible. In this study, we directly applied MSC-absorbed gelatin matrices to the aneurysm site. Thus, we can ascertain whether MSCs contribute to the healing of connective tissue by replacing the fibroblasts. It is also thought that MSCs can differentiate into muscle cells and

contribute to the reformation of the smooth muscle layer. The reconstruction of the tunica adventitia and media and the reformation of the elastic fibers support this notion.

In conclusion, in the present study, a new aneurysm model was established and adipose tissue-derived MSCs were applied to the outer surface of the aneuritic carotid artery in a degradable gelatin matrix. The application of MSCs contributed to carotid artery healing, suggesting that MSCs may have an important role in the regeneration process of aneuritic vessels. While we need to learn more about their in vivo properties to safely use MSCs in the clinic, MSCs appear to be a promising tool for clinical applications.

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