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Original Article

The effect of young plasma therapy on vascular endothelial growth factor and cell proliferation

Alper Ozbakkaloglu[1], Aysun Inan Genc[2]

[1]Özel Sağlık Hospital, Department of Cardiovascular Surgery, İzmir, Türkiye
[2]Kastamonu Univesity, Faculty of Science, Department of Biology, Kastamonu, Türkiye

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Abstract

Aim: With aging, there is a dramatic decrease in Vascular Endothelial Growth Factor (VEGF). The effects of plasma therapy applied to aged tissues have attracted attention in recent years, as young plasma is rich in rejuvenating factors that decrease in aged mice and support organ regeneration and hormone secretion. In the presented study, young plasma treatment was applied to aged rats and its effects on cellular proliferation and VEGF levels in the duodenum were evaluated.

Material and Methods: For this purpose, aged male Sprague Dawley rats (24months, n=15) were treated with pooled plasma (0.5ml/day, intravenously for 30d) collected from young (5weeks, n=51) rats. Aged rats formed the control group while aged rats that received young plasma treatment were labelled as the experimental group. At the end of the experiment, the duodenums of the groups were collected and cell proliferation index and proliferation intensity were evaluated with proliferating cell nuclear antigen (PCNA) and expressions of VEGF which plays a role in both the digestive system and cardiovascular system were investigated.

Results: VEGF expression intensity determined by immunohistochemical method showed an increase in experimental group (2.45±0.18) compared to control group (1.70±0.15) ($p<0.001$). Proliferation intensity showed a more severe immunoreaction in experimental group (2.35±0.19) compared to control group (1.20±0.08). Proliferation index was higher in experimental group (117.10±0.19) compared to control group (62.20±0.12).

Conclusion: In our study, we propose a treatment approach that can prevent the aging-related decrease in VEGF, which is known to be important in many systems.

Keywords: Duodenum, cell proliferation, vascular endothelial growth factor

INTRODUCTION

Cardiovascular diseases (CVD) are one of the leading causes of death worldwide. Among these, coronary artery disease and arrhythmias represent the most common pathological conditions. Similarly, gastrointestinal disorders such as astroesophageal reflux and inflammatory bowel diseases have a high incidence [1]. The connections between cardiac and gastrointestinal disorders are of interest to researchers because they often share similar risk factors and symptoms. Both conditions in such diseases can occur simultaneously in the same patient, thus creating problems in correct clinical diagnosis.

It is well known that gastrointestinal (GI) disorders sometimes present with chest pain and can mimic angina pectoris. On the contrary, they can also reveal heart disease, as in the case of angina-related ischemia. Therefore, there are some correlations and relationships between GI diseases and cardiovascular diseases.

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Corresponding Author: Alper Ozbakkaloglu, Özel Sağlık Hospital, Department of Cardiovascular Surgery, İzmir, Türkiye
Email: alperozbakkaloglu@outlook.com

Studies investigating the role of gastrointestinal disorders in the development of CVDs and arrhythmias such as atrial fibrillation [2], as well as gastrointestinal disorders with a high incidence such as gastroesophageal reflux and inflammatory bowel diseases (IBDs) [3] have revealed an interesting association with the onset of CVD [4].

Vascular endothelial growth factor (VEGF) is a pleiotropic growth factor with both vascular and nonvascular functions [5]. Vascular endothelial growth factor is one of the primary regulators of angiogenesis and triggers and promotes endothelial cell growth following its activation [6]. Apart from its angiogenic activity, it also supports the control of vascular permeability [7], the survival of newly formed blood vessels, the formation of capillary fenestrations, and the regulation of extracellular barrier functions [8,9]. It also plays important roles in the induction of organ-specific angiocrine factors [10]. VEGF also plays an important role in physiological processes, particularly in vascular development and wound healing in the adult stage [11]. However, it is also involved in pathological pathways, including diabetic retinopathy, tumor angiogenesis, and atherosclerotic plaque neovascularization [12].

Aging is one of the important risk factors for vascular diseases. However, the effects of vascular aging on organ function deterioration have not yet been sufficiently investigated. A recent study has highlighted the deficiency of VEGF signaling due to increased production of decoy receptors in old mice. This shows that it is the driving force of physiological aging. It has been reported that increasing the circulating level of VEGF, which is an important factor in both the digestive system and the cardiovascular system, prevents age-related capillary loss and prolongs lifespan [13].

In the present study, we aimed to evaluate the expression intensity of VEGF, which closely concerns both the digestive system and the cardiovascular system, in the duodenum of aged rats receiving young plasma therapy. We also investigated how the treatment affected cell proliferation and therefore tissue regeneration. In addition, with this study, we aimed to explain the therapeutic effects of young plasma on the aging digestive system through VEGF expression levels. In addition, this study is a preliminary study for the subsequent investigation and comparison of the multisystemic effects of microbiota in cardiovascular tissues.

MATERIAL AND METHODS

Animal Studies

A double-blind study was designed using Sprague Dawley rats as a model organism. In the study, aged male Sprague Dawley rats (24 months, control group; n=15) were treated with pooled plasma (0.5 ml/day, intravenously for 30 days) collected from young (5 weeks, n=51) rats. Aged rats that received young plasma treatment were labeled as the experimental group (24 months, n=15), while aged rats that did not receive young plasma treatment formed the control group (24 months, n=15). The transferred blood plasma was determined according to 1/10 of the animal's blood plasma amount [14]. Rats in each group were kept in separate transparent Plexiglas cages (5 rats per cage) with free access to food and water under a 12-h light/dark cycle at a constant 21°C temperature. No animals were lost during the experiment. All animals were sacrificed under ether anesthesia. Their duodenum was collected and placed in fixation solution for histological study. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health with the approval of the Ethics Committee of the Saki Yenilli Experimental Animal Production and Application Laboratory (Approval number: 2021/03, Date: 05.03.2021).

Plasma Collection

Pooled rat plasma was collected by terminal cardiac puncture during euthanasia. Plasma was prepared from blood collected with EDTA, followed by centrifugation at 1000 g. For plasma denaturation, plasma was heated for 2–3 min at 95°C, followed by a short spin at 1000 g. All plasma aliquots were stored at −80°C until use. Before administration, plasma was dialyzed using 3.5-kDa D-tube dialyzers (EMD Millipore) in PBS to remove EDTA [14].

Histological Analyzes of the Duodenum

The duodenums of sacrificed animals were taken and placed in 10% buffered neutral formalin solution, fixed for 24 hours. The duodenums that were examined manually the next day and found to be fixed were placed in tap water to remove the fixative. They were then passed through increasing alcohol series and blocked immediately after paraffin impregnation. 5 µm thick sections were cut from the paraffin blocks [15].

Immunohistochemical Analysis

Sections were stained with the indirect streptavidin-biotin-peroxidase complex method and evaluated under a light microscope. After the sections were deparaffinized, they were passed through an alcohol series. After the antigen retrieval step (pH 6- 750 W microwave (Arçelik MD 524)), hydrogen peroxide solution was used to block endogenous peroxidase activity and secondary blocking serum was used to prevent nonspecific protein binding. Primary antibodies were applied and incubated overnight at +4°C (VEGF Polyclonal Antibody # PA5-85171 dilution:1:100, PCNA: sc-7907; Santa Cruz, dilution:1/200) The tissues were incubated with secondary antibody (ImmPRESS

reagent Vector Laboratories: #MP7401) for 30 minutes the next day. After imaging with 3,3'-diaminobenzidine (DAB-Zymed Laboratories, USA) chromogen, they were then counterstained with Harris Hematoxylin and covered with entellan [16]. PCNA (in the crypt) and VEGF (in the lamina propria) expressions were evaluated by two independent observers. In the evaluation made according to the scoring system, 0 means no immunoreactivity; 1 means weak immunoreactivity; 2 means moderate immunoreactivity; 3 means strong immunoreactivity [17]. Proliferative index (PI) was obtained by calculating the ratio of the number of PCNA positive crypt cells to the total number of crypt cells. It was defined as the average of the proliferating cell numbers in 15 randomly selected crypts from the sections [18].

Statistical Analysis

IBM SPSS v29 program was used for the analyses of the study. All data were shown with means±SD: Std. Deviation. Statistical significance between the groups was analyzed by one-way ANOVA test followed by Dunn's post hoc test. In the analyses, $p<0.05$ was considered statistically significant. The results are presented as means±SD (Std. Deviation) [19].

RESULTS

At the end of the experiment, PCNA proliferation intensity and proliferation index (PI), VEGF expression intensity were evaluated in the duodenum of the control and experimental groups.

Immunohistochemical Localization

PCNA expression was observed particularly in crypt glands which are associated with cell proliferation. PCNA expression intensity was determined as moderate in the experimental group (2.35±0.19), while PCNA expression intensity was determined as weak in the control group (1.20±0.08) (Figure 1,2) (Table 1). Therefore, cell proliferation in the duodenum of aged rats treated with young plasma increased statistically ($p<0.001$).

In the PI index calculations determined in the crypt glands of the jejunum, the proliferation index in the experimental group was found to be statistically higher compared to the control group (control: 62.20±0.12- experimental: 117.10±0.19) ($p<0.001$), (Figure 1,2) (Table 1).

VEGF expression was observed particularly around capillaries in the blood vessel-rich lamina propria of the duodenal mucosa.

While VEGF expression showed a medium-low immunoreaction in the control group (1.70±0.15) (Figure 2,3) (Table 1), we determined a moderate immunoreaction in the experimental group that received young plasma therapy (2.45±0.18) (Figure 2,3) (Table 1). Statistical significance was determined between both groups ($p<0.001$).

Table 1. VEGF, PCNA expression intensity and Proliferation Index in control and experimental groups

Groups	N	PCNA expression intensity	VEGF expression intensity	Proliferation Index (PI)
Control	15	1.20±0.08[a]	1.70±0.15[a]	62.20±0.12[a]
Experimental	15	2.35±0.19[b]	2.45±0.18[b]	117.10±0.19[b]
p value		<0.001	<0.001	<0.001

PCNA: proliferating cell nuclear antigen VEGF: vascular endothelial growth factor; different letters in the same column show statistical significance (a,b)

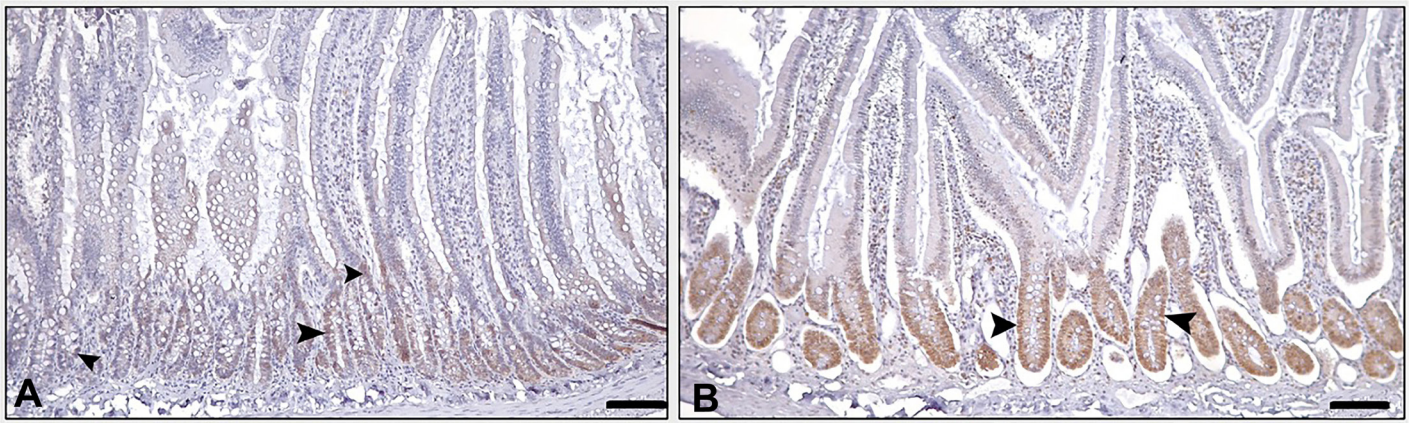

Figure 1. PCNA expression intensity in the duodenum of control and experimental groups; **A.** control group, **B.** experimental group; arrowhead: positive PCNA immunoreaction. Immunohistochemistry staining. Magnification;10x. Bar-100µm

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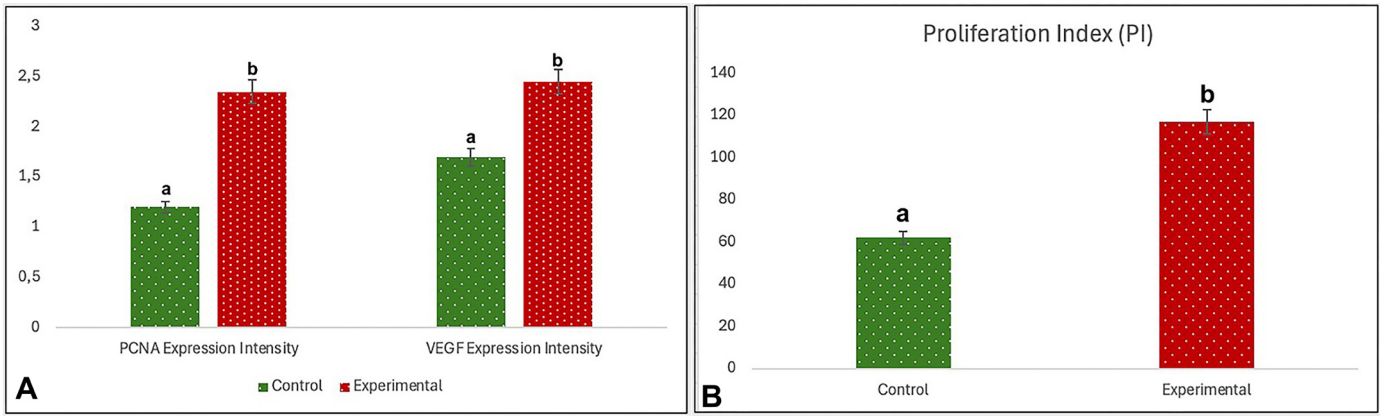

Figure 2. A. PCNA and VEGF expression in control and experimental groups; **B.** Proliferation Index in control and experimental groups; Different letters in the same column show statistical significance (a,b)

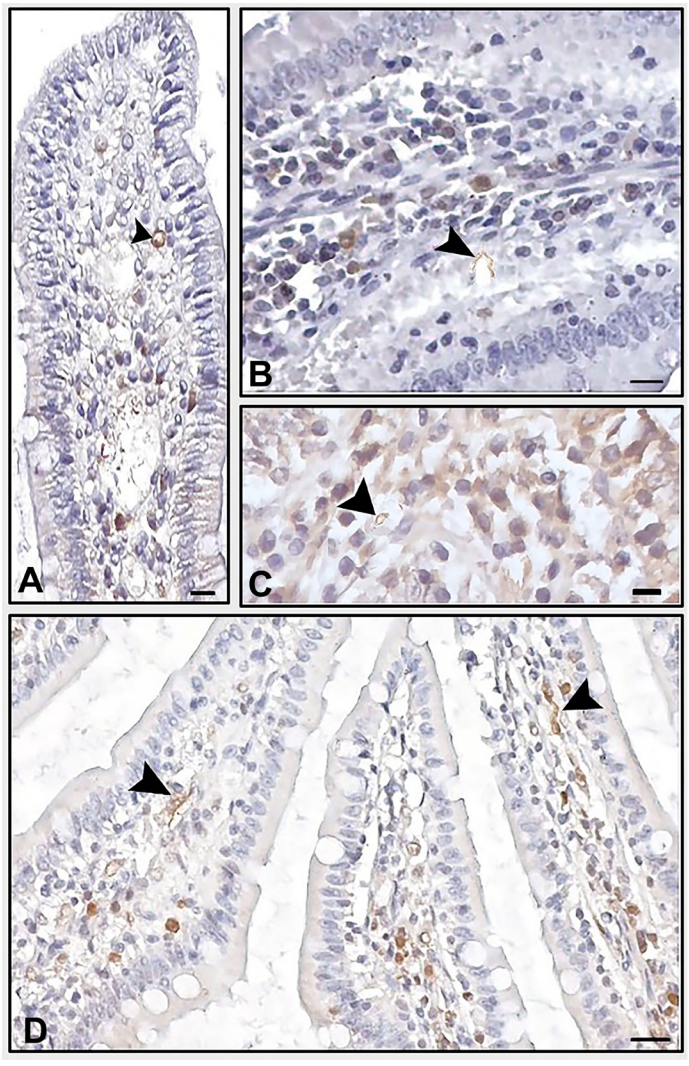

Figure 3. VEGF expression intensity in the duodenum of control and experimental groups; **A-B.** control group, **C-D.** experimental group. arrowhead: positive VEGF immunoreaction. Immunohistochemistry staining. Magnification; A-D: 20x, B-C: 40x. Bar-50µm

DISCUSSION

Plasma therapy for the digestive system is becoming increasingly attractive due to its inherent capacity to improve tissue regeneration, especially in tissue regions with high cellular turnover and more prone to injury, such as the gastrointestinal tract (GIT), with its vascular-rich structure. In our study, we aimed to investigate VEGF expressions and cell proliferation levels associated with both the cardiovascular and digestive systems by applying young plasma to old rats. In the study, we determined that cell proliferation was induced by young plasma treatment on aged duodenal tissue. Additionally, this approach supported decreased VEGF expression in the duodenum of aged rats. Although VEGF is known to play a role in vascular development and wound healing in adulthood, its expression is known to increase in pathological pathways such as tumor angiogenesis and atherosclerotic plaque neovascularization [12]. Although VEGF increase under normal conditions is seen as a precursor of negative scenarios in tumor pathologies [20], studies presented in recent years provide data that the increase in VEGF levels prevents age-related capillary loss [13]. In our study, the expression of VEGF in aged tissues increased after the treatment applied, similar to Grunewald et al. [13]. We think that this is related to the effective young plasma treatment in organ regeneration. Supporting this data, Loffredo et al. [21] showed that young plasma obtained from old mice, unlike our pooled plasma method, can slow down cardiovascular aging and improve endothelial function. This also emphasizes the potential for protecting cardiovascular health. These findings direct us to conduct studies on cardiovascular organs using our same methodology in our future studies. A study presented that increased angiogenesis (associated with VEGF) and tissue oxygenation in the intestines in the digestive system are indicators of angiogenesis and tissue oxygenation, improving mucosal growth, and nutrient absorption [14]. Also the increase in VEGF, which plays a role in the induction of angiocrine growth factors, molecules found in the endothelial cells of blood

vessels that can stimulate organ-specific repair activities in damaged or diseased organs [10], emphasizes the positive aspect of the treatment method we use. Studies have shown that VEGF expression and signalling decrease with age in old mice [13], and that there is a continuous decrease in circulating androgen levels in men with advancing age, which causes a deterioration in vascular repair mechanisms [22]. Another outcome of our study is that this increase in VEGF expression may be reversible with plasma treatment.

Evidence that young plasma application supports organ regeneration and improves cellular degeneration is remarkable. Taner et al. investigated the effect of young plasma on the liver and showed that this treatment improves hepatic fibrosis, cellular degeneration and reduces microvesicular steatosis [23]. In our study, we also determined that the same methodology increased cell proliferation and Proliferation Index in the crypt glands in the duodenum. In addition, Asmaz et al. [24] applied middle-aged plasma therapy to aged rats instead of young plasma therapy and detected a high rate of cell proliferation in the ileum, just like the results of young plasma application in our study. Another study suggests that aging-related tissue regeneration can be positively regulated by systemic factors found in young plasma [25]. However, since our study did not investigate systemic factors in the blood, we do not have sufficient information on which plasma factor young plasma therapy is effective. However, as reported by Tripathi et al. [26], the knowledge that plasma therapy not only induces organ regeneration but also is effective in improving antioxidant enzyme activities and provides potential anti-ageing benefits supports the result that the methodology we applied in our study increases both VEGF and cell proliferation.

CONCLUSION

As a result, we determined that the young plasma therapy applied induced cell proliferation, indicating that digestion was supported and facilitated. We also presented a therapeutic approach that can prevent the ageing-related decline of VEGF, which is known to be important in many systems and is especially emphasized in the cardiovascular system and digestive systems. Our study highlights the importance of plasma therapy in preventing age-related decreases in VEGF and cellular changes. We also believe that our study sheds light on the investigation of this methodology in many comparative systems, especially those related to cardiovascular organs.

Limitations

In the next stage of our study, the same methodology will be used to compare with GIS, and the same parameters will be examined in the study on cardiovascular tissues, and Sample Size will be developed, and the sham group (young rats will be included as the study itself) will be included, and proliferation data will be provided by following the increase in VEGF expression for different ages.

Ethics Committee Approval: The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health with the approval of the Ethics Committee of the Saki Yenilli Experimental Animal Production and Application Laboratory (Approval number: 2021/03, Date: 05.03.2021).

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