

Improvement of Kidney Apelin and Apelin Receptor in Nitro-L-Arginine-Methyl Ester Induced Rats

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Abstract

Background: We have investigated the effect of 8 weeks aerobic training (AT) and *Ferula gummosis* supplement (FG) on apelin and apelin receptor (APJ), nitric oxide (NO) and angiotensin converting enzyme (ACE) of hypertensive rats.

Materials and Methods: In a experimental study, 50 adult male wistar rats were classified into five groups; 1- AT, 2- FG, 3- combination of aerobic training + *Ferula Gummosa* supplement (TFG), 4- nitro-L-arginine-methyl ester (L-NAME), 5- shame (control) groups (SH). The rats in the 1 to 4 groups received L-NAME (10 mg/kg, 6 times a week for 8 weeks). Also, the 1 and 3 groups experienced the training of 15 to 22 m/min for 25 to 64 minutes, 5 times a week for 8 weeks, whereas, the 2 and 3 groups received *Ferula gummosis* supplement (90 mg/kg, 6 times a week for 8 weeks). However, rats in 5 groups received NaCl solution.

Results: At protocols resulted in a significant increase in apelin and APJ as compared to control and L-NAME groups. The TFG protocols resulted in a markedly increase in apelin, APJ and significantly decrease of ACE levels as compared to L-NAME group. Chronically administration of L-NAME resulted increased, ACE, and reduced the levels of apelin, APJ and NO, as compared to control group.

Conclusion: The results in this study show that physical regular activity with and without herbal treatment induce amplification in apelin/APJ system and down-regulation blood pressure in L-NAME induced hypertension in the rat kidney tissue.

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Introduction

Chronic kidney disease (CKD) constitutes a highly prevalent health problem worldwide. CKD patients are recognized to be at high risk for cardiovascular morbidity or mortality and end-stage renal disease (ESRD) [1]. Hypertension is present in more than 80% of patients with CKD [2]. Hypertension and its complications remain a major public health problem today. Researchers reported hypertension is an independent risk factor for cardiovascular disease. As either the cause or the consequence of CKD, hypertension contributes to progression of kidney disease towards ESRD as well as to cardiovascular events [2].

The kidney plays a major role in the regulation of arterial pressure [3]. On the other hand, apelin and APJ are recently identified multifunction peptides that regulates that have important physiological effects in several homeostatic systems, including blood pressure regulation [4]. It is well known that apelin is a strong vasodilator in various tissues and the injection of apelin significantly reduces arterial blood pressure in Wistar rats and spontaneously hypertensive rats (SHRs) [5]. Researchers reported the effect of apelin on water diuresis and regulation of blood pressure could involve not only a central effect, but also a peripheral and/or intrarenal hemodynamic effect via binding to intracranial receptors,

since apelin receptor mRNA expression was found in the rat kidney [4] and apelin immune reactivity was detected in the human collecting tubules [4]. Studies shows the injection of apelin in to the blood stream decreases arterial blood pressure [6] and this effect occurs via a mechanism dependent on nitric oxide synthase (NOS) production [6]. Moreover, APJ knockout mice display an enhanced vasoconstrictor response to systemic angiotensin II (Ang II), suggesting a counter-regulatory action of apelin on Ang II [7]. Also, apelin modulates the abnormal aortic vascular tone in response to Ang II, via endothelial NO phosphorylation pathway in diabetic mice, providing further support for a role for apelin in vascular function [8]. This led us to hypothesize that apelin and APJ could modulate actions of Ang in the renal microvasculature. Indeed, it is well established in glomerular arterioles that the vasoconstriction induced by Ang can be rapidly reversed by vasorelaxing factors such as NO that among the most effective vasodilators in the kidney [8].

The management of hypertension appears to be one of the major therapeutic goals. For example, prevention of hypertension becomes an important goal in overall efforts to control blood pressure and reduce the incidence of hypertension related cardiovascular [9]. The World Health Organization recommended the use of non-

pharmacological approaches such as; physical activity and antioxidant nutrition in the primary and adjunctive treatment for hypertension. Free radicals and reactive oxygen species are well known inducers of cellular and tissue pathogenesis and leading to several diseases such as chronic kidney disease and inflammatory disorders [10]. Antioxidants provide protection to living organisms from damage caused by the uncontrolled production of ROS [11]. Consequently, the need to identify alternative natural and safe sources of food antioxidants and the search for natural antioxidants, especially of plant origin, has notably increased in recent years [11]. *Ferula gummosa* (apiaceae) is a perennial plant native to central Asia [12]. In recent years there are some reports regarding the main effects of this plant antibacterial activity, antioxidant and anti-inflammatory activity [12, 13]. On the other hand, exercise is recognized as a useful non-pharmacological intervention to reduce blood pressure in hypertension. Wallace reported exercise to be the most promising non-pharmacological treatment of hypertension [14]. Positive cardiovascular effects of exercise are associated with beneficial changes in antioxidant systems, blood pressure, angiogenesis and inflammation. Myriad factors have been implicated, whereby exercise induces protective cardiovascular actions, including decreased sympathetic activity, reduced angiotensin levels, increased nitric oxide (NO) bioavailability, increased antioxidant capacity and expression of cardio protective factors, such as apelin [15]. However, the changes of apelin and APJ and biomarkers related to vascular dysfunction following physical activity and antioxidant turmeric supplement are poorly understood, particularly during chronic exposure to L-NAME induced hypertension.

The purpose of the current study was therefore to determine the effects of aerobic exercise, *Ferula gummosa* supplement or both on kidney apelin and APJ in rats that have been chronically exposed to L-NAME-induced hypertension. In addition, given the relationship between hypertension and vascular dysfunction, levels of the angiotensin converting enzyme (ACE) and nitric oxide (NO) were assessed.

Materials and Methods

Animals and experimental environment: The experiments were carried out with 50 male Wistar rats, (8-week-old, initially weighing 240±20 g), which were obtained from the Pasteur Institute of Iran. The present research was performed in department of exercise physiology, university of Mazandaran. Rats was housed in standard cages of polycarbonate 20×15×15 cm (length×width×height), in an air-conditioned room with a controlled temperature of 22±2°C, light-dark cycles of 12:12 h and humidity of 50±5%. The pollutant standard index (PSI) was in the acceptable range as determined by the Iranian meteorological organization. Rats were fed with a standard rat chow provided by Pars Institute for animals and poultry with a daily regimen of 10/100 g body weight for each rat. Water was available ad libitum.

All experiments were performed in accordance with the guidelines outlined by the Experimental Animal Laboratory and approved by Department of Physiology, University of Mazandaran and were performed according to guiding procedures in the care and use of animals, prepared by the Council of the American Physiological Society.

Study design and treatment of animals: The familiarization protocol was designed for 5 days, once a day for 10 min/session at a speed of 10 m/min at a slope of 0 degree. An electric stimulus (30 V, 0.5 A) was manually turned on for less than 2 s when the animals stayed on the electric grid for longer than 10 s [16]. Following this familiarization period, they were randomly assigned into five experimental groups of 8 rats each. The groups were defined as follows:

Group 1: the animals were exposed to N-nitro-L-arginine methyl ester (L-NAME) at a concentration of 10 mg/kg in the form of a solution, intra peritoneal, 6 days weekly for 8 weeks, to induce the hypertension [17];

Group 2: *Ferula gummosa* similarly received L-NAME, as well as *Ferula gummosa* be fed through gavage with 90 mg/kg dosage, 6 days weekly for 8 weeks [18];

Group 3: aerobic exercise, the rats in this group similarly received L-NAME, and in addition they performed progressive running exercise of 15 to 22 m/min for 25 to 64 min, 5 times a week; the running speed and duration of exercise were progressively increased during a graded treadmill exercise protocol [19].

Group 4: aerobic exercise and *Ferula gummosa*; the rats in this group performed an aerobic training protocol similar to that in group 3, and in addition received L-NAME and *Ferula gummosa* supplement;

Group 5: the sham (control) group; these rats received NaCl solution that is injected with 0.1 mg/kg dosage, intra peritoneal, in the same manner and for the same duration of time as other groups.

***Ferula gummosa* supplementation:** The present study, we have replicated a previously extract preparations, described by Mandegary et al. [13]. In summary, seeds and root of *Ferula gummosa* were dried at the lab temperature for a week. Aqueous and metabolic extracts were obtained by decoction. For the preparation of acetone extract, the seeds and root were macerated in acetone (2 L), in portions of 200 and 300 g, respectively, for 3 days. After filtration of mixtures, filtrates were concentrated by a rotary evaporator apparatus. The residues were then dried at room temperature. The final weight of crude acetone extracts was approximately 13.5 g which maintained at 4°C throughout the experiments.

Blood sampling, kidney biopsy and biochemical analysis: Rats in all groups were anesthetized with ketamine (100 mg/kg) and xylozine (10 mg/kg) and decapitated after 10 to 12 h overnight fasting. Blood samples were collected 24 h after the last dose of treatment. These blood samples were initially centrifuged by a refrigerated centrifuge at 3000 rpm for 15 min within 30 min of collection and then stored at -80°C for subsequent assay of ACE and NO. The thoracic cavity was then opened and the kidney was quickly excised.

Kidney tissues were weighed and was placed into Petri dishes containing cold isolation medium (0.1 M/L K₂HPO₄, 0.15 M/L NaCl, pH=7.4) to remove the blood and were frozen immediately in liquid nitrogen and stored at -80°C for subsequent analysis of apelin and APJ. Then, kidney tissue was squashed in liquid nitrogen, homogenized in a lysis buffer (5 mL/g of tissue) with a protease inhibitor cocktail for mammalian cell and tissue extracts (Sigmae Aldrich, St. Louis, U.S.A) 100 UL/1 mL, and 10 m Mtris base (Sigma-Aldrich, St. Louis, U.S.A), pH=7.4 and centrifuged at 1600 g at 4°C for 15 min. Kidney tissue supernatant was diluted 1:30. Plasma was diluted 1:10 and the fluids were used in an Apelin-13 ELISA kits (Phoenix peptides, Burlingame, California, U.S.A), following the manufacturer's instructions (Apelin-13 in rats abundant more affinity to the angiotensin receptor sub-type known as apelin receptor (APJ) and show greater biological activity) [20, 21]. The assay kit was very specific and detects apelin-13 with 100% cross reactivity. It has an inter-assay variation less than 14% and intra assay coefficient of variation less than 10%. Apelin-13 and APJ in the mentioned sample were measured using ELISA kits, too (rat apelin, ELISA, USCN Life Science Inc., Wuhan, P.R. China and APJ ELISA kit (Rat), USCN, Life Science Inc, sensitivity 0.128 ng/mL and 0.05 ng/mL respectively. Intra CV: 5% and Inter CV: 14%). The serum NO concentration was determined by first reducing the nitrate to nitrite using nitrate reductase (Sigma). Plasma levels of ACE were measured using a sandwich enzyme-linked immune sorbent assay [22].

Statistical analysis: All data have been expressed as mean±standard deviation (SD). Statistical analysis was performed using a commercial software package (SPSS-16.0), one-way ANOVA and Welch test for repeated measurements with post hoc testing by Scheffe (statistical software, Stat Soft, Inc., Tulsa, OK,) was performed to identify differences between groups. Differences were considered statistically significant at *p*-value<0.05.

Results

Table 1. Effect of aerobic training and *Ferula gummosa* supplement on apelin and APJ levels in rats during chronic exposure to L-NAME

| Groups and markers | Sham Mean±SD | Ferula gummosa Mean±SD | Training Mean±SD | Training +Ferula gummosa Mean±SD | L-NAME Mean±SD |
|-----------------------|-----------------|---------------------------|---------------------|-------------------------------------|-------------------|
| Kidney apelin (pg/mg) | 4.2675±1.52278 | 3.0412±1.10849 | 6.5425±1.74391 | 4.3000±1.27252 | 2.3200±0.93743 |
| Kidney APJ (pg/mg) | 1.0712±0.28256 | 0.8238±0.23525 | 1.8912±0.12867 | 1.2750±0.25973 | 0.6013±0.12053 |

Table 2. Effect of aerobic training and *Ferula gummosa* supplement on endothelial dysfunction levels in rats during chronic exposure to L-NAME

| Groups and markers | Sham Mean±SD | Ferula gummosa Mean±SD | Training Mean±SD | Training +Ferula gummosa Mean±SD | L-NAME Mean±SD |
|--------------------|-----------------|---------------------------|---------------------|-------------------------------------|-------------------|
| ACE (pg/mL)* | 178±19.22 | 197.25±32.25 | 204.83±34.27 | 134.43±22.67 | 247.25±36.14 |
| NO (μmol/L)* | 32.1125±2.650 | 25.7125±3.152 | 46.1500±28/806 | 45.1875±29.426 | 17.5286±7.729 |

*Angiotensin converting enzyme (ACE), nitric oxide (NO). Data are presented as the means for 8 rats

Table 1 shows changes in kidney apelin and APJ levels, in the rats exposed to L-NAME and rats in control (shame) group. Administration of L-NAME (10 mg/kg) caused a markedly decrease in apelin and significant decrease APJ by 46% and 44% (*p*<0.05) (Fig. 1), respectively, than in shame group. In contrast, aerobic training protocols resulted in a significant increase in apelin and APJ by 53%, and 79% (*p*<0.05) (Fig. 1), respectively, as compared to control and L-NAME groups. The aerobic training+ *Ferula gummosa* protocol resulted in a markedly increased in apelin by 85% (*p*<0.05) and significantly increase APJ, by 111% (*p*<0.05), as compared to L-NAME group (Fig. 1). Also, significant differences were detected in the apelin level between rats in the aerobic training group, as compared to *Ferula gummosa* group (-3.50±0.67, *p*<0.05) (Fig. 1). Moreover, insignificant increase were detected in the apelin and APJ levels between rats in the aerobic training+ *Ferula gummosa* group, as compared the only in *Ferula gummosa* group (1.25±0.67, *p*<0.05) (Fig. 1).

Data in table 2 shows changes in biomarkers related to endothelial dysfunction consisting of; ACE and NO in the rats exposed to L-NAME. Intra-peritoneal chronically administration L-NAME resulted in a significant increase, by 38% in ACE levels and a markedly decrease, by 47% in NO levels, as compared to control group (*p*<0.05) (Fig. 2). In contrast, significantly decrease of ACE levels after 8-week of the aerobic training + *Ferula gummosa* and *Ferula gummosa* protocols, by 46% and 21% (*p*<0.05) (Fig. 2), respectively than in L-NAME group were observed. On the other hand, the privacy aerobic training protocol and or the concomitant aerobic training + *Ferula gummosa* significantly increased the NO levels, by 70% and 64%, (*p*<0.05) (Fig. 2), respectively, as compared to L-NAME group. However, no significant differences were observed in NO and ACE levels between rats in the aerobic training and *Ferula gummosa* groups (*p*<0.05) (Fig. 2). However, a significant decrease was observed in ACE level between rats in the aerobic training + *Ferula gummosa* and *Ferula gummosa* groups (62.82±14.71, *p*<0.05) (Fig. 2).

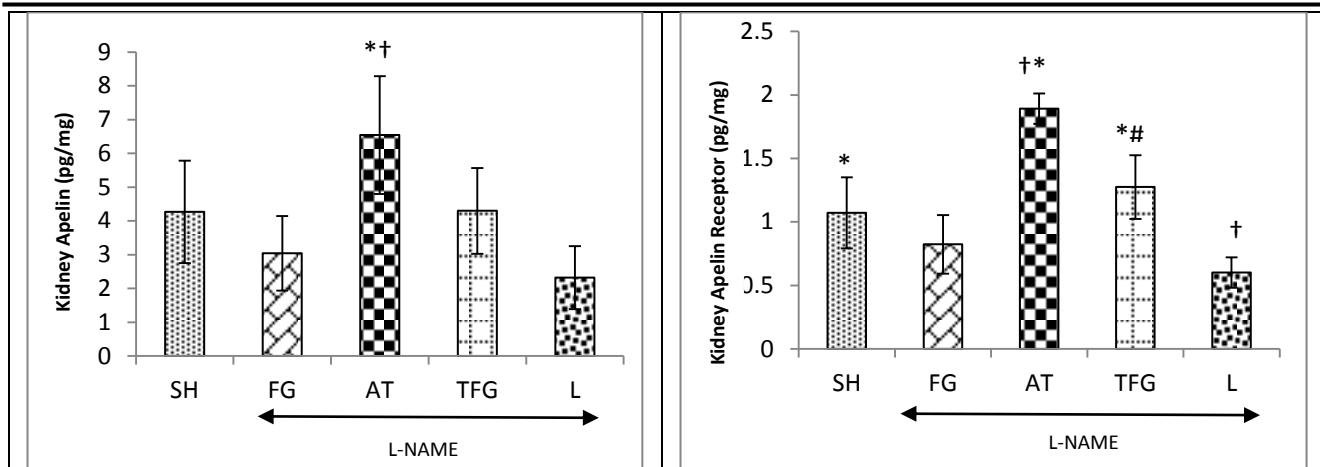


Figure 1. Apelin and APJ levels during chronic exposure to Nitro-L-arginine-methyl ester (L-NAME) and aerobic training /or *Ferula gummosa* supplement. Abbreviation: L (L-NAME), FG (Ferula gummosa), AT (Aerobic Training), TFG (Training+ Ferula gummosa), SH (Sham). Data are presented as the means for 8 Rats; †significantly different from sham group ($p<0.001$), *significantly different from L-NAME group ($p<0.001$), #significantly different between combination (training + Ferula gummosa) with Ferula gummosa groups ($p<0.05$).

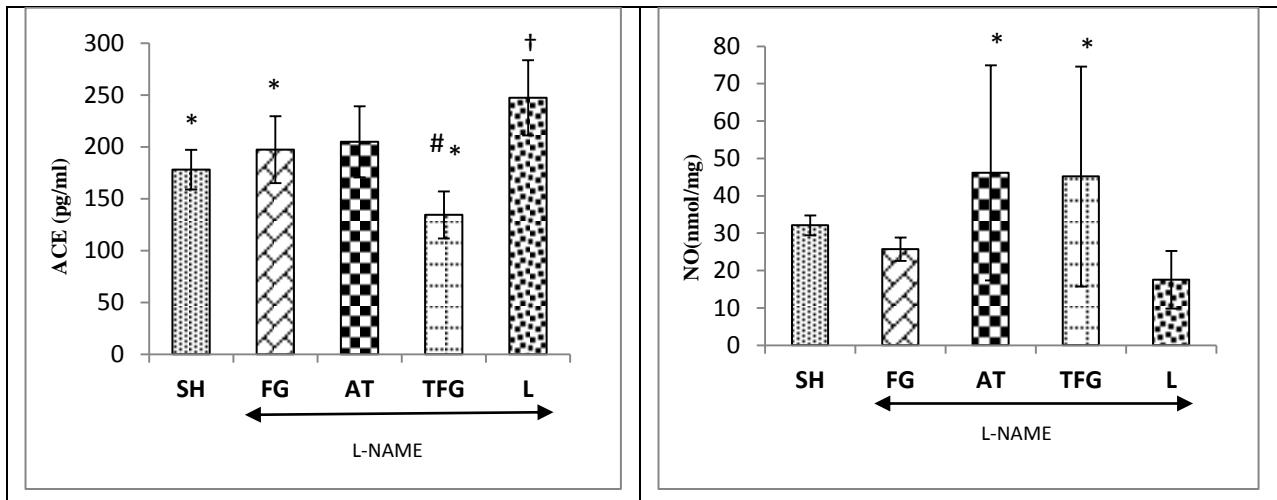


Figure 2. Markers of endothelial dysfunction during chronic exposure to Nitro-L-arginine-methyl ester (L-NAME) and aerobic training /or *Ferula gummosa* supplement. Abbreviation; angiotensin converting enzyme (ACE) and nitric oxide (NO). L (L-NAME), FG (Ferula gummosa), AT (Aerobic Training), TFG (Training+ Ferula gummosa), SH (Sham). Data are presented as the means for 8 Rats; † significantly different from sham group ($p<0.001$), *significantly different from L-NAME group ($p<0.001$), #significantly different between combination (training + Ferula gummosa) and training with Ferula gummosa groups ($p<0.05$).

Discussion

Important finding of this study was to chronic administration of L-NAME caused down-regulation of the markers such as; apelin and APJ by $(2.32 \pm 0.93$ vs. 0.60 ± 0.12), respectively (Table 1). In contrast, we demonstrated that apelin and APJ levels are elevated in rats after treatment with *Ferula gummosa*: 3.04 ± 1.10 vs. 0.82 ± 0.23 , respectively, and treadmill running: 6.54 ± 1.74 vs. 1.89 ± 0.12 , respectively, by reversing vascular dysfunction related biomarkers (Table 1). Also chronic administration of L-NAME induces increased levels ACE (247.25 ± 36.14) of (Fig. 2). Furthermore, L-NAME administration induced imbalance in vasodilator factors, as indicated by decreases in the levels of NO.

Although, the exact mechanisms of how this molecular pathway are still to be fully elucidated, there is growing evidence that apelin may be involved in the hypotensive.

The use of apelin as a diagnostic marker in human heart failure and renal seems unlikely on the basis of current evidence. The relative importance of the central and peripheral actions of apelin-APJ axis on normal blood pressure physiology and renal disease is also undetermined [23]. Animal and human studies suggest that apelin a natural ligand to APJ is a hypotensive peptide, both *in vivo* and *in vitro*, and that it acts through accepted hypotensive mechanisms and may play a role in the pathogenesis renal failure and hypertension [23].

Regular exercise favorably changes established cardiovascular risk factors such as hyperlipidemia, hypertension [24]. Zhang et al. revealed that long-term swim training reduced pathogenesis related to hypertension and reversed the down-regulation of the cardiovascular apelin and APJ induced by hypertension [24]. Studies have shown that apelin-13 injected intravenously into anesthetized rats significantly

decreased mean arterial blood pressure [25]. Another study found that apelin-12, apelin-13, and apelin-36 decreased mean arterial pressure by 26, 11, and 5 mmHg, respectively, when administered to anesthetized rats [25]. One set of experiments with apelin administered to conscious unrestrained rats showed that apelin could function as both an arterial and venous dilator *in vivo* [23–25]. This suggests that the effects of exercise training on hypertension could be mediated by up-regulating apelin and APJ [24]. In addition to preventing hypertension, training reduces oxidative markers associated with endothelial dysfunction in patients with hypertension [25]. Frederico et al. reported that 12 weeks of treadmill training increased antioxidant enzymes and decreased oxidative damage and injury in the myocardium [26]. In current study, we indicate that 8 weeks of aerobic training led to increase in NO, as compared to sham and L-NAME groups (Fig. 2).

In addition, ACE, an enzyme that is part of the renin–angiotensin system (RAS) also affects the degradation of apelin, suggesting some cross talk between apelin and the RAS. Additional support for apelin role in blood pressure regulation comes from studies in which systemic administration apelin-12 or apelin-13 was found to decrease mean arterial pressure (MAP) in anesthetized rats [27]. Interestingly, in the present study we found that level of ACE more increase and significantly in L-NAME groups, as compared to control group: 247.25 ± 36.14 vs. 178 ± 19.22 , respectively; (Table 2). Also significantly decrease of ACE levels after 8-week of the aerobic training+ferulagummosa and *Ferula gummosa* protocols, as compared to L-NAME group were also observed (Fig. 2). Our results also corroborate these findings.

The specific mechanisms by which physical activity ameliorates hypertension have not been well elucidated. Physical activity has been associated with favorable modifications of blood pressure through a reduction in sympathetic activity, improved ACE, regulated energy metabolism and increase anti-oxidant. Furthermore, exercise affects the expression and activities of vasoactive substances. It is well known that appropriate exercise inhibits the pathological overexpression of angiotensin II and endothelin [27], while simultaneously reinforcing the activities of endogenous kidney tissue defensive systems such as adrenomedullin and NO/NOS [28], to maintain and reinstate kidney tissue homeostasis. Our results also corroborate these findings.

Recent investigations have led to the discovery of some new biological activities of the plant. Together with the activities of the plant, a few activities have also been reported from essential of *Ferula* species. These include anti-microbial, anti-inflammatory, anti-convulsant, anti-oxidant, and hypotensive activities [8].

In this study, we observed that exposure to *Ferula gummosa* and training alone or together caused an increase in apelin and APJ levels (Fig. 1). Studies demonstrate that apelin can exert many kinds of physiological effects through paracrine and autocrinomodes, e.g. activate phospholipase C (PLC) via APJ receptor, increase intracellular Ca^{2+} level by way of

PLC inositol triphosphate (IP3), activate Ca^{2+}/Ca dependent nNOS, induce NO production, and exert powerful physiological effects by NO-cGMP pathway, and these effects can be greatly inhibited by NOS inhibitor [4, 17]. Studies by several groups have shown that the hemodynamic effect of apelin is abrogated in the presence of a nitric oxide (NO) synthase inhibitor, suggesting that apelin may lower blood pressure via a nitric oxide-dependent mechanism. In rodent models, exogenous apelin administration causes a rapid NO-dependent fall in blood pressure and mean capillary filling pressure, indicating powerful vasodilator effects [28].

Our results also corroborate these findings. We observed that exposure to L-NAME caused a decrease in NO level, Whereas, *Ferula gummosa* and treadmill running alone or together caused increase in NO level, as compared to the sham group (Table 2). The hypotensive effect of apelin is mediated by endothelium-derived NO, since the NO synthase inhibitor L-NAME abolished this effect both in rats [25]. In cultured mice endothelial cells, apelin stimulates the phosphorylation of endothelial NO synthase (eNOS) at Ser1176 by protein kinase B/Akt. Our data indicate a protective effect of aerobic training or *Ferula gummosa* against hypertension.

In summary, the present study demonstrated that administration of L-NAME caused down-regulation of apelin, APJ and an imbalance in endothelial function. Furthermore, *Ferula gummosa* supplementation and/or aerobic training have useful effects on reducing the L-NAME induced hypertension, probably by increase in apelin and APJ system and decrease in the endothelial dysfunction biomarkers related to hypertension. Finally, simultaneous use of *Ferula gummosa* supplementation and aerobic training is more effective than *Ferula gummosa* alone. Although augmentation of the apelinergic system in kidney tissue following *Ferula gummosa* supplementation and/or aerobic training may be safe, further research is necessary to investigate whether the observed effects are solely due to alterations in the antioxidant defenses of kidney tissue and /or other mechanisms.

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Authors' Contributions

Dr Dabidi-Roshan receives grant support from Department of Sport Physiology, University of Mazandaran. Drs Dabidi-Roshan, Gharakhanlou, are consultants for Department of Sport Physiology and Hedayati is consultants for Obesity Research Center,

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Conflict of Interest

The authors declare no conflict of interest.

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