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Plasma Collagen XVIII in Response to Intensive Aerobic Running and Aqueous Extraction of black *Crataegus elbursensis* in Male Rats

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ABSTRACT: Angiogenesis is a process that can be classified into physiological and pathophysiological forms. Collagen XVIII is a part of extracellular protein and heparan sulfate proteoglycans in vascular epithelial and endothelial basement membrane cause the release of endostatin from noncollagenous collagen XVIII. Endostatin inhibits the growth of endothelial cells, inhibits angiogenesis, weakens different types of cancer, and the growth of tumors. The purpose of the current study was to investigate the effect of intensive aerobic running with or without aqueous extraction of black *Crataegus elbursensis* on Collagen XVIII in male rats. Design: Thirty-two Wistar male rats (4-6 weeks old, 125-135 gr weight) were acquired from the Pasteur's Institute (Amol, Mazandaran), and randomly assigned into control (n = 16) and training (n = 16) groups. Rats were further divided into saline-control (SC) (n = 8), saline-training (ST) (n = 8), crataegus pentaegyna extraction -control (CPEC) (n = 8), and crataegus pentaegyna extraction - training (CPET) (n = 8). The control (SC and CPEC) groups remained sedentary; whereas the training groups underwent a high running exercise program. Plasma were excised and immediately frozen in liquid nitrogen. Statistical analysis was performed using a one way analysis of variance and Tukey test. Significance was accepted at P = 0.05. The results show that aerobic exercise group had the highest concentration collagen XVIII compared to other groups and then respectively black crataegus, training-crataegus and control groups. In general, researchers in this study concluded that the increase of collagen XVIII (albeit insignificant) as a result of physical activity and consumption of black crataegus extract could possibly serve as a regional inhibitor of angiogenesis and another evidence for the anti-cancer effects of physical activities.

Keywords: aerobic running, *Crataegus elbursensis*, Collagen XVIII

INTRODUCTION

The adaptations that occur in human body after doing exercises training are a factor to help healthy people stay away from certain diseases. One of the main adaptations is a change in blood circulation, especially in vessels. The increase of capillary density is dependent on the balance between angiogenic and angiostatic factors (O'Reilly *et al.,* 1994, Hanahan and Folkman 1996). Inappropriate angiogenesis may be accompanied by or associated with some disorders, or directly with a disease. Disorder in Angiogenesis regulators is often associated with the development of angiogenesis related to such diseases as atherosclerosis. Most studies show that the changes made to angiogenic developmental factors resulted from physical exercises indicate the low level of stimulators compared with inhibitors. It is believed that the plasma level of VEGF-A, the important angiogenic factor, is reduced after physical exercise (Folkman *et al.,* 1971, Richardson *et al.,* 2000, Asano *et al.,* 1998, Gustafsson *et al.,* 2002).

Crataegus elbursensis is the most abundant type of crataegus indigenous to Iran, which is abundantly found in the forests of northern Iran. The fruits are black or blue black, spherical, or oval shaped, and 8-10 mm long with 4-5 pyrenes. The *crataegus elbursensis* fruit is considered as a potential source of phenols and antioxidants. Crataegus is one of the oldest herbs in European medicine (Salmanian *et al.,* 2014, Weihmayr and Ernst 1996). The extract of crataegus consists of two groups of polyphenolic compounds and a mixture of monomeric flavonoids and oligomeric procyanidins (OPCs). Flavonoids are capable of inhibiting angiogenesis in vitro in micromolar concentration. The proliferation of the cells induced by bFGF and the Vasculogenesis induced by bGFF and VEGF is inhibited by flavonoids (Fürst *et al.,* 2010, Sarkar and Li, 2003). Findings indicate that the extract of crataegus plant reduces the platelet-derived growth factor receptor (PDGFR) autophosphorylation in human's fibroblast.

More importantly, crataegus (1 to 100 mg in liter) clearly leads to the inhibition of PDGFR autophosphorylation in vascular smooth muscle cells (VSMCs). Furthermore, the extract of crataegus plant severely weakens the activity (phosphorylation) of mitogen-activated protein (MAP) kinase / extracellular signal-regulated kinase (ERK), which is the effective PDGF downstream kinase and an effective factor in the proliferation and migration of VSMC (Zhan *et al.,* 2003). Migration and proliferation of VSMCs leads to the thickening of Intima, which is an important factor in pathogenesis of coronary artery restenosis.

Type XVIII collagen is a part of extracellular protein and heparan sulfate proteoglycans in vascular epithelial and endothelial basement membrane. These proteins share a 20-kilodalton part and cause the release of endostatin from noncollagenous carboxyl-terminal of collagen XVIII. Endostatin inhibits the growth of endothelial cells, inhibits angiogenesis, weakens different types of cancer, and the growth of tumors. Findings show that collagen XVIII / endostatin may regulate the interaction between endothelial cells and basement membrane. Although the release mechanisms of collagen XVIII / endostatin are yet to be known, many studies indicate that proteolytic release of endostatin from collagen XVIII is carried out by the mediation of many proteases such as cysteine proteases, matrix metalloproteinase, and aspartate protease.Most of these proteases are associated with physiological turnovers of collagen XVIII, which is increased with physical exercises (Saarela *et al.,* 1998, Sasaki *et al.,* 1998, O'Reilly *et al.,* 1997, Marneros *et al.,* 2005, Ferreras *et al.,* 2000, Thomas *et al.,* 1992, Kovanen and Suominen 1989).

The expression of pro- and anti-angiogenesis factors is regulated by cancer cells, tumor suppressor genes, and various polygraph factors. The increase of some angiogenic indices such as VEGF-A not only boosts the formation of new blood vessels, but also increases lymphatic vessels inside the tumor a trend which facilitates the development and metastases of tumor cells. Therefore, the use of angiogenic treatment in prevention of growth and metastases of tumors has recently received significant attention (Kivela *et al.,* 2008). It has been observed that the amount of collagen XVIII is increased in certain pathological conditions such as brain damage, atopic disorder and scleroderma, and it is proved to be correlated with hepatocellular cancer progress (Mueller *et al.,* 2007, Deininger *et al.,* 2002, Castro-Giner *et al.,* 2009,Passos-Bueno *et al.,* 2006, Musso *et al.,* 2001). Most reports on physical exercises have been focused on certain angiogenesis factors in muscles. There are few studies conducted on the effect of physical exercise on anti-angiogenesis factors in the heart muscle. Since ancient times, herbs have been used to prevent or treat many diseases. An overview of studies in this field indicates that there is little information on anti-angiogenic factors and their changes following physical exercises. The effect of regular aerobic physical exercise on the improvement of the structure and performance of heart and other organs is well documented in the literature; however, identifying their effects on angiogenesis inhibitors can be useful in the treatment of cancer, tumors, and angiogenesis-related diseases and in prevention of atherosclerosis. The useful effects of red crataegus plant on heart, blood pressure, immune system, and the like are well indicated in these studies. Only one study has explained the inhibitive effect of this plant on the growth of vascular smooth cells through the inhibition of growth factors (rather than anti-angiogenesis factors). Since there is little amount of information on the effect of regular and incremental physical exercise as well as the consumption of red crataegus extract on anti-angiogenic factors, and considering the fact that their possible effect on the prevention of cardiovascular diseases, cancers, and tumor mutations is not quite clear, this study seeks to explain the effect of a selective incremental aerobic exercise program along with the consumption of aqueous extract of black crataegus on plasma collagen XVIII of male Wistar rats.

MATERIAL AND METHODS

All experiment involving animals were conducted according to the policy of Iranian convention for the protection of vertebrate animals used for experimental and other scientific purposes; the protocol was approved by the Ethics Committee of the Sciences, University of Mazandaran (UMZ) and Babol University of Medical Sciences (BUMS, Mazandaran, Iran). Thirty-two Wistar male rats (4-6 weeks old, 125-135 gr weight) were acquired from the Pasteur's Institute (Amol, Mazandaran), and maintained in the Central Animal House of the Faculty of Physical Education and Sports Science of UMZ. Five rats were housed per cage (46-L) with a 12-hour: 12-hour light-dark cycle Temperature and humidity were maintained at 22°C ± 1.4°C and 50% ± 5%, respectively. Diets (a pellet form) and water were provided ad libitum. Animals were randomly assigned into control (n = 16) and training (n = 16) groups. Rats were further divided into saline-control (SC) (n = 8), saline-training (ST) (n = 8), crataegus pentaegyna extraction -control (CPEC) (n = 8), and crataegus pentaegyna extraction - training (CPET) (n = 8). The control (SC and CPEC) groups remained sedentary; whereas the training groups underwent a high running exercise program.

Exercise training protocol: At first, the animals were familiarized with the rat treadmill apparatus, every day and for 5days. The exercise groups were trained for 8 weeks on a motor driven treadmill as previously reported elsewhere [14]. The rats were submitted to run at 34 m/min for 60 minutes, 5 d/week [19]. The animals were killed 72 hours after the last exercise session.

Food but not water was removed from the cages 3hours before the sacrifices.

Plant material and Preparation of the Crataegus extraction: The ripped fruit samples of Crataegus-Pentaegyna were collected from Neka forest in the Mazandaran province of Iran, and well washed. Fruits were dried in oven at 35° C for 4 days. The extraction was prepared according to the Cai *et al*. Briefly, the whole ripped and dried fruit Crataegus-Pentaegyna were ground to fine powder. Water extraction: 5 g of the powdered sample was extracted with 100 mL distilled water at 80°C for 20 min. After cooling the extract was filtered. Then centrifuged at 3,000 rpm for 17 min and filtered through filter paper. The freshly prepared extracts were cooled and immediately used in the experiments. In last six weeks, all treatments were given in a single daily dose orally using special gavages needles. After training, 500mg/kg /or 10 ml/kg body weight liquid extraction of Crataegus-Pentaegyna was orally assigned to the Crataegus-Pentaegyna groups and the same amount of saline was fed to saline groups.

Plasma collection: Seventy-two hours after the last training session, rats were anesthetized with intra peritoneal administration of a mixture of ketamine (30-50mg / kg body weight) and xylazine (3- 5mg / kg body weight). Blood samples are directly collected from the Inferior vena cava and after transferring them to laboratory tubes containing EDTA they were centrifuged in rpm 3000 for 15 minutes and the plasma was collected. After freezing the plasma by the use of liquid nitrogen they were kept in refrigerator at -80?C for measuring collagen 18. Measurement of plasma collagen XVIII concentrations: Collagen concentration in plasma was measured by ELISA method (PHOMO ELISA Reader, China), with Company Glory kit (USA), Sensitivity 5.85 ng.l

Data Collection: The Kolmogorov-Smirnov test was used to determine the normality of distribution, and variables were found to be normally distributed. All results are expressed as means ± SEM. Statistical analysis were performed using a one way analysis of variance test. Significance was accepted at $P < 0.05$. All statistical analysis was performed with SPSS (Version 15; SPSS).

RESULTS

The results of weight samples are shown in the table1. In table 2 the results related to plasma collagen XVIII are provided in the form of indices of mean and standard deviation in different groups. The results show that aerobic exercise group had the highest concentration collagen XVIII compared to other groups and then respectively black crataegus, training-crataegus and control groups. Variance analysis results showed that a no significant difference exists between the amounts of collagen XVIII ($F = 2.950$, $P = 0.053$) (Table 3).

Table 1: Mean and SD of weight in different research groups.

	N	Before protocol	After protocol
Control- Saline	8	130±11	274±14.55
Saline -training	8	105.5±14.5	322.5±17.67
Control-Crataegus	8	131±12	294.25±14.04
Training- Crataegus	8	107±17	316.37±22.09

Table 2: Mean and SD of Collagen XVIII in different research groups.

	Control- Saline		Saline -training		Control-Crataegus		Training- Crataegus	
	mean	SD	mean	SD	mean	SD	mean	SD
Plasma collagen 18 (ng.ml)	0.721	0.142	0.839	0.079	0.761	0.080	0.648	0.160

Tale 3: Results related to the variance analysis on the amounts collagen XVIII.

		Sum of squares	df	Mean square	F	Sig.
Heart LDH	Between groups	0.132	3	0.044	2.950	0.053
	Within groups	0.359	24	0.015		
	Total	0.491	27			

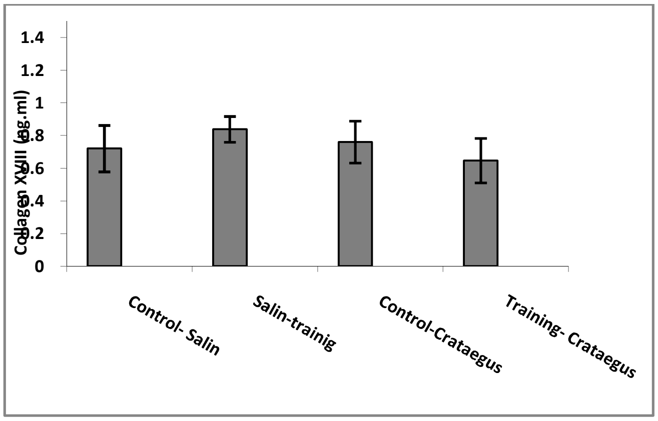

Fig. 1. Collagen XVIII in different groups.

DISCUSSIONS

Angiogenesis is a process that can be classified into physiological and pathophysiological forms. Physiological angiogenesis, which is a strongly regulated process, occurs in such cases as wound healing, placental growth, and ovulation. However, pathophysiological angiogenesis, which refers to the uncontrollable proliferation of capillary endothelium, is seen in such diseases as diabetic retinopathy, atherosclerosis, growth, and metastases of tumors (van Royen *et al.,* 2001). The inhibition of angiogenesis in these cases can leave to the improvement of the disease or its symptoms (Risau, 1997). No research has yet examined the effect of endurance exercise and the blue extract of black crataegus on the changes to plasma collagen XVIII. Generally, the present study showed that physical exercise and consumption of black crataegus extract do not cause any significant change in plasma collagen XVIII per se and in interaction with each other. Collagen XVIII is a basement membrane protein (Muragaki *et al.,* 1994, Rehn and Pihlajaniemi 1994). It is the molecule of such fragments as TSP-1 and endostatin that can control the anti-angiogenesis activity, proliferation of cells, and apoptosis, and it can be assumed to regulate the development of vascular system. It is also indicated that lack of collagen XVIII leads to the unnatural formation of basement membrane. Collagen XVIII is found in all vascular and epithelial structures of basement membrane in the entire body. It is shown that it is almost located in human's eye structure as well as in ciliary body and the iris (Quelard *et al.,* 2008, Väänänen *et al.,* 2007, Sakimoto *et al.,* 2008, Ylikärppä *et al.,* 2003, Määttä *et al.,* 2007). It is found in perisinusoidal regions and in the basement membrane. Hepatocytes and satellite cells are the most important sources of collagen XVIII in the liver (Musso *et al.,* 2001). Physical exercise seems able to increase the amount of collagen XVIII. Physical activity increases the turnovers of proteases, and proteases are one of the factors effective in the increase of collagen XVIII, which leads to the release of endostatin. Such fragments can in turn be regional inhibitors of angiogenesis. Therefore, the collagen XVIII / endostatin system may accompany the anti-angiogenic as a sensor for protolithic activities and serve as an anti-angiogenesis via negative feedback. Although there is no definitive information about different endostatin-like fragments, it is possible that the larger or smaller endostatin fragment has anti-angiogenic activity (Beck and D'Amore 1997). In this research, the increase of plasma collagen did not reach a significant level. Collagen XVIII as an effective factor in angiogenesis seems to be removed by tissues as a result of physical exercises. If such an assumption is true, the increase in the amount of collagen XVIII can possibly lead to an increase in the performance of collagen XVIII / endostatin mechanisms, and inhibit the growth of endothelial cells, inhibit angiogenesis, weaken different cancers and the growth of tumors (O'Reilly *et al.,* 1997) and can prevent the progress of primary atherosclerosis disease (Celletti *et al.,* 2001). This finding contradicts that of Thomas *et al.* (1992), which said physical activity could increase the expression of collagen XVIII in the left ventricle and LV papillary muscles. This contradiction may be concerned with the place where the collagen XVIII was studied. In the present research, collagen is studied in plasma, while heart tissue was the place of study in that of Thomas. In the work of Yuki *et al.* (2010), it was said that collagen XVIII/endostatin contribute to preserving the integrity of the extracellular matrix and capillaries in the kidney, protecting against progressive glomerulonephritis. Studies have shown that the expression of collagen XVIII is increased in many tissues in the time of growth progress. The increase of its expression was also observed in the epithelium of rats' lungs and growing kidney, in the core of endocardial connecting tissue and the components of heart valves of growing rats (Hamano *et al.,* 2010).

In the present study, the black crataegus extract led to an insignificant increase of plasma collagen XVIII.

Crataegus has various components, one of the most important ones of which are flavonoids of quercetin. Quercetin seems to have different types of biological properties like other natural polyphenols. The majority of its properties are concerned with anti-oxidant and anti-tumor activities. The anti-tumor effects of quercetin are related to its ability to inhibit the angiogenesis of tumor through the inhibition of migration and growth of endothelial cells (Verma *et al.*, 2007, Donnini *et al.,* 2006, Igura *et al.,* 2001).

In the cellular level, quercetin decreases the expression of MMP-2 and inhibits the formation of endothelial enzyme of nitric oxide synthase, while other flavonoids inhibit VEGF by affecting endothelial cells and their signaling path. Most, and not all, epidemiology studies indicate that quercetin serves as a protection against various types of cancer. The molecular mechanism through which quercetin inhibits endothelial cells seems to be the kinase protein activated by mitogen, c-Jun NH2 kinase, and focal adhesion kinase, and the expression and activation of MMP-2 (Igura *et al.,* 2001). However, the effects of this flavonoid on collagen XVIII are unknown. Most studies on crataegus have examined the inhibitive effects of quercetin flavonoid of this plant on endothelial growth factors such as VEGF and MMP-2 (rather than inhibitive factors). A deeper examination of the effects of intensive endurance trainings and the mechanism of this plant on the inhibitive factors of angiogenesis, especially plasma collagen XVIII, seems to require the examination of various vascular growth factors.

In general, researchers in this study concluded that the increase of collagen XVIII (albeit insignificant) as a result of physical activity and consumption of black crataegus extract could possibly serve as a regional inhibitor of angiogenesis and another evidence for the anti-cancer effects of physical activities. Since the research has not managed in this study to measure the amount of plasma endostatin, it is suggested that both indices are measured with important angiogenic factors so that we can have a more accurate interpretation of changes to angiogenic and angiostatic factors resulted from physical exercises.

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