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## **Liver ABCA1 Gene Expression in Male Rats: Effects of High-intensity Treadmill Running and Black Crataegus-pentaegyna (Siayh-Valik) Extraction**

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### **ABSTRACT**

The purpose of the current study was to investigate the effect of a high-intensity treadmill running (8 weeks) with or without aqueous extraction of black Crataegus-Pentaegyna (Siyah -Valik) on liver ABCA1 mRNA expression and plasma HDL-C, total, and direct bilirubin concentration in male rats. Twenty Wistar male rats (4-6 weeks old, 140-170 g weight) were used. Animals were randomly assigned into training (n =10) and control (n =10) groups and further divided into saline-control (SC, n=5), saline-training (ST, n=5), black Crataegus-Pentaegyna (Siyah-Valik) Control (SVC, n=5), and black Crataegus-Pentaegyna (Siyah-Valik)-training (SVT, n=5) groups. Training groups have performed a high-intensity running program (34 m/min on 0% grade, 60 min/day and 5 days/week) on a motor-driven treadmill for 8 weeks. Animals were orally fed with black Crataegus-Pentaegyna (Siyah -Valik) extraction (500 mg/kg body weight) and saline solution for last six weeks. A significant differences have found in liver ABCA1 gene expression between SVC and SVT ( $P<0.003$ ) and between SVC with SC groups ( $P< 0.038$ ). HDL-C levels were significantly ( $P<0.036$ ) between groups. A higher HDL level was found in SV treated groups and between SVC and SC groups. The levels of bilirubin total and bilirubin direct remained unchanged. The current results show that high-intensity treadmill running affected liver ABCA1 mRNA expression in different directions in saline (increase) and SV (decrease) treated animals. Findings also indicate an opposite pattern of change in saline and SV treated animals at rest. It seems the existence of opposite effect of exercise with supplementation of SV might be attributed to the suppression of ligands which is provided by SV supplementation at rest. This in turn might be also taking in account in lower liver ABCA1 mRNA expression and its related nuclear receptors such as LXR in SVT not ST groups.

**Key Words:** ATP-binding cassette protein-A1 (ABCA1), HDL-C, Male Rats, High-intensity running, Black Crataegus-Pentaegyna (Siyah -Valik).

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## INTRODUCTION

Liver has well been recognized as a main organ in blood glucose regulation, and lipid and lipoprotein metabolism, short energy storage by deposition of excess blood glucose in to glycogen, bile acid secretion, nitrogen fixation, ketogenesis, toxification, and HDL biogenesis (1-5). Regarding HDL biogenesis and its plasmatic formation, liver is accounted as a chief organ for a lipid-poor or lipid-free Apo lipoprotein type A (Apo-I) secretion into blood into blood circulation (6). The exercise has been shown to increase genes which are involved in HDL-C biogenesis and its plasmatic formation such as ABCA1 and ABCG1 genes. The responses of ATP-binding cassette protein transporters (ABCs) family member to different types of physical exercise in human and animal has also been investigated by several researchers (7-10). ATP-binding cassette protein transporters A, particularly, ABCA1 is a multispan molecule with high expression level in the heart and other tissues and upregulated by several factors such as cholesterol influx and physical activity (11). On the other hand, the role of various medicinal plants in reducing heart disease is well established. *Crataegus* (Valik) species is well known in phytotherapy for the treatment of many cardiovascular diseases (12). *Crataegus Pentaegyna* (Sorkh valik) is the one of best known and favorite edible wild fruit of the *Crataegus* species, (Rosaceae) which is exist in Mazandaran and other Northern states of Iran. This fruit is believed to have some medicinal beneficial effect on cardiovascular function, blood pressure, and lipid metabolism. In Mazandaran province, this fruit is consumed freshly and as jam, vinegar, and sauce (13). In despite, some information about the effects of *Crataegus* species extraction (14). There is very less

information about the effect of the black *Crataegus Pentaegyna* (Siyah-Valik) extraction on ABCA1 mRNA expression with or without a high-intensity treadmill running program. This study was conducted to evaluate of the liver ABCA1 mRNA expression response. The second purpose to see a possible change in ABCA1 could be accompanied with significant changes in plasma HDL, bilirubin total and direct concentrations to a high- intensity treadmill running programm and black *Crataegus pentaegyna* (Siyah-Valik) in male rat.

## MATERIALS AND METHODS

**Animals.** All experiments 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). Twenty Wistar male rats (4-6 weeks old, 140-170 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  $22^{\circ}\text{C} \pm 1.4^{\circ}\text{C}$  and  $50\% \pm 5\%$ , respectively. Diets (a pellet form) and water were provided ad libitum. Animals were randomly assigned into control (n =10) and training (n =10) groups. Rats were further divided into saline-control (SC, n=5), saline-training (ST, n=5), Black *Crataegus* - *Pentaegyna* (Siyah - Valik)-control, Valik - control (SVC, n =5), and Sorkh Valik - training (SVT) (n=5). The control groups remained sedentary; whereas the training groups underwent a high

intensity (35m/min) treadmill running program.

**Plant Material.** The ripped fruit and well washed samples of red Crataegus - Pentaegyna (Sorkh - Valik) were collected from the Neka forest in the Mazandaran province of Iran. Fruits were dried in oven at 35° C for 4 days and fine powdered by using a. Plant Material was identified by herbarium collection in Department of Biology, Faculty of Basic Sciences, University of Mazandaran (UMZ) Baboulsar, and Mazandaran, Iran.

**Preparation of the Crataegus extraction.** The whole ripped and dried fruits of Crataegus-Pentaegyna were grounded in house electronic grinder to a fine powder. The extract was prepared according to the Cai et al (15). Rats were orally received a single dose (500 mg/kg of body weight or 10 ml/kg of body weight) of liquid extraction of Siyah-Valik at the end of daily exercise training session (16). The saline groups were treated with the same volume of saline solution.

**Liver Biopsy and plasma HDL-C, Total and Direct Bilirubin Concentrations.** 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). The liver was excised, cleaned, divided into two pieces, washed in ice cold saline, and immediately frozen in liquid nitrogen and stored at –80° C until RNA extraction. Blood was also collected in test tubes contain EDTA (1mg/mL) as anticoagulant, and immediately processed for plasma preparation, during a 15 min centrifugation at 3000 rpm. Plasma was stored at -80C too, for future analysis. Plasma High Density Lipoprotein (HDL) was determined by enzymatic colorimetric method (Pars Azmoun, Tehran, Iran); Plasma total

bilirubin (BileT) was determined by enzymatic colorimetric method (Pars Azmoun, Tehran, Iran); intra-assay coefficient of variation and sensitivity of the method were 3.05% and 0.03 mg/dL, respectively. Plasma Direct bilirubin (BileD) was determined by enzymatic colorimetric method (Pars Azmoun, Tehran, Iran); intra-assay coefficient of variation and sensitivity of the method were 3.12% and 0.01 mg/dL, respectively.

**RNA, cDNA Synthesis and Real-time PCR.** Total RNA was extracted from 30 mg of liver tissue using RNA purification kits (QIAGEN, Cat.No: 71104). Complementary DNA (cDNA) was extended by using cDNA synthesis kit (QuantiTect Reverse Transcription Kit cDNA synthesis (Qiagen), Cat.No: 205310) according to the manufacturer's instructions. Real-time quantitative PCR was performed using Quanti Fast SYBR Green PCR Kit (Cat. No. 204052; Qiagen, GmbH, Germany) in using 10µl reaction containing 1µl single-strand cDNA, 5µl Master Mix, 1µl of the each forward and reverse primers and 2µl Transferee water. Expected fragment size and Oligonucleotide primer sequences for ABCA1, and GAPDH genes are F: 5'-ACGAGATTGATGACCGCCTC, R: 5'-GCATCCACCCACTCTCTTC, and F: 5'-GTGCCAGCCTCGTCTCATAG, R: 5'-GACTGTGCCGTTGAACTTGC, Respectively. The PCR was carried out on BIO RAD (C1000 TM Thermal Cycler). Real time PCR system is listed in Table 1. Product specificity was confirmed in the initial experiments by 1.5 % agarose gel electrophoresis and routinely by melting curve analysis.

**Statistical Analysis.** The relative levels of mRNA were analyzed by using a comparative threshold cycle method (15). Data were normalized and normal distribution was found. A two way ANOVA

were employed and significance was accepted at  $P = 0.05$ . All findings were expressed as means  $\pm$  SE and SPSS (Version

13; SPSS, Chicago, IL) software was used for data analysis.

**Table 1. Real-time Cycler Conditions**

Steps	Time	Temperature
PCR initial activation step	5 min	95 <sup>o</sup> C
Two-step cycling		
Denaturation	10 s	95 <sup>o</sup> C
45 Cycle Combined annealing/extension	30 s	95 <sup>o</sup> C
Melting Curve	6.5 min	55 to 95 <sup>o</sup> C

## RESULTS

**ABCA1 mRNA Expression.** A significant differences was found in liver ABCA1 gene expression between SVC and SVT ( $P < 0.003$ ) and between SVC with SC groups ( $P < 0.038$ ) (Fig.1).

**Plasma Biochemical Variables.** Data analysis revealed a significant differences between groups ( $F = 2.905$ ,  $P < 0.03$ ). Using a

suitable following post hoc test showed that plasma HDL-C levels were significantly ( $P < 0.036$ ) higher in SV treated rats when compared with S groups. However, a significant difference also was found between SVC and SC groups (Fig 2). The levels of bilirubin total and bilirubin direct remained unchanged (Table 2).

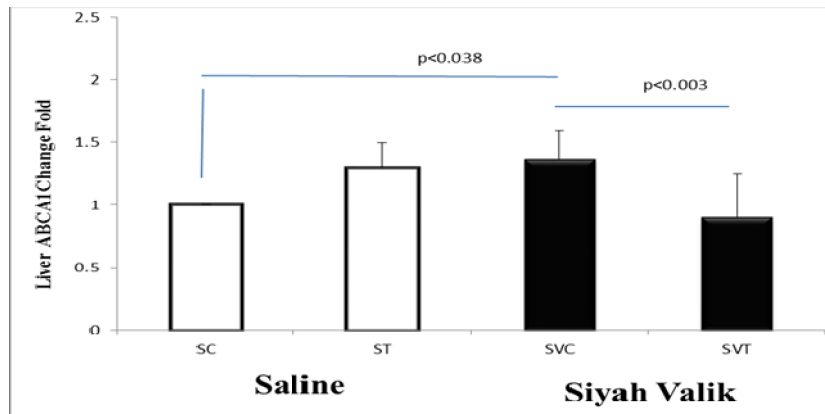


Figure1. **Real-Time PCR of Liver ABCA1 Relative mRNA Expression in Wild-Type Male Rats.** The results are expressed as mean  $\pm$  SD. Each column is for five rats per group. **SC:** Saline-Control, **ST:** Saline- Training, **SVC:** Crataegus-Pentaegyna-Control (Siyah Valik), and **SVT:** Crataegus-Pentaegyna-Training (Siyah Valik).

**Table 2. Comparative changes Plasma Variables Concentration (mg/dL)**

Variables	SC	ST	SVC	SVT	p
BileT, mg/dl	0.68 $\pm$ 0.037	0.72 $\pm$ 0.03	0.76 $\pm$ 0.024	0.78 $\pm$ 0.02	0.371
BileD mg/dL	0.18 $\pm$ 0.0	0.14 $\pm$ 0.024	0.18 $\pm$ 0.02	0.18 $\pm$ 0.02	0.061

The results are expressed as mean  $\pm$  SD. Each column is for five rats per group. **SC:** Saline-Control, **ST:** Saline- Training, **SVC:** Crataegus-Pentaegyna-Control (Siyah Valik), and **SVT:** Crataegus-Pentaegyna-Training (Siyah Valik).

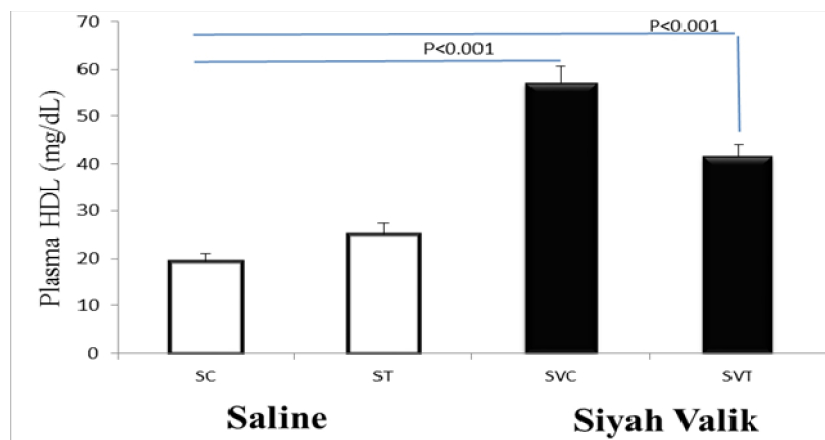


Figure 2. **Plasma HDL Concentrations (mg/dL) in Wild-Type Male Rats.** The results are expressed as mean  $\pm$  SD. Each column is for five rats per group. SC: Saline-Control, ST: Saline- Training, SVC: Crataegus-Pentaegyna-Control (Siyah Valik), and SVT: Crataegus-Pentaegyna-Training (Siyah Valik).

## DISCUSSION

The results of the present study indicate that SV extraction was able to enhance liver ABCA1mRNA expression in our experimental condition. The other main findings of the current study were a higher ABCA1 mRNA expression in SV treated groups. The results also indicated that high-intensity treadmill running program with or without SV treatment had different impact on liver ABCA1mRNA expression. Another main finding was a higher plasma HDL-C in SVC group when compared with SC, ST, and SVT groups. It should be noted that on the basis of our Knowledge, this is the first report to demonstrate alterations of male rat liver ABCA1mRNA expression in response to a high intensity training and black Crataegus - Pentaegyna (Siyah - Valik) extraction. It is well known that ABCA1 is the initiator element of reverse cholesterol transport (RCT) process which plays a crucial role in HDL biogenesis, maturation, and its plasmatic formation. The effect of moderate intensity of treadmill running with moderate and long term (60, 90 min) for 6 and 12 weeks on ABCA1mRNA expression

in rat liver, small intestine, and heart tissues have been previously reported by several investigators (8-10, 16). Our finding related to ABCA1mRNA expression in saline treated animals is in agreement with previous reports, but the results of SV trained rats is not in consistent with other reports. However, according to Ghanbari-Niaki *et al.* (2013) using the same treadmill running protocol resulted in a similar pattern but not significant changes in rat heart ABCA1mRNA expression (8). It seems that the tendency of treadmill-running induced reduction in ABCA1mRNA expression is similar in both liver and heart tissues following the same amount of SV extraction. A higher level of ABCA1mRNA expression in liver with SV treatment at rest and a reduction following the treadmill running was a novel and interested finding. The antilipidemic, antioxidative, and lowering cholesterol effects of Crataegus species very well known (11, 12), but the information about the effect of black Crataegus-Pentaegyna (Siyah-valik) on ABCA1 mRNA at rest and training is lacking. It has been suggested that the activation of PPAR- $\gamma$

ligand is resulted in a Primary activation of LXR which is in turn up-regulates the expression of transporter ABCG1 and ABCA1 and following these an increase in reverse cholesterol transport process. Would be expected as the present results showed, SV treatment resulted in a higher expression of ABCA1 in SV-treated rats, while a lower ABCA1mRNA expression was observed at the end of treadmill running program in trained liver with SV treatment (17, 18). It is not clear that by which mechanism(s) black Crataegus - pentaegyna (Siyah - Valik) could affect ABCA1mRNA, particularly at resting not training stats. However, the effect of SV treatment on ABCA1mRNA expression at rest might be due to the nature and contents of SV. The analyze of SV whole dried fruit has shown to have high levels of polyunsaturated fatty acid (PUFA) contents with the best PUFA/ (saturated fatty acids) SFA ratio, and also the high levels of phenolics components (15). On the basis of Our GC-MS data findings, the main compositions of the Crataegus-Pentaegyna (SV) sample were oleic acid (13.44%), palmitic acid (7.72%), linoleic acid (22.43%) and arashidonic acid (0.47%). On the other hand, it has been suggested that the liver X receptors (LXRs) as nuclear receptors are activated by endogenous oxysterols, oxidized derivatives of cholesterol (19). Thus it might be that the conversion of cholesterol to the oxysterol ligands for LXRs has driven the expression of ABCA1 SV treated liver. It has also shown that, unsaturated fatty acids were might also suppress the expression of ABCA1 and ABCG1 transcription by antagonizing the effects of sterols on LXR

activation (20). With consider to the present results, it might be stated that these change possibly increased the adjustment of the ABCA1 gene to justify through other signaling pathways, including activation of peroxisome proliferation activated receptors (PPARs) by fatty acids and flavonoid compounds found in hawthorn (as natural ligands of PPAR activators) (21, 22). Thus, a higher HDL levels in SV treated rats could be attributed to high level expression of ABCA1 in SV treated liver treated with SV solution.

### CONCLUSION

Taken together, the findings of the current study indicate that SV extraction enhanced plasma HDL levels, but the effect of SV at rest reduced by high intensity treadmill running in somewhat. Data also indicate that SV extraction induced an exercise-like effect on ABCA1mRNA expression at rest, while it showed opposite response to high intensity treadmill running program. The present study has shown that SV extraction could be consider as cardioprotector herb such as Crataegus oxyacantha for minimizing atherosclerotic process. Further studies are warranted to clarify the effects of different parts of this Crataegus species on other related genes which are involved in ABCA1mRNA expression.

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## پاسخ ژن ABCA1 کبدی در رت‌های نر: اثر تمرین شدید و عصاره خام کراتاگوس (سیاه ولیک)

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### چکیده

فعالیت‌های ورزشی منظم و بهره‌مندی از داروهای گیاهی بر سوخت و ساز مناسب چربی و تنظیم آن در کبد اثر دارد. از آنجایی که این تأثیر احتمالاً از طریق مسیرهای سیگنالی برخی از ژن‌های مؤثر در فرآیند انتقال معکوس کلسترول از جمله ABCA1 اعمال خواهد شد، و از طرفی چون اطلاعات کافی از مکانیسم‌های درگیر در این فرآیند در دسترس نیست، لذا هدف از پژوهش حاضر، بررسی اثر تمرین شدید روی نوارگردان (۳۴ متر در دقیقه و شیب صفر درجه) با و بدون عصاره ولیک بر بیان ژن مذکور، سطوح پلاسمایی HDL، بیلی روبین در رت‌های صحرائی نر سالم است. ۲۰ سر رت نر (۶-۴ هفته‌ای و ۱۴۰-۱۷۳ گرم وزن) از نژاد ویستار به طور تصادفی به دو گروه تمرین (۱۰ سر رت) و کنترل (۱۰ سر رت) تقسیم شدند. پس از آشنایی مقدماتی با محیط و نوار گردان به گروه‌های سالیین- شاهد، سالیین- تمرین، سیاه ولیک- شاهد، و سیاه ولیک- تمرین تقسیم شدند. گروه‌های تمرین به مدت ۸ هفته (۵ روز در هفته، ۶۰ دقیقه در جلسه) تمرین روی نوارگردان دویند. محلول سالیین و عصاره آبی میوه ولیک به طور دهانی در ۶ هفته پایانی داده شد. ۷۲ ساعت پس از آخرین جلسه تمرین حیوانات قربانی و جهت اندازه‌گیری متغیرهای مورد نظر بافت کبد سریع جدا، تمیز و به سرعت در نیتروژن مایع منجمد گردید. تحلیل داده‌های مربوط به بیان نسبی ژن ABCA1 در بافت کبد اختلاف معنی‌داری ( $p < 0.003$ ) را بین گروه‌های تمرین و تیمار سیاه ولیک نشان داد. همچنین، افزایش سطح HDL در گروه‌های سیاه ولیک در مقایسه با شاهد معنی‌دار (به ترتیب:  $p < 0.01$  و  $p < 0.001$ ) بود، در حالیکه در سطوح بیلی روبین T و D تغییرات معنی‌داری مشاهده نشد. نتایج پژوهش حاضر نشان می‌دهد که تمرین شدید احتمالاً از طریق سازوکارهای متفاوتی بر ژن‌های درگیر اثر می‌گذارد. داده‌ها همچنین حاکی از آن است که گروه سیاه ولیک بدون تمرین قادر بود تا موجب افزایش سطح بیانی ژن ABCA1 و افزایش HDL گردد.

واژگان کلیدی: HDL، ABCA1، بیلی روبین، تمرین ورزشی شدید و عصاره ولیک.

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