

## Original Article

# Involvement of IGF/IGFBP/Erk axis in the exercise-mediated preventive effects on colorectal cancer in rats

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**Abstract:** Recent studies have indicated that downregulation of insulin-like growth factor (IGF)-1 and its downstream targets are the main mechanisms underlying the anti-cancer impact of exercise. Therefore, we examined the impact of exercise on chemically induced-aberrant crypt foci (ACF), the earliest step of colorectal carcinogenesis, in rats and involvement of the IGF-1/IGF binding protein (IGFBP)-3/Erk axis. Twenty-four male Wistar rats were assigned into two groups (n=12): the control and exercise group. After eight weeks of training intervention, 6 rats were randomly selected from each group and received four injections of 1,2-dimethylhydrazine (DMH; 40 mg/kg), for two weeks. 0.2% methylene blue staining was used to evaluate the number of ACF in the colon. IGF-1 and IGFBP-3 protein levels in the serum were measured using commercially available ELISA kits for rat. The expression levels of proliferating cell nuclear antigen (PCNA), Erk1/2 and p-Erk1/2 were evaluated in colon tissue. Histological assessments were also performed in all groups. We found that the total number of ACF was significantly lowered after eight-week exercise ( $P<0.05$ ). Moreover, the exercise program downregulated the IGF-1, PCNA, and p-Erk1/2 expressions and upregulated IGFBP-3 expression. Exercise was also found to increase the goblet cell number and improved colon architecture. Our finding demonstrated reduced ACF number in rat colons following exercise training, and this function may be associated with the inhibition of IGF-1/IGFBP-3/Erk1/2 signaling. Therefore, exercise appears to result in a lower number of ACF for preventing colon cancer.

**Keywords:** ACF, exercise, IGF-1, IGFBP-3, Erk1/2, DMH

## Introduction

Colorectal cancer, as one of the most lethal human malignancies, has an increasing rate of morbidity and mortality [1]. Under normal physiological conditions, the proliferation, differentiation, and apoptosis of stem cells, present at the base of colonic crypts are strictly regulated, and easily predisposed to various genetic mutations [2]. Some important risk factors for genetic mutations, and colon carcinogenesis include: lack of physical activity, diet, obesity, smoking, stress, and excessive consumption of alcohol and red meat [2, 3]. Despite huge improvement in the diagnostic techniques and

efforts for understanding the molecular pathogenesis of this type of malignancy, the precise etiology of colorectal cancer is still unclear [3]. Recently, there has been intense attention to positive impacts of physical activity on reducing the risk of numerous cancers, especially colorectal cancer in humans and rodents [4]. For instance, exercise leads to a significant decline in the development of colon tumors in experimental mouse models [5]. In another animal model study, 4 weeks of treadmill-running decreased the manifestation of colon preneoplastic lesions [6]. Aberrant crypt foci (ACF), as the initial phase of colorectal cancer, characterized by a thicker epithelial lining, increased size,

and increased pericryptal zone, has often been used as biomarker for the initiation of colorectal cancer in rats [7, 8].

Furthermore, it has been hypothesized that multiple biological mechanisms such as hyperinsulinemia, obesity, variation in prostaglandin ratio, lowered bile acid secretion, reduced gut transit time and altered gut flora explain the possible correlation between colorectal cancer and physical activity [9]. Insulin-like growth factor-1 (IGF-1) hormonal axis is a substantial factor, that links the beneficial effects of exercise and cancer [9, 10]. More importantly, IGF-1 is a strong mitogen with stimulatory effects on cell proliferation, as well as inhibitory impacts on apoptosis in normal and cancer cells through modulating various related signaling pathways [11]. An increasing body of epidemiological investigations have demonstrated that elevated IGF-1 or decreased IGFBP, its binding protein, are related to a higher risk of several cancers [12]. Thus, the down-regulation of IGF-1 and up-regulation of IGFBP-3, because of the exercise intervention, may have protective effects against the development of colon cancer. Therefore, we aimed to assess the impact of exercise on the ACF number, the levels of IGF-1 and IGFBP-3 and their downstream signaling, MAPK pathway, in colon tissues of the rats with 1,2-dimethylhydrazine (DMH)-induced colorectal cancer.

### Materials and methods

#### *Animals*

Twenty-four male Wistar rats (4-week-old) were provided from the Pasteur Institute of Iran. All animals were kept in plastic cages (three rats/cage) under standard conditions of temperature ( $22 \pm 2^\circ\text{C}$ ) and luminosity (dark 12 h/12 h light cycle) with free access to water and food. The Animal Care and Use Committee of the Urmia University Medical of Sciences (Urmia, Iran) approved the experimental protocol (Ethical Code: Ir.umsu.rec.1397.336).

#### *Experimental design*

After one-week acclimatization to the housing environment, all animals were familiarized with a motor-driven treadmill (Maze Router, Tabriz, Iran) for 3 days, 5 min/day, on a 0% grade in a room with controlled temperature condition ( $23 \pm 1^\circ\text{C}$ ). The running speed was initially 15 m/min and was gradually increased to 25 m/min.

Then, the rats were randomly divided into two groups ( $n=12$ ) including: control group and exercise group. The weight of all rats was measured weekly until the end of the experiment.

#### *Exercise protocol*

The animals in the exercise group were trained for 8 weeks at 25 m/min on the treadmill and performed weekly five exercise sessions of 60-min duration at 10.00 am. At the start of the session, five minutes were used as warm-up, and at the end, five minutes were used as cool-down [13].

#### *Neoplastic induction, animal autopsy and tissue preparation*

After the last session of training, 6 rats were randomly selected from each group. 40 mg/kg body weight 1,2-dimethylhydrazine (DMH; Sigma-Aldrich, St. Louis, MO, United States) was injected twice a week for 2 weeks, subcutaneously. Therefore, the examined groups included Control group: only received EDTA-vehicle, Exercise group: received EDTA-vehicle; performed exercise program, DMH group: only received DMH, Exercise + DMH group: performed exercise training; received DMH.

Two weeks after the last injection of DMH, the rats were sacrificed. After overnight fasting, the rats were anesthetized using ketamine (100 mg/kg body weight) + xylazine (10 mg/kg body weight), intraperitoneally. Blood samples were taken from the heart and centrifuged. For histopathologic analysis, the entire colon was removed and fixed in 10% buffered formalin for 24 h.

#### *Quantification of ACF*

For ACF quantification, 0.2% methylene blue (Sigma Chemical Co. St. Louis, MO) was used to stain fixed colon specimens. The number of ACF was assessed under the light microscope. A thicker epithelial cell layer, increased size, and increased pericryptic zone were three main criteria for identification of the ACF in colon specimens [7].

#### *Hematoxylin and eosin (H&E) staining*

H&E staining was performed following the method of Darband et al [14]. Briefly, after dehydration in 80% alcohol, colon tissues were stained by Hematoxylin for 10 min and then

Eosin for 5 min. At the end, the samples were evaluated with the use of Olympus light microscope and photographed at a magnification  $\times 100$ .

### *Periodic acid-schiff (PAS) staining*

The periodic acid-Schiff (PAS) staining technique (Pajohesh Asia, Iran) was used to evaluate the carbohydrate ratio. All steps were performed in accordance to the manufacturer's guidelines. In brief, the hydrated specimens were oxidized in 5% periodic acid solution for 5 min and then placed in Schiff reagent for 15 min. After incubation with borax solution slides were counterstained with hematoxylin. Then, the samples were evaluated with the use of Olympus light microscope and photographed at a magnification  $\times 100$  [14].

### *Immunohistochemical (IHC) staining for PCNA*

The sections were exposed to 60°C for about half an hour. The paraffin was removed by xylene and the samples were hydrated by passing through an alcohol gradient. IHC staining was performed using IHC kit rabbit specific HRP/DAB (Abcam, UK) in accordance with manufacturer's guidelines. Finally, the PCNA-positive stained cells were counted per one mm<sup>2</sup> of the tissue in 18 sections from each group and compared between groups. The scale bars were based on a morphometric lens (Olympus, Germany, CH-2). All images were captured under 100 $\times$  magnification [14].

### *Serum IGF-1 and IGFBP-3 measurement*

The serum levels of IGF-1 (Elabscience, Cat: E-EL-R0010) and IGFBP-3 (Elabscience, Cat: E-EL-R0533) were measured using rat-specific ELISA kits. All steps were followed in accordance with the manufacturer's guidelines.

### *Western blotting*

RIPA buffer (Sigma-Aldrich) was used to prepare colon tissue protein samples for western blotting. Bradford method was also used to determine protein concentration in samples and then in order to separate proteins, 10% SDS-polyacrylamide gel electrophoresis was employed. This step was followed by transfer of separated proteins into PVDF membrane (Sigma, St. Louis, MO, USA). Then, this membrane was placed in a buffer containing 5%

bovine serum albumin at room temperature for 2 h and after that, polyclonal primary antibodies for Erk1/2, p-Erk1/2 and  $\beta$ -actin (Santa Cruz, UK, 1:500) were added and incubated at 4°C overnight. This step was followed by addition of secondary antibodies (Santa Cruz, UK) and incubation for 2 h at room temperature and finally finished by exposure and photography [14].

### *Statistical analysis*

All data were expressed as mean  $\pm$  SD. Kolmogorov-Smirnov test was used to assess normality of the data and one-way analysis of variance (ANOVA), then Tukey's test was performed for evaluation of multiple comparisons SPSS for windows (version 20, SPSS Inc., Chicago, IL, USA). The values were considered significant if  $P < 0.05$ .

## Results

### *Effect of exercise program on rats' weight*

The mean weight of rats in the Exercise group was significantly lowered compared with the control group (control group,  $228 \pm 11$  g; Exercise group,  $201 \pm 17$  g) ( $P < 0.05$ ) (**Figure 1**). In addition, there was no significant difference between Exercise + DMH and DMH group ( $206 \pm 22$  g vs.  $215 \pm 15$  g).

### *The effect of exercise program on ACF number*

ACF were observed in all groups that received DMH (**Figure 2A**). The number of total ACF was less in the Exercise + DMH group than in the DMH group (Exercise + DMH,  $24 \pm 3.68$ ; DMH,  $46.67 \pm 9.17$ ) ( $P < 0.05$ ) (**Figure 2B**). The numbers of ACF for proximal, medial, and distal regions were also significantly lower in the Exercise + DMH group compared with the DMH group (Exercise + DMH; proximal,  $5.33 \pm 1.86$ ; medial,  $7.83 \pm 2.04$ ; distal,  $10.83 \pm 2.48$  vs. DMH; proximal,  $10.16 \pm 3.43$ ; medial,  $15.33 \pm 3.61$ ; distal,  $21.16 \pm 4.16$ ) ( $P < 0.05$ ) (**Figure 2C**).

### *Effects of exercise program on the colon architecture and goblet cells*

Significant histopathologic alterations were observed in the H&E stained-colonic tissues of rats treated with DMH. These alterations included ACF, hyperplasia, and dysplasia, elon-

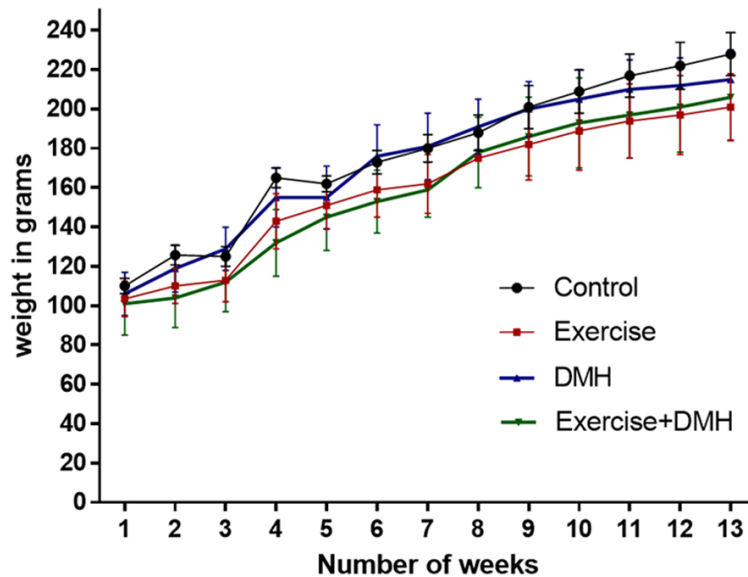


Figure 1. Body weight of rats in control and treated groups.

gated nuclei in the colonic mucosa and abnormally shaped lumens. Exercise training resulted in a significant improvement in the colonic architecture (Figure 3A).

The results of PAS staining also showed a decreased number of goblet cells in the colon tissue of the rats with DMH-induced colorectal cancer. On the other hand, exercise training in rats led to an increased number of goblet cells in the Exercise + DMH group as compared to DMH group (Figure 3B and 3C).

#### Effects of exercise on cell proliferation

Evaluating the expression levels of PCNA using IHC was used to measure cell proliferation in colonic mucosa of all groups. It is suggested that upregulation in PCNA protein levels has a strong correlation to the elevated cell proliferation. Our results showed that the number of PCNA-positive cells increased significantly in the DMH group in comparison to the control group (Figure 4A and 4B). Exercise program significantly reduced the cell number with positive staining for PCNA in comparison to the DMH group.

#### The effects of exercise program on the serum levels of IGF-1 and IGFBP-3 proteins

For evaluating the underlying mechanism of the effects of exercise on the colorectal cancer, we

assessed the serum levels of IGF-1 in all groups. As shown in Figure 5A, IGF-1 protein levels were significantly higher in the DMH group than the control group ( $326.91 \pm 32.37$  vs.  $190.83 \pm 23.87$  pg/mL;  $P < 0.05$ ). Additionally, IGF-1 appeared to be significantly lower in the Exercise + DMH group, in comparison to DMH ( $233.68 \pm 36.74$  vs.  $326.91 \pm 32.37$  pg/mL;  $P < 0.05$ ).

IGFBP-3 is one of the IGF-1 binding proteins, which increases its bioavailability. As shown in Figure 5B, IGFBP-3 was significantly lower in the DMH than the control group ( $334.40 \pm 52.47$  vs.  $862.86 \pm 59.79$  pg/mL;  $P < 0.001$ ). IGFBP-3 levels were significantly higher in Exercise + DMH in comparison with the DMH group ( $572.51 \pm 57.25$  vs.  $334.40 \pm 52.47$  pg/mL;  $P < 0.001$ ).

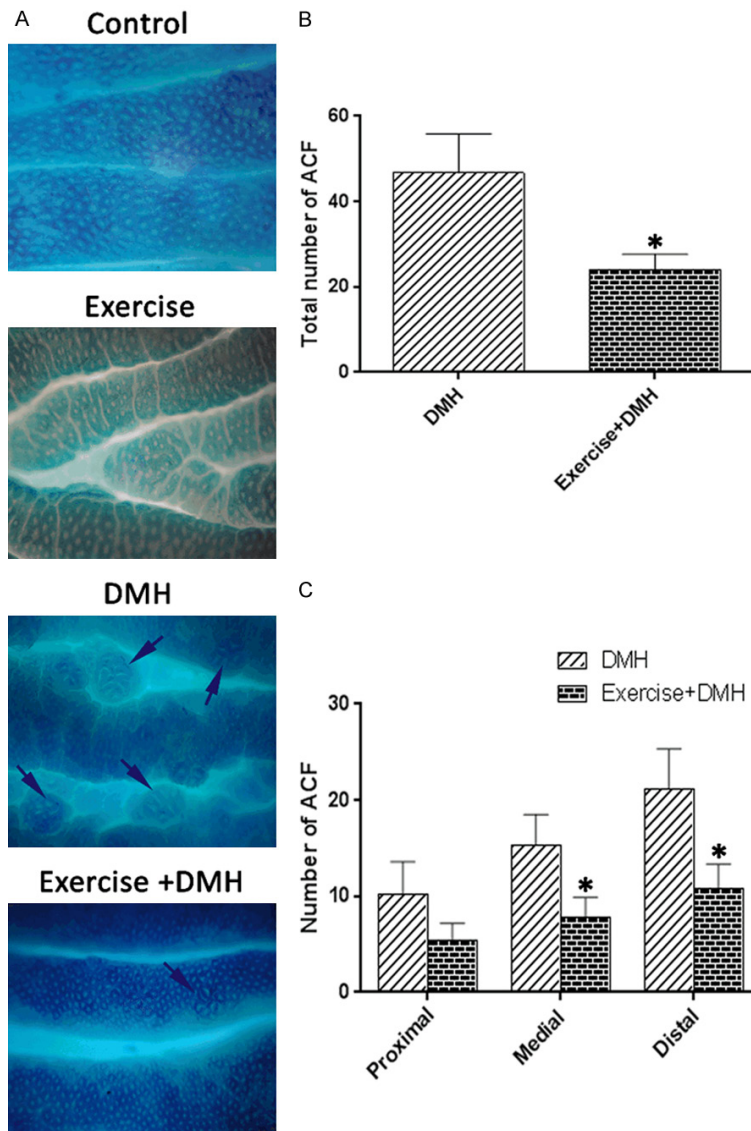
#### Effects of exercise program on the expression levels of Erk1/2 and p-Erk1/2

Next, we measured the expression levels of Erk1/2 and its phosphorylated form, p-Erk1/2, the important downstream effectors of the IGF-1 axis (Figure 6A and 6B). When the level of p-Erk1/2 in the control group was set at 1.00, the level of p-Erk1/2 was  $1.68 \pm 0.03$  in the Exercise group,  $5.04 \pm 0.23$  in the DMH group, and  $2.01 \pm 0.07$  in the Exercise + DMH group. Hence, the expression levels of p-Erk1/2 were significantly higher in the DMH group, as compared to other groups ( $P < 0.001$ ). Exercise decreased the expression of p-Erk1/2 in the Exercise + DMH ( $P < 0.001$ ).

#### Discussion

The main finding of present study was that the number of DMH-induced ACF in the rat colon decreased in response to exercise, suggesting a protective effect against the progression of colorectal cancer. Our findings also suggest that IGF1/IGFBP3 pathway and its downstream effector Erk1/2 may be one of the most important factors mediating the beneficial effects of exercise in prevention of colorectal cancer,





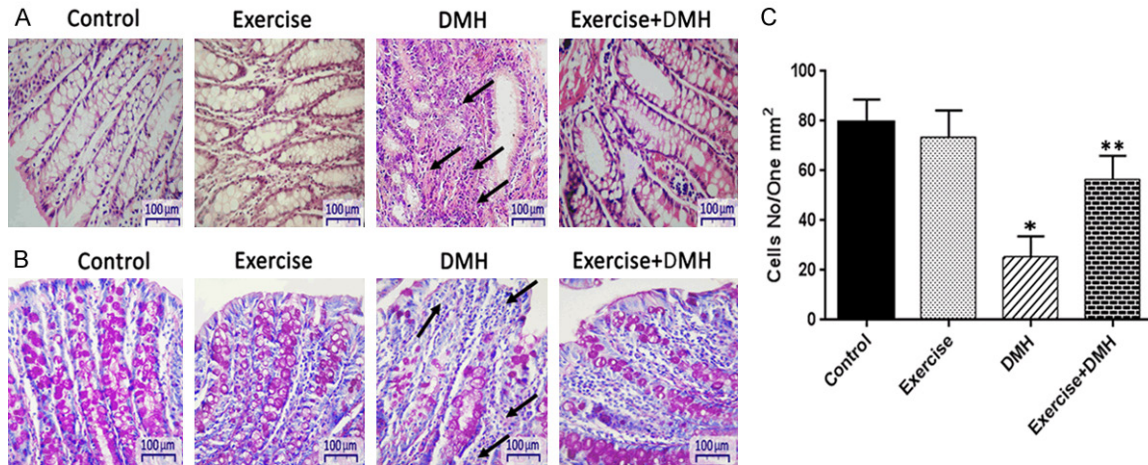
**Figure 2.** Inhibitory effect of exercise on ACF number in DMH-treated rats. DMH-induced ACF in methylene blue stained colonic mucosa (400×) (A). The effect of exercise training on the total number of DMH-induced ACF in the rat colon (B). The effect of exercise training on the DMH-induced ACF number in the proximal, medial, and distal colon (C). \* $P < 0.05$  for exercise + DMH group in comparison to DMH group.  $n = 6$ /group. All data are presented as mean  $\pm$  SD. DMH, 1,2-Dimethylhydrazine; ACF, Aberrant crypt foci.

since an exercise program decreased the serum levels of IGF-1 and Erk1/2 expression levels and increased the IGFBP-3 levels.

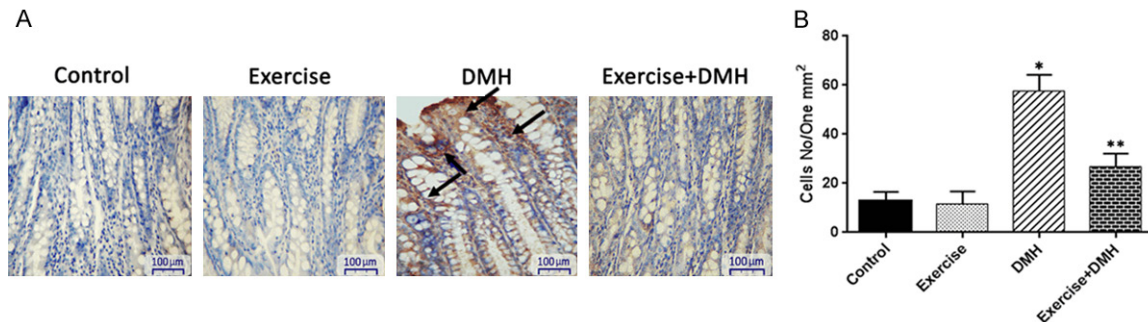
The effects of physical activity, as a tumor preventive modulation of colorectal cancer, as well as other types of human malignancies, have been extensively documented in previous studies [15]. In human studies, various training programs have been reported to inhibit cancer ini-

tiation. For example, Wen et al [16] showed that 15-min/day of vigorous-intensity exercise could inhibit cancer initiation and significantly increase lifespan. In line with this, Inoue et al [17] reported a similar effect when a training program with 1 h/day exercise was performed. Additional studies have demonstrated protective effects of training against cancer development throughout the human lifespan [18, 19]. Similar results were reported in animal studies particularly, in experiments on the various models of colorectal cancer. Fuku et al [20] showed the suppressive effects of running (10 m/min; for 4 weeks) on the number of ACF and total aberrant crypts (AC) in F344 rats with DMH-induced colon cancer. In another study by Andrianopoulos et al [21] it was observed that in animals with voluntary exercise training, the colonic tumor incidence was significantly decreased. Treadmill exercise (1 h/d, 3 d/wk) was also shown to decrease the number of ACF associated in the large intestine of obese mice [6]. Matsuo et al [22] showed that swimming promoted anti-carcinogenic effects, as indicated by a decrease in ACF number through increase in the secreted protein acidic and rich in cysteine (SPARC), which has been suggested as

an exercise-related factor while another study reported the same results for swimming training in rats, which exerted protective effects against DMH-induced ACF [23]. In agreement with all the above studies, our results showed that moderate intensity training (25 m/min for 8 weeks) significantly decreased the ACF number. One possibility for the observed effects is that calorie restriction-induced weight loss and an exercise-induced negative energy balance



**Figure 3.** Effect of exercise on colon architecture and goblet cells in DMH-treated rats. H&E stains of colon sections in different groups (400×), show histological abnormalities such as ACF including dysplasia and hyperplasia, abnormally shaped lumens and elongated nuclei in the colonic mucosa of the treated DMH group, but an improved colonic architecture in exercise treated group (A). PAS staining in the colon tissues of all groups (400×) (B). The numbers of positively stained cells were determined by counting the absolute number of positive stained cells in at least 300 colonic crypts for each rat (C). \* $P < 0.05$  for DMH group in comparison to control group, \*\* $P < 0.05$  for exercise + DMH group in comparison to DMH group.  $n = 6/\text{group}$ . All data are presented as mean  $\pm$  SD. DMH, 1,2-Dimethylhydrazine.

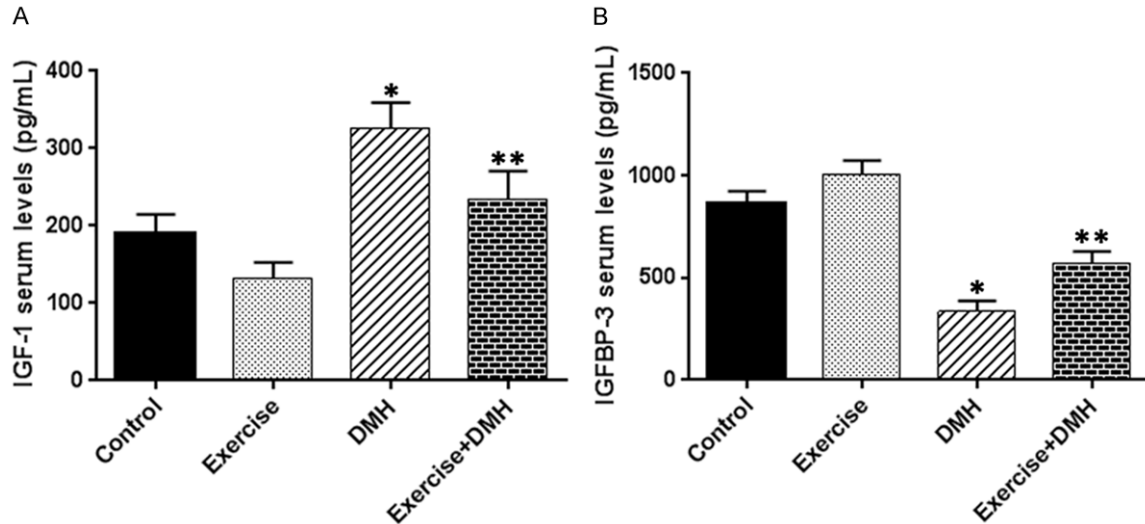


**Figure 4.** Photomicrograph representing immunohistochemical staining techniques of PCNA. Note decreased expression of PCNA in exercise + DMH group (A). Mean  $\pm$  SD of PCNA-positive cells/mm² of tissue is also included (400×) (B). \* $P < 0.05$  for DMH group in comparison to control group, \*\* $P < 0.05$  for exercise + DMH group in comparison to DMH group.  $n = 6/\text{group}$ . All data are presented as mean  $\pm$  SD. DMH, 1,2-Dimethylhydrazine; PCNA, proliferating cell nuclear antigen.

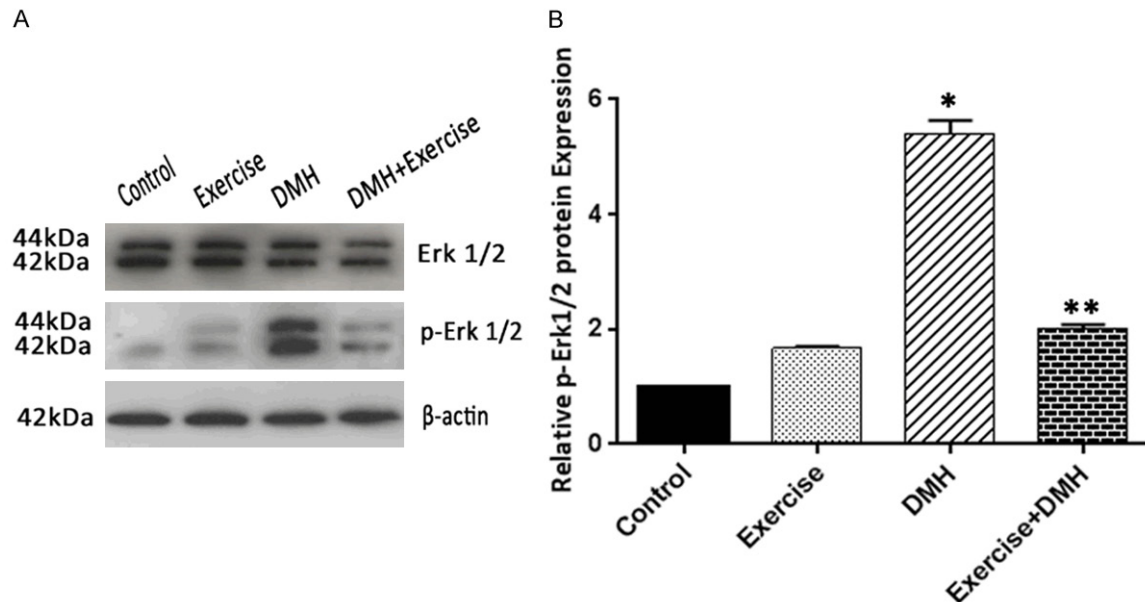
were suggested to inhibit the initiation or proliferation of ACF on colonic mucosa [23], which is consistent with significant weight loss from training in our study. In addition, histological evaluations revealed that performing exercise programs before colorectal cancer induction resulted in better colon architecture, which was lost in the DMH group. An exercise program also had positive effects on the numbers of goblet cells, which were decreased in the DMH group.

Several underlying mechanisms have been proposed for the protective impact of exercise pro-

grams on human malignancy. One is the exercised mediated-decrease in IGF axis, when a high level of IGF-1 is circulating in the bound form to the one of the binding proteins, IGFBP, and only ~2% of IGF-1 is present in free form in the circulation, which could be altered in response to different stimuli such as exercise [24]. In this context, an increasing body of studies has reported mixed results regarding circulating IGF-1 levels after different exercise periods in rats or humans [25, 26]. Therefore, the results from the effects of exercise on the IGF/IGFBP axis in healthy conditions are very inconsistent, mostly due to different exercise mod-



**Figure 5.** Effect of exercise on the expression levels of IGF-1 and IGFBP-3 in DMH-treated rats. ELISA results for the serum levels of IGF-1 (A) and IGFBP-3 (B). \* $P < 0.05$  for DMH group in comparison to control group, \*\* $P < 0.05$  for exercise + DMH group in comparison to DMH group.  $n = 6/\text{group}$ . All data are presented as mean  $\pm$  SD. DMH, 1,2-Dimethylhydrazine; IGH-1, insulin like growth factor-1; IGFBP-3, IGF binding protein-3.



**Figure 6.** Effects of exercise on the expression levels of Erk1/2 and p-Erk1/2 in DMH-treated rats. Western blot analysis was used for evaluating the colonic expression levels of p-Erk1/2. Separately, 5  $\mu\text{g}$  of each sample were run for  $\beta$ -actin to confirm comparable protein loading. \* $P < 0.05$  for DMH group in comparison to control group, \*\* $P < 0.05$  for exercise + DMH group in comparison to DMH group.  $n = 6/\text{group}$ . All data are presented as mean  $\pm$  SD. DMH, 1,2-Dimethylhydrazine.

els, as well as the different protocols used in the studies [27, 28]. However, in the case of malignancies, exercise-induced reduction in IGF-1 levels is considered as a favorable outcome, since IGF-1 is identified as a pivotal risk factor for cancer initiation/progression through

activating cell proliferation and suppressing apoptosis [29]. Piringner et al [30] reported that one-year controlled exercise training resulted in a significant reduction in the IGF-1, IGF-2, and IGF-BP3 levels after adjuvant chemotherapy in colorectal cancer patients. In another study by



Haydon et al [24] it was demonstrated that exercise training significantly increased IGFBP-3, which was associated with a 48% reduction in colorectal cancer-specific death. Lee et al [31] reported an upregulation of IGFBP-3 protein levels after 12-week exercise training. Additionally, the IGF-1/IGFBP-3 ratio was also reduced after exercise intervention. Similar results were also reported for the effects of voluntary exercise, which inhibited intestinal tumorigenesis in *Apc<sup>Min/+</sup>* mice [5]. Consistent with these studies, we found that an exercise program significantly decreased serum IGF-1 levels, as well as significantly increased serum IGFBP-3 levels, in rats with DMH-induced colorectal cancer. Thus, the anti-carcinogenesis effect of exercise may be mediated by its inhibitory action on IGF-1 signaling.

For further elucidation of IGF-1 axis involvement in the chemopreventive function of exercise in colorectal cancer, Erk1/2/p-Erk1/2 expression levels were also evaluated in groups. Erk signaling pathway, which acts downstream of IGF-1 proliferative signaling, has a crucial function in the proliferation of numerous human malignancies, such as breast, prostate, and colorectal cancer [32-34]. Teng et al [35] reported that IGF-1, as mitogen of colon cancer cells, activated the Erk1/2 signaling, resulting in cell growth and anti-apoptotic effects in colon cancer cells. In addition, Licato et al [36] showed a 23-fold and 29-fold increase in JNK and ERK activity in the colon tissue of DMH-treated rats in comparison with controls. These results are consistent with our results, which showed an increased expression level of p-Erk1/2 in the DMH group. Furthermore, various studies have reported that the Erk signaling pathway is one of the most substantial targets of exercise in the liver [37], skeletal muscle [38], hippocampus [39], heart [40], and skin [41]. Alizadeh et al [42] showed that exercise training led to a significant decrease in phosphorylation of Akt and Erk in the tumor tissues of patients with breast cancer. In agreement with this result, the innovative finding of the present study is that exercise training contributes to inhibition of Erk1/2 phosphorylation and activation, hence inhibition of cell growth, which was apparent from the decreased expression levels of PCNA. Therefore, our results demonstrated that the preventive effects of exercise training

on cancer cell growth and proliferation may be mediated, in part, by the IGF-1/IGFBP-3/Erk axis.

### Conclusion

Exercise training decreased the ACF number in the rat colon, and this inhibitory effect may be related to reduce IGF-1/IGFBP-3/Erk signaling. Therefore, exercise training can be recommended for preventing colorectal cancer. Further investigation is needed to better elucidate the effects of exercise programs and downstream signaling pathways in colorectal cancer prevention.

### Disclosure of conflict of interest

None.

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