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Voluntary wheel running and testosterone replacement increases heart angiogenesis through miR-132 in castrated diabetic rats

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Objective: Low levels of testosterone in men with diabetes are associated with cardiovascular complications. We investigated the effect of testosterone and voluntary exercise on heart angiogenesis in castrated diabetic rats. *Methods:* Sixty-three diabetic rats were treated with testosterone 2 mg/kg/day or voluntary exercise alone or combination of these two for 6 weeks. At the end of the study, heart tissue samples were collected and used for CD31 detection by immunohistochemical method and determination of miR-132 levels. *Results:* miR-132 levels and CD31 of heart tissue were higher after testosterone administration and in the voluntary exercise group in diabetic rats after 6 weeks. Combination of testosterone and voluntary exercise had synergistic effect on angiogenesis and miR-132 level. In castrated diabetic rats, there were significantly lower levels of miR-132 and CD31 in heart tissue compared to the diabetic group, whereas testosterone and exercise reversed these effects. In addition, testosterone supplementation plus exercise had an additive effect on miR-132 levels and CD31 in castrated diabetic rats. *Conclusions:* It was concluded that castration in rats leads to reduced miR-132 levels and subsequently decreased angiogenesis in diabetes. Testosterone plus voluntary exercise improved angiogenesis possibly through enhancement of miR-132 levels in heart of castrated diabetic rats.

Keywords: testosterone, voluntary exercise, miR-132, angiogenesis, diabetes

Introduction

Type 1 diabetes is a chronic and autoimmune disorder characterized by inappropriate hyperglycemia due to the lack of insulin (24). It was estimated that in 2017, 451 million people worldwide were affected by diabetes. Numbers are expected to rise to 693 million by 2045 (7). It was found that diabetics in general show an increased morbidity and mortality, predominantly with cardiovascular mortality as a specific cause of death (10). Actually, insufficient myocardial angiogenesis due to high glucose levels in the bloodstream is the major manifestation of diabetes-affected cardiovascular complications (11). Nevertheless, increasing heart angiogenesis and improving quality of life for diabetic patients with heart failure are an ongoing challenge for health-care providers.

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Testosterone is the main androgen that is needed for the development of normal sperm production and it also contributes to sex drive (28). Testosterone deficiency is common in men with diabetes (3). Although a direct relationship between testosterone deficiency and cardiovascular health remains controversial, there is evidence that patients with diabetes having a low serum testosterone level possess a higher incidence of coronary artery disease than those with a normal testosterone level (5). Numerous studies have shown that testosterone administration increased cardiac angiogenesis, highlighting the cardioprotective role of testosterone (6, 9, 29, 31).

Voluntary exercise has been shown to reduce risk of diabetes and is associated with various cardiovascular benefits (13). Voluntary exercise has tremendous potential to be used as an approach to promote cardiac angiogenesis by stimulating pro-angiogenic factors (15, 16). However, data on the effects of voluntary exercise on cardiac angiogenesis and related effects on expression of heart tissue miRNAs in diabetic animal models remain limited.

MiRs are small non-coding RNA molecules that are ~22 nucleotides long and modulate both physiological and pathological pathways by transcriptional and post-transcriptional gene expression (34). miR-132, also known as angiomiR, has recently been studied for its role in angiogenesis and cardiovascular disease (20). miR-132 promotes endothelial cell neovascularization by various pro-angiogenic stimuli such as VEGF (1, 20). Reports from preclinical animal models as well as recent clinical trials have shown that the downregulation of miR-132 is a major underlying mechanism for the development of microangiopathy in diabetic hearts (26).

Hence, considering the important role of exercise and testosterone in vascular formation, we examine the effect of voluntary exercise and testosterone replacement on heart angiogenesis by stimulating miR-132 in type 1 diabetic rats.

Materials and Methods

Animals

Sixty-three adult male Wistar rats each weighing 200–250 g were used. The rats were procured from the Tabriz Medical Faculty (Tabriz, Iran) and were kept under standard laboratory conditions (12-h light–dark cycle and 24 ± 3 °C) during experimentation period. The sedentary rats were group housed in normal plastic cages, whereas rats in the exercise group were placed in individual wheel-cage units. They were fed with standard rat pellet diet and water ad libitum. All procedures described were reviewed and approved by the Institutional Animal Ethics Committee (no. 92198).

Experimental design

The rats were bilaterally castrated, or sham operated under a ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (5 mg/kg) anesthesia. Some of the castrated male rats were injected with testosterone propionate (Unigen Life Sciences, Fremont, CA, USA), at a physiological dose of 2 mg/kg/day dissolved in dimethyl sulfoxide (DMSO) once daily for 6 weeks (6). Rats in placebo groups received DMSO vehicle with the same amount. Exercising male rats were housed individually in cages containing a stainless-steel running wheel. This running wheel was equipped with a digital magnetic counter that was activated by wheel rotation and wheel revolutions. Rats with running distance lower than ~2,000 m per day were eliminated before statistical analysis (32). Animals were randomly allocated to nine

treatment groups ($n = 7$): group 1, sham operation group (Sham): the operated diabetic rats received placebo; group 2, diabetic group (Dia): diabetic rats receiving placebo; group 3, testosterone group (Tes): diabetic rats receiving testosterone; group 4, exercise group (Exe): diabetic rats performing exercise; group 5, testosterone and exercise group (Tes-Exe): diabetic rats receiving testosterone and performing exercise; group 6, castrated group (Cas): castrated diabetic rats receiving placebo; group 7, testosterone–castrated group (Cas-Tes): castrated diabetic rats receiving testosterone; group 8, exercise–castrated group (Cas-Exe): castrated diabetic rats performing exercise; and group 9, testosterone and exercise–castrated group (Cas–Tes-Exe): castrated diabetic rats receiving testosterone and performing exercise.

Induction of type 1 diabetes

All animals received a single dose intraperitoneal (i.p.) injection of streptozotocin (STZ; 50 mg/kg, i.p.; Sigma-Aldrich, Oakville, ON, Canada) to induce diabetes. STZ was freshly dissolved in 0.1 M citrate buffer, pH 4.2. Two days after STZ treatment, type 1 diabetes was confirmed by blood glucose levels higher than 250 mg/dl (19). Induction of diabetes in rats was performed 7 days after castration surgery.

Preparation of tissue samples

After 6 weeks, the animals were anesthetized by i.p. injection of sodium pentobarbital (30 mg/kg b.w.) and heart tissues were excised and frozen in liquid nitrogen immediately. Tissue samples were weighted, homogenized in phosphate-buffered saline (pH 7.2–7.4), and centrifuged for 20 min at the speed of 3,000 rpm and 4 °C. Then, supernatants were collected in new tubes for miR-132 measurement.

Immunohistochemical assessments

Tissue samples from left ventricles were immediately collected for histopathology, fixed in buffered paraformaldehyde solution (4%), and embedded in paraffin. Then, sections were deparaffinized by sequential washing with xylene and dehydrated in a graded series of ethanol. Slides were treated respectively with proteinase K and treated by 0.3% hydrogen peroxide to block endogenous peroxidase activity. Sections were incubated with primary antibody CD31 (Santa Cruz, USA), used as an angiogenesis marker to represent the capillary vessels in myocardium, overnight at 4 °C. Slides were washed and incubated with standard avidin–biotin complex (Santa Cruz) according to the manufacturer's instructions. Next, the slides were developed with di-amino-benzidine (DAB; Santa Cruz), as the chromogen, and counterstained with Mayer's hematoxylin. Following the development with DAB, sections were cleared in xylene, mounted with Entellan, and assessed by light microscopy (Olympus BX 40, Japan). Capillaries were visualized in the myocardium as a brown precipitate. Vascular structure positive cells that were stained with the anti-CD31 antibody were counted for 5–6 slides per rat and 10 random fields per slide.

miRNA expression by real-time polymerase chain reaction (PCR)

For quantitative real-time PCR for miR-132, qRT-PCR was assessed. For each RNA sample, triplicate assays were carried out. MiRCURYTM RNA Isolation Kit (Exiqon, Denmark) was used for isolation of microRNA from the heart tissue according to the manufacturer's protocol. The procedure was performed based on a spin column using a proprietary resin as a separation matrix for RNA from other cell components. Nanodrop 1000 spectrophotometeric analysis

(Thermo Scientific, Wilmington, DE, USA) at a wavelength of 260–280 nm was performed to determine RNA content and purity. For cDNA synthesis, LNA universal RT miRNA PCR kit (Exiqon) was used. Briefly, total RNA containing microRNA was polyadenylated and cDNA was synthesized using a poly (T) primer with a 3′ degenerate anchor, and a 5′ universal tag. SYBR Green qPCR Mix (Exiqon) was used to monitor the amplification of cDNA. Real-time PCR was carried out using Rotor-Gene™ 6000 (Corbett Life Science, Sydney, Australia). Analysis was performed using the $2^{-(\Delta\Delta Ct)}$ method to determine relative quantitative levels of miR-132, and mir-1 was used as the endogenous control miRNA (22).

Statistical analysis

All data were tested against a normal distribution using the Kolmogorov–Smirnov test. Results are presented as means ± standard error of the mean. Significance was assessed using the two-way analysis of variance followed by multiple comparisons with Tukey's *post hoc* test. Statistical analysis was performed using SPSS software version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). Statistical significance was defined as $p < 0.05$.

Results

Effect of testosterone and voluntary exercise on miR-132 levels

We observed statistically significant difference in gene expression of miR-132 in both testosterone and exercise groups. Compared to the diabetic group, gonadectomized rats showed a decrease in miR-132 levels with a significant difference ($p < 0.001$; Fig. 1A). After 6 weeks of testosterone supplementation, the levels of miR-132 in the Tes ($p < 0.001$) and the Cas-Tes ($p < 0.01$) groups were significantly higher compared to the Dia and Cas (Fig. 1A). Six-week treatment of diabetic and castrated diabetic rats with exercise significantly raised the heart levels of miR-132 compared to the Dia and Cas groups ($p < 0.05$ and $p < 0.001$, respectively; Fig. 1B and C). *Post hoc* analysis revealed that combined treatment with testosterone and exercise increased miR-132 levels in diabetic animals. Actually, combination therapy with testosterone and exercise in diabetic and castrated diabetic rats increased miR-132 in comparison with the Dia and Cas groups ($p < 0.001$; Fig. 1B and C). Furthermore, combination therapy with testosterone and exercise in diabetic and castrated diabetic rats elevated heart levels of miR-132 compared to the testosterone and exercise group ($p < 0.05$ and $p < 0.01$, respectively; Fig. 1B and C). No significant difference was detected between sham and diabetic groups (data are not shown).

Immunohistochemical results

The representative images of CD31-positive microvascular blood vessel in the cardiomyocytes are presented in Fig. 2. The CD31-positive cell count was higher in the testosterone groups (Tes and Cas-Tes) when compared to the Dia ($p < 0.001$) and to the Cas groups ($p < 0.001$ and $p < 0.05$, respectively; Fig. 2A–D and I). As shown in Fig. 2F, H, J, and K, 6-week treatment of castrated diabetic rats with exercise along with testosterone supplementation significantly enhanced capillary density in the Cas-Tes-Exe and in the Tes-Exe groups compared to the Cas and the Dia ($p < 0.001$) groups. There is significant difference in the Cas-Tes-Exe group when compared to the Cas-Tes and the Cas-Exe groups ($p < 0.001$; Fig. 2D–F). Similarly, increased capillary density is also observed in the Tes-Exe group as compared to the Tes ($p < 0.05$) and the Exe ($p < 0.01$) groups (Fig. 2C, G, H, and K). No significant difference was detected between sham and diabetic groups (data are not shown).

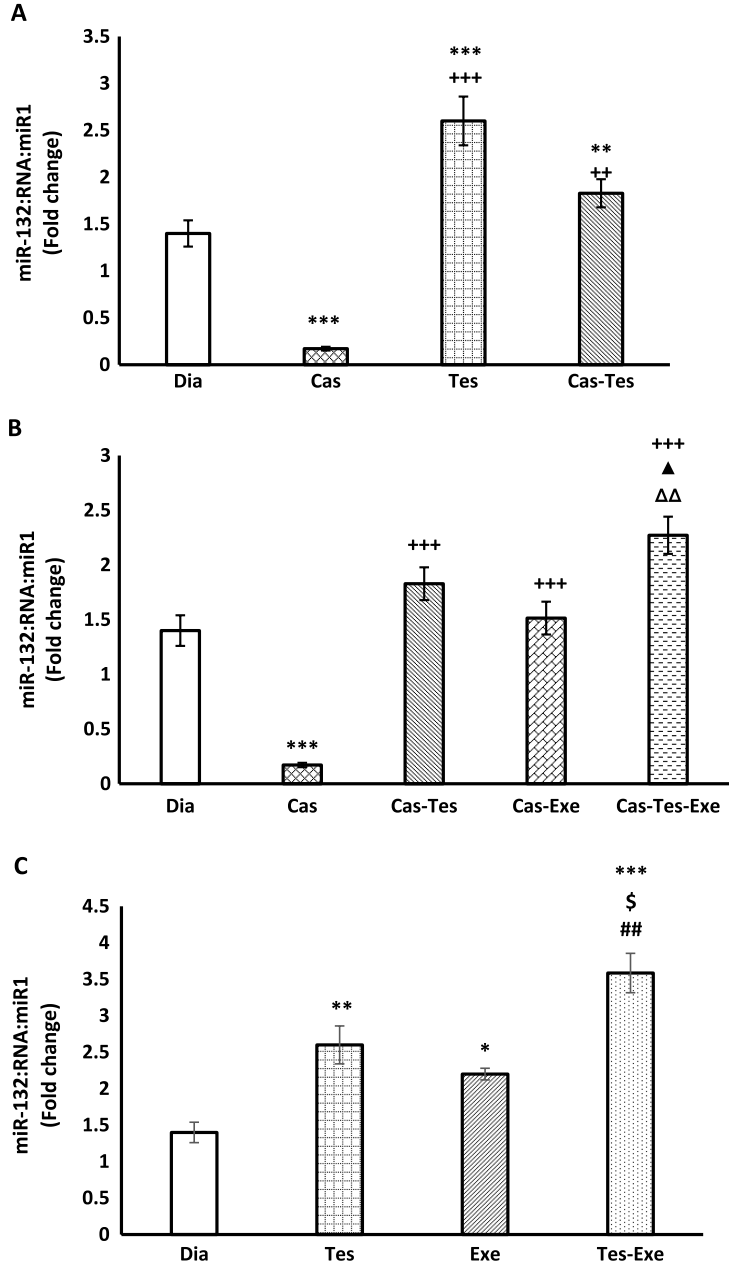

Fig. 1. Testosterone and voluntary exercise increased miR-132 levels in type 1 diabetic rats. (A) Effect of testosterone therapy on miR-132 levels in diabetic and castrated diabetic rats. (B) Effect of testosterone combined with voluntary exercise on miR-132 in castrated diabetic rats. (C) Effect of testosterone combined with voluntary exercise on miR-132 in diabetic rats. Data were represented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. Dia group. $^{++}p < 0.01$ and $^{+++}p < 0.001$ vs. Cas group. $^{▲}p < 0.05$ vs. Cas-Tes group. $^{ΔΔ}p < 0.01$ vs. Cas-Exe group. $^{\$}p < 0.05$ vs. Tes group. $^{\#\#}p < 0.01$ vs. Exe group

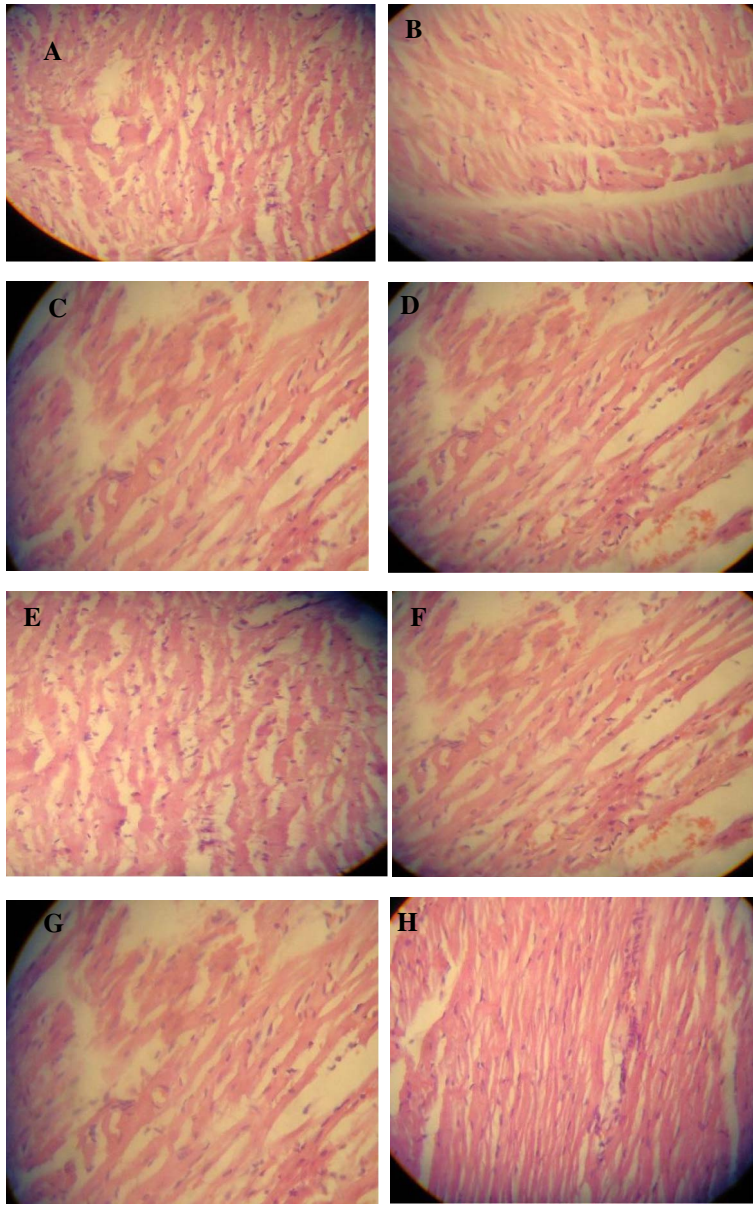

Fig. 2. Representative immunohistochemical CD31 staining of heart sections in different experimental groups (40× magnification and scale bar 50 mm). Arrow indicates the positive reaction for CD31. (A) Dia, (B) Cas, (C) Tes, (D) Cas-Tes, (E) Cas-Exe, (F) Cas-Tes-Exe, (G) Exe, and (H) Tes-Exe (I–K). Quantitative analysis of capillary density in counts/mm^2. Microvessel density was assessed by counting the number of microvessels in 10 randomly chosen high-power fields (40×) from three sections. Data were represented as mean ± SEM. ***$p < 0.001$ vs. Dia group. $^{+}p < 0.05$ and $^{+++}p < 0.001$ vs. Cas group. $^{\blacktriangle\blacktriangle\blacktriangle}p < 0.001$ vs. Cas-Tes group. $^{\Delta\Delta\Delta}p < 0.001$ vs. Cas-Exe group. $^{\$}p < 0.05$ vs. Tes group. $^{\#\#}p < 0.01$ vs. Exe group

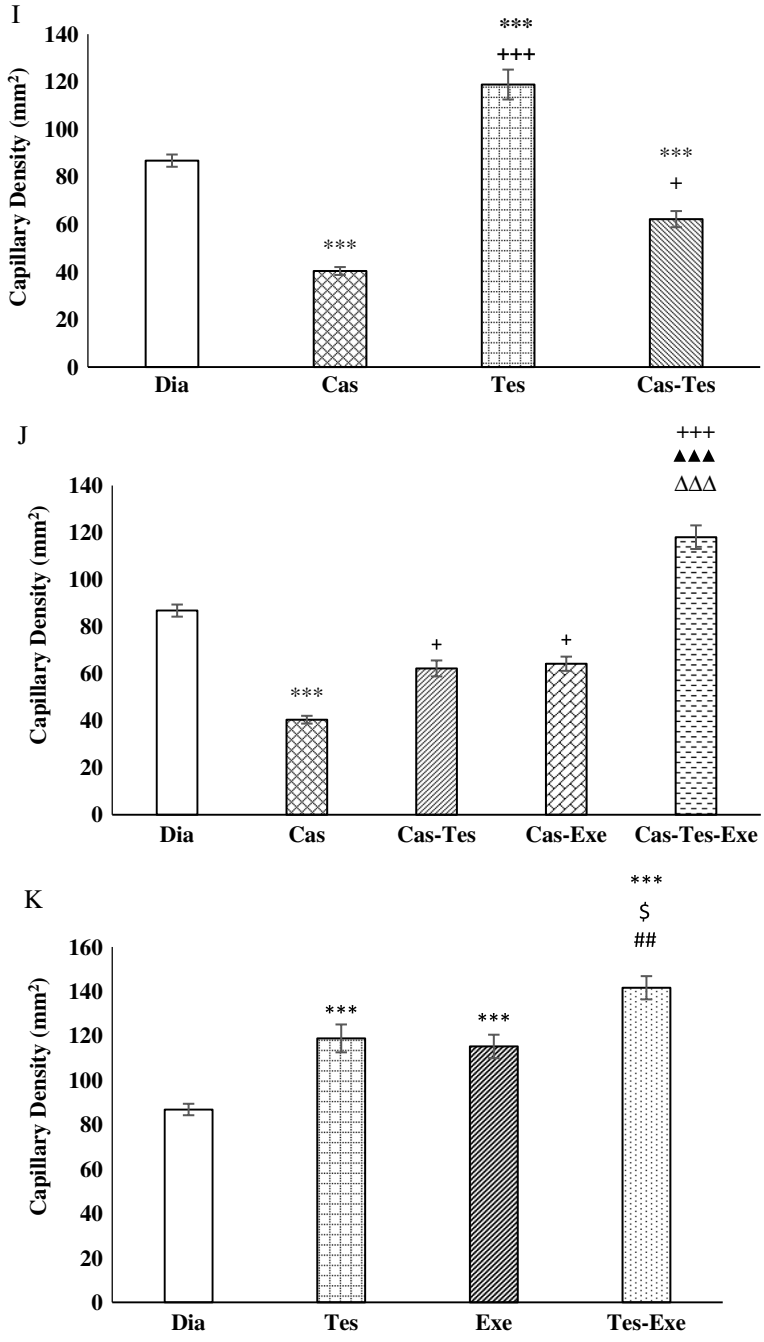

Fig. 2. (Continued)

Discussion

The novel finding of this study is that combined voluntary exercise and testosterone administration, according to our hypothesis, resulted in enhancement of heart angiogenesis in type 1 diabetes via expression of the miR-132, a proangiogenic microRNA in endothelial cells of the heart tissue.

Diabetes is a serious and common chronic disease that leads to severe complications. Impaired collateralization and angiogenesis in coronary artery are major complications of diabetes (2, 25). As previously reported by our group, diabetic rats in castrated condition show a further decrease in cardiac angiogenesis when compared to diabetic non-castrated rats (8). Testosterone levels showed a decrease in both types of diabetes, and diminished level of this hormone is the main factor for cardiovascular complications of diabetes (27, 33, 36). There is a potential relationship between low testosterone levels and loss of heart angiogenesis (6, 30). Thus, androgen replacement therapy in castrated type 1 diabetic rats could potentially improve angiogenesis and cardiovascular events. In relation to improve microvascular density in diabetes, previous studies showed that exercise induces fiber-type-specific angiogenesis and reduces cardiovascular disease-specific mortality in humans with diabetes (14, 17). Rats in our voluntary wheel-running model were able to select the time, duration, and intensity of exercise in a non-stressful environment. Therefore, we propose that combining voluntary exercise with testosterone has potential as a method to combat diabetes-related cardiovascular complications. Although regular exercise training and testosterone have been confirmed as cardioprotective treatments, the precise underlying mechanisms for their beneficial angiogenic effects remain to be defined.

This study demonstrates that voluntary exercise and testosterone increase miR-132 levels, which exert proangiogenic activity in a rat model of diabetes. miR-132 is a highly conserved miRNA transcribed from an intergenic region on human chromosome 17 that is induced in endothelial cells in response to vascular endothelial growth factor (VEGF) (23). Recently, we have shown that exercise and testosterone enhanced expression of VEGF-A in the heart tissue of castrated diabetic rats (8). VEGF can rapidly induce the transcription factor cAMP response element-binding protein that increases expression of miR-132 in endothelial cells (35). Overexpression of miR-132 increased endothelial cell proliferation by targeting p120RasGAP, a GTPase-activating protein (1). In this study, we have also demonstrated downregulation of the proangiogenic miR-132 and the reduction of cardiac angiogenesis in the heart of diabetic rats. With the progression of the disease and the downregulation of miR-132, there was a consequent upregulation of p120RasGAP in the diabetic heart and repaired angiogenesis. In this study, we have shown that voluntary exercise and testosterone were able to increase miR-132 in activated endothelial cells, where it can suppress p120RasGAP expression leading to neovascularization. Moreover, this effect was augmented when exercise and testosterone were combined. In agreement with our result, Malkin et al. (21) reported that administration of low physiological replacement doses of testosterone with exercise can delay the time to ischemia in men with coronary artery disease. Bhasin et al. (4) demonstrated that high intramuscular dosages of testosterone combined with resistance exercise result in significantly greater increase in muscle size and repletion of lean body mass than either intervention alone. Dos Santos et al. showed that testosterone treatment combined with exercise in heart failure patients reduced muscle atrophy. These authors stated that testosterone treatment alone did not seem to be useful in these patients in the absence of exercise (12). In contrast with our results, Hildreth et al. (18) reported that there were no

additive effects of testosterone therapy plus resistance training on strength or physical function in healthy older men with low testosterone levels. This contradiction may be due to the difference in both the type and the duration of exercise. Regarding the limitation of this study, we did not measure other miRNAs involved in heart angiogenesis.

Conclusions

In this study of castrated diabetic rats, 6 weeks of testosterone administration and voluntary wheel running increased miR-132 that led to heart angiogenesis. Moreover, testosterone treatment plus exercise produced greater improvements in heart angiogenesis than either intervention alone. These findings may be of importance for future therapeutic interventions of cardiac angiogenesis in diabetes, where signaling pathways involved in heart angiogenesis can be targeted.

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