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Investigating the Potential of Sildenafil in Mitigating Fetal Growth Restriction: A Study on Experimentally Restricted Fetal Growth Rats

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ABSTRACT

Intrauterine growth restriction (IUGR) is a common pregnancy complication. Sildenafil is also emerging as a potential candidate for the treatment of IUGR. The objective of this study was to evaluate the effect of sildenafil in the prevention of IUGR due to impaired uterine blood supply in rats. Female rats were randomly divided into three groups once the pregnancy was confirmed. The control group received normal saline, and the Sild40 and Sild80 groups received 40 and 80 mg/kg of sildenafil. The uterine artery was clamped for 60 minutes on day 17 of gestation. Amniotic fluid was collected from viable and nonviable fetuses and subjected to metabolite measurement. The live and dead fetuses, the weight and size of the placenta and fetus were counted during the first celiotomy and Cesarean section on day 21 of gestation. 45%, 90%, and 83.88% of fetuses were alive in the Control, Sild40, and Sild80 groups, respectively. No significant difference in placenta weight, fetus weight, and fetus length, were found. However, differences of the liver-to-body and brain-to-body ratio were significant. Amniotic fluid metabolites showed no significant difference between the groups except for lactate. In addition, post-ischemia glucose was reduced significantly in the Control group. Furthermore, pyruvate was significantly increased and lactate was significantly reduced post-ischemia in the Sild40 and Sild80 groups, respectively. In conclusion, sildenafil seems to counteract the effects of uterine ischemia in pregnant rats and prevent IUGR which results from temporary occlusion of the uterine artery.

Introduction

Fetal growth restriction (FGR) or intrauterine growth restriction (IUGR) refers to the failure of the fetus to reach its full growth potential. It affects up to 8% of pregnancies and is the second leading cause of infant mortality after premature birth. There is currently no treatment available for FGR. Fetuses that encounter growth restriction in the womb are at risk of prenatal and postnatal events, including stillbirth, premature birth, and negative long-term outcomes. Therefore, identification and monitoring fetal growth restriction is an important part of prenatal care.[1,2,3] The primary cause of IUGR, when there is no structural or genetic defect in the fetus, is related to placental insufficiency. In such a situation, the fetus has a problem receiving enough nutrients and oxygen. This factor is influenced by various factors, such as changes in maternal or fetal blood flow, reduced transport of nutrients, or changes in the placenta, such as an increase in thickness, which hinders the nutrient transfer.[4]

Phosphodiesterase 5 (PDE5), like some other phosphodiesterase isoforms, hydrolyzes cyclic guanosine triphosphate (cGMP) to GMP. cGMP activates protein kinase G (PKG), leading to decreased calcium

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influx, resulting in smooth muscle relaxation. Sildenafil is a PDE5 inhibitor that raises the cGMP level.[5] Sildenafil is known for its strong relaxing effect on the smooth muscles of blood vessels by prolonging the guanosine monophosphate (cGMP) cycle. It is commonly used to treat erectile dysfunction in men. Although it has also been used to treat sexual dysfunction in women, the results of a review of 3000 women were not satisfactory.[6] Experimental studies have suggested that sildenafil could be a potential drug for the treatment of IUGR in both animal and human models.[7-9] The results of *in vitro* studies showed that sildenafil inhibits the uterotonic potential caused by oxytocin, and this effect could be related to the action of the drug on cGMP.[10]

Given the significant importance of prevention and treatment of IUGR, this study aimed to investigate whether sildenafil could mitigate the adverse effects of ischemia on fetuses because of its vascular dilation effects, in experimentally restricted fetal growth rats.

Materials and Methods

Animals

Fifteen adult female Wistar rats with the same weight range (250 ± 20 gr) were used in this study. They were housed in 22-25 °C temperature and 12 hours of dark/light cycle in separate cages. Semi-synthetic pellets and drinking water were provided *ad libitum*. To acclimate the animals to the manipulations, they were exposed to restraining towel once a week and intragastric gavage of water was done. All animal procedures complied with the ARRIVE guidelines and carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Experimental Design and Surgical Procedure

To make female rats pregnant, they were placed in a cage with male rats overnight for mating, and after observing the vaginal plugs in the early morning, randomly divided into three equal groups. The control group received normal saline (0.9% sodium chloride solution, Shahid Ghazi Co., Iran) from the first day to the 21st day of pregnancy using an intragastric gavage tube. Sildenafil 100 mg coated tablets (SDF 100, Marham Daru Co., Tehran, Iran) at doses of 40 and 80 mg/kg was gavage orally in the Sild40 and Sild80 groups, respectively, after dissolving in the normal saline.

All pregnant females were anesthetized on the 17th day of pregnancy by intraperitoneal injection of 5% ketamine HCl (80 mg/kg, Rotexmedice, Trittau, Germany) and 2% xylazine (5 mg/kg, Alfasan, Woerden, Holland).[11] Ventral midline laparotomy was performed after aseptic preparation of the area. The horns of the pregnant uterus were identified, and the number of implantations was counted. Using a vascular atraumatic clamp (Codman 20-1200 DRAKE Aneurysm Clip, 16 mm),[12] the uterine artery on the side that had more fetuses was clamped for 60 minutes (Figure 1). The abdominal incision was temporarily closed using towel clamps. The clamps were removed 60 minutes after the onset of ischemia. The *linea alba* and skin incisions were sutured in simple continuous pattern with 4-0 polyglycolic acid suture material (Dexon II, U.S. Surgical, Norwalk, CT, USA), and 3-0 nylon suture material (Monofil Polyamide, SUPA, Iran), respectively. Cefazolin (50 mg/kg, Cefazolin-Exir, 500 mg vials, Exir Pharmaceutical Co., Iran) was administered intraperitoneally.

Fetal Evaluation

On the 21st day of pregnancy, a Cesarean section was performed after general anesthesia. The number of alive, dead, and absorbed neonates was counted and compared with the number of implantations counted on day 17. The reabsorption rate of fetuses was determined from the number of fetuses counted on the 17th day of pregnancy minus the number of fetuses found during Cesarean section on the 21st day. Fetal survival was calculated from the total number of live fetuses found during Cesarean section.

Neonatal survival was evaluated using macroscopic parameters such as color, body consistency, and size. The rats were euthanized by intracardiac overdose injection of sodium thiopental. If the fetus was alive, its weight was measured by a digital scale and the head-to-tail distance, and the size of their abdomen were measured by a digital caliper. The placenta weight was measured after separation from the fetus. In addition, the kidney, liver, spleen, lungs, and heart were examined for color, consistency, and presence of blood spots. Liver and brain weight were measured and recorded and their ratio to total body weight was calculated.

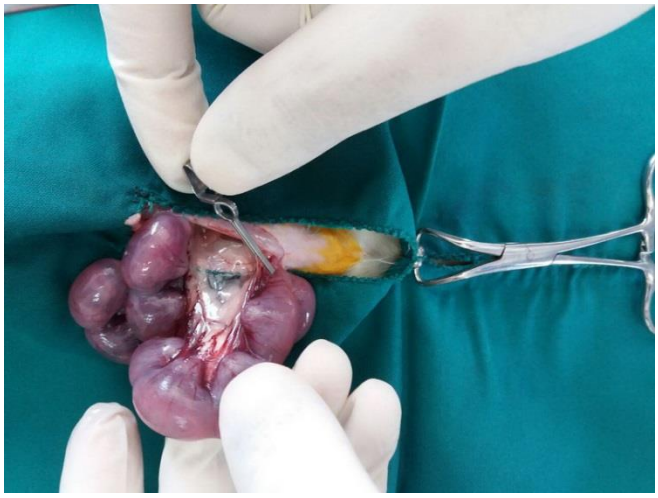

Figure 1. Surgical intrauterine growth restriction with temporary uterine artery occlusion with a vascular atraumatic clamp.

Biochemical Analyses

Samples of the amniotic fluid (AF) were obtained before (day 17) and after (day 21) induction of ischemia from viable and dead fetuses and stored at -20 °C. However, no samples were collected from absorbed fetuses as they had shrunk and lacked any fluid. Glucose, urea, lactate, and pyruvate were measured using commercial kits (Pars Azmoon, Iran) and according to the manufacturer's guides on an Auto Analyzer (Hitachi 917, Roche Diagnostics, Indianapolis, IN, USA).

Statistical Analysis

For statistical analysis, Minitab software (version 16.2.0, Minitab Inc., State College, PA, USA) was utilized. The analysis of variance (ANOVA) statistical method was employed to analyze all numerical data except for pre- and post-ischemia biochemical parameters comparisons, in which t-test was employed. Tukey's post hoc test was used to compare between groups. A p-value of <0.05 was considered indicative of a statistically significant difference.

Results

From the total of 20 fetuses in the uterine horn in which ischemia occurred and were counted in the first celiotomy, there were 9 alive (45%), 6 dead (30%) and 5 (25%) were absorbed in the control group. The body consistency of dead fetuses in this group was lost and they had a darker color (Figure 2). They were mostly near the site of the uterine artery clamp. In the Sild40 group, 18 alive (90%) and only 2 absorbed fetuses (10%) were observed, and no dead fetuses were detected out of a total of 20 fetuses in the ischemic horn. In the Sild80 group, the number of 26 alive fetuses (83.88%), only 2 dead (6.45%), and 3 absorbed fetuses (9.67%) were seen from the total of 31 fetuses in the ischemic horn. There was a statistically significant difference between the number of alive and dead fetuses in the Sild40 and Sild80 groups in comparison to the control group ($p \leq 0.05$). The number of absorbed fetuses in the Sild40 and Sild80 groups was lower than in the control group, however, the differences were not statistically significant ($p \geq 0.05$). (Figure 3).

The results related to the fetal and placental weights and sizes are shown in Table 1. There was no statistically significant difference ($p \geq 0.05$) between the treatment groups and the control group in parameters related to placenta weight, fetal weight, fetal length, and fetal back-to-abdomen distance. However, these differences were statistically significant between the control and treatment groups regarding the brain-to-body weight ratio and the liver-to-body weight ratio ($p \leq 0.05$). The brain-to-body weight ratio in the Sild40 and Sild80 groups was less than the control group, and the liver-to-body weight ratio in the Sild40 and Sild80 groups was more than the control group.

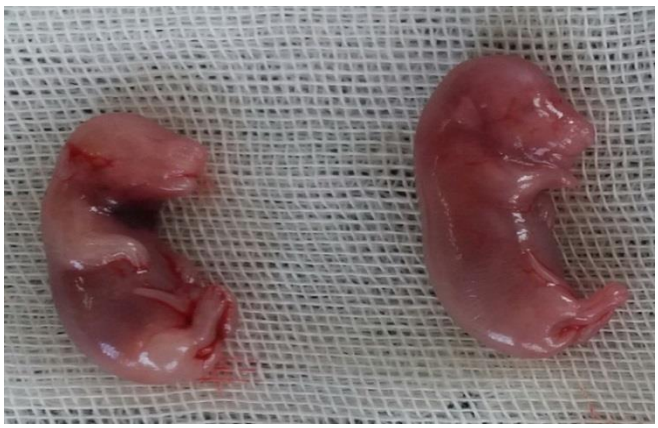

Figure 2. Dead fetuses with dark color in the control group on the 21st day of pregnancy.

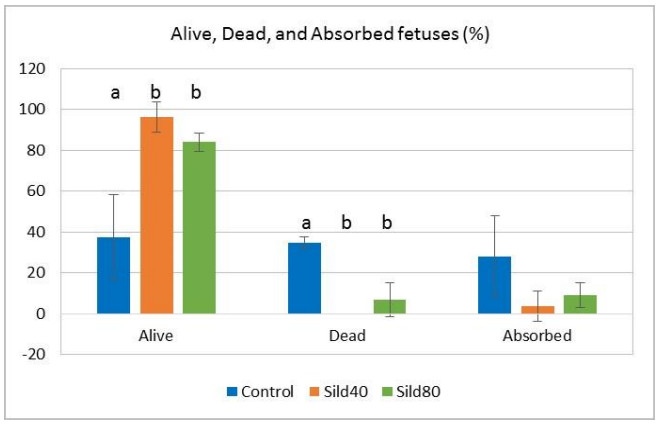

Figure 3. The percentage of alive, dead, and absorbed fetuses (mean ± SD) in day 21 of gestation after uterine artery ischemia in day 17 of gestation in three experimental groups of rats. a, b: different letters in each line indicate statistically significant difference ($p < 0.05$).

Amniotic fluid analyzes (Figures 4 and 5) showed not any statistically significant difference among the groups both pre- and post-ischemia, except for post-ischemia lactate, in which the metabolite concentration in the Sild40 group was found to be higher than that in the Sild80 group ($p = 0.034$) (Figure 5). In addition, post-ischemia glucose was reduced compared to pre-ischemia. However, this difference was only statistically significant in the Control group. Furthermore, pyruvate was significantly increased post-ischemia in the Sild40 group, and lactate was significantly reduced post-ischemia in Sild80 group (Table 2).

Discussion

In this study, sildenafil in pregnant rats that suffered uterine ischemia significantly increased fetus survival rate and promoted their growth. Moreover, the ratio of the fetal brain-to-body weight was significantly different between the groups, so that the highest ratio was in the control group and the lowest ratio was in the Sild40 group. Regarding the ratio of liver-to-body weight, the differences were significant only in the Sild80 group compared to the control group.

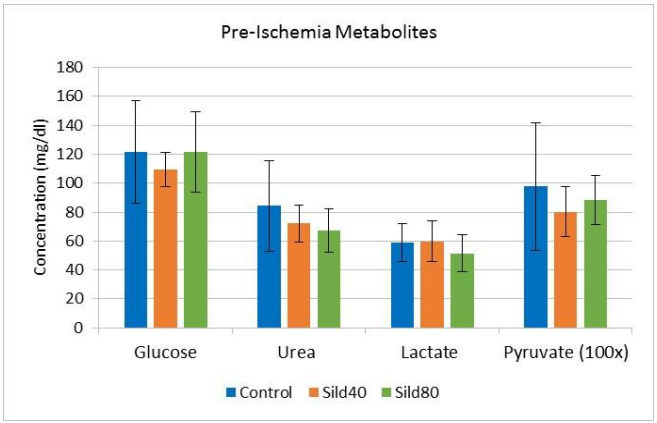

Figure 4. Amniotic fluid metabolites including glucose, urea, lactate, and pyruvate (10×) concentration (mean ± SD) before ischemia in experimental groups.

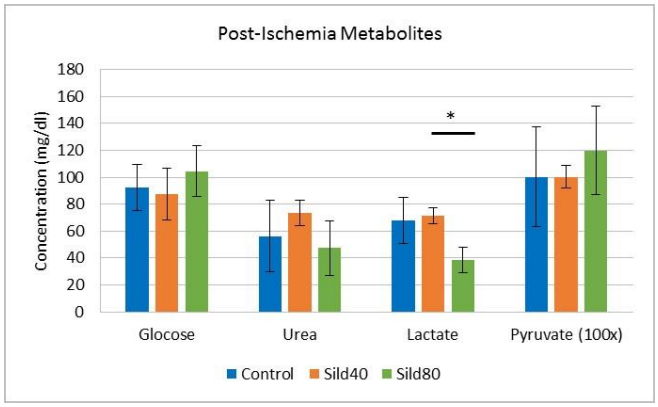

Figure 5. Amniotic fluid metabolites including glucose, urea, lactate, and pyruvate (10×) concentration (mean ± SD) post ischemia in experimental groups. * indicates statistically significant difference between two groups.

Table 1. The weight of the placenta, the weight of the fetus, the fetal head-to-tail distance, the fetal back-to-abdomen distance, the brain-to-body weight ratio, and the liver-to-body weight ratio (mean ± SD) in day 21 of gestation after uterine artery ischemia in day 17 of gestation in three experimental groups of rats.

Parameter	Groups			p-value
	Control	Sild40	Sild80	
Weight of the placenta (gr)	0.58 ± 0.12	0.58 ± 0.17	0.49 ± 0.06	0.578
Weight of the fetus (gr)	1.86 ± 0.11	2.90 ± 0.62	2.33 ± 1.24	0.243
Head-to-tail distance (mm)	27.35 ± 1.62	29.95 ± 3.03	29.23 ± 5.64	0.624
Back-to-abdomen distance (mm)	10.05 ± 1.55	11.60 ± 0.70	11.65 ± 1.46	0.193
Brain-to-body weight ratio (gr)	0.09 ± 0.00[a]	0.04 ± 0.01[b]	0.06 ± 0.01[c]	0.000
Liver-to-body weight ratio (gr)	0.05 ± 0.01[a]	0.06 ± 0.01[ab]	0.08 ± 0.01[b]	0.022

a,b,c: different letters in each line indicate statistically significant difference ($p < 0.05$).

Table 2. Comparison of glucose, urea, lactate and pyruvate levels before (day 17) and after (day 21) ischemia in the studied groups . Mean ± SD, mg/dl

		Control	Sild40	Sild80
Glucose	Pre	121.63 ± 35.24[a]	109.45 ± 11.60	121.60 ± 27.87
	Post	92.30 ± 17.29[b]	87.70 ± 19.19	104.32 ± 18.83
	p-value	0.029	0.294	0.127
Urea	Pre	84.33 ± 31.29	72.05 ± 12.63	67.40 ± 15.15
	Post	56.10 ± 26.60	73.60 ± 9.23	47.28 ±20.17
	p-value	0.06	0.895	0.103
Lactate	Pre	59.06 ± 13.18	59.82 ± 14.1	51.52 ± 12.56[a]
	Post	68.03 ± 17.01	71.30 ± 5.92	38.50 ± 9.43[b]
	p-value	0.205	0.294	0.017
Pyruvate	Pre	1.03 ± 0.48	0.80 ± 0.17[a]	0.88 ± 0.16
	Post	1.00 ± 0.37	1.00 ± 0.08[b]	1.19 ± 0.32
	p-value	0.926	0.032	0.218

a,b: different letters in each line indicate statistically significant difference ($p < 0.05$).

Intrauterine growth restriction can either result in symmetric or asymmetric growth restrictions. Symmetrical growth restriction is a condition in which the entire fetal body is small. In asymmetric growth restriction, the growth of vital organs such as the brain and heart is maintained and preserved in normal size, but other structures such as the liver, muscles, and fat are destroyed as a result of insufficient intake of nutrients. This situation can turn into symmetric growth restriction if the growth restriction factor is severe or stable enough.[13] In this study, it was found that 60 minutes of ischemia in the uterine artery of a pregnant rat causes asymmetric growth, so that the ratio of the brain-to-body weight of the fetus was increased, and on the other hand, the ratio of the fetus liver-to-body weight was decreased in the control group. However, this ratio was lower in the brain and higher in the liver in the treatment groups compared to the control group. It was also found that a low dose of sildenafil (40 mg/kg) has a better effect than a high dose (80 mg/kg) in improving asymmetric growth.

Uterine artery ligation has long been used in animal models such as sheep, rabbits, or rats to induce experimental IUGR.[14] However, this method also has some weaknesses, including high embryo absorption rate (at least 30%) and inability to cause IUGR in some cases.[15] In our study, fetal reabsorption in the control group was similar to the reported rate, and the model was able to create IUGR.

Wigglesworth and coworkers (1974) first stated that unilateral occlusion of the uterine artery in pregnant rats causes a 40% decrease in fetal weight and an increase in brain weight and is associated with mortality and high fetal absorption. In addition, fetuses with severe growth disorders are close to the ligature site.[16] Tanaka *et al*. (1994) found that 60 minutes of ischemia cause a significant decrease in fetal weight and the liver-to-body weight ratio. However, they found that ischemia does not affect the placenta-to-body weight ratio. In their study, the rate of fetal death was reported as 14%.[17] Singh *et al.* (1983) stated a decade earlier that 30 minutes of ischemia on day 14 of pregnancy causes histopathologic changes in the rat placenta on day 21.[4] In the current study, with uterine artery ischemia for 60 minutes, a significant decrease in the liver-to-body weight ratio and a significant increase in the brain-to-body weight ratio were observed in the control group compared to the treatment groups, which is consistence with the previous works. However, the mortality and the absorption rate of the fetuses in the control group were slightly higher compared to the above-mentioned studies, which can be attributed to the manipulation of the animal during intragastric gavage. Furthermore, our results showed that sildenafil has prevented the effects of ischemia in fetuses by increasing placental blood flow.

Price and coworkers (1992) stated that rat uterine artery occlusion caused a significant decrease in fetal weight, liver weight, and placental weight.[18]

A research showed that sildenafil increases uterine blood flow on non-pregnant ewes.[19] Administration of sildenafil neither caused weight or histopathologic changes in the rat testes and ovaries, nor emerged embryotoxicity. Additionally, it has been stated that there is no effect on mating behavior, pregnancy success, and other reproductive parameters in studies conducted on the fertility of mice.[20] It is suggested that sildenafil reduces intrauterine pressure during labor and prevents premature birth.[8] Belik (2005) stated that sildenafil increases the weight of fetuses during pregnancy in rats.[7] Refuerzo *et al.* (2006) found that sildenafil increased the weight of rat newborns in hypoxic conditions.[21] Sher and Fisch (2000) stated that intravaginal sildenafil in combination with estradiol improves uterine blood flow and endometrial thickness women with miscarriage.[22] The results of our study showed that sildenafil can prevent the effects of ischemia by increasing placental blood flow compared to the control group.

Some studies report that sildenafil could not be effective in this field. The results of Nassar and coworkers's study (2012) showed sildenafil did not reduce L-NAME-induced mortality and IUGR, and may even have a negative synergistic effect on infant weight. Also, the rate of fetal mortality in the group that received only sildenafil was similar to the control group, and the weight of the fetuses was lower than the control group.[23] However, the findings of this current study, unlike the above research, showed a positive effect in improving the condition of the fetus after ischemia.

Dilworth *et al.* (2013) showed that oral sildenafil in pregnant mice suffered from growth restriction due to the elimination of IGF2 causes an increase in fetal weight and back-to-abdomen distance, but it does not create a significant effect on placental weight, head-to-tail size, and head circumference.[24] Herraiz and coworkers (2012) investigated the effects of sildenafil on fetuses in pre-eclamptic rats and stated that sildenafil normalizes blood pressure, cell count, and proteinuria of the mother. Additionally, sildenafil increases weight and survival rate without any phototoxic effects in litters.[25] Maharaj *et al.* (2009) found that phosphodiesterase-5 (PDE-5) exists in the muscular layer of chorionic arteries and sildenafil causes dose-dependent vasodilation in this circulation. They also stated that incubation with direct cGMP inhibitor (methylene blue) and cGMP-dependent protein kinase inhibitor (Rp-8-Br-Pet-cGMPs) significantly reduces the vasodilation effect of sildenafil. Additionally, inhibition of nitric oxide production by incubation with L-NAME (non-specific inhibitor of nitric oxide synthase) does not reduce the effect of sildenafil. In contrast, sildenafil significantly and dose-dependently increases the vasodilation produced by sodium nitroprusside (a NO donor).[26] The results of a study by Wareing *et al.* (2005) showed that vascular contraction is increased, and endothelium-dependent relaxation is reduced in pregnancies with FGR compared to normal pregnancy.[27] Wareing *et al.* (2006) noted that in pre-eclampsia women the endothelial function of these vessels is disturbed. However, they stated that one hour of incubation with sildenafil could not change the contractile response to vasoconstrictors (e.g. AVP and U46619) or endothelial vasodilators (e.g. acetylcholine and bradykinin) in the small arteries of the chorionic plate and omental arteries.[28] The results of *in vitro* studies on uterine tissue in non-pregnant rats showed that sildenafil inhibits the uterotonic potential caused by factors such as oxytocin, and it was stated that this effect could be related to the action of the drug on cGMP.[10] In addition, *in vitro* studies were conducted on uterine myometrium tissue of women who underwent Cesarean section and it was stated that the relaxation of myometrium is independent of cGMP and probably dependent on K channels.[29] Trapani *et al*. (2016) concluded that sildenafil decreases the pulsatility index in the uterine-placental (uterine artery) and fetoplacental (umbilical artery) blood circulation and a moderately decreased maternal blood pressure in women with IUGR.[30]

Amniotic fluid is a complex substance that is

generated by various tissues within the mother, fetus, and placenta. It serves as a reflection of the physiological processes involved in fetal development and holds significant diagnostic value for assessing fetal health.[31] Although, we did not notice any significant difference for amniotic fluid metabolites before induction of ischemia, sildenafil decreased lactate 72 hours after ischemia. This effect was pronounced for higher dose of sildenafil. PDE5 inhibitors do not considerably influence physical performance in normoxia, while, they may increase exercise capacity in hypoxic conditions.[32] Sildenafil improved microcirculation and redistributed blood flow, and thus reduces hypoxia-ischemia damages.[33] 24 exposure to 1 μM tadalafil (a PDE5 inhibitor) increased the activity of citrate synthase and hydroxy acyl-CoA dehydrogenase, two enzyme including in aerobic oxidation of metabolic fuels, in C2C12 myocytes. This effect was not observed for lower dose of tadalafil and shorter exposure times.[34] 2 weeks administration of 10 mg/kg sildenafil reduced the expression and activity of the key glycolytic enzyme LDH in rat model of hepatocellular carcinoma.[35] Sildenafil may enhance aerobic metabolism, increase blood flow, and thus increase the energy yield of the tissue in a dose- and time-dependent manner.

In conclusion, it can be stated that 40 mg/kg sildenafil citrate can neutralize the effects of uterine ischemia in pregnant rats and prevent the intrauterine growth restriction of fetuses caused by the temporary blockage of the uterine artery.

Conflict of Interest

The authors declare no conflicts of interest.

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