Role or synergistic interaction of adenosine and vitamin D3 versus high intensity interval training and isocaloric moderate intensity training: An experimental protocol

Abstract

Background: Obesity is known as one of the most major cause of epidemiologic disease worldwide. Therefore, introducing treatment strategies including various medicines to reduce fat or prevent obesity in addition to methods of exercise programs by medical professionals and exercise scientists is on the increase. Recently, researchers have shown special interest in assessing the effect of lipolytic adenosine and vitamin D deficiency. On the other hand, the effect of exercise on decreasing body fat percent has been indicated by many researchers.

Objective: This research has been designed to examine the effect of injection of adenosine, vitamin D3 and High intensity interval training and isocaloric Moderate intensity training on metabolic parameters in obesity induced by high-fat diet.

Methods: This is an experimental study. We will select 79 rats and then after getting weight till to 6 weeks will divide in 12 groups. After one week of adaptation to a new environment, 12 groups of Wistar rats will participate in two stages of the experimental intervention including 13 weeks of fattening diet followed by 12 weeks of exercise program and injection of adenosine and vitamin D3. All the rats in the normal diet(except 1 and 2 groups) will consume 40% fat and have free access to food and water up to the second half of the second stage (end of the sixth week of training). After termination of the interventions, tissue collection and molecular assessments (blood for biochemical, tissues for gene expression analyses and anthropometrical indexes) will be performed.

Results: The project was founded in April 2017 and completed in December 2017. Data analysis is under way, and the first results are expected to be submitted for publication in July 2018.

Conclusion: We supposed that weight loss induced molecular changes and up regulation will be observed in line with increase in lipolysis and beta oxidation in muscle and fat tissue as a result of performing isocaloric training in drug receiving rats and groups on high fat diet.
**Key words:** Obesity, high intensity interval training, isocaloric moderate intensity training, vitamin D3, adenosine, lipolysis, weight loss.

**Introduction**

Obesity is one of the main health risk factors [1,2] and the major cause of diseases including metabolic syndrome [3], type II diabetes, high blood pressure and cardiovascular incidence worldwide. The High intake of energy within the body results in abnormal accumulation of fat in adipose tissues [4] which has deteriorating effects on health, life quality and aging [5]. Thus, the damage of glucose and fat metabolism pathways and disturbance in metabolic balance of these interrupted conditions [6-8] result to the incidence or development of fat related diseases [9].

Plant based substances [4,10,11] and medicines [12-16] have been proposed as strategic treatment and preventive measures for obesity. Presently, one of the most challenging issues in the field of pharmacy is the discovery of the most effective anti-obesity intervention with the least negative side effects on humans. Recently, researchers have focused on the effect of most active forms of vitamin D and have shown its controlling role in the incidence of obesity [13-17]. On the other hand, while some studies have shown that the adipogenesis [24,25] continues through different mechanisms, others have focused on identifying intervention factors that can lead to weight loss, particularly, fat weight. The researchers, revealing the undetected effects of molecules driven from ATP, ADP and AMP called adenosine [26].

Depending on the type and dependency of adenosine on specific G protein, it has link with one of the 4 receptors named A1, A2A, A2B and A3 in different tissues showing different functions [27-31]. In contrast to the clinical findings, exercise science training experts rely on effective, preventive and treatment effect of different types of exercise program on obesity which is relatively consistence without any harmful side effect. On the other hand, considering the significance of intensity and duration of exercise training programs [32], HIIT programs have been identified as a fat controlling intervention [33]. The control of weight increase due to the high fat content of diet [34] compared to the stable aerobic activity, improvement in fat distribution and insulin with similar energy cost [35], has an effect on obesity. Regardless of the benefits of physical activities in improvement of obesity, there are inconsistent findings with regards to decrease in fat indices through participation in physical activities without calorie restriction. In addition, there are limited number of studies on the significance of calorie
consumption based on exercise training volume in contrast to response to different types of training, leading to the same changes in metabolic conditions of obesity induced high fat diet with participation in HIIT and isocaloric MIT program as observed earlier [36]. Thus, considering the significance of finding an anti-obesity medicine to reduce weight with less harmful side effects and the undeniable effect of exercise as medicine [37] for health and longevity. It seems necessary to examine and compare the effect of medicine, exercise and their interaction on health. Therefore, due to the inadequate knowledge to introduce harmless medicine to control the increase of volume and size of fat cell and enhance fat burning activities in high fat diet. it seems adenosine (by activating adenosine receptors in response to the density and release of adenosine within the cell that leads to different processes of fat burning) and vitamin D in interaction and synergy with isocaloric sport training may play a significant role in the reduction of fat accumulation, lipolysis regulation and insulin sensitivity in vital metabolic organs including the liver, muscle, different fat tissues, which may eventually lead to weight loss.

Study Objective
The primary objective of the study is to compare the incidence of anthropometrical parameters outcomes after drug and exercise program interventions.
The secondary objective is to investigate the effects of exercise volume specially exercise volume control with and without adenosine injection and VD3 on gene expression between metabolic organs.
In addition, we aim to determine the new impact adenosine and VD3 related to the fat weight loss.

Methods
Animals
In our experimental study, eighty- nine male Wistar rats will be prepared by the Biomedical and experimental sports research center of Shahid Mirghani. They will be kept in similar laboratory environment condition at 22±3°C in 12 hour day-night cycle. All the rat will be fed on a normal diet till 5 to 6 weeks to gain of 182.32 g weight. After one week of adaptation to a new environment, 12 groups of Wistar rats will participate in two stages of the experimental intervention including 13 weeks of fattening diet plan (will consume 40% fat) followed by 12 weeks of exercise program. All the rats in the normal diet (except 1 and 2 groups) have free
access to food and water up to the second half of the second stage (end of the sixth week of training). In the beginning of the seventh week, the amount of food given to all the groups will be prepared based on gram scale (based on the mean value of food in groups 3 and 6) for 6 weeks in an identical scale. This process will continue until the end of the training stage. Anthropometric measures will be assessed for all the groups including: weight per week, body mass index, waist and chest size and their ratio, height, Lee index, calorie consumed, the ratio of weight gain to the total amount of food consumed and the ratio of weight gain to the total calorie in a specific time, monthly and also the amount of food consumed daily by every rat.

**Diet**

Normal diet will contain 4.30 kcal per gram including 3.87% fat (Soy oil), 17.46% casein protein, 68.7% carbohydrate, 8.97% minerals and 1% vitamin. High fat diet will contain 5.81 kcal per gram with 40% fat (20% soy oil and 20% (animal fat) subcutaneous fat oil), 14.1% casein protein, 36.58% carbohydrate, 8.4% mineral and 0.72% vitamin.

**Experimental Groups**

Prior to any intervention, all 89 rats will be assigned randomly to 12 groups while matched for their weights. Two of these groups will serve as the control group and receive normal diet. The remaining groups will go through two treatment stages; in the first one, they will consume a 40% fat content diet for 13 weeks. In the second stage of the protocol (training phase), one of the control groups (n=5), named group 1, will be slaughtered. The 11 remaining groups will include: group 2- the second control group (n=5) still fed normal diet, group 3-will remain on high fat diet (n=5), group 4- will be fed by high fat diet and vitamin D3 injection (n=5), group 5- will have high fat diet and adenosine injection (n=8), group 6- fed high fat diet and placebo injection (n=5), group 7-with continued high fat diet and undergoing HIIT program (n=11), group 8- will remain on high fat diet and undergoing HIIT program and placebo injection(n=10), group 9-remaining on high fat diet and undergoing endurance exercise program and placebo injection(n=10), group 11- will consume high fat diet while undergoing endurance exercise with D3 injection (n=7) (Figures 1).

Exercise Training phase: 12 weeks of HIIT and isocaloric moderate intensity training (MIT), food will be provided ad libitum for the first 6 weeks, then homogenized in the second 6 weeks. Two types of exercise training: HIIT and MIT 5 times/week. Adenosine will be injected intraperitoneal (ip) per day with 0.2 Mg/ml/Kg dose in the first 6 weeks, then Adenosine will be
injected (ip) per day with 0.4 Mg/ml/Kg dose in the second 6 weeks. Vitamin D3 will be injected intraperitoneal (ip) once with 10000 units dose at the first 6 weeks and then injected ip once with 20000 units dose at the second 6 weeks.

**Anthropometric assessments**
The abdominal circumference (AC) (immediately anterior to the forefoot), thoracic circumference (immediately behind the foreleg), body length (nose-to-anus or nose–anus length) will be determined in all the rats every month. The measurements will be made in anaesthetized rats (0.1mL intraperitoneally of 1% sodium barbiturate). The body weight and body length will be determined with the following anthropometrical parameters: _ Body mass index (BMI)=body weight(g)/length² (cm²). Lee index = cube root of body weight (g) / nose-to-anus length (cm) (Bernardis, 1970) [38]. Specific rate of body mass gain (g/kg=dM/Mdt), Where, dM represents the gain of body weight during dt= t2−t1 and M is the rat body weight at t1.

**Body mass and food intake**
Body mass and food intake (difference between the feed offered and the remaining feed) of each animal will be measured daily throughout the experimental period by precision balance (Gehaka, model BG 2000, Brazil). Feed efficiency and energy efficiency will be calculated using the following formulas:

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\text{Feed Efficiency} = \frac{\text{Body mass gain (g)}}{\text{Total food intake (g)}}
\]

\[
\text{Energy Efficiency} = \frac{\text{Body mass gain (g)}}{\text{Total caloric intake (kcal)}}
\]

**Drug treatment**
One hundred and ninety doses of 3mg/ml adenosine packs will be purchased from the College of Pharmacy, Tehran University of Medical Sciences. During the first 6 weeks of the training phase, every rat will be injected intraperitoneal, 0.2 mg/ml/kg adenosine dose and vitamin D3 with 10000 unit dose. After 6 weeks of training, in order to assess the rate of effectiveness of the drug, a cross-over design will be employed by introducing a dose of 0.4/Mg/ml/Kg adenosine injection per day and increased unit dose of vitamin D3 to 20000 once in the beginning of the second six weeks training. These doses will not change further up to the end of the protocol.
**Exercise protocol**

The rats in the training groups will be placed on an animal-treadmill to run at various speeds of 6, 8 and 10 m/min in a trial period of a week prior to the main exercise protocol to become acquainted with the procedures. Then, every rat will be placed on the treadmill to continue running at maximum speed up to the exhaustion point. Following the recording of the maximum speed during exhaustion for every rat, the mean value of speed of the exercising rats will be calculated. Then, the exercise protocol will be designed, [Table 1]. The designed program will be based on the data obtained through the pilot phase. The high intensity interval training [HIIT] will include an 85 to 90% Vmax intensity, whereas the moderate intensity training program [MIT] will be set at 60 to 65% Vmax level. The warmup period included will be 3 min of running at a speed of 10 m/min and cool down period will include 2 min of running at a speed of 15 m/min. Both exercise protocols will be matched for the training volume [consumed calorie] to determine the effect of types of exercise program [isocaloric exercise].

**Tissue collection**

After 24 h of rest and 8 min of fasting, the rats will go through anesthetic phase by applying pentobarbital sodium (40Mg/Kg;ip). Then, after complete anesthetics condition, blood sample will be drawn directly from the heart and transferred into tubes for serum separation by centrifugation procedure. The samples will be frozen to -80°C for fat and fat burning markers analysis. The white fat samples (kidney circumference, visceral), mesenteric (visceral), thigh fat (subcutaneous), inter scapular (as brown fat), Epicardial fat, liver (from the inferior right lube), Gastrocnemius muscle, planetarius, heart epics and superior part of thigh will be isolated in 2 x 2 mm size. All the samples collection will be performed at 2 to 4 pm after 7 to 8 h of fasting. After placing the samples in nitrogen for RNA extraction and gene analysis, they will be transferred at -80°C.

**Quantitative PCR**

**RNA extraction and real-time PCR**

Adipose tissue samples will be homogenized in TRIzol solution using a tissue homogenizer (Tissue-Lyser LT; Qiagen, Valencia, CA). Total RNA will be assayed using a
Nanodropspectrophotometer (ThermoScientific, Wilmington, DE) to assess purity and concentration. First-strand cDNA will be synthesized from total RNA using the high-capacity cDNA reverse transcription kit (Applied system (Applied Biosystems). Primer sequences (available upon request) will be designed using the NCBI primer design tool. All primers will be purchased from Pishgam (Pishgam, Iran). A 20 µl reaction mixture containing 10 µlSYBR Green Mastermix (Amplicon) and appropriate concentrations of gene specific primers plus 1000 ng/µl of cDNA template will be loaded in each well of a 96-well plate. All PCR reactions will be performed in duplicate. PCR will be performed with thermal conditions as follows: 95°C for 10min, followed by 40 cycles of 95°C for 15 s, and 60°C for 45 s. A dissociation melt curve analysis will be performed to verify the specificity of the PCR products. GAPDH primers will be used to amplify the endogenous control product. mRNA expression values will be presented as 2^-ΔΔCT. Data will be expressed as the fold difference relative to GAPDH.

For examination of the gene expression in every group, Real Time PCR will be employed by ABI Applied Biosystems, Real-Time PCR Systems, StepOne™, - Systems, Hettich Centrifuges, UNIVERSAL 320, Capacity: 4x100 mL | 32x15 mL, RPM/RCF: 15,000 / 21,382, Temp. Control: -20 to +40°C, cDNA Synthesis Kits—Thermo Scientific, Revert Aid First Strand cDNA Synthesis Kit.

**Western blot analysis**

RIPA buffer cell lysates will be used to produce Western blot-ready samples. Samples will be separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and will be incubated with primary antibodies. HRP conjugated mouse or rabbit secondary antibody will be used to detect primary antibodies and will be stained with DAB (Sigma-Aldrich, USA). Protein loading will be measured by Bradford (Sigma) staining to determine total protein concentration. The total protein will be loaded in each lane and quantified. These values will be used to adjust for any difference in protein loading or transfer of all band densities. Individual protein bands will be quantified using image j software (NIH, USA) and data will be expressed relative to rabbit polyclonal beta Actin antibody. Antibodies will be purchased from Abcam (Abcam, Germany).

**Biochemical analytics**
The concentrations of glucose, total TG, TC, HDL-c, LDL-c and VLDL-C in serum will be determined by the Clinical Pathology Laboratory using an automated analyzer (Alpha Classic) – tajhizatsanjesh (Alpha-Classic is an ideal biochemistry solution for Automation needing advanced and medium class laboratories). Glycerol, insulin and FFA will be measured respectively with rat-specific ELISA kits (cat No:ZB-GCL48A. Lot.No:ZB-OC717210), Cat No: 10-1250-01. Lot No.25692. (ZB-A1515818) according to the manufacturer’s instructions. Quantitative insulin sensitivity check index (QUICKI) and HOMA will be calculated as described previously using the equation: QUICKI51/[log (I0)1 log (G0)], where I0 is fasting insulin (IU/ml) and G0 is fasting glucose (mg/dl) [40].

**Statistical analysis**

The sample size for this research protocol will be estimated based on the effect size which was effective in previous research and G*power software will be used to determine the needed number. For the descriptive results, mean and standard deviation will be calculated and reported in appropriate tables. For any variable showing non-symmetric or lack of normality, median, 25 and 75 percentile will be calculated. For determination of the interaction effect, the mean differences and confidence interval will be calculated and for estimation of the effect size, Cohen method will be employed to calculate the standardized mean differences.

**Expected results**

The researcher expects to observe a decrease in anthropometric indices as apparent changes due to the isocaloric training. It is also likely that anthropometric indices will change as a result of medication. In addition, it is likely that molecular changes and up regulation will be observed in line with increase in lipolysis and beta oxidation in muscle and fat tissue as a result of performing isocaloric training in drug receiving rats and groups on high fat diet.

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**Authors' Contributions**
SJM, MAA, MP, and AK conceived the study design; SJM, AK and MAA contributed to design data collection tools and wrote the statistical analysis plan. SJM, MS, SSH and OYY will performed exercise training programs and monitor data collection for the whole trial and analysis of the data. SJM, AK and MAA drafted the manuscript, and all authors reviewed the draft of the manuscript and revised it. All authors approved the final manuscript to be published.

**Conflicts of Interest**

None declared.

**References**


