Original article

Down-regulation of Sirtuin 1 gene expression correlated with higher atherogenic fatty acid level in the liver of male mice offspring born from high fat-fed mothers

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Abstract. *Background:* Maternal diet can alter metabolism of offspring through changes in Sirtuin 1 (SIRT1) expression, as a metabolic sensor. However, its correlation with hepatic fatty acid profile is not clear. Herein, the correlation of SIRT1 expression was assessed with hepatic fatty acid profile in offspring born from isocalorie high fat-fed mothers during gestation and lactation. *Methods:* C57BL/6 female mice were randomized to AIN93G (CG) and high-fat AIN93G (HFG) diets after insemination. Diets were isocaloric by change in fat and carbohydrate percentages. Protein content was similar. After weaning, all offspring received a CG diet. At the adolescence, liver tissue was extracted for assessing SIRT1 expression and fatty acid profile. *Results:* Gene and protein of SIRT1 decreased in both sexes born from HFG-fed mothers compared with the controls ($p<0.001$). In female offspring born from the CG-fed mothers, SIRT1 gene expression correlated with lower linoleic acid ($r= -0.98$, $p=0.002$) and higher eicosapentaenoic acid ($r=0.91$, $p=0.02$) level in the liver. In male offspring born from HFG-fed mothers, down-regulation of SIRT1 gene expression was correlated with higher palmitic ($r=0.9$, $p=0.03$), linoleic ($r=0.95$, $p=0.01$), arachidonic ($r=0.9$, $p=0.04$), acids and cholesterol ($r=0.94$, $p=0.01$) in the liver. At the protein level, no significant correlation was observed. *Conclusions:* Maternal dietary fat and carbohydrate distribution, regardless of calorie intake, effect on hepatic fatty acid profile by SIRT1 gene changes. Atherogenic fatty acids increased in the liver of male mice offspring born from HFG-fed mothers.

Key words: calorie, dietary fats, pregnancy, embryonic programming, sirtuin 1

Introduction

Silent mating type information regulation 2 homolog 1 (SIRT1) is a nuclear metabolic sensor which is mostly conserved in mammals. It is an NAD^+-dependent enzyme that regulates epigenetic modifications, as well as gene expression through de-acetylation of histones, transcription factors, and transcription co-factors (1). Studies reported that nicotinamide mononucleotide, as a main coenzyme of SIRT-1, promote neurovascular rejuvenation; activates SIRT1 at the transcriptional level, protects mitochondria from damage, reduces apoptosis, and oxidative stress (2, 3). Recent studies proposed that SIRT1 regulates carbohydrate and lipid metabolism including gluconeogenesis, fatty acid oxidation, cholesterol efflux, bile

acid synthesis, and lipogenesis in the liver (4-6). An increase in SIRT1 activity improves liver insulin sensitivity and decreases energy requirements (7). Recently, attention to SIRT1 is increasing due to its role in the damaged sites by promoting DNA repair (8).

Maternal diet during gestation and lactation creates persistent alterations in fetal metabolism according to the "developmental origins of health and disease" (DOHaD) hypothesis that is reported by David Barker, et al (9). Hence, early life nutrition can result in developmental adaptations that produce permanent metabolic, physiologic and phenotypic changes without DNA sequence alterations (10). Previous studies have shown that type and amount of maternal dietary oil can change gene expression in fat tissue and bone of mice offspring in a sex-dependent manner (11, 12). Moreover, one non-human primate study reported that maternal high fat-high calorie diet acetylates histone H3 (H3K14ac) in the liver of offspring via SIRT1 pathway followed by abnormal cytoplasmic lipid accumulation and homeostasis (13). Previous animal studies assessed the impacts of restricted- and high-calorie diets on SIRT 1 gene and protein level, however effects of isocalorie diet with various carbohydrate, protein and fat ratio is not clear [14, 15]. In addition, there is no animal study to assess this effect in critical periods of life, such as gestation and lactation. Moreover, correlation of SIRT1 gene and protein expression is not studied with hepatic fatty acid profile. Therefore, we designed the present study to assess the effects of changes in maternal dietary fat and carbohydrate distribution in an isocalorie diet on SIRT1 gene and protein level, and their correlation with the profile of fatty acids in liver of male and female mice offspring.

Materials and methods

Animal experiments

The present animal study was conducted in accordance with the ARRIVE guidelines and the national institutes of health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and approved by ethical committee of Tehran University of Medical Sciences, Iran (IR.TUMS.VCR.REC.1396.3008). Twenty female C57BL/6 mice (21 ± 1.5 g) were housed according to the standard protocol for maintenance of laboratory animals (21–23 °C; 50 ± 5 % humidity; and 12 h artificial light cycle). After two weeks of adaptation with AIN-93M (rodent maintenance diet), female mice were mated overnight. The vaginal plug was confirmed, and mothers were randomly divided to the controls and intervention groups that received AIN-93G (rodent growth diet). The controls received AIN-93G (CG), and the intervention group received a high fat AIN-93G (HFG) diet, which contain 16% and 48% of calories as fat, respectively. The CG prepared 64% of calorie as carbohydrate compared with 32% in the HFG. Both diets had 3.97 kcal/g (Table 1). Mothers received these diets during gestation and lactation in a pair-fed model (16). The number of offspring were equaled in all cages (n=4), nursed, and lactated with their mothers for three weeks. Mice were separated according to the sex, and all offspring received the control diet up to 6 weeks. At the end, one male and one female offspring were randomly selected from each cage (total number in each group =10) and euthanized by ketamine and xylazine. Liver tissues were removed; snap frozen and kept at -80 °C refrigerator for final gene and protein expression, as well as gas chromatography- mass spectroscopy (GC/MS) analysis. The schematic overview of the study is illustrated in Figure 1.

SIRT1 gene expression

Frozen liver tissues (1 gr) were powdered in liquid nitrogen (N2), and total RNA was extracted using TRIzol Lysis Reagent (QIAGEN Inc., Valencia, CA 91355, USA). After tissue washing by PBS, 300 µl of TRIzol was added to prepare the lysates. Then, chloroform was added, and samples were centrifuged (10000 rpm) for 15 min at 4 °C. RNA supernatants were collected, isopropanol was added, and samples were stored for 30-60 min at -20 °C. Finally, the samples were centrifuged (10000 rpm) for 15 min at 4 °C. To omit the possible contaminants such as lipids, 700 µl of alcohol (70-80%) was added and centrifuged (7500 rpm) for 10 min at 4 °C. RNA

Table 1. Composition of diets (per 1 kg).

Ingredients (g/kg)	Diets	Casein	Corn starch	Sucrose	Soybean oil	Fiber	Mineral mix	Vitamin mix	L-cysteine	Choline tartrate	tert-butyl hydroquinone
AIN-93M		140	620.692	100	40	50	35	10	1.8	2.5	0.008
AIN-93G	CG	200	529.486	100	70	50	35	10	3	2.5	0.014
	HFG	200	216.986	100	210	222.5	35	10	3	2.5	0.014

Abbreviations: AIN 93M: diet during maintenance; AIN 93G: diet during growth; CG: low fat-high carbohydrate diet; HFG: high fat-low carbohydrate diet. Ingredients were prepared as follows: L-cysteine (W326305, Sigma Aldrich, Germany), AIN 93 M mineral mix (296040002, MP Biomedicals, USA), AIN 93 vitamin mix (296040201, MP Biomedicals, USA), choline bitartrate (C1629, Sigma Aldrich, Germany), tert-butyl hydroquinone (112941, Sigma Aldrich, Germany). Casein lactate, corn starch, sugar, soybean oil and fiber were prepared from local products.

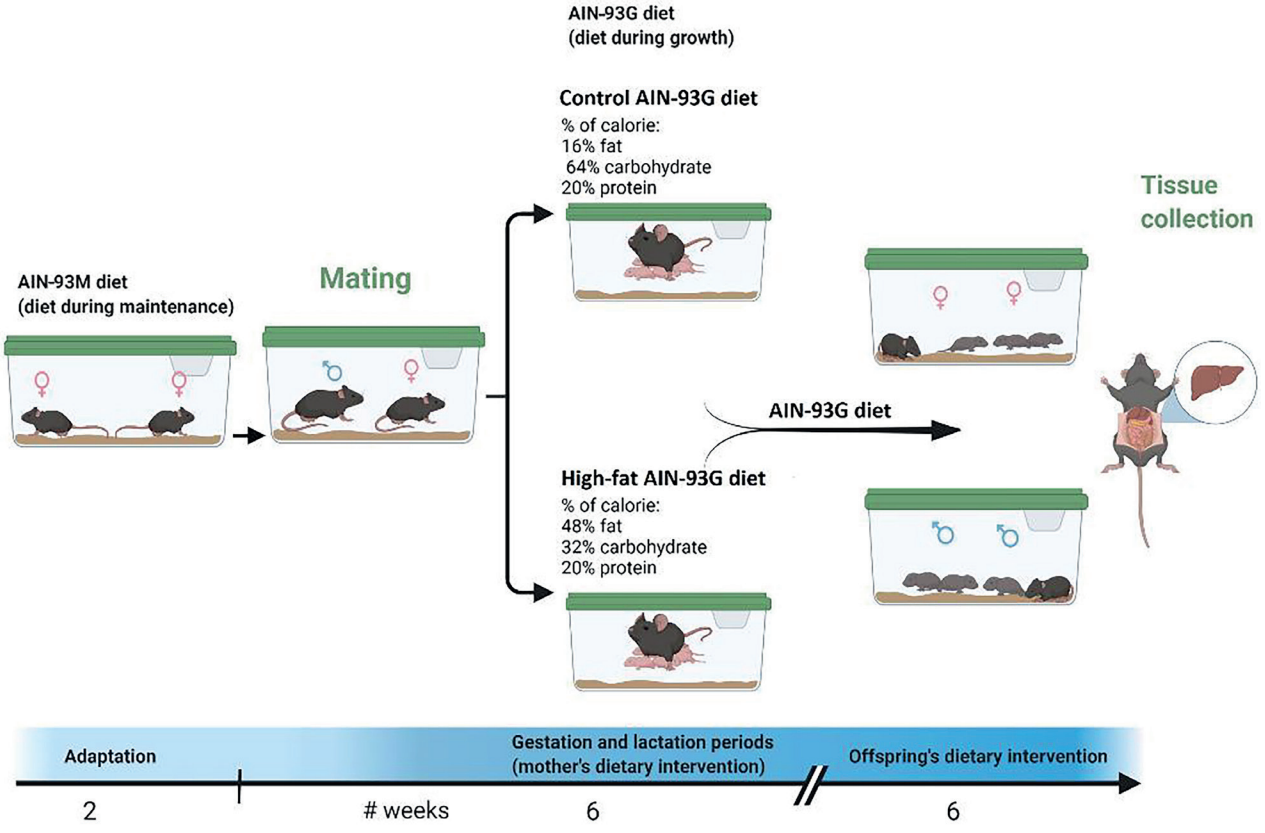

Figure 1. Schematic overview of the study protocol is created with Biorender.

sediments were dissolved in 20 µl of distilled water for 5 min at 45-55 °C. The ND-1000 spectrophotometer (DPI-1, QIAGEN Inc., USA) was used to assess RNA quantity at 260 nm. Quality (integrity) of the extracted RNA was checked by agarose gel electrophoresis. The cDNA was synthesized from one microgram of total RNA using Fermentas protocols (Fermentas Co. USA).

ABI StepOne sequence detection system (Applied Biosystems, California, USA) was used for the real-time polymerase chain reaction (RT-PCR) with 10 pmol of the forward and reverse primers for SIRT1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, housekeeping gene), 1 µl of the synthesized cDNA and SYBR Green I Master Mix (Roche), containing a thermostable DNA polymerase, dNTPs, $MgCl_2$, and proprietary additives in a buffer optimized, in duplicate runs. Both primers were designed by the Primer Express software 2.0.0 as bellows: SIRT1 (Forward: GTGTCATAGGATAGGTGGTG; Reverse

TATGAAGAGGTGTTGGTGG) and GAPDH gene (Forward: CTATGTTTGTGATGGGTGTGA; Reverse AGTGGATGCAGGGATGATGT).

The cycle threshold (CT) values were normalized against GAPDH mRNA as a control. The amplification profile included 40 three-step cycles: 94°C for 20 s, 58-60 °C for 30 s and 72 °C for 30s. The results were generated and analyzed using the $2^{\wedge\text{-}\Delta\Delta Ct}$ method in which ΔΔCT was computed as follows:

$$\Delta\Delta CT = (CT_{SIRTI} - CT_{GAPDH})_{TIMES\ X} - (CT_{SIRTI} - CT_{GAPDH})_{Time\ 0}$$

Western blotting

Liver tissues (1 gr) were washed by PBS, powdered on ice, and transferred to a micro tube. Then, 200 µl of RIPA, as a lysis and extraction buffer, was added and stored for 90 min at 4 °C. Ethylene diamine tetra acetic acid (EDTA) was used as a protease inhibitor. The lysates were centrifuged by a refrigerator centrifuge. 50 mg of protein (from each sample) was added to the same amounts of loading buffer for 5 min at 95 °C. Then, denaturized proteins were loaded on wells, inserted in the western pons and running buffer was added at 90-100 mV for 220-230 min. Proteins were separated by 10% SDS-PAGE (PH=6.8) and transferred to nitrocellulose filter membrane at 80 mV for 70-80 min at 4 °C. After overnight incubation in blocking buffer (skim dried milk), diluted SIRT1 antibodies were diluted by TBS-Tween buffer (1:200), added to the membrane and shaken for 120 min. Samples were washed three times with TBS-Tween buffer and shaken for 10 min in each step. Anti-SIRT1 antibody (Abcam Co., UK) (1:3000) was added and stored on the shaker for 90 min. Then, samples were washed by TBS-Tween for three times and shaken for 10 min. GAPDH antibody (Abcam Co., UK) was used as the housekeeping protein. Chemiluminescence detection system and ImageJ software were used for detection of protein bands and analyzing the data, respectively.

Gas chromatography–mass spectrometry (GC/MS)

Frozen samples were powdered in N_2, and a mixture of chloroform-methanol (1 mL, 2:1; v/v) was added to the 0.5 g of each sample and shaken for 10 min. The chloroform phase containing the lipids was separated, and the aqueous phase was extracted again, similarly. Then, samples were centrifuged (4500 rpm) for 5 min and supernatants collected for the derivatization step. The 2% H_2SO_4-methanol, as a methylation/transesterification solution, was added to the extracted samples and refluxed for 45 min at 80 °C. After neutralization with NaOH (1 M; PH=7), n-hexane was added to each sample, and supernatant was collected for GC/MS analyses.

Analyses were performed on a 5977A MS and 7890B GC (Aligent Co., USA) equipped with the split/splitless column as injector system and HP5-MS (60 m × 0.25 m × 0.25 µm) column for fatty acid profile analysis. The oven temperature was kept at 70 °C for 5 min, then programmed at 15 °C /min to 150 °C and kept for 2 min. Finally, the system programmed at 20 °C /min to 290 °C and kept for 10 min. Electron impact ionization (EI+, 70 eV) was used for all samples. The split ratio was settled on 1:20.

Statistical analysis

To detect a difference in total body fat mass between two groups, we used a 90% confidence interval with a two-sided test with α=0.05 (type I error). On the basis of SDs reported in a similar study (12), the required subjects were six per group. Data were tested for normal distribution using the Kolmogorov-Smirnov test and did not have normal distributions in gene expression even after all of the transformation methods. The differences of gene, protein and fatty acid profile among groups were measured by One-way ANOVA followed by the Tukey's test. Correlation coefficients were analyzed by the Pearson test. All data are expressed as means ± SD. The level of significance was set at $p<0.05$.

Results

Maternal weight gain during gestation

Maternal weight gain was significantly different in week three of gestation. Mothers in the CG group were significantly heavier than the HFG ($p<0.001$).

Weight gain of mothers had no significant difference at week 1 and 2. The trend of weight gain is shown in Figure 2a.

Effect of isocalorie CG and HFG diets on weight of offspring

Birth and adolescence weight of offspring born from the CG-fed mothers was significantly higher in males than females (1.85±0.1 g *vs.* 1.42±0.14 g, p<0.001; and 23.2±1.06 g *vs.* 19.1±0.7 g, p<0.001, respectively). Similarly, the adolescence weight was significantly higher in the male offspring born from HFG-fed mothers than females (24.2±1.6 g *vs.* 21.8±1.3 g, p=0.01). But birth weight of male offspring significantly reduced in the HFG group compared to females (1±0.14 g *vs.* 1.45±0.1 g, p<0.001). Birth weight of male offspring born form CG-fed mothers was significantly higher than the HFG-fed one (p<0.001). Moreover, adolescent weight of female offspring was significantly higher in the HFG-fed mothers than CG-fed group (p<0.001). The trend of weight gain is shown in Figures 2b and 2c.

Effect of isocalorie CG and HFG diets on liver SIRT1 gene and protein expression

In male (0.06±0.007 *vs.* 1; p<0.001) and female (0.05±0.003 *vs.* 1; p=0.006) offspring born from HFG-fed mothers, SIRT1 gene expression significantly decreased compared with the CG. Protein expression of SIRT1 significantly increased in male offspring born from CG- and HFG-fed mothers compared to females (1.6±0.08 *vs.* 1; p<0.001 and 1.25±0.1 *vs.* 0.57±0.08; p<0.001). In contrast, female offspring born from HFG-fed mothers had lower protein levels than the CG (0.57±0.08 *vs.* 1; p<0.001).

Effect of isocalorie CG and HFG diets on liver fatty acid profile

Meristic ($C_{14:0}$) and palmitic ($C_{16:0}$) acid were significantly higher in the liver of female offspring born from HFG-fed mothers (p<0.001). Stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), linolenic ($C_{18:3}$), dihomo-γ-linolenic ($C_{20:3}$), arachidonic ($C_{20:4}$) and docosahexaenoic ($C_{22:6}$) acids were significantly higher in female offspring of CG- than HFG-fed group (p<0.001). Fatty acid profile in the liver of male offspring was similar to females. Moreover, eicosapentaenoic acid ($C_{20:5}$) was significantly higher in male offspring of CG group (p<0.001). Palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids were significantly lower in the liver of male offspring born from HFG-fed mothers than females (p=0.03 and p=0.01, respectively), but oleic acid ($C_{18:1}$) was significantly higher in the males than females (p<0.001). Liver cholesterol significantly increased in male and female offspring born from HFG-fed mothers (p<0.001 in all cases). Also, cholesterol was significantly higher in the liver of male offspring born from HFG-fed mothers than females (p<0.001). (Table 2)

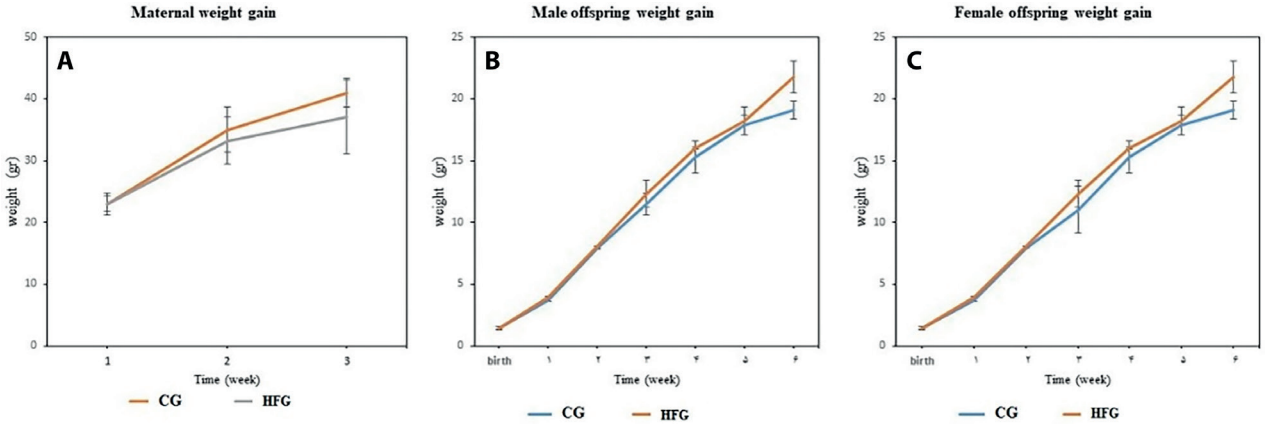

Figure 2. Trend of maternal (a), male offspring (b) and female offspring (c) weight gain during the study is shown.

Table 2. Liver fatty acid profile in the studied groups.

Groups Fatty acids	CG Female	CG Male	HFG Female	HFG Male	p value
Meristic acid ($C_{14:0}$)	0.36±0.04	0.31±0.03	3.5±0.35	3.2±0.2	<0.001
Palmitic acid ($C_{16:0}$)	2.83±0.2	2.4±0.1	7.13±0.32	6.44±0.29	<0.001
Stearic acid ($C_{18:0}$)	15.38±0.81	19.45±1.4	5.6±1.1	2.5±0.55	<0.001
Arachidic acid ($C_{20:0}$)	1.53±0.4	0.35±0.1	0.54±0.08	0.04±0.2	0.1
Oleic acid ($C_{18:1}$)	3.84±0.29	6.7±0.71	0.52±0.07	2.93±0.45	<0.001
Linoleic acid ($C_{18:2}$)	2.62±1.64	27.7±1.95	9.4±0.08	10.75±1.32	<0.001
Linolenic acid ($C_{18:3}$)	0.43±0.06	0.3±0.04	0.1±0.015	0.06±0.02	<0.001
Dihomo-γ-linolenic acid ($C_{20:3}$)	2.83±0.24	1.88±0.28	0.61±0.47	0.06±0.02	<0.001
Arachidonic acid ($C_{20:4}$)	13.7±1.36	10.9±0.6	2.94±0.1	2.4±0.5	<0.001
Eicosapentaenoic acid ($C_{20:5}$)	1.36±0.56	2.8±0.82	0.22±0.03	0.1±0.04	<0.001
Docosahexaenoic acid ($C_{22:6}$)	13.67±2.07	8.97±0.61	2.21±0.3	1.3±0.18	<0.001
Cholesterol ($C_{27}H_{46}O$)	1.13±0.22	2.43±0.44	45.24±1.87	69.5±1.08	<0.001

Data are expressed as means ± SD

Correlation between liver SIRT1 gene and protein level with fatty acid profile

In female offspring born from the CG-fed mothers, a significant negative correlation was seen between the liver SIRT1 gene expression and linoleic acid (r= -0.98, p=0.002) level. In male offspring born from HFG-fed mothers, a significant positive correlation was seen between SIRT1 gene expression with palmitic (r=0.9, p=0.03), linoleic (r=0.95, p=0.01), arachidonic (r=0.9, p=0.04) acid, and cholesterol (r=0.94, p=0.01) level in the liver tissue. At the protein level, no significant correlation was observed.

Discussion

For the first time, we showed that maternal high fat diet, regardless of calorie intake, during pregnancy and lactation alters fatty acid profile, as well as SIRT1 gene and protein expression in the liver of offspring. SIRT1 gene and protein expression were significantly higher in male than females. Gene expression of SIRT1 was significantly down-regulated in the liver of male and female offspring born from HFG-fed mothers compared to the controls. Higher SIRT1 gene expression correlated with lower linoleic, but higher eicosapentaenoic acid level in the liver of female offspring born from CG-fed mothers. Although, SIRT1 down-regulation correlated with higher palmitic, linoleic, arachidonic acids, and cholesterol level in the liver of male offspring born from HFG-fed mothers. Down-regulation of SIRT1 gene and protein expression showed no correlation with hepatic fatty acid profile in females. In addition, protein alterations showed no significant correlation with hepatic fatty acid profile in male offspring. Male offspring born from CG-fed mothers were heavier than females, both at the birth and adolescence. Similarly, adolescence weight was significantly higher in the male offspring born from HFG-fed mothers than females. However, birth weight of male offspring significantly reduced in the HFG-fed mothers compared to females. Saturated fatty acids (meristic and palmitic acids) were significantly higher in the liver of male and female offspring born from HFG-fed mothers. Stearic and unsaturated fatty acids significantly increased in the liver of male and female offspring of the CG compared to the HFG group. Liver cholesterol was significantly higher in males than females. In the present study, the inbred C57BL/6 mice were used due to exclude the effect of gene diversity and its effect on outcomes. Totally, studies on the effect of maternal high fat-high calorie diets on SIRT1 are scarce (17- 19), and there is no

study on isocalorie diets. Previous animal studies have shown that SIRT1 expression reduced in the fetus, especially liver, by maternal obesity (20, 21). One animal study showed that a high fat diet increases hypothalamic SIRT1 gene expression (22). But, the correlation between SIRT1 gene and protein expression in the liver with hepatic fatty acid profile is not studied, up to date.

According to the DOHaD hypothesis, maternal nutrition has permanent effects on fetal protein levels followed by changes in gene expression through epigenetic pathways, which increases susceptibility to chronic diseases at the adulthood (9). All of these changes can be inherited to the next generation, permanently (23). One study compared the effects of long-term high-fat vs. energy-restricted diet on endothelial Sirtuin-1 in the cavernous tissue. Long-term high-fat diet increased SIRT1 deacetylation that showed a detrimental effect on tissues (24). Our results showed that hepatic SIRT1 down-regulation correlated with higher atherogenic fatty acid accumulation including palmitic and arachidonic acid, as well as cholesterol level in the liver of male offspring born from HFG-fed mothers. Diets in our study were isocaloric and the percentages of fat and carbohydrate only changed. The difference in the dietary composition and the studied life period can be some of the reasons for the disagreement results in various studies. SIRT1 is a nutrient-responsive, NAD^+-dependent histone deacetylase that response to energy availability by inducing catabolic and repressing anabolic pathways, as well as inflammation (25). In the present study, SIRT 1 gene and protein level significantly decreased in offspring born from HFG-fed mothers compared to the controls. These changes lead to increase in cholesterol and saturated fatty acids in the liver, however omega-3, -6 and-9 fatty acids decreased. Moreover, SIRT1 in a normal (control) diet correlated with higher eicosapentaenoic acid, but lower linoleic acid in the liver. SIRT1 down-regulation at the gene level correlated with higher atherogenic fatty acids including palmitic, linoleic and arachidonic acids, as well as cholesterol in the liver of male offspring born from high fat-fed mothers. Fatty acids have various vital roles, and the liver is the main and primary site for their metabolism. The most biologically important omega-3 fatty acids are eicosapentaenoic and docosahexaenoic acids, which are metabolic derivatives of α-linolenic acid via desaturation and elongation pathways. Increase in omega-6 fatty acids in a high-fat diet blocks this conversion (26, 27). Di-homo-γ-linolenic and arachidonic acids are metabolic derivatives of linoleic acid, the main omega-6 fatty acid (28). These are precursors of eicosanoids, as signaling molecules, which have important roles in the regulation of inflammation. Intake of high amounts of fat in diet suppresses linoleic conversion to Di-homo-γ-linolenic and arachidonic acids (28). During the past few decades, the nutritional transition has been occurred. There is a notable increase in fat intake, especially sources of omega-6 fatty acids compared to the omega-3 (~15:1), that is associated with inflammatory diseases (27, 28). Decrease in SIRT1 gene expression in the HFG group may be related to the liver fatty acid disturbances. We resulted that female offspring are more susceptible to metabolic changes than males, which is in agree with the previous studies (28, 29). There are some limitations; this was an animal study, then results are not generalizable to human. Moreover, intervention was performed during gestation and lactation and offspring received the control diet from weaning to the adolescent. Future studies are suggested to assess interventions in various periods of life to assess the outcomes. Moreover, gene and protein levels only analyzed at the adolescent. More studies with changes in macronutrient distribution in different ages of offspring are needed. It is suggested that future studies focus on epigenetic changes on SIRT1 to determine the exact mechanisms.

Conclusion

In summary, maternal high-fat isocalorie diet during gestation and lactation effect on SIRT1 gene and protein expression, as well as hepatic fatty acid profile in the liver of male and female offspring at the adolescents. Down-regulation of SIRT1 gene expression in the liver of male offspring born from HFG-fed mothers was correlated to higher atherogenic fatty acid profile that may predispose them to chronic diseases in later periods of life. This correlation was not observed in female offspring. Alterations in SIRT1

protein expression showed no correlation with hepatic fatty acid profile.

Acknowledgement: Authors are very thankful from staffs of animal lab at Zanjan University of Medical Sciences for caring during the study.

Ethics Committee Approval Code: IR.TUMS.VCR.REC.1396.3008.

Authors' Contribution: SN. M and F.K designed the study and critically revised the manuscript; SNM designed the study, contributed to data analysis and drafting the manuscript; S.Gh and MS. SD contributed in data acquisition, and drafting the manuscript; E.H contributed in data acquisition, and drafting the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Received: 12 December 2022
Accepted: 15 June 2023
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