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Investigating the changes of the components of the Krebs cycle in patients with type 2 diabetes treated with squalene

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ABSTRACT

Background: Type 2 diabetes mellitus is a chronic disease that impairs the body's ability to regulate glucose. Recent studies have shown that squalene, a bioactive compound, has shown promising potential in increasing ATP levels for diabetic patients and aged individuals.

Objective: Our main goal was to evaluate the cellular effects of different doses of squalene on the intermediates and enzymes of Krebs cycle, in order to determine if squalene increases ATP production among groups of people with type 2 diabetes. The intermediates and enzymes that are being studied are acetyl coenzyme (A-CoA), alpha ketoglutarate dehydrogenase (AKGDH), calcium ion (Ca^{2+}), citrate synthase (CS), isocitrate dehydrogenase, oxaloacetate, and pyruvate dehydrogenase complex component (PDH).

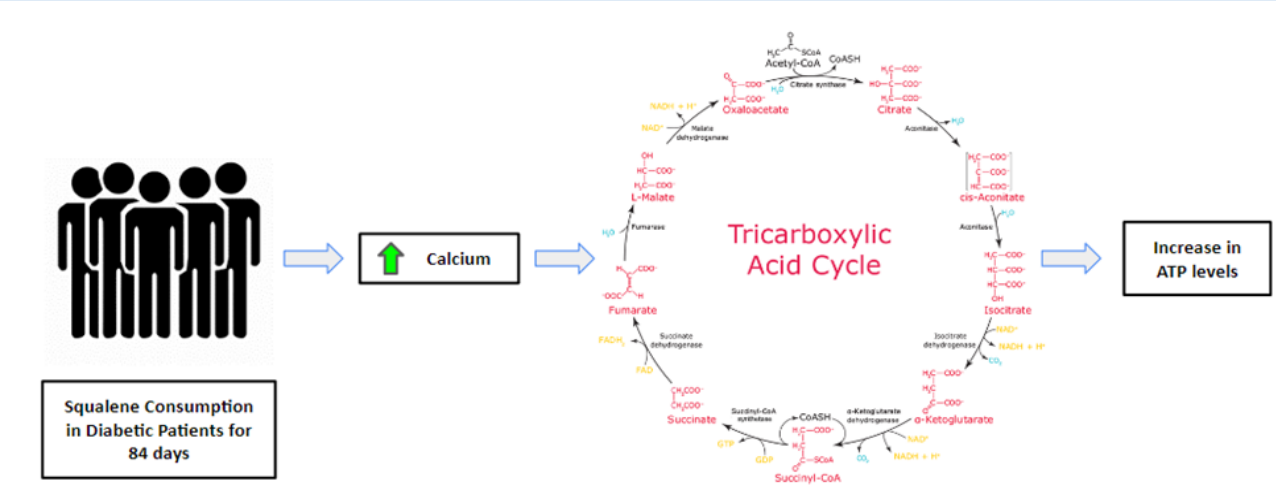
Methods: In this study, 30 healthy volunteers were selected as the healthy control group (group 1) and 120 volunteers with type 2 diabetes mellitus were selected. Subjects with diabetes were randomly divided into 4 groups. Group 2 was untreated with squalene and groups 3, 4, and 5 were treated with 200, 400 and 600 mg of squalene, respectively for 84

days. Intermediates and enzymes of the Krebs cycle as well as calcium ion were assayed on days 1, 14, 28, 56, and 84 according to the relevant protocols in all groups.

Results: The squalene-treated diabetic groups were compared to group 2 that was not treated any squalene to determine the differences of the parameters. Throughout these 84 days, it was observed that only calcium levels increased in the diabetic patients with high statistical difference ($P < 0.05$). The other parameters: acetyl coenzyme, alpha ketoglutarate dehydrogenase, citrate synthase, isocitrate dehydrogenase, oxaloacetate, and pyruvate dehydrogenase did not have a significant difference ($P > 0.05$).

Conclusion: Based on the findings of this study, the addition of various doses of squalene to a diabetic patient's diet increases the amount of calcium found in their metabolic process in relation to the Krebs cycle. As calcium is responsible for stimulating the Krebs Cycle, it is evident that squalene plays an important part in ATP production.

Keywords: squalene, type 2 diabetes, Krebs cycle, calcium, ATP



INTRODUCTION

Squalene is a bioactive compound that is classified as a 30-carbon triterpenoid ($C_{30}H_{50}$) and is most notably found in shark oils and olive oils. The abundance of this compound makes it an optimal molecule for research as a subject to learn the beneficial effects on the human body. It serves as an intermediate for biosynthesis of phytosterol or cholesterol in both plants and animals. For humans, the concentration of squalene is highest in newborns and the concentration begins to decrease drastically between ages 30 and 40 [1]. The majority of squalene produced within humans is synthesized in the liver and the skin. It is then transported through the blood by low density lipoproteins (LDL) and finally secreted by the sebaceous glands in large quantities [2].

Type 2 diabetes mellitus causes the body use insulin ineffectively as a result of having excess body weight and lack of physical activity. The implementation of a healthy lifestyle and diet can prevent and help alleviate the effects of diabetes. The Functional Food Center (FFC)

proposes the use of functional foods to help mitigate the symptoms of type 2 diabetes. The FFC defines functional foods as "natural or processed food that contain biologically active compounds, which, in defined, effective, non-toxic amounts, provide a clinically proven and documented health benefit utilizing specific biomarkers, to promote optimal health and reduce the risk of chronic/viral diseases and manage their symptoms" [21]. In recent years, studies have stated that the consumption of squalene had numerous beneficial effects to its consumers.

Squalene has been reported to have beneficial health effects due to its antioxidant and anti-inflammatory properties [3, 4]. It was reported that antioxidant enzymes such as SOD, CAT, and GPx activity were increased in the diabetic groups treated with squalene, bringing the studied parameters to have closer levels to those of the healthy control group [3]. In a study, squalene was observed to help reduce proteinuria levels. Proteinuria indicates that there are high levels of protein present in the urine, and it serves as an indicator if there could be damage to the kidney. It was observed that the proteinuria levels within the diabetic patients had decreased in relation to the amount of squalene given to the patients [4].

A recent study sought to determine the effects of squalene on certain parameters for energy production and inflammation in the human body, especially those with type 2 diabetes mellitus (T2DM). It was reported that the control group that consisted of healthy individuals who did not have T2DM had higher ATP levels than the groups without T2DM [5]. It was confirmed there was an increase in the production of ATP levels for patients with T2DM who consumed squalene while the ATP levels for the patients who did not consume squalene remained the same. Also, the amount of increase in ATP levels correlates with the amount of squalene consumed, the more squalene consumed, the higher the ATP levels are in the diabetic patients [5].

In another study, young and aged rats were treated with squalene in order to determine if squalene will improve the mitochondrial function within the liver. The mitochondria were the area of study due to the fact it serves an important role in energy production and the liver produces the majority of the squalene in the body. It was believed the lifespan of a species is influenced by the condition of mitochondria. Among the control aged and young groups of rats, it was observed that the aged rats had lower levels of ATP than the young rats [6]. The supplementation of squalene served as a protective agent, improving the mitochondrial function with the liver by maintaining the antioxidant defense system and minimizing aging alterations to the mitochondria It was observed that the levels of ATP were maintained, and the respiratory enzymes are similar to [6].

It is evident with these numerous studies that squalene has provided beneficial health effects to humans and other animals. In order to determine more information about the potential effects on affecting energy levels in human beings, we created an experiment to analyze the effects of squalene on the Krebs cycle.

The Krebs cycle is also known as the tricarboxylic acid cycle or the citric acid cycle. It serves as an important function in obtaining energy through the use of enzymes and serves a major metabolic pathway for cells. It consists of 8 enzymes and most of these are found within the mitochondrial matrix. [7]. Before entering the Krebs cycle, organic molecules like carbohydrates, lipids, and proteins are converted into pyruvate. After the pyruvate enters the mitochondria, pyruvate dehydrogenase complex (PDH) converts pyruvate to Acetyl-CoA (A-CoA). The cycle uses the chemical energy from a molecule of A-

CoA and yields ATP, NADH, and FADH2 molecules, the intermediates of the Krebs cycle are citrate, isocitrate, cis-Aconitate, oxoglutarate, succinyl-CoA, succinate, fumarate, malate, and oxaloacetate [8].

In order to determine the enhancing effect of squalene on the production of ATP, our study will focus on measuring the effects on the molecular components and enzymes of the Krebs cycle. The 7 components of the Krebs cycle that are being observed are: acetyl coenzyme (A-CoA), alpha ketoglutarate dehydrogenase (AKGDH), calcium ion (Ca^{2+}), citrate synthase (CS), isocitrate dehydrogenase, and oxaloacetate, pyruvate dehydrogenase complex component (PDH).

MATERIALS AND METHODS

Materials: Squalene (S3626) was purchased from Sigma Company (USA). The ELISA kits of human glucose, Alpha Ketoglutarate Dehydrogenase (AKGDH), Acetyl Coenzyme A (A-CoA), Citrate Synthase (CS), Isocitrate Dehydrogenase (ID) and Pyruvate Dehydrogenase (PDH) were procured from MyBioSource Inc (USA). The colorimetric detection kits of calcium ion (Ca^{2+}) and oxaloacetate (OAA) were purchased from Elabscience Biotechnology Inc (USA) and abcam Company (USA), respectively.

Methods

Participants: 150 volunteers participated in this study. Five groups of 30 of them were assessed: group 1- Healthy volunteers, as healthy control; group 2- T2DM patients who didn't receive squalene, as diabetic control; group 3- T2DM patients with consumption of 200 mg/day squalene; group 4- T2DM patients with consumption of 400 mg/day squalene; group 5- T2DM patients with consumption of 600 mg/day squalene. Patients in groups 3, 4, and 5 consumed squalene (as an oral capsule, liquid filled oral) once a day, during lunch for 84 days. Volunteers with diabetes were patients who referred to Vali-Asr medical laboratory (Tehran, Iran). According to World Health Organization (WHO), inclusion criteria contained fasting plasma glucose ≥ 126 mg/dL, glycated hemoglobin (HbA1c) ≥ 6.5%, and not taking corticosteroids. Patients with T1DM and other diseases, a history of surgery, as well as young patients with T2DM were excluded from the study. All mentioned volunteers filled the consent form. Groups 3, 4 and 5 were informed about how to conduct the study and the type of substances they consume.

General Features and Sampling: After arrangement of groups, blood samples were taken from all participants under sterile condition. The sampling was performed in five time periods on days 1, 14, 28, 56 and 84. In each period, biochemical parameters of all groups were evaluated. Also, anthropometric items including age, sex, weight, height, and body mass index (BMI**)** of all volunteers were recorded in each period. After 12 hours of nighttime fasting, a blood sample was taken from each volunteer. The indicated blood samples were centrifuged to prepare the serum (250 g for 10 min). Then, biochemical parameters in serum samples were quantified.

Statistical Analysis: Statistical analysis was done by SPSS (version 23, IBM, USA) software for Windows. All results were expressed as mean ± standard deviation (SD). Independent-sample T-test and one-way ANOVA was used to compare the results obtained of the obtained data of the participants. After the one-way ANOVA test, Tukey post hoc was used. The Kolmogorov-Smirnov test was used to analyze the normal distribution of data. P-values < 0.05 were considered significant.

RESULTS

In the comparison of some intermediates (acetyl CoA and oxaloacetate) and some enzymes related to the citric acid cycle (such as alpha-ketoglutarate dehydrogenase, pyruvate dehydrogenase, citrate synthase and isocitrate

dehydrogenase) as well as calcium ion between the diabetic groups (groups 2, 3, 4 and 5) and the healthy control group (group 1) was observed a statistical difference significant ($P < 0.05$). These results are shown in Table 1.

Table 1: Comparison of some intermediates and enzymes of the Krebs cycle as well as ca^{2+} between the control group and the others

Parameter / Group	A-CoA	OAA	AKGDH	PDH	CS	IDH	Ca^{2+}
Healthy control	62.27 ± 7.08	128.23 ± 6.87	58.33 ± 6.57	62.57 ± 6.87	15.42 ± 1.22	160.37 ± 10.01	0.82 ± 0.07
Diabetic control (No squalene)	46.97 ± 7.92	109.80 ± 9.84	44.73 ± 6.71	50.13 ± 8.23	10.90 ± 1.23	126.03 ± 9.17	0.64 ± 0.07
	P value = 0.001						
Diabetic + 200 mg/day squ (14th day)	47.23 ± 8.02	110.23 ± 8.71	44.87 ± 6.81	50.37 ± 8.03	11.04 ± 1.23	127.93 ± 9.10	0.65 ± 0.07
Diabetic + 400 mg/day squ (14th day)	48.27 ± 8.51	111.37 ± 8.39	45.03 ± 6.42	50.53 ± 7.97	11.13 ± 1.24	128.07 ± 9.17	0.66 ± 0.07
Diabetic + 600 mg/day squ (14th day)	49.03 ± 9.16	112.50 ± 7.24	45.17 ± 6.99	50.77 ± 8.03	11.29 ± 1.28	128.23 ± 9.16	0.67 ± 0.07
Diabetic + 200 mg/day squ (28th day)	47.83 ± 9.10	110.63 ± 8.60	44.93 ± 6.67	50.43 ± 8.30	11.06 ± 1.23	128.03 ± 9.64	0.66 ± 0.07
Diabetic + 400 mg/day squ (28th day)	49.47 ± 8.39	112.67 ± 8.65	45.23 ± 6.56	50.87 ± 8.03	11.29 ± 1.25	128.37 ± 9.08	0.68 ± 0.07
Diabetic + 600 mg/day squ (28th day)	49.93 ± 8.35	112.83 ± 9.41	45.27 ± 5.71	50.93 ± 7.70	11.38 ± 1.16	128.93 ± 9.38	0.69 ± 0.07
Diabetic + 200 mg/day squ (56th day)	50.47 ± 8.94	113.53 ± 9.41	45.33 ± 6.70	51.27 ± 8.60	11.39 ± 1.23	129.03 ± 9.07	0.70 ± 0.07
Diabetic + 400 mg/day squ (56th day)	50.53 ± 9.90	114.27 ± 9.49	45.47 ± 5.91	51.53 ± 8.03	11.40 ± 1.25	129.37 ± 9.30	0.71 ± 0.07
Diabetic + 600 mg/day squ (56th day)	50.77 ± 9.07	114.63 ± 8.24	45.53 ± 5.88	51.77 ± 7.90	11.40 ± 1.12	129.93 ± 9.36	0.72 ± 0.07
Diabetic + 200 mg/day squ (84th day)	50.50 ± 9.38	113.77 ± 9.87	45.43 ± 6.24	51.33 ± 8.15	11.40 ± 1.23	129.07 ± 9.35	0.73 ± 0.07
Diabetic + 400 mg/day squ (84th day)	50.63 ± 9.07	114.33 ± 8.96	45.57 ± 5.94	51.47 ± 8.14	11.40 ± 1.20	129.83 ± 8.78	0.74 ± 0.07
Diabetic + 600 mg/day squ (84th day)	50.83 ± 8.64	114.47 ± 9.25	45.63 ± 6.59	51.63 ± 8.02	11.41 ± 1.22	130.27± 8.56	0.75 ± 0.07

Data are given as mean ± SD. A-CoA, acetyl coenzyme A; OAA, oxaloacetate; AKGDH, alpha-ketoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; CS, citrate synthase; IDH, isocitrate dehydrogenase; Ca^{2+}, calcium ion.

At the end of 14 days of treatment of patients with different doses of squalene, a significant difference ($P < 0.05$) was observed in the comparison of intermediates and enzymes mentioned above as well as calcium ion between the group 1 and groups 3, 4, and 5 (received a dose of 200, 400 and 600 mg of squalene, respectively). At the end of 14 days of treatment of patients with squalene, in the comparison of the mentioned parameters between groups 2 and 3, 2 and 4, and also 2 and 5 statistically significant differences were not observed in any of the parameters ($P > 0.05$). No significant difference was observed in the comparison of

parameters between groups 3 and 4 and also 3 and 5 (P > 0.05). At the end of 14 days of squalene consumption by patients, comparing the mentioned parameters between groups 4 and 5, no statistically significant difference (P > 0.05) was observed in any of them. At the end of 28 days of treatment of patients with different doses of squalene, a significant difference (P < 0.05) was observed in the comparison of intermediates and enzymes mentioned above as well as calcium ion between the group 1 and groups 3, 4, and 5. At the end of 28 days of treatment of patients with squalene, in the comparison of the mentioned parameters (except for calcium ion levels between groups 2 and 4 (P = 0.04) and groups 2 and 5 (P = 0.009)) between groups 2 and 3, 2 and 4, and also 2 and 5 statistically significant differences were not observed in any of the parameters (P > 0.05). No significant difference was observed in the comparison of parameters between groups 3 and 4 and also 3 and 5 (P > 0.05). At the end of 28 days of squalene consumption by patients, comparing the mentioned parameters between groups 4 and 5, no statistically significant difference (P > 0.05) was observed in any of them.

At the end of 56 days of treatment of patients with different doses of squalene, a significant difference (P < 0.05) was observed in the comparison of intermediates and enzymes mentioned above as well as calcium ion between the group 1 and groups 3, 4 and 5. At the end of 56 days of treatment of patients with squalene, in the comparison of the mentioned parameters (except for calcium ion levels between groups 2 and 3 (P = 0.02), groups 2 and 4 (P = 0.001), and calcium ion and oxaloacetate levels between groups 2 and 5 (P = 0.001 and P = 0.04, respectively)) between groups 2 and 3, 2 and 4, and also 2 and 5, statistically significant differences were not observed in any of the parameters (P > 0.05). The significance of the analysis of the results of calcium and oxaloacetate levels, in the mentioned groups, has also been shown in figures 1 and 2. No significant difference was observed in the comparison of parameters between groups 3 and 4 and also 3 and 5 (P > 0.05). At the end of 56 days of squalene consumption by patients, comparing the mentioned parameters between groups 4 and 5, no statistically significant difference (P > 0.05) was observed in any of them.

At the end of 84 days of treatment of patients with different doses of squalene, a significant difference (P < 0.05) was observed in the comparison of intermediates and enzymes mentioned above as well as calcium ion between the group 1 and groups 3, 4, and 5. At the end of 84 days of treatment of patients with squalene, in the comparison of the mentioned parameters (except for calcium ion levels between group 2 and 3, groups 2 and 4, and groups 2 and 5 (P = 0.001)) between groups 2 and 3, 2 and 4, and also 2 and 5, statistically significant differences were not observed in any of the parameters (P > 0.05). No significant difference was observed in the comparison of parameters between groups 3 and 4 and also 3 and 5 (P > 0.05). At the end of 56 days of squalene consumption by patients, comparing the mentioned parameters between groups 4 and 5, no statistically significant difference (P > 0.05) was observed in any of them.

After the intra-group comparison between the groups on different days, the mentioned parameters were compared between the groups that received different doses of squalene (groups 3, 4, and 5) on different days. Analysis of the results of acetyl CoA, oxaloacetate, alpha-ketoglutarate dehydrogenase, pyruvate dehydrogenase, citrate synthase, and isocitrate dehydrogenase levels in patients who received the dose of 200 mg (group 3) was compared between different days. Statistically, no significant (P > 0.05) relationship was observed in the comparison of the results of the levels of the mentioned parameters in group 3 on

different days. Statistically significant differences were observed only in calcium ion levels between days 14 and 56 (P = 0.04), 14 and 84 (P = 0.001), and 28 and 84 (P = 0.002). As in group 3, the results of the mentioned parameters were compared on different days in group 4 as well. Same as group 3, statistically, no significant (P > 0.05) relationship was observed in the comparison of the results of the levels of the mentioned parameters in group 4 on different days. Statistically, significant differences were observed only in calcium ion levels between days 14 and 84 (P = 0.001), and 28 and 84 (P = 0.009). The analysis and comparison of the results of the stated parameters between different days in group 5 was the same as groups 3 and 4. Only in the comparison of calcium ion levels between days 14 and 84 (P = 0.001), as well as 28 and 84 (P = 0.01), statistically significant differences were observed. The analysis of the results on different days and different doses is shown in Table 2.

Table 2: Multiple comparisons between some intermediates and enzymes of the Krebs cycle as well as Ca^{2+} between the groups in different days

Parameter / Group	A-CoA	OAA	AKGDH	PDH	CS	IDH	Ca^{2+}
Diabetic first day vs. Diabetic 14 day (200squ)	0.86	0.85	0.94	0.91	0.66	0.42	0.60
Diabetic first day vs. Diabetic 28 day (200squ)	0.69	0.72	0.90	0.89	0.61	0.41	0.27
Diabetic first day vs. Diabetic 56 day (200squ)	0.11	0.14	0.73	0.60	0.12	0.20	**0.00**
Diabetic first day vs. Diabetic 84 day (200squ)	0.12	0.12	0.67	0.57	0.12	0.21	**0.00**
Diabetic 14 day vs. Diabetic 28 day (200squ)	0.99	0.99	1.00	1.00	1.00	1.00	0.93
Diabetic 14 day vs. Diabetic 56 day (200squ)	0.52	0.50	0.99	0.97	0.68	0.96	**0.04**
Diabetic 14 day vs. Diabetic 84 day (200squ)	0.51	0.44	0.98	0.97	0.67	0.96	**0.00**
Diabetic 28 day vs. Diabetic 56 day (200squ)	0.66	0.61	0.99	0.98	0.72	0.97	0.16
Diabetic 28day vs. Diabetic 84 day (200squ)	0.65	0.55	0.99	0.97	0.71	0.97	**0.00**
Diabetic 56 day vs. Diabetic 84 day (200squ)	1.00	1.00	1.00	1.00	1.00	1.00	0.40
Diabetic first day vs. Diabetic 14 day (400squ)	0.54	0.51	0.86	0.85	0.47	0.39	0.26
Diabetic first day vs. Diabetic 28 day (400squ)	0.24	0.23	0.77	0.72	0.22	0.32	**0.04**
Diabetic first day vs. Diabetic 56 day (400squ)	0.13	0.08	0.65	0.50	0.12	0.16	**0.00**
Diabetic first day vs. Diabetic 84 day (400squ)	0.10	0.06	0.61	0.53	0.11	0.10	**0.00**
Diabetic 14 day vs. Diabetic 28 day (400squ)	0.95	0.94	0.99	0.99	0.95	0.99	0.79
Diabetic 14 day vs. Diabetic 56 day (400squ)	0.76	0.58	0.99	0.96	0.83	0.94	0.05
Diabetic 14 day vs. Diabetic 84 day (400squ)	0.73	0.59	0.98	0.97	0.83	0.87	**0.00**
Diabetic 28 day vs. Diabetic 56 day (400squ)	0.96	0.89	0.99	0.98	0.98	0.97	0.33

Parameter Group	A-CoA	OAA	AKGDH	PDH	CS	IDH	Ca^{2+}
Diabetic 28 day vs. Diabetic 84 day (400squ)	0.95	0.88	0.99	0.99	0.98	0.92	**0.00**
Diabetic 56 day vs. Diabetic 84 day (400squ)	1.00	1.00	1.00	1.00	1.00	0.99	0.42
Diabetic first day vs. Diabetic14 day (600squ)	0.35	0.23	0.80	0.76	0.24	0.37	0.09
Diabetic first day vs. Diabetic 28 day (600squ)	0.16	0.22	0.74	0.70	0.12	0.23	**0.00**
Diabetic first day vs. Diabetic 56 day (600squ)	0.09	**0.04**	0.62	0.43	0.10	0.11	**0.00**
Diabetic first day vs. Diabetic 84 day (600squ)	0.95	0.81	0.93	0.95	0.98	0.74	0.59
Diabetic 14 day vs. Diabetic 28 day (600squ)	0.98	0.99	1.00	1.00	0.99	0.99	0.74
Diabetic 14 day vs. Diabetic 56 day (600squ)	0.87	0.77	0.99	0.96	0.98	0.88	0.05
Diabetic 14 day vs. Diabetic 84 day (600squ)	0.86	0.81	0.99	0.97	0.97	0.82	**0.00**
Diabetic 28 day vs. Diabetic 56 day (600squ)	0.98	0.85	0.99	0.97	1.00	0.97	0.38
Diabetic 28 day vs. Diabetic 84 day (600squ)	0.98	0.88	0.99	0.98	1.00	0.94	**0.01**
Diabetic 56 day vs. Diabetic 84 day (600squ)	1.00	1.00	1.00	1.00	1.00	0.99	0.46
P value*							

*P value < 0.05 is significant. A-CoA, acetyl coenzyme A; OAA, oxaloacetate; AKGDH, alpha-ketoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; CS, citrate synthase; IDH, isocitrate dehydrogenase; Ca^{2+}, calcium ion.

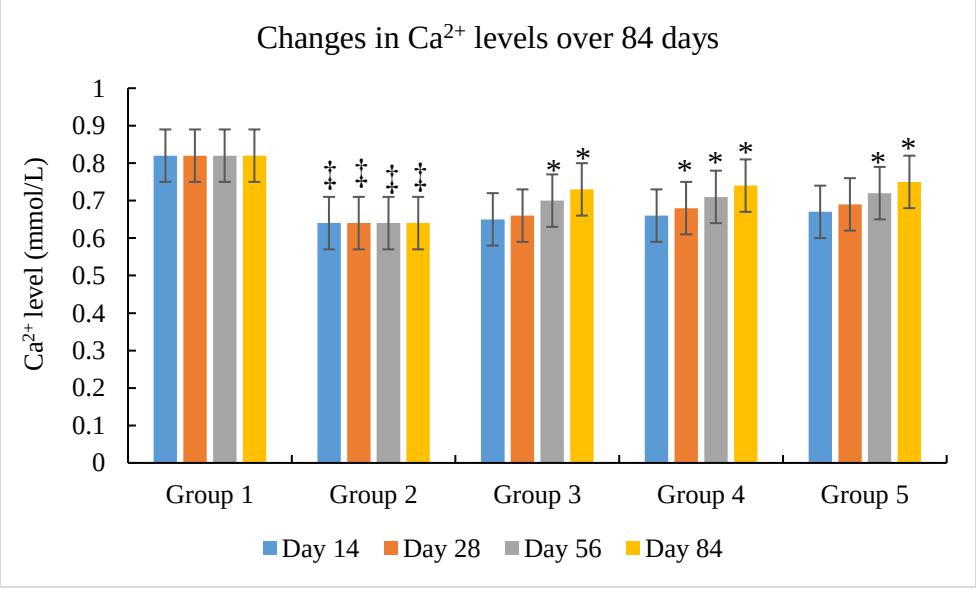


Figure 1: Changes in Ca^{2+} level in all five experimental groups throughout 84 days. Data are given as mean ± SD. * Significances of data comparing groups 3, 4 and 5 vs. the group 2. ‡ Significances of data comparing group 2 vs. the group 1.

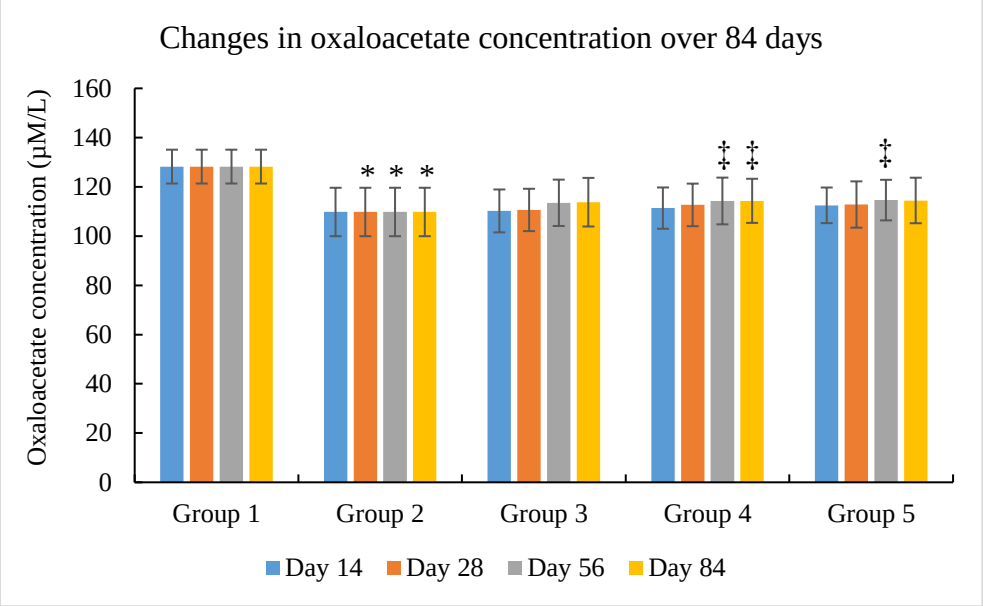


Figure 2: Changes in oxaloacetate concentration in all five experimental groups throughout 84 days. Data are given as mean ± SD. * Significances of data comparing group 2 vs. the group 1. ‡ Significances of data comparing groups 4 and 5 vs. the group 2.

DISCUSSION

In this study, the effect of squalene in different doses and days on the activity of some tricarboxylic acid (Krebs) cycle intermediates and enzymes as well as calcium ion (Ca^{2+}) in different groups of patients with T2DM was investigated and compared with each other. The Krebs cycle in the mitochondria provides energy for cells by producing energy in the form of ATP. DM can affect the function, size, and morphology of mitochondria [9]. It has been reported that skeletal muscle mitochondria in patients with T2DM and obesity are smaller in size and less in number than healthy subjects [10]. Studies on humans and animal models have shown that the oxidative phosphorylation of mitochondria in muscle cells is disrupted in insulin-resistant diabetes [11]. A decrease was observed in the activities of both complex number I and citrate synthase in mitochondria in subjects with DM and obesity when compared with healthy and lean subjects which can be the cause of this disruption [10]. Studies at the protein level report that the muscle of individuals with T2DM has impaired ATP production, which may be a result of decreased ATP synthase and creatine kinase B activity [12].

According to clinical studies, it is possible that a defect in mitochondrial function is the main cause of insulin resistance. Studies have shown that some genes and cofactors related to oxidative phosphorylation are decreased in the family members of patients with T2DM. This decrease in expression has also been reported in subjects with impaired glucose tolerance or pre-diabetes [13].

In this study, we investigated the effect of different doses of squalene on the activity of a number of Krebs cycle intermediates and enzymes, as well as calcium ion levels on patients with T2DM. The volunteers received different doses of squalene on different days and the parameters expressed in their serum samples were investigated. As stated in the results section, a significant difference was observed between the diabetic groups (with and without receiving squalene) and the healthy

control group. In comparing the diabetic groups that received squalene with the diabetic group that did not receive squalene, a significant difference was observed in the levels of calcium ions and oxaloacetate only on certain days and doses. The increase or decrease of levels in other parameters was not significant. Squalene is one of the constituents of argan and amaranth oils and we have shown in previous studies that squalene can play the main role [4, 14-16, 23-24]. In the study conducted by Aydin, the effects of argan oil on mitochondrial functions, antioxidant system and the activity of NADPH-producing enzymes were investigated in the brain of rats [17]. Aydin's study showed that argan oil has a significant effect on the activity of mitochondrial complexes as well as isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase, and malate dehydrogenase enzymes. According to these findings, argan oil (maybe by its important component, squalene) can increase the production of energy in the form of ATP in the brain of rats.

A study by Er et al. was conducted after Aydin's study on the effect of argan oil on mitochondrial function and oxidative stress [14]. By inducing liver and kidney injury in rats and treating them with argan oil, they investigated mitochondrial function and oxidative stress in these tissues. They found that argan oil increases the activity of complexes I, II, and IV in liver tissue compared with kidney tissue. The activity of isocitrate dehydrogenase enzyme in liver tissue was generally higher than the activity in the kidney. Investigation of this enzyme in the group treated with argan oil did not show a significant increase in the liver compared with the control group. The activity of alpha-ketoglutarate dehydrogenase enzyme in both liver and kidney was significantly increased in the group treated with argan oil compared with the control group. In our study, the increase of these two enzymes in the serum of squalene receiving groups was not significant compared with the diabetic group which did not receive squalene.

In a study conducted by Buddhan et al., the effect of 2% dietary squalene supplementation on liver mitochondrial function in aged rats was investigated [6]. Their study showed that 30 days of treatment of aged rats with squalene had a significant increase in ATP production compared to the control group. This significant increase in ATP production was also observed in young rats. They also showed that the activity of Krebs cycle enzymes, such as isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase in liver mitochondria of aged and young rat groups was significantly increased compared with control groups. In our previous study, we showed that the production of ATP in the groups receiving squalene had a significant increase compared with the group without squalene [5, 25]. But in this study, there was no significant increase in the activities of Krebs cycle enzymes in the groups that received squalene compared with the group that did not receive squalene. Cardiovascular disease can be one of the consequences of uncontrolled DM.

A review article, conducted by Ibrahim et al. in 2020, studied the effect of squalene on the risk of cardiovascular disease [18]. In their article, there was only one human study that reported the positive effect of squalene on cardiovascular disease. This study reported that squalene can stop cholesterol biosynthesis by inhibiting the HMG-CoA reductase. This reduces high total cholesterol and LDL-C levels in the body which are important risk factors of cardiovascular disease.

In the study conducted by Motawi et al., it was reported that the treatment of cardiotoxic-induced rats with squalene is effective in normalizing calcium ions and nitric oxide compared to the control group [19]. Squalene maintains the Ca^{2+} ATPase transporter activity close to

normal in heart tissue. The main responsibility of this transporter is to maintain normal intracellular levels of calcium in different cells [20]. In our study, a significant increase in the level of calcium ions was observed in subjects receiving squalene. Because Krebs cycle has a direct relationship with ATP and squalene increases the amount of ATP. Also, Ca^{2+} is an energy activator (for example, the contraction of our muscles depends on Ca^{2+}). So, we can say that the main point of squalene effect on ATP is through the effect on Ca^{2+} in the Krebs cycle. The effect of squalene on the increase of Krebs cycle regulating enzymes (although this increase is not significant), can justify the effect of squalene on the increase of ATP.

CONCLUSION

The data obtained in this study show that squalene with the doses used and on different days does not have a significant effect on some enzymes and intermediates of the Krebs cycle and only has a significant effect on calcium and oxaloacetate levels. Considering the effect of squalene on energy production, it can be concluded that probably squalene affects energy production through an indirect mechanism on the Krebs cycle. To further investigate this role of squalene, molecular studies seem necessary.

List of Abbreviations: T2DM: type 2 diabetes mellitus, ATP: Adenosine triphosphate, Ca^{2+}: Calcium ion, NADPH; Nicotinamide adenine dinucleotide phosphate, A-CoA: acetyl coenzyme A, OAA: oxaloacetate, AKGDH: alpha-ketoglutarate dehydrogenase, PDH: pyruvate dehydrogenase, CS: citrate synthase, IDH: isocitrate dehydrogenase, LDL: low density lipoproteins, SOD: Superoxide dismutase, CAT: catalase.

Authors' contributions: HM and DM discussed the idea of Squalene effects to the cellular energy level for diabetic patients. HM contributed to the selection of volunteers to participate in the study and doing the experimental and clinical work. DM participated in discussion and editing manuscript. BC contributed to writing the abstract and introduction. MRA and ASM participated in data collection and analysis of the results and drawing the graphs. All authors read and approved the final manuscript.

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