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Investigating the changes of some enzymes and metabolites of the Urea 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 diminishes the body's ability to regulate glucose levels due to the lack of insulin produced. In recent studies, squalene has been reported to have beneficial effects for diabetic patients, especially within the liver where the urea cycle takes place.

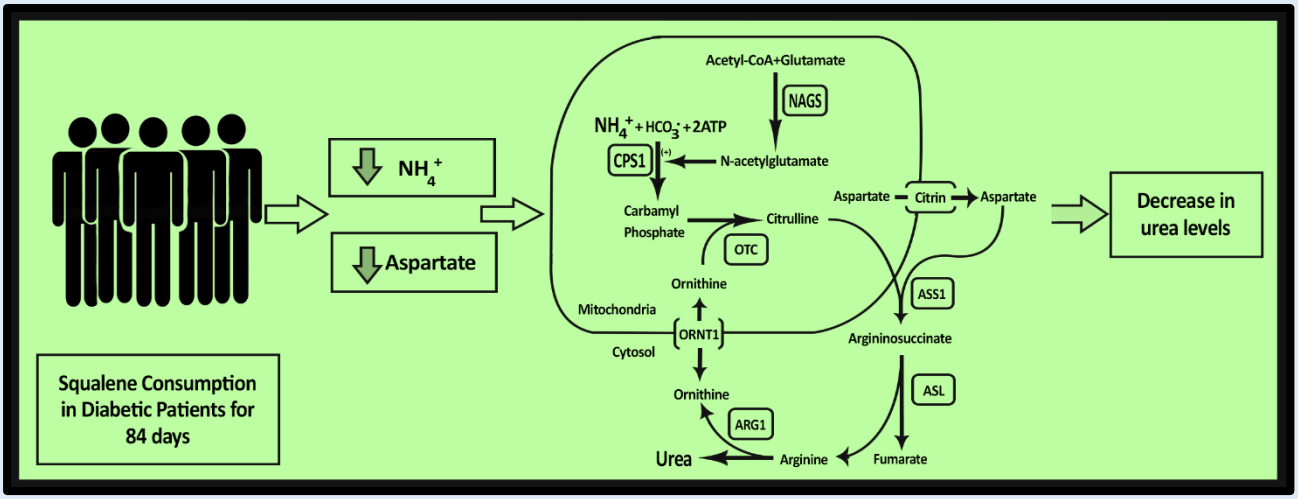
Objective: Our main goal was to evaluate the molecular effects of different doses of squalene on the enzymes, intermediates, and molecules of the urea cycle, in order to determine if squalene has beneficial effects among groups of people with type 2 diabetes mellitus. The enzymes and molecules that are being studied are ornithine transcarbamylase (OTC), arginosuccinate synthetase (ASS), arginase, carbamoyl-phosphate synthetase 1 (CSP1), urea, aspartate, and ammonium ion (NH_4^+).

Methods: In this study, healthy volunteers were categorized as the healthy control (group 1) and volunteers with type 2 diabetes mellitus were selected. The patients with diabetes were divided up into 4 groups. Group 2 consists of the patients that will not be treated with squalene. Groups 3, 4, 5 were treated with 200, 400, 600 mg, respectively. The patients were treated with their respective amounts every 14 days for the duration of 84 days. The enzymes and molecules were measured on days 1, 14, 28, 56, and 84.

Results: The squalene-treated diabetic groups were compared to group 2, who was not treated with any squalene to determine the differences between the parameters. Throughout the 84 days, it was observed that NH_4^+ or ammonium molecules decreased in all treated diabetic patients with high statistical difference ($P < 0.05$). For the majority of the diabetic patients treated with squalene, there was also a decrease in aspartate. The other parameters did not have consistent significant differences ($P > 0.05$).

Conclusion: Based on the findings of this study, the addition of various doses of squalene to a diabetic patient's diet decreased the amount of ammonium and aspartate in the body. As ammonium is the direct product of the urea cycle, it is evident that squalene does play a key role in reducing the amount of ammonium in a diabetic patient to a healthier level.

Keywords: Diabetes mellitus, urea cycle, enzyme, metabolite, squalene.



INTRODUCTION

Squalene is a 30-carbon triterpenoid ($C_{30}H_{50}$) bioactive compound that is commonly found in natural sources such as olive oil and shark liver oil [1, 2]. This compound is an abundant resource which makes it an optimal choice for research in order to learn more of its beneficial effects on the human body. Squalene serves as an intermediate in biosynthesis of phytosterol or cholesterol in plants and animals [3]. Recent studies have shown that squalene has various health benefits on the human body, such as acting as a protective agent towards cancer, boosting the immune system with anti-inflammatory effects, and providing protection against some chronic diseases [4].

Diabetes is one of the leading causes of mortality globally, resulting in millions of deaths over the past few years. In more developed countries, prevalence of

diabetes increases at a much faster rate as a result of rapid economic development and urbanization [5]. According to the World Health Organization (WHO), diabetes is a "chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces [6]. Type 2 diabetes mellitus (T2DM) is the result of the body ineffectively using insulin which is mainly caused by having excess body weight and lacking physical activity. Studies reported that modifications to form a healthy diet and have a healthy lifestyle will help prevent and alleviate T2DM [7, 8]. Uncontrolled DM leads to many complications on the tissues [9, 10]. To help combat the increasing threat of DM, Functional Food Center (FFC) proposes to use functional foods to mitigate the effects of diabetes. The FFC defines functional foods as "natural or processed foods 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'' [11]. Squalene serves as an excellent candidate as a functional food ingredient [12]. Several recent studies on the effects of squalene have shown promising beneficial effects to the patients that have consumed squalene [13-17].

In a study, the effects of squalene were observed to determine if it reduces proteinuria levels in patients with T2DM. Proteinuria refers to the body having elevated levels of protein in urine and maintaining these levels could be an indicator that there could be damage in the kidneys. It was reported that the proteinuria levels of the diabetic patients had decreased based on the amount of squalene that the patient had consumed [18].

It has been reported in a study that healthy individuals that did not have T2DM had higher ATP levels than the individuals that have T2DM [19]. Also, it was observed that the patients with T2DM that consumed squalene experienced an increase of ATP levels. Another study observed the effects of squalene in young and aged rats in order to determine how squalene will affect the mitochondrial functions that occur in the liver [20]. The mitochondria serves a key role in energy production and is also an important site where part of the urea cycle takes place. It was first observed that the older rats had lower ATP levels than the young rats. The addition of squalene acted as a protective agent that improved the mitochondrial function within the liver [20].

As shown with these studies, it is evident that squalene has provided beneficial health effects for humans, especially those who have diabetes. In order to determine more information about the potential effects on people with diabetes, we designed an experiment to analyze the effects of squalene on the urea cycle.

The urea cycle is the process that is responsible for eliminating excess nitrogen, in the form of ammonia, from the body in the form of urea [21]. Toxic ammonia is a product of protein catabolism, the breakdown of proteins in the intestinal tract and absorption of peptides and free amino acids. These peptides and proteins mainly come from a person's diet. Anything that is not broken down and used for protein synthesis is then used to produce ammonia [21]. If the urea cycle is not functioning properly, it will lead to the accumulation of ammonia within the body and result in harmful effects to the human body [22].

The cycle mainly occurs in the liver where around 80% of the urea is synthesized from ammonia. The liver's mitochondria and cytoplasm serve as important sites for the urea cycle as these are where the main five enzymes that are part of this cycle are located; two of the enzymes are located in the mitochondria and three of the enzymes are located in the cytoplasm [19]. These enzymes are: carbamoyl-phosphate synthetase (CPS1), ornithine transcarbamylase (OTC), arginosuccinate synthetase (ASS), and arginase. The first step of the urea cycle involves CPS1 converting CO and ammonia into carbamoyl phosphate. Then the OTC forms citrulline with the carbamoyl phosphate and ornithine [21]. Citrulline is transported from the mitochondria to the cytoplasm where the other three enzymes are located. Then, the citrulline is combined with aspartate and synthesized by

arginosuccinate synthetase to become arginosuccinate. The arginosuccinate lyase converts the arginosuccinate into arginine and fumarate. Fumarate is used in the citric acid cycle for NADH generation while arginine is used in the next step of the urea cycle. The arginine is hydrolyzed by the arginase to form urea and arginase [22]. The urea is finally excreted from the body by the kidneys.

In order to determine the effects of squalene, our study will focus on measuring the effects of the enzymes and molecular components of the urea cycle. The 8 components of the urea cycle that are being observed are: ornithine transcarbamylase (OTC), arginosuccinate synthetase (ASS), arginase, carbamoyl-phosphate synthetase 1 (CSP1), urea, aspartate, and ammonium ion (NH_4^+).

MATERIALS AND METHODS

Materials: Squalene (S3626) was purchased from Sigma Company (USA). The ELISA kits of human glucose, urea and arginase were purchased from My BioSource Inc (USA). The colorimetric detection kits of NH4+ and aspartate were procured from Arigo Company (Canada) and Abcam Company (USA), respectively. The ELISA kits of Ornithine Transcarboxylase (OTC) were purchased from BIOMATIK Company (USA). The ELISA kits of Arginosuccinate Synthase (ASS) and Arginosuccinate (AS) were procured from Assay Genie Company (Ireland). The ELISA kits of Carbamoyl Phosphate Synthase 1 (CPS 1) were purchased from CUSABIO Company (USA).

METHODS

Participants: In this study, 150 volunteers were involved. The assessment was performed for five groups of 30 participants. These groups include group 1-or healthy volunteers (healthy control), group 2 (diabetic control)-or T2DM patients who didn't receive squalene, group 3-or T2DM patients with consumption of 200 mg/day squalene, group 4-or T2DM patients with consumption of 400 mg/day squalene, and group 5-or T2DM patients with consumption of 600 mg/day squalene. Groups 3, 4, and 5 used up squalene (as an oral capsule including relevant liquid) once a day during lunch for 84 days. All volunteers with T2DM took their medications during the study and were monitored for possible side effects of squalene. Fortunately, no side effects were observed. Participants with diabetes were patients who were referred to Vali-Asr medical laboratory (Tehran, Iran). Inclusion criteria contained fasting plasma glucose ≥ 126 mg/dL, glycated hemoglobin (HbA1c) ≥ 6.5% and not taking corticosteroids, according to the World Health Organization (WHO). Exclusion criteria included a history of surgery, young patients with T2DM, T1DM and other diseases. All contributors filled in the consent form. The conduction of study and the kind of consumable substances was explained to groups 3, 4 and 5.

General Features and Sampling: After the arrangement of groups and under sterile condition, blood samples of all participants were taken. On days 1, 14, 28, 56 and 84, the sampling was performed. The biochemical parameters of all the groups were evaluated in each period. Anthropometric items of all of the volunteers, including age, sex, weight, height, and body mass index (BMI**)** were recorded in the above periods. A blood sample was taken from each volunteer after 12 hours of nighttime fasting. For preparing serum (250 g for 10 min), the indicated blood samples were centrifuged. After that, the relevant biochemical parameters in each serum sample were quantified.

Statistical Analysis: A statistical analysis was done by SPSS (version 26, IBM, USA) software for Windows. All results were expressed as mean ± standard deviation (SD). An Independent-sample T-test was used to compare the mean of general characteristics of the participants. Statistical significance was analyzed by one-way ANOVA to compare the mean of the obtained data. After the one-way ANOVA test, the 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

The results of the statistical analysis of the data obtained from measuring the activity of OTC, ASS, AS, arginase, CPS1 and the concentration of urea, aspartate, and ammonium ion in the control group (group 1) and diabetic groups (2, 3, 4 and 5) are shown in Table 1. As shown in Table 1, from the comparison of the mean obtained data from the measurement of the mentioned parameters between the control group and the diabetic groups (group 2 who did not receive squalene and groups 3, 4 and 5 who received squalene in different doses and days) significant difference was observed (P-values < 0.05). Figures 1 to 5 also show the differences in significance in the comparison of the previously mentioned parameters between the groups.

Figures 1 and 2 show the changes of arginase and CPS1 activities in the studied groups, respectively. Arginase and CPS1 activities in the groups with and without squalene (groups 2, 3, 4 and 5) showed a significant increase compared to the healthy control group (group 1). Changes in urea levels, as a graph, in Figure 3 show a significant increase between groups 2, 3, 4 and 5 in comparison to group 1.

Table 1: Comparison of some intermediates and enzymes of the urea cycle between the control group and the others.

Parameter / Group	OTC (ng/ml)	ASS (ng/ml)	AS (ng/ml)	Arginase (ng/ml)	CPS1 (pg/ml)	Urea (mg/dl)	Aspartate (µM)	NH_4^+ (µM)
Healthy control	11.94 ± 0.92	7.41 ± 0.71	7.47 ± 0.83	283.17 ± 8.64*	2850.53 ± 49.71*	37.09 ± 1.65*	101.37 ± 3.15*	499.97 ± 15.18*
Diabetic control (No squalene)	12.21 ± 0.89	7.83 ± 1.08	7.86 ± 1.06	301.80 ± 9.14	2932.03 ± 63.89	42.80 ± 2.17	117.33 ± 4.60	564.90 ± 7.29
	*P value < 0.05							
Diabetic + 200 mg/day squ (14th day)	12.21 ± 0.99	7.71 ± 1.08	7. 47 ± 1.06	299.73 ± 9.17	2906.97 ± 63.87	41.84 ± 2.20	114.57 ± 4.44	554.87 ± 7.34
Diabetic + 400 mg/day squ (14th day)	12.19 ± 0.91	7.71 ± 1.08	7.74 ± 1.06	297.77 ± 9.11	2903.07 ± 63.92	41.64 ± 2.17	113.30 ± 4.63	549.83 ± 7.30
Diabetic + 600 mg/day squ (14th day)	12.18 ± 0.99	7.70 ± 1.07	7.73 ± 1.06	296.67 ± 9.14	2901.10 ± 63.94	41.42 ± 2.20	112.37 ± 4.62	543.80 ± 7.41
Diabetic + 200 mg/day squ (28th day)	12.00 ± 0.89	7.70 ± 1.08	7.73 ± 1.05	296.57 ± 10.35	2900.97 ± 63.42	41.10 ± 2.22	111.67 ± 4.23	542.83 ± 7.36
Diabetic + 400 mg/day squ (28th day)	11.99 ± 0.89	7.69 ± 1.08	7.72 ± 1.06	295.77 ± 9.20	2898.03 ± 63.89	40.90 ± 2.17	111.33 ± 4.60	540.97 ± 7.28
Diabetic + 600 mg/day squ (28th day)	11.99 ± 0.89	7.69 ± 1.08	7.72 ± 1.06	294.73 ± 9.16	2894.10 ± 63.77	40.64 ± 2.16	110.20 ± 4.55	535.10 ± 7.29
Diabetic + 200 mg/day squ (56th day)	11.98 ± 0.89	7.68 ± 1.08	7.72 ± 1.06	293.77 ± 9.13	2887.97 ± 63.91	40.51 ± 2.13	109.30 ± 4.61	530.77 ± 7.50
Diabetic + 400 mg/day squ (56th day)	11.98 ± 0.89	7.68 ± 1.08	7.71 ± 1.06	292.73 ± 9.15	2885.93 ± 64.00	40.49 ± 1.97	108.27 ± 4.51	526.90 ± 7.23
Diabetic + 600 mg/day squ (56th day)	11.97 ± 0.89	7.67 ± 1.08	7.71 ± 1.06	291.67 ± 9.19	2882.03 ± 63.71	40.41 ± 1.36	107.53 ± 4.63	522.73 ± 7.24
Diabetic + 200 mg/day squ (84th day)	11.97 ± 0.89	7.67 ± 1.08	7.71 ± 1.06	291.23 ± 9.15	2877.97 ± 64.00	40.35 ± 2.20	106.77 ± 4.23	521.87 ± 7.24
Diabetic + 400 mg/day squ (84th day)	11.96 ± 0.89	7.67 ± 1.08	7.70 ± 1.06	290.67 ± 9.30	2873.83 ± 64.07	40.20 ± 2.20	106.23 ± 4.63	518.80 ± 7.17
Diabetic + 600 mg/day squ (84th day)	11.96 ± 0.89	7.66 ± 1.08	7.70 ± 1.06	289.63 ± 9.17	2869.90 ± 63.75	40.01 ± 2.08	105.30 ± 4.55	515.73 ± 7.11
	*P value < 0.05							

*Indicates the comparison of the results between the groups 2, 3 4 and 5 with the control group. Data is given as mean ± SD. OTC, ornithine transcarbamylase; ASS, arginosuccinate synthetase; AS, arginosuccinate; CPS1, carbamoyl phosphate synthase 1; NH_4^+, ammonium ion.

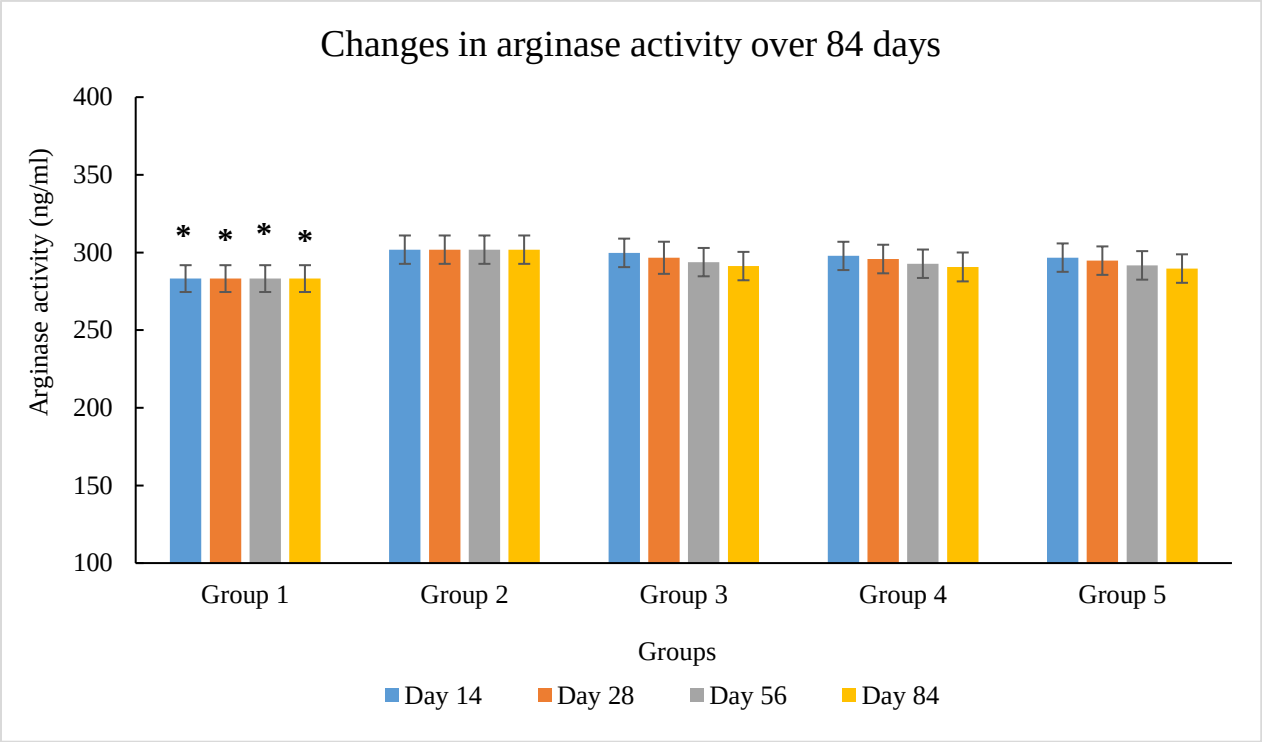


Figure 1. Changes in Arginase activity in all five experimental groups throughout 84 days. Data is given as mean ± SD. *Significances of data comparing the group 1 vs. groups 2, 3, 4 and 5.

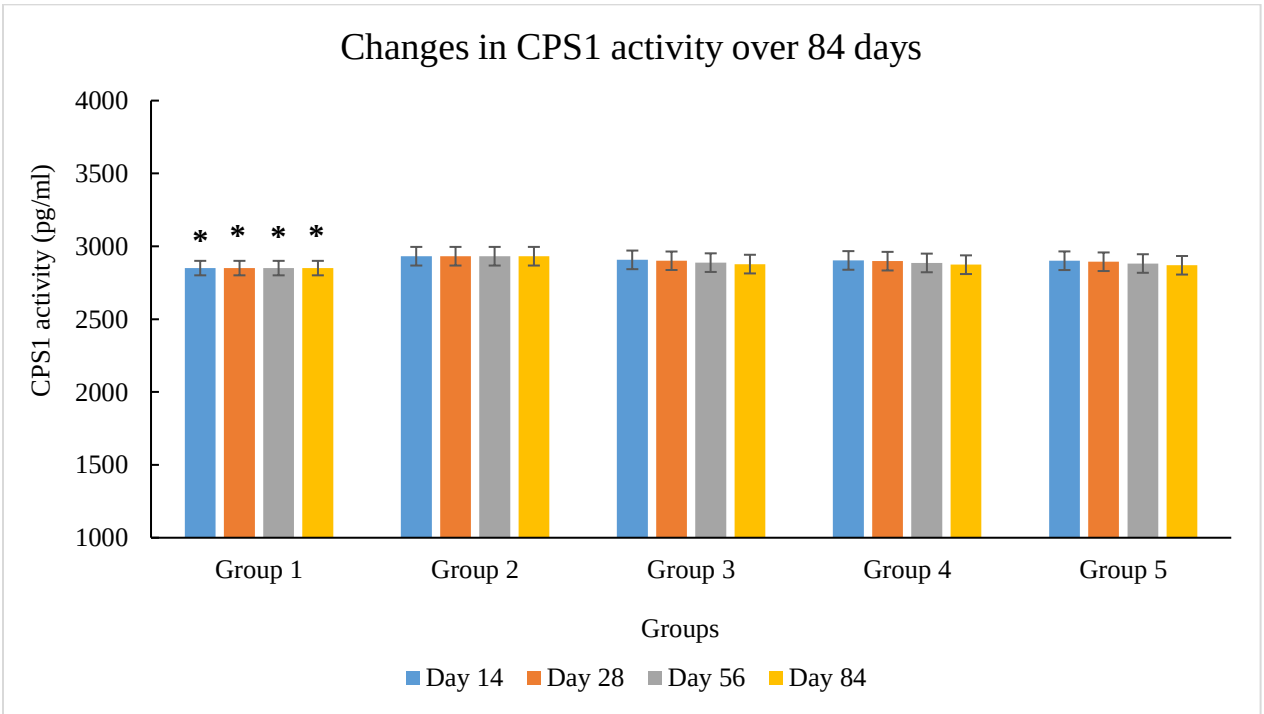


Figure 2. Changes in carbamoyl phosphate synthase 1(CPS1) activity in all five experimental groups throughout 84 days. Data is given as mean ± SD. *Significances of data comparing the group 1 vs. groups 2, 3, 4 and 5.

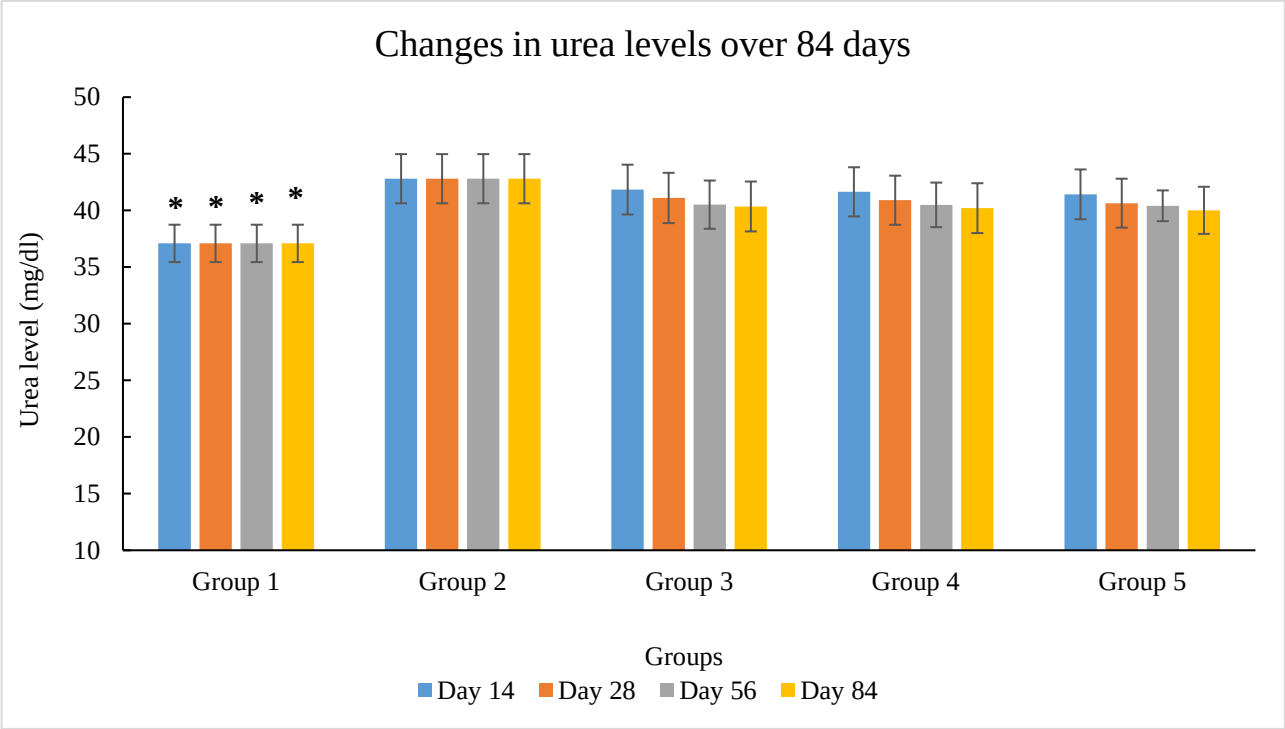


Figure 3. Changes in urea levels in all five experimental groups throughout 84 days. Data is given as mean ± SD. *Significances of data comparing the group 1 vs. groups 2, 3, 4 and 5.

Table 2 shows the statistical comparison of the obtained results between groups 2, 3, 4 and 5 with each other on different days and with different doses of squalene. Comparing the mentioned enzymes and parameters between group 3 and group 2 on day 14, significant differences were observed only between aspartate and NH_4^+ (P-values < 0.05). Comparing the mentioned enzymes and parameters between group 3 and group 2 on day 28, significant differences were observed only between urea, arginase, aspartate and NH_4^+ (P-values < 0.05). Comparing the mentioned enzymes and parameters between group 3 and group 2 on days 56 and 84, a significant difference was observed between urea, arginase, aspartate, $NH_4^+$and CPS 1 (P-values < 0.05).

Comparing the mentioned enzymes and parameters between group 4 and group 2 on day 14, a significant difference was observed between urea, aspartate and NH_4^+ levels (P-values < 0.05). Comparing the mentioned enzymes and parameters between group 4 and group 2 on days 28, 56 and 84, a significant difference was observed between arginase, CPS 1 activity and aspartate, $NH_4^+$and urea levels (P-values < 0.05).

Comparing the mentioned enzymes and parameters between group 5 and group 2 on day 14, a significant difference was observed between urea, aspartate and NH_4^+ levels as well as arginase activity (P-values < 0.05). Comparing the mentioned enzymes and parameters between group 5 and group 2 on days 28, 56 and 84, a significant difference was observed between arginase, CPS 1 activity and aspartate, NH_4^+ and urea levels (P-values < 0.05). A comparison between some enzymes of the urea cycle and parameters related to the urea cycle between groups 3, 4 and 5 was also done on different days, which is shown in Table 2.

Table 2: Multiple comparisons between some intermediates and enzymes of the urea cycle between the groups in different days.

Parameter Group	OTC (ng/ml)	ASS (ng/ml)	AS (ng/ml)	Arginase (ng/ml)	CPS1 (pg/ml)	Urea (mg/dl)	Aspartate (µM)	NH_4^+ (µM)
Diabetic first day vs. Diabetic 14 day (200squ)	0.99	0.67	0.66	0.38	0.13	0.09	**0.02**	**0.00**
Diabetic first day vs. Diabetic 28 day (200squ)	0.35	0.64	0.63	**0.04**	0.06	**0.00**	**0.00**	**0.00**
Diabetic first day vs. Diabetic 56 day (200squ)	0.31	0.60	0.59	**0.00**	**0.01**	**0.00**	**0.00**	**0.00**
Diabetic first day vs. Diabetic 84 day (200squ)	0.29	0.57	0.56	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Diabetic 14 day vs. Diabetic 28 day (200squ)	0.80	1.00	1.00	0.56	0.98	0.56	0.05	**0.00**
Diabetic 14 day vs. Diabetic 56 day (200squ)	0.76	1.00	1.00	0.07	0.65	0.09	**0.00**	**0.00**
Diabetic 14 day vs. Diabetic 84 day (200squ)	0.73	0.99	0.99	**0.00**	0.29	**0.04**	**0.00**	**0.00**
Diabetic 28 day vs. Diabetic 56 day (200squ)	1.00	1.00	1.00	0.66	0.86	0.73	0.16	**0.00**
Diabetic 28 day vs. Diabetic 84 day (200squ)	0.99	1.00	1.00	0.13	0.50	0.55	**0.00**	**0.00**
Diabetic 56 day vs. Diabetic 84 day (200squ)	1.00	1.00	1.00	0.72	0.93	0.99	0.11	**0.00**
Diabetic first day vs. Diabetic 14 day (400squ)	0.92	0.66	0.64	0.09	0.08	**0.04**	**0.00**	**0.00**
Diabetic first day vs. Diabetic 28 day (400squ)	0.33	0.63	0.61	**0.01**	**0.04**	**0.00**	**0.00**	**0.00**
Diabetic first day vs. Diabetic 56 day (400squ)	0.31	0.59	0.58	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Diabetic first day vs. Diabetic 84 day (400squ)	0.27	0.56	0.55	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Diabetic 14 day vs. Diabetic 28 day (400squ)	0.82	1.00	1.00	0.83	0.99	0.53	0.35	**0.00**
Diabetic 14 day vs. Diabetic 56 day (400squ)	0.79	1.00	1.00	0.15	0.72	0.16	**0.00**	**0.00**
Diabetic 14 day vs. Diabetic 84 day (400squ)	0.75	0.99	0.99	**0.01**	0.29	**0.04**	**0.00**	**0.00**
Diabetic 28 day vs. Diabetic 56 day (400squ)	1.00	1.00	1.00	0.58	0.88	0.87	0.05	**0.00**
Diabetic 28 day vs. Diabetic 84 day (400squ)	0.99	1.00	1.00	0.14	0.46	0.58	**0.00**	**0.00**
Diabetic 56 day vs. Diabetic 84 day (400squ)	1.00	1.00	1.00	0.82	0.88	0.95	0.32	**0.00**

Diabetic first day vs. Diabetic14 day (600squ)	0.89	0.65	0.63	**0.03**	0.06	**0.01**	**0.00**	**0.00**
Diabetic first day vs. Diabetic 28 day (600squ)	0.33	0.62	0.61	**0.00**	**0.02**	**0.00**	**0.00**	**0.00**
Diabetic first day vs. Diabetic 56 day (600squ)	0.30	0.58	0.57	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Diabetic first day vs. Diabetic 84 day (600squ)	0.27	0.55	0.54	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
Diabetic 14 day vs. Diabetic 28 day (600squ)	0.85	1.00	1.00	0.84	0.97	0.42	0.26	**0.00**
Diabetic 14 day vs. Diabetic 56 day (600squ)	0.82	1.00	1.00	0.15	0.65	0.20	**0.00**	**0.00**
Diabetic 14 day vs. Diabetic 84 day (600squ)	0.78	0.99	0.99	**0.02**	0.23	**0.03**	**0.00**	**0.00**
Diabetic 28 day vs. Diabetic 56 day (600squ)	1.00	1.00	1.00	0.56	0.88	0.97	0.11	**0.00**
Diabetic 28 day vs. Diabetic 84 day (600squ)	0.99	1.00	1.00	0.14	0.46	0.61	**0.00**	**0.00**
Diabetic 56 day vs. Diabetic 84 day (600squ)	1.00	1.00	1.00	0.82	0.88	0.86	0.24	**0.00**
P value*								

*P value < 0.05 is significant. OTC, ornithine transcarbamylase; ASS, arginosuccinate synthetase; AS, arginosuccinate; CPS1, carbamoyl phosphate synthase 1; NH_4^+, ammonium ion.

Figures 4 and 5 show the changes in aspartate and NH_4^+ concentrations in the studied groups, respectively. A significant increase was observed in the comparison of aspartate and NH_4^+ between groups 2, 3, 4 and 5 to group 1. A significant decrease in the levels of these two parameters was observed on days 28, 56 and 84 in comparison between groups 3, 4 and 5 to group 2.

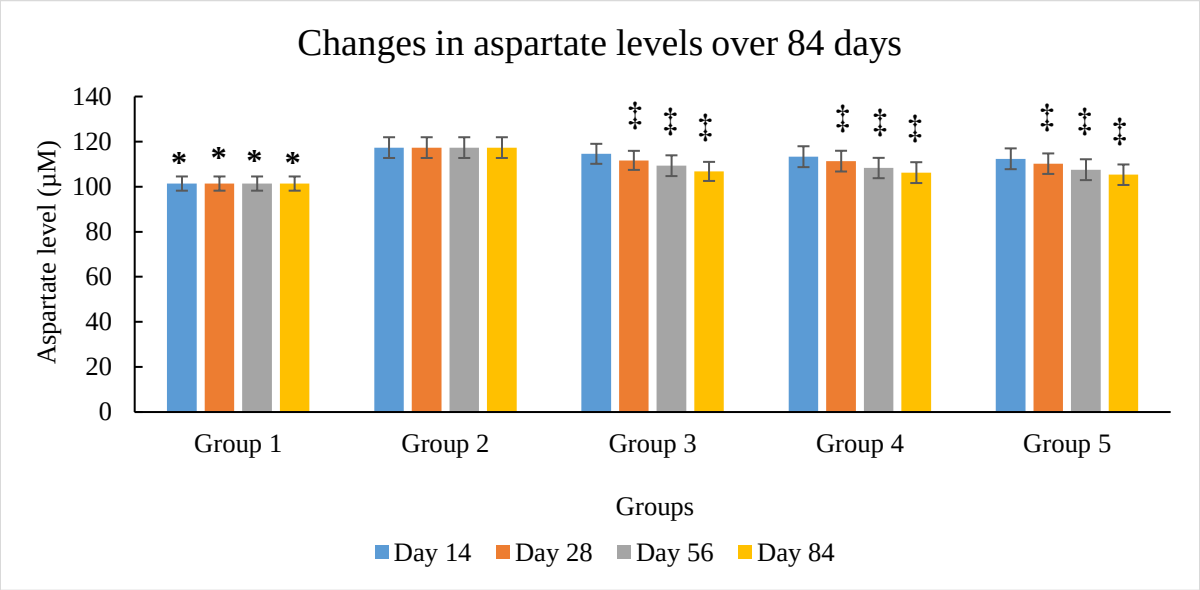


Figure 4. Changes in aspartate levels in all five experimental groups throughout 84 days. Data is given as mean ± SD. *Significances of data comparing the group 1 vs. groups 2, 3, 4 and 5. ‡ Significances of data comparing groups 3, 4 and 5 vs. the group 2.

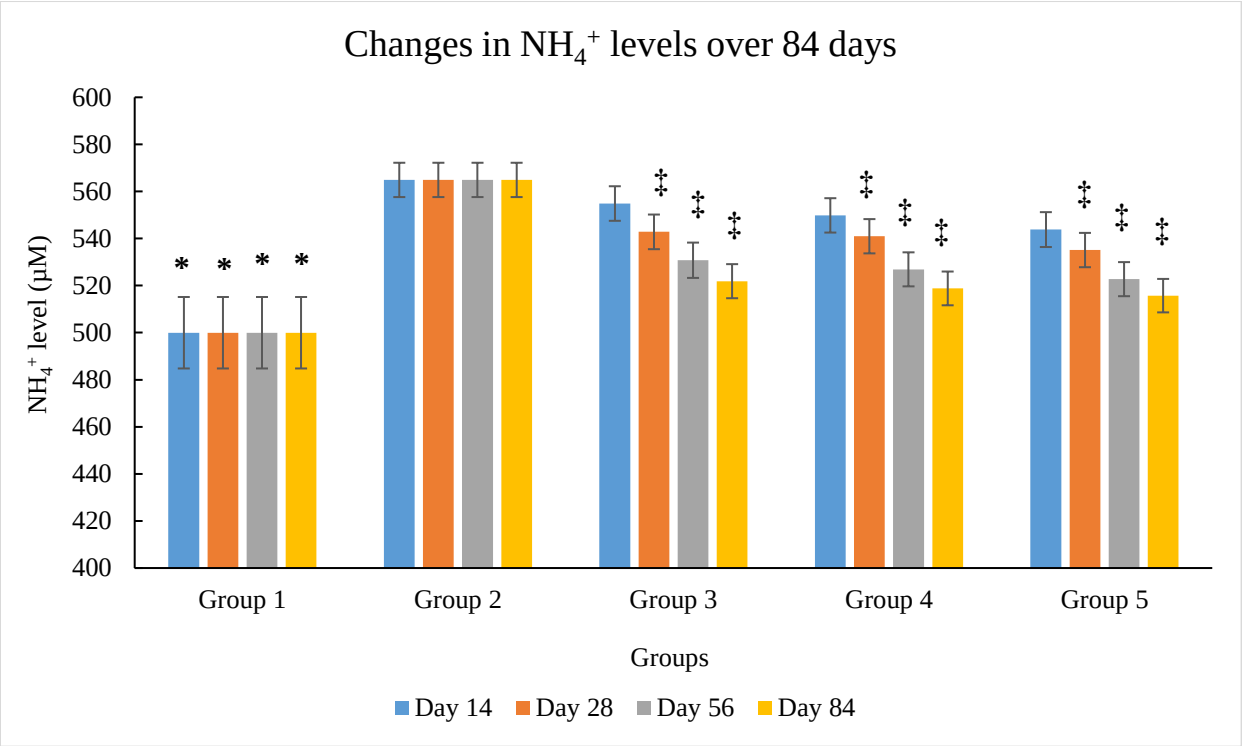


Figure 5. Changes in NH_4^+ levels in all five experimental groups throughout 84 days. Data is given as mean ± SD. *Significances of data comparing the group 1 vs. groups 2, 3, 4 and 5. ‡ Significances of data comparing groups 3, 4 and 5 vs. the group 2.

DISCUSSION

Ammonia is a toxic compound for humans and other organisms. Urea cycle activities prevent the accumulation of ammonia in the human body in the liver and kidneys [23]. Uncontrolled and untreated DM can lead to many acute and chronic complications. One of the complications of DM is non-alcoholic fatty liver disease (NAFLD). If this disease is not treated, the liver will lose its function and the patient's life will be threatened [24]. As an essentially metabolic disease, renal cell carcinoma (RCC) develops in metabolically altered conditions such as DM, obesity, and chronic kidney disease (CKD) [23]. Obesity is a risk factor for T2DM, and CKD is one of the complications of uncontrolled DM. In this study, for the first time, we investigated the activity of some urea cycle enzymes as well as the level of ammonium ions in healthy volunteers and subjects with T2DM. Subjects with T2DM took squalene in different doses and on different days, and the mentioned parameters were also investigated between these subjects and subjects who did not receive squalene. It was reported that in RCC there are changes in ammonia metabolism and the expression of urea cycle enzymes such as arginase, AS and ASS is downregulated. Downregulation of these enzymes is directly related to tumor growth [25].Elevated levels of blood urea nitrogen are associated with an increased risk of T2DM [26]. In the urea cycle, several enzymes involved in removing nitrogen from amino acids are regulated by glucagon and insulin. For example, insulin affects the activity of arginase, a key enzyme that also synthesizes nitric oxide [27]. Arginase is one of the important enzymes in the urea cycle. This enzyme plays a role in converting L-arginine into urea and ornithine. Arginine is the substrate of the nitric oxide synthase enzyme. Nitric oxide decreases with the increase of ammonium ions and urea production. Nitric oxide depletion can affect endothelial

function. It has been reported that arginase activity is related to DM and vascular complications related to DM [28]. In a study conducted by Kashyap et al. [29], it was shown that plasma arginase activity increased in individuals with T2DM and impaired nitric oxide synthase activity. Their cross-sectional study was conducted on 73 patients with T2DM. In this study, the comparison between some enzymes of the urea cycle and ammonium ions in the serum of volunteers was investigated. Regarding the increase of arginase activity in patients with T2DM compared to healthy control subjects, our study agreed with Kashyap's study. In our study, a significant ($P < 0.05$) difference was observed in the comparison of arginase, OTC, ASS, AS and CPS1 enzymes between the healthy control group and the groups with T2DM (the groups that received squalene and the group that did not receive squalene). This significant difference in the levels of urea, aspartate and ammonium ions was also observed between the control group and the groups with T2DM. This shows that the production of ammonium ions and urea in patients with T2DM is higher than in healthy subjects.

In a study conducted by Cao et al. on 401 patients with type 2 diabetes, the plasma levels of amino acids related to the urea cycle and the risk of T2DM in Chinese adults were investigated [30]. They found that amino acids involved in the urea cycle were associated with T2DM. In this research, only aspartate amino acid levels were investigated. They reported that increased levels of arginine and decreased levels of ornithine and their related ratios are associated with newly diagnosed T2DM. Our data showed that there is a significant ($P < 0.05$) difference in the level of this amino acid between the control and groups with T2DM. Consumption of squalene with different doses and on different days in groups 3, 4 and 5 decreased the levels of aspartate. A decrease in aspartate level is associated with a decrease in urea production. Our data showed that diabetes increases the activity of some urea cycle enzymes, ammonium ions and urea in patients with T2DM. With the consumption of squalene in different doses and on different days, a significant decrease was observed in the activity of these enzymes and the levels of ammonium ions and urea. De Chiara et al. [31] found that in subjects with NAFLD, the enzymes that convert excess nitrogen into urea are affected and ammonium accumulates in the blood. They reported that gene and protein expression of OTC and CPS1, as well as OTC activity were reversibly decreased.

Dogan et al. [32] studied the antioxidant effect of Quercus brantii extract on diabetic rats. An increase in urea levels was observed in diabetic rats. They reported that treating diabetic rats with Quercus brantii extract at a dose of 500 mg/kg has a significant effect in reducing urea levels. In our study, a decrease in urea level was observed with squalene consumption in patients with T2DM.

CONCLUSION

According to the obtained results in this study, it can be concluded that the consumption of bioactive compound such as squalene play a key role in reducing the production of excess nitrogen and subsequently reducing the production of urea. Both the increase and decrease of ammonium ions and urea can be consequences of uncontrolled DM.

List of Abbreviations: DM, diabetes mellitus; T2DM, type 2 diabetes mellitus; OTC, ornithine transcarbamylase; ASS, arginosuccinate synthetase; AS, arginosuccinate; CSP1, carbamoyl-phosphate synthetase 1; NH_4^+, ammonium ion; WHO, World Health Organization; FFC, Functional Food Center; HbA1c, glycated hemoglobin.

Competing interests: The authors declare that they have no competing interests.

Authors' contributions: HM and DM discussed the idea of squalene effects and 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, participated in the study design. DM edited and finalized the manuscript for submission, participated in the study design and supervised the project. MRA and ASM participated in data collection and analysis of the results and drawing the graphs. BC contributed to writing the abstract and introduction.

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