Squalene modulates fatty acid metabolism: Enhanced EPA/DHA, in obese/diabetic mice (*KK-A^y*) model

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Running title: Squalene effect on EPA/DHA levels in obese/diabetic mice

Abbreviations:

ALA - α-Linolenic Acid; Con – Control; DPA – Docosapentanoic Acid; FAME – Fatty Acid Methyl Ester; SO – Soybean Oil; SQ – Squalene; WAT – White Adipose Tissue

Abstract

Biosynthesis of long-chain n-3 fatty acids from precursors is limited. *In-vivo* effect of squalene (SQ) on the metabolic fate of n-3 fatty acid precursors in obese/diabetic*KK-A*^{y} rodent model was evaluated in our work. Soybean oil, being rich in ALA (18:3 n-3; a known precursor of EPA/DHA), was chosen as the n-3 fatty acid precursor rich source. *A* high-fat diet (20%) containing 7% soybean oil (SO) was fed to obesity/diabetes-prone male KK-Ay mice (control). In the case of diets fed to test groups, soybean oil was replaced with 5% SO & 2% SQ. Hepatic DHA (6 fold) levels increased in SQ fed group over control ($p<0.05$). Gene and protein expressions of Δ^5 and Δ^6 desaturases, key enzymes involved in the fatty acid metabolism, further supported the results. Also, SQ exhibited a hypotriglyceridemic and hypoglycemic effect. The results clearly indicated the effect of SQ in modulating the n-3 fatty acid metabolism, including EPA/DHA synthesis in the presence of n-3 fatty acid precursor. This is the first report of enhancement of *in-vivo* DHA/EPA by SQand in turn, modulating the physiological fatty acid profile.

Practical Applications (150 words)

Squalene (SQ) is an important marine biofunctional material that is found in some terrestrial sources as well. Squalene, being a cholesterol precursor, forms an interesting subject of research for its effect in-vivo. SQ significantly enhanced proportions of EPA and/or DHA when their n-3 fatty acid precursors were available in the diet. The study further establishes the usefulness of SQ in functional food formulations. The work provides an important basis for further evaluation of the role of SQ in normal and disease conditions.

Keywords: Docosahexanoic acid; eicosapentanoicacid; *KK-A*^{y} mice, n-3 PUFA, squalene

1 Introduction

Long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) have drawn considerable attention due to their diverse and dynamic biofunctional properties. Among n-3 PUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to be essential for brain and retinal development during infancy, a factor for longer/completion of gestation in pregnant women in addition to maintenance of homeostasis of various functions and organs in adults [1]. Also, EPA and DHA are essential components of the membrane, necessary for retinal and neuronal function, and can reduce the risk of inflammatory, cardiovascular diseases, cancer, hypertension and diabetes [1-3].The primary sources of pre-formed long chain n-3 fatty acids such as EPA and DHA include foods of marine origin such as unicellular phytoplankton, seaweeds and fish [1]. The precursor of n-3 fatty acids, α-linolenic acid (ALA) is abundant in plant sources such as linseed/flaxseed oil [2]. Though humans and other animals have a biosynthetic pathway for the synthesis of EPA, docosapentaenoic acid (DPA) and DHA, the rate of conversion to EPA has been found to be very limited (up to 8%) [4]. Further, the bioconversion is still more meagre in the case of DHA (0.01%) while pregnant women may exhibit greater (9%) bioconversion [5-6]. Hence, while biosynthesis of EPA from precursors may be feasible, DHA production is grossly inadequate [7]. Also, a sex dependent difference with greater conversion to DHA in women as compared to men has also been reported [8]. Overall, preformed DHA has been the only effective means to increase DHA to desirable levels in blood and target organs [7-11].

Squalene is a triterpenoid that is a precursor in the cholesterol/sterol biosynthetic pathway. Skin is the major organ for squalene storage (13%), and adipose tissue is also known to store considerable amounts of squalene. Since squalene is a sterol/cholesterol precursor compound, it is an important intermediate component in the formation of eukaryotic sterols and bacterial hopanoids and is present in almost all cells [12]. Some reports attribute anticancerous

properties to squalene as it enhances cellular antioxidant status due to its antioxidant activity [13-15]. Our recent study in macrophages demonstrated increased protection against lipid peroxidation by the xanthophyll carotenoid, astaxanthin, in the presence of squalene [16]. Besides, many researchers have attributed the anti-cancerous properties of squalene to the down-regulation of phenylation action of the RAS oncogene [17,18]. Though varied in results, several reports are available on the effect of squalene on lipid components as a result of feeding including hypocholesterolemic effect [19-22] and hypercholesterolemic effect [23-25]. These have led the scientific community to suggest exercising caution with regards to squalene. On the other hand, recently, deCastro et al [26] and Smith et al [27] have reported that squalene had no effect on plasma lipid parameters; while, enhancement of glucose-induced insulin secretion and insulin content indicating its possible hypoglycemic effect is also reported[28].

From the above published reports, squalene's role in altering the lipid profile, positively or negatively, is probable. However, none of the studies have attempted to decipher the role of squalene in fatty acid metabolism, biosynthesis in particular, especially in the presence of n-3 fatty acids precursor. We hypothesized that squalene could affect fatty acid metabolism and EPA/DHA in particular. A model with altered metabolic status (*KK-A^y* mice) was fed a high fat diet and used for the experiments. *KK-A^y* mice were chosen as it would reflect better any effect of squalene feeding on lipid related parameters as this mice model responds to altered metabolic status with rapid development of obesity and diabetes. Based on previous studies evaluating other effects of squalene that reported dosages varying from 1 to 4% [22, 26, 27], a 2% squalene dosage was employed in our study. Against this background, our study was designed to analyse the effect of orally administered squalene (2% of diet) on the fatty acid profile in the presence of an n-3 fatty acids precursor (ALA) responsible for EPA/DHA

formation. In addition to this, other lipid parameters and effect of orally fed squalene on glucose levels was also monitored.

2Materials and Methods

2.1 Materials

Squalene (SQ) was from Wako Pure Chemicals, Ltd., Osaka, Japan. Dietary lipids, soybean oil and lard, were obtained from Wako, Summit Oil Mill Co. Ltd., Chiba, Japan, and Junsei Chemical Co. Inc., Tokyo, Japan, respectively. All the other chemicals and solvents used in the study were of analytical grade.

2.2 Animals and diets

Obese/diabetic model KK-A^y mice (male, four weeks age) employed in the study were obtained from Japan CREA Co., Osaka, Japan. The diet preparation was as per the recommendations of American Institute of Nutrition (AIN-93G) [29]. All the procedures and protocols for the use and care of animals were approved by the Ethical Committee for Experimental Animal Care of the Hokkaido University, Japan (Approval no. 14-0072).

After acclimation for 1 week, the mice were randomly divided into control and experimental groups (n=7). Mice fed with 20% fat diet [13% lard and 7% soybean oil (SO)] formed the control (Con) (Table 1). The test diet (SO+SQ) contained 13% lard,5% SO and 2% SQ. The animals were maintained at 23 ± 2°C and 60 ± 5 % humidity under a 12 hour light/dark cycle. All the animals had free access to food and water.

The food and water intakes along with body weights were measured and recorded on a daily basis. At the end of the experimental period of 4 weeks, both control and test animals were sacrificed to collect blood and other organs. The animals were fasted overnight before culling. The blood collected in vacuettes was used for separation of serum. Organs were excised, weighed, divided and stored in liquid nitrogen, RNA later solution (Life Technologies, USA) and stored in the deep freezer (-40°C) until further analyses.

2.3 Fatty acid analysis of dietary lipids

Total lipid was extracted with chloroform/methanol (2:1,v/v) from each diet. Fatty acyl groups in the lipid of diets and dietary oils were transmethylated to obtain methyl esters. The fatty acid methyl esters (FAME) were prepared according to the method of Prevot and Mordret[30] and used for determining the fatty acid composition by GC. Briefly, 1 mL of n-hexane and 0.2 mL of 2 N NaOH in methanol were added to an aliquot of total lipid (ca.20 mg), vortexed and incubated at 50 °C for 30 min. Post incubation, 0.2 mL of 2 N HCl in methanol was added to the solution and vortexed. The mixture was separated by centrifugation at 3000 rpm for 5 min. The upper hexane layer containing FAME was recovered and subjected to GC analysis on a Shimadzu GC-14B (Shimadzu Seisakusho, Kyoto, Japan) equipped with a flame-ionization detector(FID) and a capillary column(Omegawax-320; 30 m x0.32 mm i.d.; Supelco, Bellefonte, PA, USA). The detector, injector, and column temperatures were 260, 250, and 200 °C, respectively. The carrier gas was helium at a flow rate of 50 kPa. The peaks were identified by comparing with retention of FAME of different fatty acid standards (Nu-Check-Prep, Inc, Elysian MN) and were quantified by an online integrator (Shimadzu Chromatopack C-R8A). The fatty acids content was expressed as weight % of total fatty acids by comparing the retention times with standard fatty acid mix and heptadecanoic acid (C17:0) was used as an internal standard (Supelco, Bellefonte, PA, USA)

2.4 Profiling lipids of tissues and blood glucose

Total lipids from serum and tissues were extracted by the method of Folch et al [31]. An aliquot of the total lipids was subjected to transmethylation with 2M methanolic sodium hydroxide followed by 2M methanolic hydrochloric acid as described above [30]. The FAME thus obtained were dissolved in hexane and analysed on a GC (Shimadzu GC-14B, Shimadzu) under the same conditions as described in section 2.3. The relative amount of each

fatty acid was expressed as weight % of total fatty acids. Fatty acid composition of the liver of *KK-A*^{y} mice was determined by the method as mentioned earlier.

Triglycerides (TG) and cholesterol content in tissue and serum were measured by LabAssay Kits (Wako Pure Chemical Industries, Osaka, Japan) by spectrophotometric method using a plate reader. Blood glucose levels (overnight fasting) were measured in the live animals by using tail blood on a glucose monitor, the Glutest Neo Sensor (Sanwa Kagaku Kenkyusho Co. Ltd., Aichi, Japan). This sensor is an amperometric sensor with flavin adenine dinucleotide (FAD)-dependent glucose dehydrogenase and Fe $(CN)_6^{3-}$.

2.4 Quantitative real-time PCR

Total RNA was extracted from the liver of mice using the RNeasy Lipid Tissue Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The cDNA was then synthesised from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan Ltd., Tokyo, Japan). Quantitative real-time PCR analysis of individual cDNA was performed with ABI Prism 7500 (Applied Biosystems Japan Ltd., Tokyo, Japan) using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd., Tokyo, Japan; Acox1: Mm01246834_m1, Acox2: Mm00446408_m1, Elovl2: Mm00517086_m1 Elovl5: Mm00506717_m1, Elovl6: Mm00851223_s1, Fads1: Mm00507605_m1, Fads2: Mm00517221_m1, Fasn: Mm00662319_m1, GAPDH: Mm99999915_g1. PCR cycling conditions were 50 _C for 2 min, 95 _C for 10 min, followed by 40 cycles of 95 _C for 15 s and 60 _C for 1 min.

2.5 Western Blotting

Livers of mice (30 mg) were homogenised in 5-10 volumes of a solution containing 10 mM Tris‑HCl, 1 mM EDTA (pH 7.4) and 1% protease inhibitor for 30 s with a Polytron. The homogenate was subjected to centrifugation (15000 rpm, 20 min, 4°C) and the fat-free supernatant was collected and used for Western Blot analysis of Δ^5 desaturase (FADS1) and

Δ^6 desaturase (FADS2). The total protein concentration of liver was estimated with the DCprotein assay kit (Bio-Rad). Separation of proteins in the solution (30 mg protein/lane) was achieved by 10% SDS‑polyacrylamidegel electrophoresis. The separated proteins were blotted onto polyvinylidene difluoride membranes and incubated with the antibody against FADS 1 or FADS 2(1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour. Next step involved incubation with a secondary antibody mouse IgG-conjugated horseradish peroxidase (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hr at room temperature. Visualisation of bands was achieved by treating the membranes with the reagents in the chemiluminescence detection kit (ECL system, Amersham PharmaciaBiotech, Piscataway, NJ, USA) as per the protocol provided by the manufacturer. β-Actin served as a loading control with τηε β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.6 Statistical analysis

Mean separation of the data in the groups was achieved using Student's t-test with a confidence interval of 95% (significant when $p<0.05$). **3 Results**

3.1 Fatty acid profile of diets and dietary oils

Fatty acid composition of dietary oils and the lipids from the diet are shown in Table 2. The 20% fat diet with and without squalene fed to *KK-A*^{y} mice was found to contain common fatty acids such as palmitic, stearic, oleic, linoleic and ALA, which were contributed by the lard and SO present in the diet.

3.2 Weight gain in the mice and weight of organs

The gain in body weight of the mice, monitored on a daily basis, showed no significant difference ($p>0.05$) between the groups as a result of feeding the experimental diet (data not shown). Similarly, the weight of major organs (data not shown) also was not significantly different($p>0.05$), except for a slight increase in the liver weight (18%) of the SO+SQ($p>0.05$) -fed group ($p>0.05$).

3.3 Fatty acid composition of liver

Fatty acid profiles of the liver lipids of KK-A^y mice fed SO, or SO+SQ diets are presented in Table 3. The results reveal increased proportions ($p<0.05$) of the long-chain n-3 fatty acid, DHA, in the squalene fed group when compared to control (SO), indicating the possible modulation of fatty acid metabolism. The results demonstrated a significant ($p<0.05$) increase in the long chain n-3 fatty acids, EPA and/or DHA, in the experimental group that was fed squalene.

3.4 Triglycerides(TG), cholesterol, and glucose levels

In addition to the fatty acid profile, it was of interest to study the effect of squalene on other lipid parameters including the cholesterol and TG, together with blood glucose. The results obtained with KK-A^y mice revealed significant decrease ($p<0.05$) in TG (Figure 1a) in the liver and epididymal WAT of squalene fed experimental groups as compared to the control. Whereas, TG levels in serum and cholesterol levels in serum, liver and epididymal WAT were not significantly ($p>0.05$) different from control (data not shown).. On the other hand, a marked decrease ($p<0.05$) in the blood glucose levels of mice in the group fed with the diet containing SQ with that of control was observed, as can be seen in Figure 1 (c).

3.5 mRNA expression of elongase, desaturase and peroxisomal enzymes involved in EPA/DHA synthesis

Elongases and desaturases are essential for the biosynthesis of long chain PUFA. Many of these are common to the n-3 and n-6 fatty acid pathways. The effect of squalene feeding on the mRNA expression of important elongases and desaturases as well as other enzymes such as fatty acid synthase and peroxisomal acyl-CoA oxidases associated with DHA synthesis from DPA is shown in Figure 2 (a). Increase ($p<0.05$) was observed in the Δ^5-desaturase enzymes in liver of mice fed squalene (Figure 2a).

3.6 Protein expression of Δ^5 and Δ^6 desaturase enzymes involved in EPA/DHA synthesis

225 The protein expressions for the Δ^5 and Δ^6 desaturases was determined using Western blot
226 from the protein extract from liver of control and experimental mice. The results indicate a
227 significant increase in the protein expression of Δ^5 desaturase. Besides, the protein expression
228 of the Δ^6 desaturase (Figure 2b) showed an increase ($p<0.05$).

229 **4 Discussion**

230 The results of our study indicated that squalene influenced the lipid metabolism as seen by
231 the TG levels and fatty acid profiles in test diet fed to KK-A^y mice. No significant differences
232 ($p>0.05$) were observed in organ as a result of squalene feeding. Though several previous
233 studies have shown the conversion of n-3 fatty acid precursor, ALA to EPA and DPA,
234 increase in the DHA levels have rarely been reported [4-6]. In a previous study by our
235 research team, fucoxanthin, a marine carotenoid, was observed to increase the DHA levels in
236 the liver of KK-A^y mice [32]. In the present study also, an increase in the proportion of DHA
237 in the fatty acid profile of the liver with a 4.0 fold increase ($p<0.05$) and elevation ($p<0.05$) in
238 liver DHA levels (6 fold) was observed in the squalene fed *KK-A*^{y} mice. Several studies have
239 reported the conversion of ALA to EPA and DPA in different experimental models. However,
240 the reports have demonstrated very limited conversion to DHA [4-6]. Squalene is a naturally
241 occurring lipid substance that is a precursor to cholesterol and is present in many foods with
242 shark liver oil being the primary source. It is also present in other food sources such as olive
243 oil, amaranth seed oil, etc. The results of this study offer another evidence for the utility of
244 squalene as a dietary component to enhance the long chain n-3 fatty acids from their
245 precursors present in food, *in vivo*.

246 A minor but interesting observation was the decrease in arachidonic acid proportions on
247 feeding squalene. A possible reason for this occurrence could be the increased levels of EPA
248 and DHA that have been previously associated with decreased arachidonic acid levels [9].
249 The same Δ^5-desaturase enzyme - that is responsible for the formation of arachidonic acid

(AA, $C_{20:4,n-6}$) from $C_{20:3,n-6}$ (intermediate formed during the synthesis of $C_{20:4}$ from $C_{18:2,n-6}$) - is involved in the formation of EPA ($C_{20:5,n-3}$) from $C_{20:4,n-3}$ (intermediate formed during synthesis of EPA from ALA) [9]. The role of uncontrolled synthesis of arachidonic acid metabolites in the development of CVD and cancer is well documented[18] and, squalene by reducing the AA content effectively prevents those ill effects.

An attempt was made to study the effect of squalene on mRNA gene expression levels of desaturases and elongases and related molecules involved in the synthesis of the long chain n-3 fatty acids. Moreover, the protein expression of the desaturases (Δ^5 and Δ^6) were also ascertained. The mRNA gene expression of EPA synthesis related Δ^5-desaturase enzyme was increased ($p<0.05$) in experimental group fed squalene. However, slight increase in expression of elongases - Elovl2 and Elovl5 - in experimental groups was not significant ($p>0.05$). While this suggests the involvement of squalene in the fatty acid metabolism, no definite link could be established between the increase in the fatty acids and the mRNA expression of other related genes such as the elongases, fatty acid synthase or Δ^6-desaturase. Similar explanation is seen in the instance of Barceló-Coblijn and Murphy [33] who have stated that measures of gene expression of fatty acid metabolism may not directly relate to enzyme activity and accumulated products. The lack of consistency in the mRNA expression of the involved genes observed in our study can be explained by their theory.However, the protein expression studies revealed an increased expression of Δ^5 and Δ^6 desaturases, lending support to the gene expression studies and confirming that squalene is capable of modulating the fatty acid metabolism.

In addition to its regulation of fatty acid metabolism resulting in increased long chain n-3 fatty acids, squalene also was found to regulate the TG in the tissues of KK-A^y mice. While previous studies have reported the hypocholesterolemic effect of squalene [19-22], squalene was not found to decrease cholesterol levels in the present study. Contradictory results

resulting in hypercholesterolemia have also been reported as a result of feeding trials with squalene [23-25]. Squalene has also been reported to protect the long chain fatty acids from oxidation, in particular, linolenic, arachidonic and docosahexaenoic acids [33], affording an added benefit of squalene.

Another significant observation was the ability of squalene to exhibit hypoglycemic effect. Squalene was found to decrease blood glucose levels ($p<0.05$) in high fat/sugar fed *KK-A*^{y} mice, as compared to control in the present study. The ability of squalene to enhance glucose stimulated insulin secretion as well as insulin content could be the reasons [28].

5 Conclusion

The results collated from this study clearly indicate that squalene significantly enhances liver DHA levels when n-3 fatty acid precursors are available in the diet. For the first time, the results show the fatty acid modulation by squalene. Further, squalene also exhibits hypotriglyceridemic effect apart from decreasing the blood glucose levels. Further studies on oral feeding of squalene in other *in-vivo* models including normal phenotypes (e.g., C57BL/6J) are warranted so that the mechanism of squalene action can be understood better and comparisons may be drawn to its role in normal and disease conditions.

Conflict of interest

Authors declare that there are no conflicts of interest.

Acknowledgement

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Table 1. Composition (g/kg) of the control and experimental (SO+SQ) diets.

Ingredient	Control	SO+SQ
β-Corn starch	224.6	224.6
α-Corn starch	84.9	84.9
Milk casein	260	260
Sucrose	130	130
Cellulose	50	50
Soybean oil	70	50
Lard	130	130
Squalene	-	20
L-cystine	3	3
AIN93G mineral mix	35	35
AIN93G vitamin mix	10	10
Choline bitartrate	2.5	2.5
Tert-butyl hydroquinone	0.014	0.014

SO=Soybean oil, SQ=Squalene

309 Table 2. Fatty acid composition (g/100g total fatty acids) of control (con) and experimental
310 (SO+SQ) diets and fatty acid profile of oils used in the diets.
311

FA	Control	SO+SQ	Lard	SO
16:0	4.1	3.9	25.39	10.98
16:1	ND	ND	2.64	0.11
17:0	ND	ND	ND	0.1
18:0	2.1	2.0	13.67	4.02
18:1	7.7	7.2	46.03	24.66
18:2	4.8	3.8	9.19	51.66
18:3	0.4	0.3	0.48	4.92
20:0	ND	ND	ND	0.33
20:1	ND	ND	0.97	0.34
20:5	ND	ND	ND	ND
22:0	ND	ND	ND	0.34
22:6	ND	ND	ND	ND
24:0	ND	ND	ND	0.12

312 SO=Soybean oil, SQ=Squalene, ND=Not Detected
313
314

315 Table 3. Fatty acid composition (%) of liver in high fat fedcontrol and experimental (squalene

316 supplemented) KK-A^y mice.

FA	Control	SO+SQ
$C_{16:0}$	19.63±0.8	19.02±0.8
$C_{18:0}$	5.49±1.3	3.98±0.88[x]
$C_{18:1}$	36.76±3.4	36.16±1.5
$C_{18:2}$ n-6	22.35±1.2	17.62±1.9[x]
$C_{18:3}$ n-3	0.82±0.07	0.66±0.07[x]
$C_{20:4}$ n-6	4.15±1.3	2.67±0.72[x]
$C_{20:5}$ n-3	0.05±0.05	0.02±0.02[x]
$C_{22:5}$ n-3	0.27±0.04	0.20±0.04[x]
$C_{22:6}$ n-3	2.56±0.5	10.30±1.4[x]

317 SO=Soybean oil, SQ=Squalene

318 [x]Statistically significant ($p<0.05$) compared to control.

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Figure titles

Figure 1.Effect of squalene on:

(a) Triglycerides (TG) in per g liver (L) and epididymal white adipose tissue (WAT),

(b) Fasting glucose levels per dl blood.

SO=soybean oil, SQ=squalene

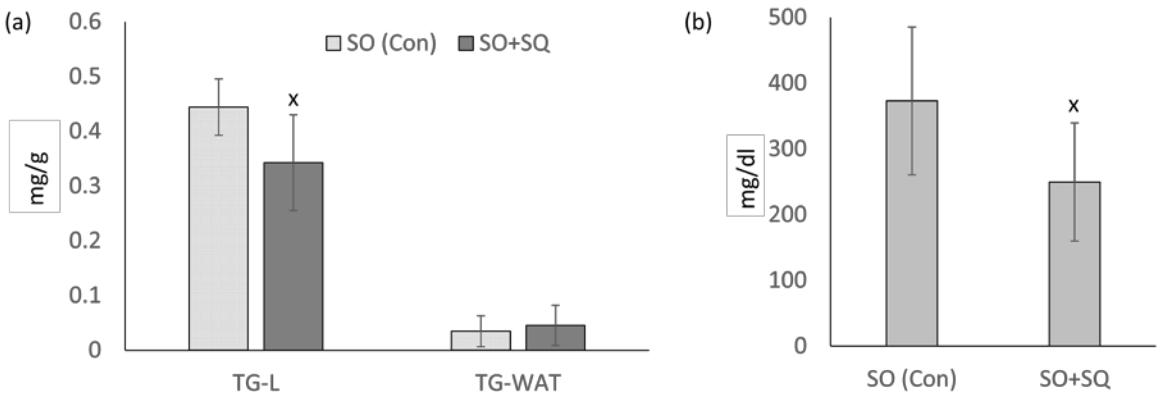
x = Statistically significant ($p<0.05$) compared to SO (Con)

Figure 2.(a) mRNA expression of genes associated with biosynthesis of long chain n-3 PUFA in control (con) and experimental (SO+SQ) KK-Ay mice.

(b) Protein expression of Δ^5 and Δ^6 desaturases: L1 = Δ^5 in con, L2 = Δ^5 in SO+SQ, L3 = Δ^6 in con, L4 = Δ^6 in SO+SQ. Representative bands for each group are represented in L1 to L4.

SO=soybean oil, SQ=squalene, D5-D=Δ5-Desaturase, D6-D=Δ6-Desaturase.

x = Statistically significant ($p<0.05$) compared to SO (Con)



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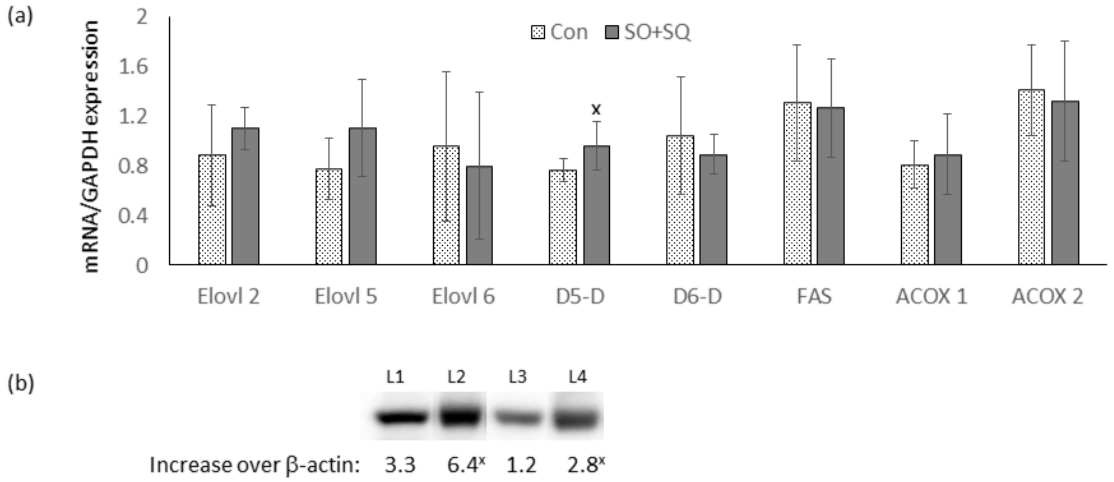

Figure 2.(a) mRNA expression of genes associated with biosynthesis of long chain n-3 PUFA in control (con) and experimental (SO+SQ) KK-Ay mice.

(b) Protein expression of Δ^5 and Δ^6 desaturases: L1 = Δ^5 in con, L2 = Δ^5 in SO+SQ, L3 = Δ^6 in con, L4 = Δ^6 in SO+SQ. Representative bands for each group are represented in L1 to L4.

SO=soybean oil, SQ=squalene, D5-D=Δ5-Desaturase, D6-D=Δ6-Desaturase.

x = Statistically significant ($p<0.05$) compared to SO (Con)