COMBINED EFFECT OF ASTAXANTHIN AND SQUALENE ON OXIDATIVE STRESS IN VIVO

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Abstract

Obesity and diabetes, risk factors for metabolic syndrome, are characterized by oxidative stress and inflammatory responses. Marine biofunctionals, astaxanthin (Ax) and squalene (SQ), were evaluated for their combined effect. Groups of male *KK-A*^{y} mice were fed high fat/sucrose diet for 4 weeks, supplemented with either 0.1%Ax, 2%SQ or 0.1 %Ax+2%SQ. In comparison to control, Sod was elevated in only Ax+SQ. while Gpx was highest in Ax+SQ, indicating the combined antioxidant effect of Ax and SQ. This was supported by elevated mRNA expression of Sod1 and Gpx1. Except adiponectin (elevated in Ax and Ax+SQ), expression of other inflammatory markers was not altered. Blood glucose levels were decreased in SQ and Ax+SQ while liver triglycerides decreased in SQ group. This is the first *in-vivo* study demonstrating combined effects of Ax and SQ resulting in antioxidant effects and modulation of glucose/cholesterol levels. This study highlights the benefit of utilizing Ax and SQ together for management of obesity/diabetes.

Keywords: Antioxidant enzymes; astaxanthin; *KK-A*^{y} mice; lipid hydroperoxides; squalene

1 Introduction

The metabolic syndrome - a complex condition involving obesity, insulin resistance, hypertension, diabetes, hyperinsulinemia and dyslipidemia - is a major risk factor for atherosclerosis and coronary artery disease. The conditions involved in metabolic syndrome are believed to lead to insulin resistance and clinical and biochemical changes [1]. Further, obesity is a common risk factor for diabetes along with advanced age, undesirable diet and lifestyle changes. These contribute to an environment of increased oxidative stress which is believed to be a major risk factor for the onset of diabetes as well as progression of the disease. Diabetes is characterized by hyperglycemia which contributes to the progression and maintenance of an oxidative environment [2]. Hence, compounds exerting antioxidant, hypoglycemic, anti-obesity or anti-inflammatory effect and/or attenuate lipid parameters would be beneficial in these conditions.

Astaxanthin (Ax) is a red-colored xanthophyll that is primarily found in the marine environment. Ax is biosynthesized by microalgae/ phytoplankton, accumulates in zooplankton and crustaceans and subsequently in fish from where they are added to the higher levels in the food chain. Ax is shown to exert several benefits to human health such as antioxidant, anti-inflammatory, anti-cancer, anti-diabetic effects as well as protection against cardiovascular, neurological, gastro-intestinal disorders and in ocular health and drug toxicity [3]. The potential of Ax to act as an antioxidant has been established *in-vitro* and *in-vivo* by several researchers [4-9].

Squalene (SQ) is a triterpenoid that is ubiquitously found in the plant and animal kingdoms by virtue of its importance as an intermediate in the sterol pathway. Although the most abundant source of squalene is shark liver oil, it is also found in terrestrial oil sources such as olive, amaranth seed, rice bran etc. In humans, the main storage organ is the skin where it is

62 believed to protect against the oxidative stress caused by exposure to ionizing rays [10]. Sq is
63 reported to act as an antioxidant, anti-inflammatory, anti-cancer, hypoglycemic and
64 hypocholesterolemicagent [11,12]. However, several researchers have reported the negative
65 effect of feeding squalene, viz. hypercholesterolemia [13,14].
66 Saw et al [15] carried out a combination study involving Ax and PUFAs (EPA/DHA) and
67 reported their synergistic effect *in-vitro*. Dhandapani et al [16] have reported the synergistic
68 effect of SQ and PUFA (fish oil concentrate with ~65% EPA/DHA) on lipid peroxidation and
69 increase in activities of antioxidant enzymes such as superoxide dismutase (Sod), catalase
70 (Cat), glutathione peroxidase (Gpx), and glutathione transferase. However, apart from an *in-*
71 *vitro* report by our group very recently [17], there are no studies available detailing the effect
72 of Ax and SQ when present together. The results of the *in-vitro* study by our group had
73 revealed the combined effect of Ax and SQ in suppressing lipid hydroperoxides in RAW
74 264.7 cells stimulated by lipopolysaccharide [17]. It is evident from the reports in literature
75 that both Ax and SQ exert antioxidant and anti-inflammatory effects and this may involve
76 common factors such as NF-kB, ERK, MAPK, iNOS, Cox-2, P13/Akt and related
77 downstream molecules like interleukin-1β (Il-1β), interleukin-6 (Il-6), tumor necrosis factor-
78 α (TNF-α), interferon-γ (INF), monocyte chemoattractant protein-1 (MCP-1), peroxisome
79 proliferator activated receptor-alpha (PPAR-α) and PPAR-γ [11,18-21]. With this
80 background, the present study was undertaken to study the effect of Ax and SQ, individually
81 and in combination, in obese/diabetic *KK-A*^{y} mice fed a high fat (20%) diet. The activity of
82 antioxidant enzymes, cholesterol and triglyceride levels was measured in liver and white
83 adipose tissue (WAT). Serum glucose levels as well as blood cholesterol profile was
84 determined. Molecules pertaining to antioxidant activity of enzymes as well as markers of
85 inflammation such as TNF-α, PPAR-α, PPAR-γ, IL-6, IL-1β, IFN, Cox-1, Cox-2 and
86 adiponectin were studied.

2 Materials and Methods

2.1 Materials

Squalene (SQ) and dietary lipids were purchased from Wako Pure Chemicals, Ltd., Osaka (Japan). Astaxanthin (Ax) (5%) was obtained from AstaReal, Japan. Soybean oil and lardwere from 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, unless mentioned otherwise and were purchased from Wako Pure Chemicals, Ltd., Osaka (Japan).

2.2 Animals and diets

Male obese/diabetic model, *KK-A*^{y} mice (4 weeks old, n=28) were acclimatized with basal diet for a week following which they were divided into 4 groups and fed a high fat (20%) diet based on the the recommendations of American Institute of Nutrition (AIN-93G), for 4 weeks [22]. The different groups (n=7/group) included control, astaxanthin (Ax-0.1%), squalene (SQ-2%) and combination of astaxanthin and squalene (Ax 0.1% + SQ 2%). The dosage of SQ was fixed at the mid level of dosages reported (1-4%) in previous works [12, 23-25]. Ax dosage was based on the work carried out in our laboratory (unpublished). The composition of the control and experimental diets is given in Table 1. The animals were maintained at 23 ± 2°C temperature and 60 ± 5 % humidity with a 12 hour light/dark cycle. All the animals had free access to food and water. 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. 113 14-0072).

Daily measurements of body weight and food intake were recorded. At the end of the 4 weeks feeding trial, the animals were sacrificed and blood and organs were sampled. . The blood was collected in vacuettes for separation of serum. Organs were excised, weighed, divided and stored in liquid nitrogen, RNA later solution (Life Technologies, USA) and

stored in deep freezer (-40°C) till further analyses. Analyses were carried out with serum, liver and epididymal white adipose tissue (WAT) tissues. Hereafter, reference to WAT would indicate epididymal WAT unless mentioned otherwise.

2.3 Lipid Hydroperoxides

Total lipid extracts were obtained from liver and WAT by the method of Folch et al [26]and the lipid hydroperoxides present were allowed to react with non-fluorescent diphenyl-1-pyrenylphosphine (DPPP) to give fluorescent DPPP oxide which could be measured by the fluorescence detector of the HPLC system [17]. In brief, the weighed lipid extract was dissolved in chloroform: methanol (2:1, v/v) (containing 10 mg butylhydroxytoluene per mL chloroform) and 100 µl of this sample solution was allowed to react with 50 µL of DPPP solution (1 mg/10 ml chloroform)in a water bath for 60 min at 60°C. The reaction mixture was cooled on ice and isopropyl alcohol (3 ml) was added to it. An aliquot was drawn, diluted with mobile phase and injected to the HPLC system (reverse phase). The HPLC system used was Hitachi L-2350 HPLC system (Hitachi, Tokyo, Japan) with a pump (L-2130), an auto-sampler (L-2200) and a fluoresence detector (L-2485). The DPPP oxide was measured at 40°C with a Develosil-ODS column (UG-5, Nomura Chemicals, Aichi, Japan), protected by a ODS guard column (10 x 4.0 mm i.d.). The mobile phase contained HPLC grade butanol and methanol (10:90, v/v), and the flow rate was 1.0 ml/min. The fluorescence detector was set at Ex. 352 nm and Em. 380 nm. The lipid hydroperoxide concentration in the samples were calculated using the DPPP standard curve and expressed as pmol/g lipid.

2.4 Antioxidant Enzymes and Molecules

Antioxidant enzymes, superoxide dismutase (Sod), catalase (Cat), glutathione peroxidase (Gpx) as well as reduced glutathione (GSH) and oxidised glutathione levels (GSSG) were estimated in liver tissue while Sod and Gpx were measured in WAT, with the help of research kits. Kits for estimation of SOD and GSH/GSSG were obtained from Dojindo

Molecular TechnologiesInc (MD, USA), protocols as defined by the supplier were followed and results were obtained by reading at 450 and 405/415 nm, respectively. Cat and Gpxkits were procured from Cayman Chemical Company (MI, USA), instructions of the supplier were followed and final readings were recorded at 540 and 340 nm, respectively.

2.5 Profiling of lipids and glucose in tissues and serum

Lipids extracted from liver and WAT by the method of Folch et al [26] were subjected to cholesterol and triglyceride (TG) analysis by LabAssay Kits (Wako Pure Chemical Industries, Osaka, Japan) by spectrophotometric method using a plate reader. Serum samples were outsourced to Hakodate Medical Association Health Examination and Clinical Laboratory Centre for estimation of cholesterol, LDL-C and HDL-C. Blood glucose levels (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 ferricyanide ($Fe(CN)_6^{3-}$).

2.6 Quantitative real time PCR

Total RNA was extracted from liver and WAT of mice using the Rneasy Lipid Tissue Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The cDNA was then synthesized 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 primers for TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd.), Tokyo, Japan; [Sod1: Mm01344233_g1, Sod2: Mm01313000_m1, Cat: Mm00437992_m1GPX1: Mm00656767_g1,Adiponectin: Mm00456425_m1, PPARα: Mm00440939_m1, PPARγ: Mm00440940_m1, Cox1: Mm00477214_m1, Cox2: Mm00478374_m1, Il-1β: Mm00434228_m1, Il-6: Mm00446190_m1, MCP-1:

Mm0041242_m1, TNFα: Mm00443258_m1, IFN: Mm01168134_m1, GAPDH: Mm99999915_g1]. PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles 15s at 95°C and 60°C for 1 min.

2.7 Statistical analysis

All data were analysed by one way analysis of variance (ANOVA) and wherever significant, mean separation was accomplished using Student's t-test at 95% confidence level.

3 Results

3.1 Body and organ weights

Body weight of the animals was measured daily. A slight decrease ($p>0.05$) was observed in the average body weight of the SQ and Ax+SQ groups as compared to the Ax and Con groups. A significant increase ($p<0.05$) was observed in the weight of liver in SQ group and brown adipose tissue (BAT) in Ax group, while BAT and retroperitoneal WAT was significantly decreased ($p<0.05$) in SQ group, compared to control. Weights of other organs such as spleen, kidney, brain and epididymal WAT were unaffected ($p>0.05$) by feeding Ax and/or SQ in the diet. Body weight and weight of the various organs in different groups is given in Table 2.

3.2 Lipid hydroperoxides and anti-oxidant molecules

Lipid hydroperoxide levels, measured as pmol DPPP/g lipid, were significantly suppressed ($p<0.05$) in liver of all the experimental groups, whereas, in WAT, the decrease was not significant ($p>0.05$), when compared to control. No significant difference ($p>0.05$) in lipid hydroperoxide levels was observed amongst the experimental groups in liver and WAT. Lipid hydroperoxide levels as well as activities of antioxidant enzymes in are shown in Figure 1. Alteration of antioxidant molecules/activity was observed in liver of mice, but not in WAT. Sod activity was significantly ($p<0.05$) increased in liver of Ax+SQ groups while there was only slight ($p>0.05$) increase in the case of Ax group. No significant change

187 ($p>0.05$) was observed for Sod activity in WAT. Liver Cat activity was significantly elevated
188 ($p<0.05$) in Ax, SQ and Ax+SQ groups when compared to control, and this rise was higher
189 ($p<0.05$) in Ax group than SQ and Ax+SQ groups. Liver Gpx activity was also significantly
190 increased ($p<0.05$) in the experimental groups when compared to control and this was in the
191 order of Ax<SQ<Ax+SQ. GSSG/GSH, considered to be an indicator of oxidative stress status,
192 was significantly ($p<0.05$) lower in the experimental groups as compared to control but were
193 not significantly different ($p>0.05$) amongst the test groups.

194 **3.3 Expression of mRNA relevant to antioxidant and immunomodulatory molecules**

195 Quantitative RT-PCR analysis results for liver mRNA expression of genes relevant to
196 antioxidant activity and immunomodulatory pathways are shown in Figures 2 and 3
197 respectively. The results demonstrated an increase in mRNA expression of liver Sod1 and
198 adiponectin in the experimental groups when compared to control, though significant
199 ($p<0.05$) only for Ax and Ax+SQ groups. In addition, the mRNA expression of liver Gpx1
200 was significantly elevated for Ax+SQ over control ($p<0.05$). Whereas, only a slight ($p>0.05$)
201 increase in mRNA expression was observed for liver Sod2 and Cat; no significant changes
202 ($p>0.05$) were observed in expression of genes involved in the antioxidant and
203 immunomodulatory pathways in epididymal WAT tissue (data not shown). No significant
204 change ($p>0.05$) was observed for immunomodulatory factors such as MCP-1, IL-6, IL-1β,
205 TNF-α, IFN, PPAR-α, PPAR-γ, Cox-1and Cox-2 on feeding Ax, SQ or Ax+SQ to mice
206 while adiponectin expression was at increased levels ($p<0.05$) in Ax and Ax+SQ groups as
207 compared to control.

208 **3.4 Lipids and glucose profile of serum and tissue**

209 The lipid profile of blood and tissues of the different groups as well as fasting blood glucose
210 levels are presented in Table 3. Increase in serum cholesterol and HDL-C was observed in
211 mice fed either Ax or SQ as compared to control ($p<0.05$), whereas, the cholesterol and

HDL-C levels of Ax+SQ as well as LDL-c levels in all groups were comparable with control. In addition, blood glucose levels saw a significant ($p<0.05$) decrease in the SQ and Ax+SQ groups while the decrease in Ax group was not significant ($p>0.05$). Although statistically significant ($p<0.05$), changes in liver and WAT cholesterol were not apparent on feeding SQ and Ax+SQ. With regard to TG, levels in liver were significantly decreased ($p<0.05$) in SQ and Ax+SQ groups while in Ax, it was significantly increased ($p<0.05$), when compared to control. TG levels in WAT were unaffected ($p<0.05$) by feeding Ax and/or SQ.

4. Discussion

Obese/diabetic *KK-A*^{y} mice were fed 20% fat diet supplemented with either Ax, SQ or Ax+SQ. Feeding Ax and/or SQ did not have any significant effect ($p>0.05$) on weight gain although average weight of SQ and Ax+SQ fed mice appeared to be lower than control ($p>0.05$). Organs weights were also not affected except for liver and BAT which were significantly ($p<0.05$) increased in SQ and Ax respectively, while BAT and retro-peritoneal WAT were decreased ($p<0.05$) in Ax+SQ group. Gabas-Rivera et al [27] have reported a similar effect with no change in weight and increase in liver weight of mice fed SQ at a dose of 1g/kg while a decrease in body weight of rats was observed with SQ feeding by Liu et al [28].

Lipids, present in the cells are prone to peroxidation by free radicals. The effects of these are neutralized by antioxidant molecules including endogenous enzymes and molecules as well as externally supplied antioxidants. Feeding Ax, SQ and Ax+SQ resulted in decrease in lipid hydroperoxides in liver. Activity of antioxidant enzymes like Sod, Cat and Gpx in liver were elevated on feeding Ax, SQ and Ax+SQ. Hashimoto et al [5] and Choi et al [6] have also reported Ax to be a potent antioxidant with increase in Sod activity and other antioxidant enzymes and decrease in hydroperoxides in human subjects. Similarly, Tripathi and Jena [7] have reported the protective effect of Ax resulting in increased Sod activity and GSH levels

in murine model. Whereas, Augusti et al [8] have reported Ax to increase in Sod and decrease in Cat and Gpx activities in mercuric chloride treated rats. The present results also corroborate with the results of Buddhan et al [29] and Farvin et al [30], who have also reported suppression of lipid peroxidation and improvement in the antioxidant enzymes as a result of feeding 2% SQ to rats, while Moreno [31] reported the lack of antioxidant activity for SQ in murine cell lines. Das et al [10] have also demonstrated increased GSH levels and increased activity of Gpx and glutathione transferase antioxidant enzymes with SQ in mice. In the present study, antioxidant enzymes and the ratio of the indigenous antioxidant molecules, GSSG/GSH was also decreased in the experimental groups. The GSSG/GSH ratio is accepted as a marker of oxidative stress status [32]. Collectively, this implies that feeding Ax, SQ or Ax+SQ protected the cells from oxidative stress. In particular, Ax+SQ was more potent ($p<0.05$), than Ax and SQ individually, in elevating the activity of Sod and Gpx while increase in activity of Cat was significantly more in Ax. The clearly indicates that Ax and SQ are more potent in combination than when present alone. This surmise is further supported by the mRNA expression of Sod1, Gpx1 and adiponectin in liver, where Ax and Ax+SQ showed a significant elevation over control and in addition, Ax +SQ had a markedly higher expression of Gpx1. This is the first study reporting the combined effect of carotenoids and SQ*in-vivo*. Previously, Saw et al [15] have reported that the combination of Ax with EPA/DHA resulted in synergistic antioxidant effects in Hep-G2-C8 cells and Dhandapani et al [16] have reported on the synergistic effect of SQ and PUFA as antioxidants in rats. We had previously reported the combined effect of Ax and SQ on lipid peroxidation in macrophages [17] and speculated that it may involve regulation of inflammatory markers common to Ax and SQ. However, feeding Ax, SQ or Ax+SQ did not significantly influence mRNA expression of IL-1β, IL-6, MCP-1, TNF-α, IFN, Cox-1, Cox-2, PPAR-α and PPAR-γ. A significant increase ($p<0.05$) was observed in the mRNA expression of adiponectin for Ax

and Ax+SQ. Previously, Cardeno et al [11] have reported SQ to decrease the mRNA expression of TNF-α, IL-1β, IL-6, IFN, Cox-2 and other markers of inflammatory processes in neutrophils and monocytes stimulated with lipopolysaccharide. Similarly, Ax was reported to suppress mRNA expression of NF-κB,Cox-1, Cox-2, MCP-1, TNF-α, IL-1β *in-vitro* and *in-vivo*[18-21]. Jia et al [33] have reported that Ax activated PPAR-α but suppressed PPAR-γin mice. Park et al [34] have reported increase in IFN and decrease in IL-6 in human subjects fed high dose of Ax (8mg/day) but not for those fed lower dose (2 mg/day). A possible reason for the discrepancy in our results from those of others may be due to the use of a very low dosage of Ax (0.01%) in the diet of the mice. A longer duration study may be considered for confirming the true effect of Ax and SQ in the mice.

In addition to the protective effects of feeding Ax, SQ and Ax+SQ on oxidative stress related parameters, the experimental diets also had an effect on the cholesterol and TG in the blood and tissues as well as fasting blood glucose levels. While Ax and SQ groups had increased ($p<0.05$) levels of cholesterol and HDL-C compared to control, the levels in Ax+SQ group was comparable ($p>0.05$) with control. An interesting observation made from Table 3 is that while cholesterol levels increased in Ax and SQ fed groups, there was a corresponding rise in HDL-C and LDL-C in Ax but a rise in HDL-C and fall in LDL-c levels in SQ. The statistically significant decrease observed for cholesterol in liver and WAT of SQ and Ax+SQ could be misleading as it was not apparent when viewed holistically. Liver TG decreased ($p<0.05$) in SQ group, whereas, Ax was found to result in increased liver TG. Aguilera et al [35] have reported increased cholesterol and TG levels in chick embryos treated with alcohol and this effect was attenuated by feeding SQ indicating its hypocholesterolemic and hypotriglyceridemic effect. Farvin et al [12] have also reported the ability of SQ to suppress increase in cholesterol and TG levels in rats. de Castro et al [23] whereas, have reported no change in the lipid profile as a result of feeding SQ to hamsters. Contrary results have been

reported by Relas et al [13] and Zhang et al [14] who have reported increase in cholesterol and TG levels as a result of SQ feeding, *in-vivo*. The decreased serum cholesterol and liver TG observed in our study may have been due to a squalene-induced suppression of the HMG-CoA reductase activity, by a feedback mechanism [35]. In addition to lipids, blood glucose levels were significantly decreased in SQ and Ax+SQ groups. Liu et al [28] have also reported lowering of plasma cholesterol, TG and glucose levels on feeding SQ to rats. Tsuchiya et al [36] have suggested that this may be due to glucose stimulated insulin secretion and increased insulin levels. Overall, the results indicate that while Ax, SQ and Ax+SQ protected the cells from oxidative stress, the effect was more pronounced when Ax and SQ were administered in combination. Moreover, the combination Ax+SQ resulted in a significant decrease in tissue cholesterol and blood glucose levels, indicating its multifaceted beneficial effect in diabetes/obesity.

This is the first study *in-vivo* to highlight the advantage of using them in combination as exhibited by their superior antioxidant effects and ability to modulate glucose and cholesterol levels in blood and tissues. Dietary supplementation with Ax or/and SQ resulted in decreased lipid hydroperoxide levels as well as increased activity of antioxidant enzymes and molecules in *KK-A*^{y} mice fed a high fat/sucrose diet. In combination, the elevation in the activities of Sod and Gpx antioxidant enzymes was greater than when Ax and SQ were present individually and this was supported by the increased mRNA gene expression of Sod1, Gpx1 and adiponectin. In addition, tissue cholesterol and triglyceride levels as well as serum glucose levels were lowered in SQ and Ax+SQ groups. This study thus confirms the antioxidant effects of Ax and SQ. Given that Ax and SQ are obtained naturally from marine and other food sources and have multifarious effects, especially when used in combination, they may be considered for improving the outcome of obesity/diabetes management regimes.

Further studies are required for establishing the combined effect of Ax and SQ in normal mice models.

List of abbreviations

Ax : Astaxanthin, BAT: Brown Adipose Tissue, Cat : catalase, Cox : Cyclooxygenase, DHA : Docosahexaenoic acid, DPPP: diphenyl-1-pyrenylphosphine, EPA : Eicosapentaenoic acid, ERK : Extracellular-signal regulated kinase, Gpx : glutathione peroxidase, GSH : Reduced glutathione, GSSG : Oxidised glutathione, HDL-C: High density cholesterol, iNOS : Inducible nitric oxide synthase, MAPK : Mitogen activated protein kinase, MCP : Monocyte chemoattractant protein, NF : Nuclear factor, PPAR : Peroxisome proliferator activated receptor, Sod : Superoxide dismutase, SQ : Squalene, TG: Triglycerides, TNF : Tumor necrosis factor, WAT : White adipose tissue

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Conflict Of Interest

The authors have no conflict of interest to report.

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Table 1. Composition of control and experimental diets

Ingredient (g/kg)	Control	Ax*	SQ*	Ax+SQ
β-Corn starch	224.6	224.6	224.6	224.6
α-Corn starch	84.9	84.9	84.9	84.9
Milk casein	260	260	260	260
Sucrose	130	130	130	130
Cellulose	50	50	50	50
Soybean oil	70	50	50	30
Lard	130	130	130	130
Ax oil#	-	20	-	20
Squalene	-	-	20	20
L-cystine	3	3	3	3
AIN93G mineral mix	35	35	35	35
AIN93G vitamin mix	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
Tert-butyl hydroquinone	0.014	0.014	0.014	0.014

*Ax - Astaxanthin, SQ - Squalene; #Ax oil contained 5% Ax

Table 2. Effect of feeding Astaxanthin (Ax) or/and Squalene (SQ) on body and organ weights.

	Control	Ax	SQ	Ax+SQ
Body weight, g				
Initial	26.12±0.99[a]	26.02±1.56[a]	25.78±1.45[a]	25.70±1.02[a]
Final	42.77±1.83[a]	43.13±1.59[a]	42.01±1.40[a]	40.94±1.63[a]
Organ weight, g				
Liver	2.24±0.35[a]	2.00±0.18[a,b]	2.64±0.29[b,c]	2.39±0.34[a,c]
Spleen	0.12±0.03[a]	0.10±0.01[a]	0.10±0.03[a]	0.12±0.05[a]
BAT[*]	0.23±0.03[a]	0.26±0.03[b]	0.20±0.04[a]	0.20±0.04[c]
Epididymal WAT[#]	1.92±0.29[a]	2.05±0.24[a]	1.96±0.25[a]	1.94±0.47[a]
Retroperitoneal WAT[#]	0.81±0.14[a]	0.81±0.18[a]	0.78±0.17[a]	0.64±0.08[b]
Kidney	0.59±0.05[a]	0.64±0.05[a]	0.60±0.07[a]	0.57±0.04[a]
Brain	0.35±0.05[a]	0.37±0.02[a]	0.38±0.03[a]	0.35±0.02[a]

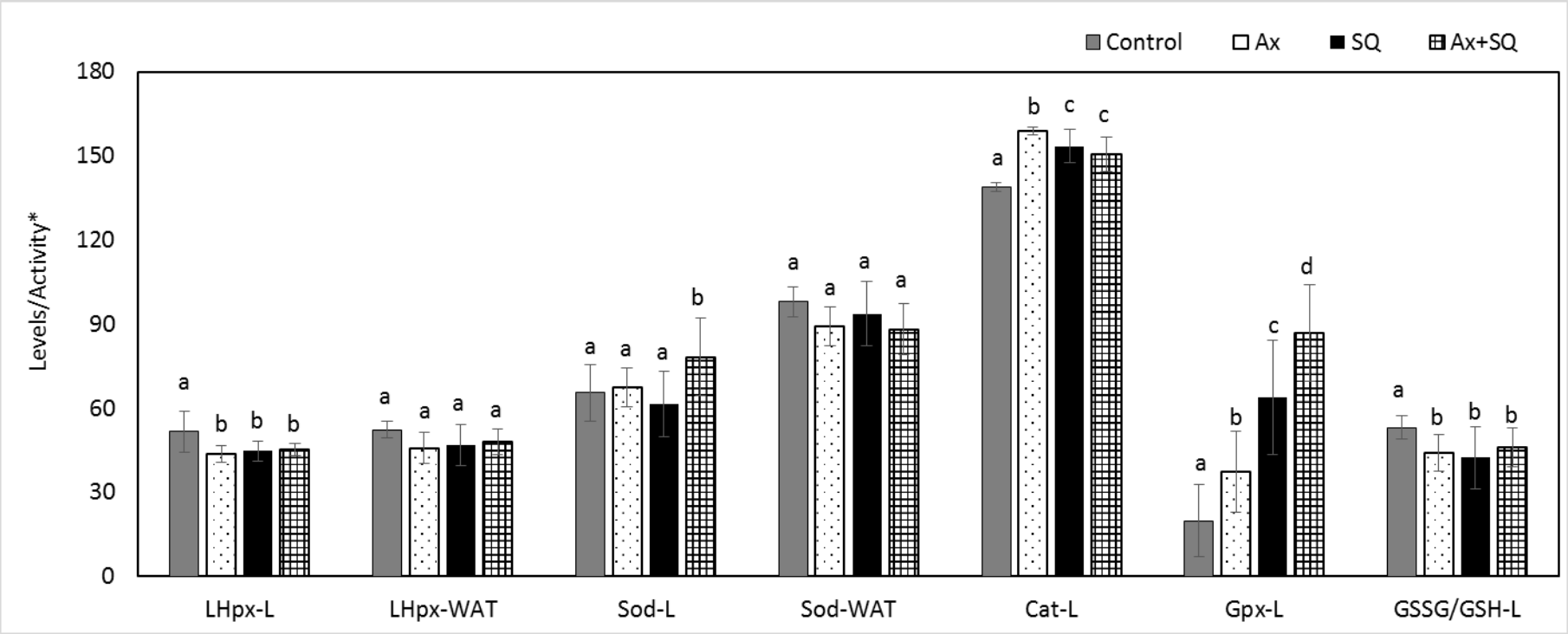
[*]BAT – Brown Adipose Tissue; [#]WAT – White Adipose Tissue; Columns not sharing common alphabets are significantly different ($p<0.01$)

Table 3. Effect of feeding astaxanthin (Ax) or/and squalene (SQ) on lipid profile and fasting blood glucose in serum, liver and epididymal white adipose tissue (WAT).

Parameter	Control	Ax	SQ	Ax+SQ
Serum (all parameters in mg/dl)				
Cholesterol	146.71±10.7 [a]	173.00±23.5 [b]	164.29±16.4 [b]	152.57±24.0 [a]
LDL-C	12.57±6.3 [a]	14.00±1.6 [a,b]	10.14±4.5 [a,c]	9.57±2.8 [a,c]
HDL-C	72.29±7.9 [a]	85.14±7.4 [b]	82.00±11.9 [b]	77.00±11.4 [a]
HDL:LDL	6.7±2.4[a]	6.2±0.5[a,b]	9.3±3.2[a,c]	8.8±3.3[a,c]
Glucose	372.86±112.4 [a]	299±77.9 [a]	249.4±89.9 [b]	226.7±120.6 [b]
Liver (all parameters in mg/g)				
Cholesterol	$2.1312\pm0.5\times10^{-3}$ [a]	$2.1320\pm0.9\times10^{-3}$ [a]	$2.1297\pm0.3\times10^{-3}$ [b]	$2.1299\pm0.8\times10^{-3}$ [b]
Triglycerides	0.444±0.05 [a]	0.716±0.17 [b]	0.343±0.09 [c]	0.450±0.08[a,c]
Epididymal WAT (all parameters in mg/g)				
Cholesterol	$2.1306\pm0.9\times10^{-3}$ [a]	$2.1298\pm0.5\times10^{-3}$ [a]	$2.1282\pm0.2\times10^{-6}$ [b]	$2.1282\pm0.3\times10^{-6}$ [b]
Triglycerides	0.035±0.0 [a]	0.029±0.0 [a]	0.045±0.02 [a]	0.047±0.02 [a]

Columns not sharing common alphabets are significantly different ($p<0.01$)

List of Figures


Control
Ax
SQ
Ax+SQ
Levels/Activity*
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150
120
90
60
30
0
a
b
b
b
a
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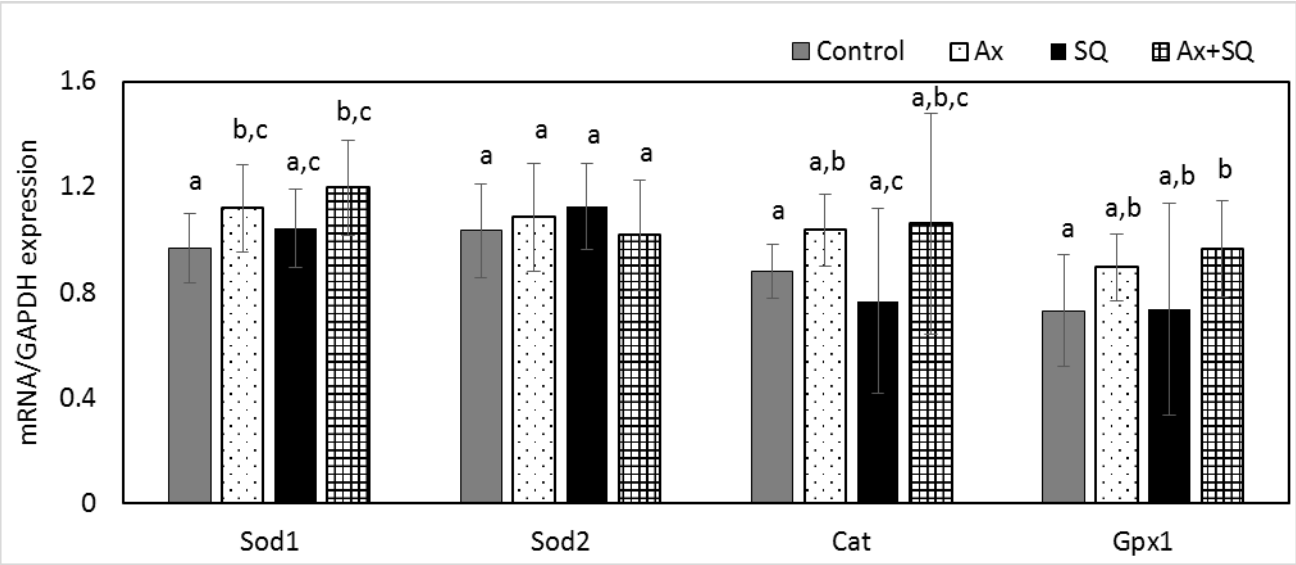
a
a
a
a
b
a
a
a
a
a
b
c
c
a
b
c
d
a
b
b
b
LHpx-L
LHpx-WAT
Sod-L
Sod-WAT
Cat-L
Gpx-L
GSSG/GSH-L

Figure 1.


Control
Ax
SQ
Ax+SQ
mRNA/GAPDH expression
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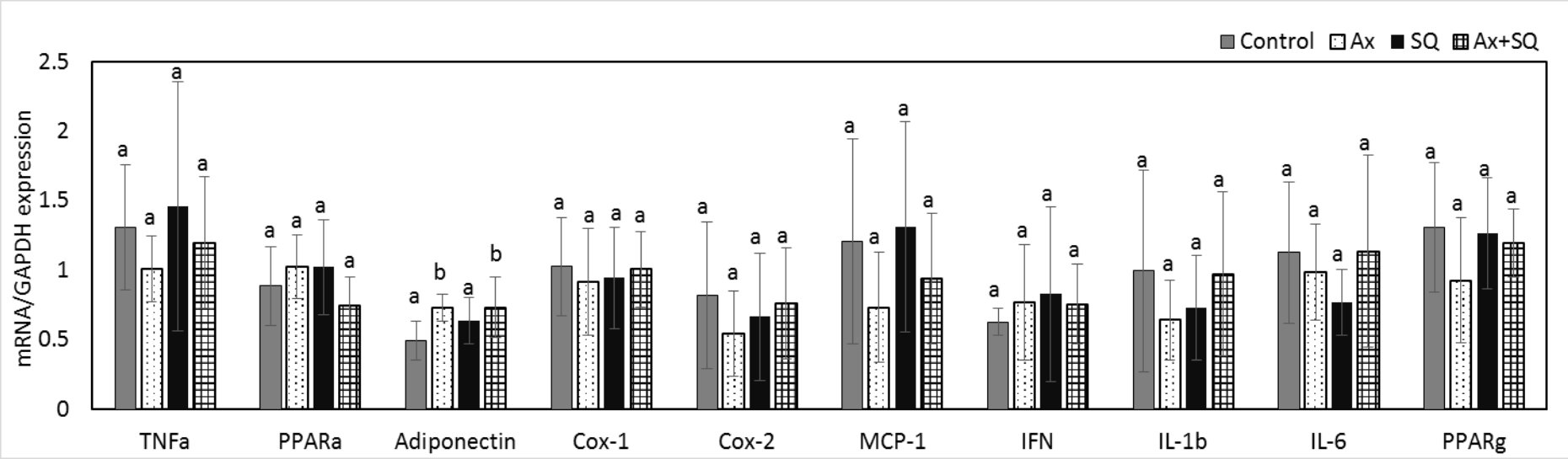
1.2
0.8
0.4
0
a
b,c
a,c
b,c
a
a
a
a
a
a,b
a,c
a,b,c
a
a,b
a,b
b
Sod1
Sod2
Cat
Gpx1

Figure 2.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Ax
SQ
Ax+SQ
mRNA/GAPDH expression
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2
1.5
1
0.5
0
TNFa
PPARa
Adiponectin
Cox-1
Cox-2
MCP-1
IFN
IL-1b
IL-6
PPARg

Figure 3.