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Impact of Oxidative Stress and Lithogenic Diet on Experimentally Induced Cholelithiasis in Rabbits

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ABSTRACT

This study aimed at determining the influence of oxidative stress and lithogenic diet on rabbits' gallstone formation. Thirty-six healthy local mature rabbits of both genders were divided into six equal groups. 100% gallstones were formed in the GIII and 50% in the GIV. Serum levels of MDA, lipid profile, ALT, AST and ALP were significantly higher in GIII and GV when compared with the GI, while the serum levels of GR-activity and HDL-C were significantly lower in GIII and GV when compared with GI. It was concluded that rabbits exposed to imbalanced oxidative stress and fed a high-cholesterol diet could induce gallstone disease 100%.

Key words : Gallbladder stone, glutathione reductase, MDA, oxidative stress, lithogenic diet

INTRODUCTION

Oxidative stress has been studied since the 1970s, but its concept can be traced back to the fifties in the last century (Yu and Xiao, 2021). It is determined as an imbalance between the generation of free radicals and the cell's antioxidant capacity, causing potential cellular prejudice to proteins, fat, nucleic acids and carbohydrates (Adwas *et al.*, 2019; Aranda-Rivera *et al.*, 2022). Free radicals are usually known as any chemical species that exist independently and are characterized by unstable and high reactivity in chemical reactions for electrons exchange due to the existence of unpaired electrons in the outer orbitals (Kurutas, 2016; García-Ruiz and Fernández-Checa, 2018). There are two kinds of free radicals in the biological system, reactive oxygen species (ROS) and reactive nitrogen species (RNS) commonly known together as RONS (Li *et al.*, 2015; Ito *et al.*, 2019), that are produced in the liver as a byproduct through the processes of metabolizing of several compounds also through a mitochondrial electron chain reaction (Lennicke *et al.*, 2015; Jeong *et al.*, 2021). Under normal physiological conditions, the body has enough defense and repair systems of antioxidant capacity which recognize and expel molecules injured by RONS and oxidation, so that a mild level of oxidative stress can be afforded by most cells of the body (Yadav *et al.*, 2016). The excessive amounts of cellular free radicals production and/or impairment or limitation in the action of antioxidants can result in the overproduction of RONS. It exhausts the endogenous antioxidants that thence fail to oppose all the RONS, exerting deleterious effects on cell function. These react well with all biological substances such as proteins, nucleic acids, and lipid peroxidation, therefore, the balance between the generation rate of RONS and their removal by specific antioxidants is important (Schieber and Chandel, 2014; Karkkainen *et al.*, 2017). Poly-unsaturated fatty acids degradation occurs by RONS forming malondialdehyde (MDA) which are considered one of the reliable and popular biomarkers to measure oxidative stress level in an organism (Atamer *et al.*, 2014; Ayala *et al.*, 2014; Yousif *et al*,, 2019). Cholelithiasis is a highly prevalent gastroenterological disorder (Onuk *et al.*, 2019), affecting about 10-15% of the global adult population (Günay *et al.*, 2019; Chen and Wu, 2020; Silina *et al.*, 2022). GD has the big burden of substantial financial on the healthcare economy due to its associated complications and comorbidities. GS is affected by an interaction of complicated environmental and genetic factors (Lin *et al.,* 2016). Depending

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on the main biochemical components, gallstones are classified into three types: cholesterol GSs, pigment GSs, as well as mixed GSs (Saadati *et al.*, 2021). Anatomically cholelithiasis can be categorized as intrahepatic and extrahepatic bile duct stones (Chen *et al.*, 2019). The pathogenesis of cholesterol gallstone disease (CGD) has long been a mystery, for many years ago it had been believed that cholelithiasis was due to an inflammatory disease of the gallbladder, that yields in the cell desquamate and the production of abnormal materials. Now-a-days, the hepatic production of cholesterol-hyper saturated bile is considered as a pre-requisite for CGD (Chen *et al.*, 2015). Gallstones are mainly formed in the gallbladder from equilibrium between major bile constituents, cholesterol, phospholipids and bile salts (Tharp *et al.,* 2016). The cholesterol precipitation and the supersaturation of bile are required when there is too much cholesterol and there is a shortage of the salts or phospholipids, or there is a combination of these factors so that the homeostasis of cholesterol is important to stop the formation of gallstone. There are main important factors to maintain homeostasis of cholesterol like intake/biosynthesis of cholesterol and biliary secretion of cholesterol (Lee *et al.*, 2018).

MATERIALS AND METHODS

Dihydro cholesterol (Alfa Aesar Thermo Fisher, Scientific, Germany), cholic acid (Alfa Aesar Thermo Fisher, Scientific, Germany), hydrogen peroxide (H_2O_2) (Scharlab S.L, Gato Perez, Sentmenat Spain) and. vitamins AD3E injection (Interchemie, Netherlands) were precurred.

Thirty-six healthy local mature rabbits regardless of sex were used in this study, with weights between 1-1.5 kg, for at least a week prior to the experiment. The adaptation of all rabbits was done environmentally in the animal holding room and kept under standard laboratory conditions. They were put in cages and fed with standard locally prepared diets with free access to tap water. They were divided into six equal groups, each group consisting of three males and three females separated from each other, and every one individual rabbit was housed in the stainless cage. G1 (control group) was fed a standard rabbit diet and water for six weeks and did not receive any medications. GII (H_2O_2 group) was administrated 1% H_2O_2 with drinking water in a dark bottle prepared daily and a normal diet for six weeks. GIII (lithogenic group) in addition to 1% H_2O_2 with their drinking water was fed with a lithogenic diet consisting of 1% dihydro cholesterol which began from the start of the 4th week and continued till the end of the 6th week for induction of gallstone. The GIV (Vit. AD3E group) was injected intramuscularly twice weekly with 0.1 ml of vitamin AD3E and fed with 1% dihydro cholesterol in their food starting from the fourth week till the end of the experiment. Group V was administrated with 1% H_2O_2 for six weeks and fed with 0.5% cholic acid in their diet from the fourth week until the end of the experiment for the induction of stones. Group VI was injected intramuscularly twice weekly with 0.1 ml of vitamin AD3E and fed with 0.5% cholic acid in their food starting from the fourth week till the end of the six weeks.

According to the ethical guidelines that were approved according to the American Veterinary Medical Association, all animals were euthanized before killing. All rabbits fasted overnight at the end of the 6-week feeding period, a blood sample was drawn immediately and directly from the heart by a sterile disposable syringe (10 ml) into a heparinized test tube, and the isolation of the serum was done by centrifugation of the blood samples at 3500 rpm using a cooling centrifuge at 4°C for 20 min. The separated serum samples were right away put in a labelled Eppendorf tube and stored in deep freezers at -20 ^{0}C for biochemical assays. All rabbits were sacrificed and an immediate autopsy and cholecystectomy were performed. The bile and stones were collected from the gallbladder. A piece of the gallbladder was put on a slide to take an immediate image for seeing the gallbladder tissues and the presence of any remaining gallstones under different magnification lens (Fig. 1: A, B, C, D, E and F).

The Double Antibody Sandwich Technique was used to determine malondialdehyde in the rabbit's serum which was considered a marker of lipid peroxidation by using a rabbit malondialdehyde ELISA Kit by BT LAB (Bioassay Technology Laboratory, UK). Values of MDA were expressed as nmol/ml.

The Double Antibody Sandwich Technique was used to determine glutathione reductase

activity in the serum of the rabbits using a rabbit glutathione reductase ELISA Kit by BT LAB (Bioassay Technology Laboratory, UK), Values of GR were expressed as nmol/Ml.

The colorimetric method was used for the estimation of AST, ALT and ALP activities using a standard enzymatic kit supplied by Roche (USA- AST; REF: 20764949, ALT; REF: 20764957, and ALP; REF: 03333752) using Roche/Hitachi Cobas c 311, Cobas c 501/502. A standard commercial kits provided by Roche (USA- Serum total cholesterol: REF: 03039773. serum Triglyceride: REF: 20767107 322. serum low-density lipoprotein-cholesterol: REF: 03038866. serum high-density lipoprotein-cholesterol: REF 07528566) were used to estimate TC, TG, LDL-C and HDL-C.

All statistical analyses were carried out by one-way analysis of variance (ANOVA), and differences between groups were estimated using Duncan multiple range tests by using version (23) of the SPSS. The results were expressed as mean ± standard error of the mean (SEM) of six rabbits per group at $P < 0.05$ for difference between the two groups.

RESULTS AND DISCUSSION

Gallstones were not formed in any rabbits of groups GI, GII, V and VI despite that groups V, and VI were fed with a lithogenic diet of 0.5% cholic acid for the last three weeks of the experiment, however, the gallbladders were filled with stones in GIII which was fed dihydro cholesterol for three weeks after exposure to 1% of H_2O_2 for six weeks so it was so difficult to withdraw the bile from their gallbladder (Fig. 2B). The GS was 100% formed in this group (all Three females as well as males) regardless of their sex. While in GIV where rabbits were fed with dihydro cholesterol and injected with 0.1 ml of vitamins AD3E twice weekly, the GSs were formed only in 50% and their gallbladders were half-filled with stones (Fig. 2C).

The serum levels of glutathione reductase (GR) activity of all groups as an antioxidant and MDA as an oxidative stress biomarker were compared with those of the normal control group. There was a significant decrease in the serum GR activity levels in the groups GIII, GV and GII when compared with G1, the mean

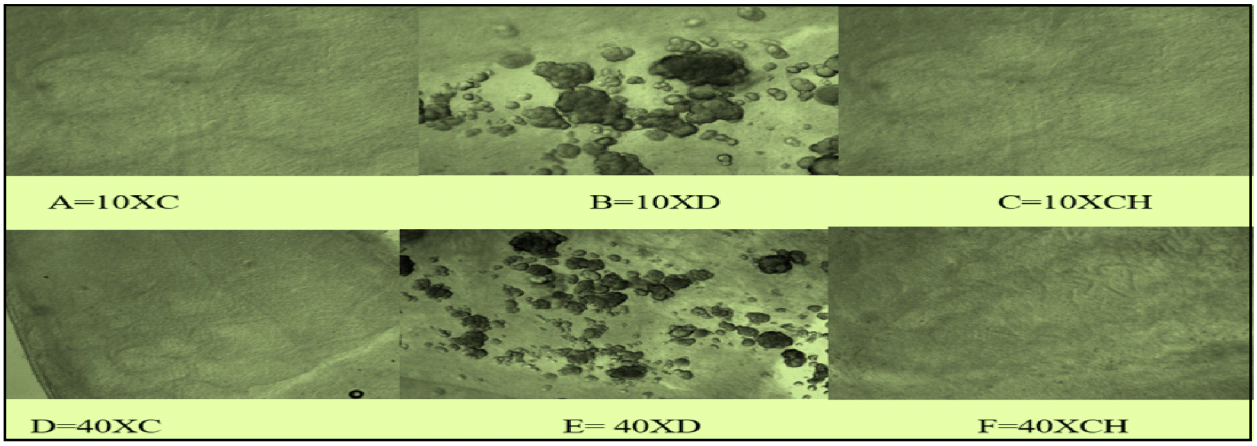

Fig. 1. Microscopic appearance under two magnifications power (40 and 10X) of the opened gallbladder cyst, C – control, D – dihydro cholesterol and CH – cholic acid.

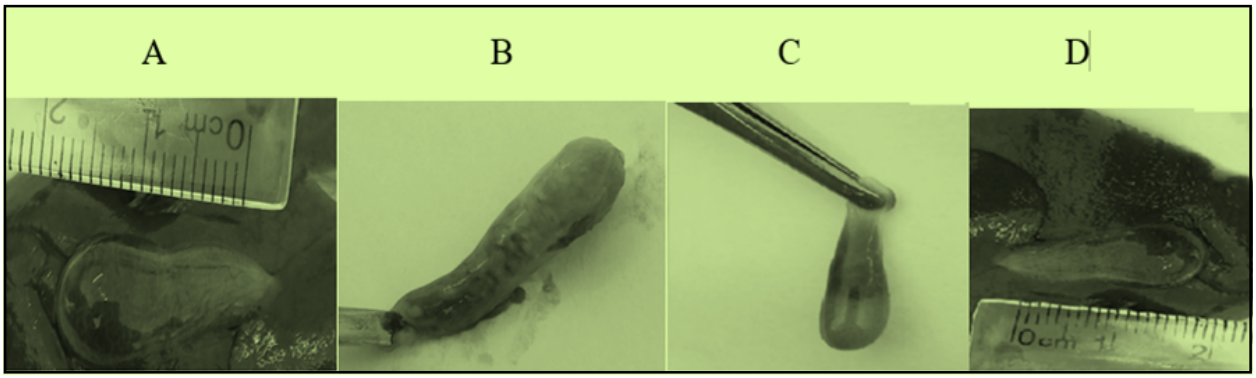

Fig. 2. The gallbladder in different groups, A – G 1, B – G III, C – G IV and D – G V.

values ±SE of GR in serum of GIII, GV, GII and GI were 4.316±0.653, 5.29±.927, 5.763±0.752 and 9.720±1.460, respectively. There was a difference in the serum of GR activity levels in GIV and GVI when compared with GI, however, it was insignificant. The mean values±SE of GR. in serum of G IV, GVI and G1 were 8.57±0.593, 8.97±.914 and 9.720±1.460, respectively (Table 1).

The serum MDA values as an oxidative stress biomarker showed a significantly higher in GIII and GV than in GI. The mean values±SE of GR in serum of GIII, GV and GI were 19.581±1.31, 18.89±1.26 and 9.711±0.91. There was significantly an increase in the serum MDA levels in GII than in GI, the mean values±SE of GR in serum of GII and GI were 16.486±1.28 and 9.711±0.91, respectively (Table 1). When compared the serum MDA levels in GV I with G IV there was insignificant difference, the mean values±SE of GR in serum of GV I and G IV were 13.056±.639 and 12.73±0.231, respectively, while when comparing of serum MDA levels in GV I with G I the difference was significant. The mean values±SE of GR in serum of GV I and G I were 13.056±.639 and 9.711±0.91, respectively, while there was an insignificant increase in the serum MDA levels in the G IV than in G I, the mean values±SE of GR in serum of G IV and GI were 12.73±0.231 and 9.711±0.91, respectively.

In the current study, the serum total cholesterol (TC) level was significantly increased in groups G V, G III and G II when compared with the normal control group. The mean values±SE of serum total cholesterol levels of G V, G III, G II and G 1 were 114.40±4.69, 112.8±11.36, 99.60±6.20 and 58.60±2.65, respectively. While the serum total cholesterol level in G 1V and GV I was increased when compared with the control group but its increase was statistically insignificant; the mean values±SE of serum total cholesterol levels of G IV, G VI and G 1 were 72.20±7.996, 71.80±9.707 and 58.60±2.65, respectively (Table 2).

The serum triglyceride (TG) level was significantly increased in groups G V, G III and G II, when compared with the G I, the mean values±SE of serum. Triglyceride levels of groups G V, G III, G II and G 1 were 129.40±4.50, 127.6±14.93, 104.6±7.73 and 57.80±9.906, respectively. Regarding the G IV, there was an insignificant increase in serum triglyceride level when compared with G 1. The mean values±SE of serum triglyceride level of groups G IV and G 1 were 72.2±13.77 and 57.80±9.906, respectively. The G IV was statistically located on the borderline between significant and insignificant when compared with G 1; the mean values±SE of serum triglyceride level in G IV and G I were 77.80±4.55 and 57.80±9.90. There was a lower level of serum HDL-C in all groups when compared with the normal control group, however, it was statistically insignificant. the mean values ± SE of serum HDL-C of G III, G V, G II, G IV, GV I and G 1

Table 1. The mean±SE of the serum levels of glutathione reductase (GR) and malondialdehyde (MDA) in the experimental groups

Parameter	G I	G II	G III	G IV	G V	GV I
GR	9.720±1.460^{a}	5.763±0.752^{b}	4.316±0.653^{b}	8.57±0.593^{a}	5.29±.927^{b}	8.97±.914^{a}
MDA	9.711±0.91^{d}	16.486±1.28^{b}	19.581±1.31^{a}	12.73±0.231^{c,d}	18.89±1.26^{a,b}	13.056±.639^{c}

*Different superscripts indicate statistically significant differences at P≤0.05.

Table 2. The mean±SE of lipid profile in the experimental groups

Parameter (mg/dl)	G I	G II	G III	G IV	G V	GV I
S. TC	58.60±2.65^{b}	99.60±6.20^{a}	112.8±11.36^{a}	72.20±7.996^{b}	114.40±4.69^{a}	71.80±9.707^{b}
S. TG	57.80±9.90^{c}	104.6±7.73^{a,b}	127.6±14.93^{a}	77.80±4.55^{b,c}	129.40±4.50^{a}	72.2±13.77^{c}
S. HDL-C	34.20±3.99^{a}	29.40±1.20^{a}	26.0±2.529^{a}	30.60±.509^{a}	26.40±1.60^{a}	31.4±4.60^{a}
S. LDL-C	18.20±.734^{b}	71.20±16.67^{a}	87.80±9.759^{a}	74.40±9.479^{a}	94.4±5.99^{a}	63.20±21.51^{a}
S. VLDL-C	13.40±1.43^{c}	26.80±1.28^{a}	29.80±2.90^{a}	20.40±1.469^{b}	29.0±1.89^{a}	17.80±1.46^{b,c}
TC/HDL ratio	1.82±.338^{c}	3.38±.288^{a,b}	4.33±.279^{a}	2.416±.311^{b,c}	3.96±.176^{a}	2.68±.636^{b,c}
LDL/HDL ratio	.572±.102^{c}	2.60±.558^{a,b}	3.558±.500^{a}	2.172±.2406^{a,b}	2.566±.520^{a,b}	1.404±.516^{b,c}

*Different superscripts in a row indicate statistically significant differences at P≤0.05.

were 26.0±2.529, 26.40±1.60, 29.40±1.20, 30.60±.509, 31.4±4.60 and 34.20±3.99, respectively (Table 2).

The serum LDL-C level in all treated groups was significantly higher than the control group, the mean values±SE of serum of groups G V, G III, G IV, G II, GV I and G 1 were 94.4±5.99, 87.80±9.759, 74.40±9.479, 71.20±16.67, 63.20±21.51 and 18.20±.734, respectively.

The serum VLDL-C level in groups GIII, GV, GII, and GIV, were significantly higher than G1. The mean values ± SE of serum VLDL-C levels in G III, G V, G II, G IV and G 1 were 29.80±2.90, 29.0±1.89, 26.80±1.28, 20.40±1.469 and 13.40±1.43, respectively. While the serum levels of VLDL-C in both groups' G IV, and G VI were significantly lower when compared with the G III, G V and G II.

The ratio of serum level of total cholesterol/ HDL was significantly increased in G III, G V and G 11 when compared with G 1. The mean values±SE of serum TC/HDL levels of GIII, GV, and G 11 and G 1 were 4.33±.279, 3.96±.176, 3.38±.288 and 1.82±.338, respectively. While, there was an insignificant increase in serum level of TC/HDL ratio in GV I and GIV than in G1. The mean values ± SE of serum TC/HDL levels of G V, G VI, and G 1 were 2.68±.636, 2.416±.311 and 1.82±.338, respectively.

The serum level of LDL/HDL ratio in all treated groups except GV I was statistically higher than the G I, the mean values±SE of serum LDL/ HDL ratio in G III, G II, G V, G IV, G VI and G I were 3.558±.500, 2.60±.558, 2.566±.520, 2.172±.2406, 1.404±.516 and .572±.102, respectively.

The serum ALT level of G V and G III was significantly increased when compared with G 1; the mean values ± SE of serum ALT levels of G V, G III, and G 1 were 159.80±3.104, 151.4±12.1, and 105±12.7, respectively, while the levels of serum ALT in G II, GV I and G IV were insignificantly higher than G 1. The mean values±SE of serum ALT levels of G II, G VI, G IV and G 1 were 129.00±3.42, 122.80±18.786, 117±1.7 and 105±12.7, respectively (Table 3).

The serum AST level of all treated groups was significantly increased when compared with the G1; the mean values±SE of serum AST levels of G III, G V, G II, G IV, G VI and G I were 80.2±34.5, 77.40±2.52, 67.2±20.5, 51±8.9, 47.80±1.62 and 28.6±.1.9, respectively. The serum ALP level of G V and G III were significantly higher than that of G 1; the mean values±SE of serum of ALP of G V, G III and G 1 were 52.0±5.403, 46.2±2.55 and 35.4±1.5, respectively, while the levels of serum ALP in G II, G IV and G VI were insignificantly higher than the G I. The mean values±SE of serum of ALP of G II, G IV, G VI and GI were 42.4±4.36, 37.2±1.77, 36.80±3.86 and 35.4±1.5, respectively.

The gallstones were experimentally formed in rabbits exposed to oxidative stress and by adding a lithogenic agent (1% dihydro cholesterol) in their diet. The formation of gallstones was a multifactorial and complicated process. It was related to different metabolic and genetic factors like Lith gene mutation, deficiency of cholecystokinin (CCK), hypomotility of gallbladder and aging. Studies showed that cholesterol GSs produced by feeding animals with cholesterol and cholic acid (Jabeen *et al.*, 2018).

The results of this work showed that the prevalence rate of the GSs formation was 100% in G III rabbits, whereas 50% of the rabbits of the G IV developed GSs after the cessation of oxidative stress and continuing administration of the lithogenic agent (1% dihydro cholesterol) and multiple vitamins. The anti-lithogenic effects of vitamin AD3E were for the antioxidants effects that increased the carrying effect of cholesterol to the liver for destruction and the lowering of its level in the blood resulting in dilution of the bile acid concentration. Both of which shared in decreasing the index of cholesterol saturation and, hence, reduction of crystallization and low

Table 3. The mean±SE of the ALT, AST and ALP enzymes

Parameter IU/L	G I	G II	G III	G IV	G V	G VI
ALT	105±12.7^{c}	129.00±3.42^{b,c}	151.4±12.1^{a,b}	117±1.7^{C}	159.80±3.104^{a}	122.80±18.786^{c}
AST	28.6±.1.9^{d}	67.2±20.5^{b}	80.2±34.5^{a}	51±8.9^{c}	77.40±2.52190^{a}	47.80±1.624^{c}
ALP	35.4±1.5^{c}	42.4±4.36^{b,c}	46.2±2.55^{a,b}	37.2±1.77^{c}	52.0±5.403^{a}	36.80±3.86^{c}

*Different superscript in a row indicate statistically significant differences at P≤0.05.

incidence of GS formation. While in the other groups G V, when rabbits were fed with a lithogenic diet (0.5%) of cholic acid and G VI animals fed with (0.5% + vitamins AD3E) no stones were formed in any of them. These may be due to the short duration of giving cholic acid or giving it alone with no other combination or due to its low amount. Jabeen *et al.* (2018) in their study carried out on albino mice fed with a high-fat diet (HFD) (a diet containing 1% cholesterol and 0.5% cholic acid) for eight weeks, the GSs were clustered in the gall bladder more than half of it in size.

The results of this study showed that the prevalence of gallstone formation in both males and females was similar, this finding was in consistent with Lin *et al.* (2016), who in mice feeding with a lithogenic diet consisting of 15% fat, 2% cholesterol and 0.5% cholic acid up to eight weeks, mentioned that the per cent of GSs formation was the same in both the genders, 80% (4 of 5 females and 4 of 5 males) of mice developed GS when compared with the mice that fed on a normal diet.

Regarding oxidative stress, this finding demonstrated that the serum malondialdehyde (MDA) levels were considerably higher, and the serum levels of GR enzyme activity were lower in rabbits with cholelithiasis than in the control group. A similar finding was reported by Sadiem *et al.* (2014). They also estimated oxidative stress biomarker myeloperoxidase (MPO) and antioxidants activity as superoxide dismutase (SOD) in a human patient with cholelithiasis. They found in their results that both the control and patient groups had significant differences in SOD and MPO serum level. They also demonstrated decrease SOD in serum level in the patient group when compared with a control group. Also they found a significant decrease in the MPO serum level in the control group when compared with patients' group. Atamer *et al.* (2014) reported in their study that the oxidative stress in serum as MDA in the control group was decreased significantly when compared with the GS group, while the serum levels of paraoxonase-1 (PON-1) as the activity of the antioxidant enzyme were increased significantly in the control groups when compared with the GS group.

In present study, a positive relationship was found between serum lipid profile and GSs formation in G III where the incidence of GSs was 100%, the same finding was estimated by Atamer *et al.* (2014) and Chen and Wu (2022) who found a positive relation between hyperlipidemia and GSD and a significant increase in serum levels of TG, TC and LDL-C in the GS group, while the serum level of HDL-C was significantly decreased in the GS group. However, in this research that there was no relation between hyperlipidemia and GSs formation like in G V (fed with 0.5% cholic acid) despite that there was no incidence of GSs, however, there was hyperlipidemia in G V.

Regarding the liver enzymes ALT, AST and ALP present results showed that there was a significantly higher level of these enzymes in the cholelithiasis group (G III) than those in the control group. It was revealed that there was a lack of correlation between GSs formation and significantly high levels of liver enzymes. Though significantly high levels of ALT, AST and ALP in G V (fed with 0.5% cholic acid) when compared with G I was due to the pathophysiological changes that affect normal liver functions. While in both G IV and G VI there were only increased significant levels of AST when compared with the normal control group. Omer (2014) in his study, compared 150 GS patients to 150 normal healthy controls and concluded that insulin resistance may exist in metabolic-GS patients suffering from high serum TGs and low serum HDL-Ch. This could lead to dysmotility of the biliary system, which was regarded as a risk factor in the formation of gallstones. He found serum levels of ALP were significantly higher in metabolic-GS patients than in the metabolic controls, There was an increase in the serum levels of ALT and AST in GS patients than in the control groups however, the increase was insignificant.

CONCLUSION

It was concluded that gallbladder stone disease can be developed experimentally when there was a state of imbalanced oxidative stress in presence of a lithogenic agent with high cholesterol or fat diet content. This study could explain why people who are exposed to high oxidative stress especially those with obesity, insulin resistance, diabetes and other diseases that affected the cellular metabolic mechanisms, develop a high oxidative stress state, decreasing antioxidants defense

mechanism, and low HDL-C with higher TC, TG, LDL-C, VLDL-C and liver enzymes, which contribute to disturb liver functions and GS disease can develop. While supplemental vitamins and antioxidants can elevate the body's defense mechanism, improving liver functions, rising HDL-C, and balancing oxidative stress may help cure the disease and slow its progression or decrease its occurrence.

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