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Histopathological Assessment of Gallbladder Health and Gallstone Formation in Rabbits: The Interplay of Oxidative Stress, Lithogenic Diets, and Antioxidant Intervention

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Abstract Gallstones are crystalline deposits that form in the gallbladder, often due to bile stasis, dietary factors, and oxidative stress, leading to significant gallbladder dysfunction. This study aimed to evaluate the pathological impact of oxidative stress, lithogenic diets, and antioxidant therapy on gallbladder health and gallstone formation in rabbits. The working hypothesis proposed that gallbladder dyskinesia, linked to pathological wall changes and bile stasis, plays a central role in gallstone formation. Thirty-six healthy local rabbits (10–14 months old, 900–1480 g) were allocated into six groups, each with three males and three females. The **control group** received a standard diet and water. The **H2O2 group** was given a standard diet plus 1% hydrogen peroxide in water to induce oxidative stress. The **DHC + H2O2 group** received 1% dihydrocholesterol (starting week 4) and 1% H2O2 to simulate cholesterol gallstone development. The **DHC + vitamin AD3E group** followed the same lithogenic diet and were injected with vitamin AD3E (0.1 ml/kg) twice weekly as antioxidant therapy. The **cholic +** H2O2 **group** was administered 0.5% cholic acid (from week 4) and 1% H2O2. The **cholic + vitamin AD3E group** had a similar cholic acid diet along with vitamin AD3E injections. At week six, all rabbits were euthanized, and gallbladders were collected for analysis. The results showed that combining a lithogenic diet with oxidative stress significantly induced gallstone formation and gallbladder damage. Antioxidant therapy with vitamin AD3E offered partial protection, reducing oxidative stress and inflammation. However, 50% of rabbits in the DHC + vitamin AD3E group still developed gallstones, compared to 100% in the DHC + **H2O2** group. In conclusion, lithogenic diets combined with oxidative stress accelerate gallstone development and cause epithelial damage, while antioxidant therapy mitigates but does not entirely prevent these effects. Further studies are needed to explore the protective mechanisms of antioxidants.

Keyword: Gall bladder, lithogenic diets, Gallstone, Histopathological changes, Rabbit

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Introduction:

Gallstone diseases (GDs) affect approximately 10–20% of adults worldwide (2), placing a substantial financial burden on healthcare systems due to associated complications and health issues. The development of GDs is driven by a complex interplay of genetic and environmental factors (3), among which oxidative stress (OS) has emerged as a potential contributor. OS refers to an imbalance between the production of free radicals and the body's antioxidant defenses (4), leading to potential damage to cellular proteins, lipids, nucleic acids, and carbohydrates (5), which may play a role in the pathogenesis of GDs. Diets rich in fat and protein are strongly associated with oxidative stress, as they contribute to increased protein carbonylation and lipid per oxidation while weakening antioxidant defense mechanisms (6; 7). Under normal conditions, the body's antioxidant systems and repair mechanisms are sufficient to identify and eliminate molecules damaged by free radical activity (8; 9; 10). Additionally, mild oxidative stress can typically be managed by most cells in the body (11). The balance between the production of free radicals and their elimination by specific antioxidants is crucial (12). Disruption of this balance, either through reduced antioxidant activity or excessive production of free radicals, can lead to an overproduction of reactive oxygen species (ROS), which may cause harm to cells. The precise cause of cholesterol gallstone disease (CGD) has long been a subject of scientific investigation. Initially, it was believed to result from an inflammatory condition of

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the gallbladder, characterized by cellular shedding and the formation of abnormal substances (13). However, current understanding highlights that the liver's production of cholesterol-supersaturated bile is a central factor and a necessary prerequisite for the development of CGD (13). Gallstones primarily produced by imbalance among the main components of bile—cholesterol, phospholipids, and bile salts (14). Cholesterol precipitation and bile super saturation occur when there is excessive cholesterol, insufficient bile salts or phospholipids, or a combination of both. Maintaining cholesterol homeostasis is crucial to preventing gallstone formation, with factors such as dietary intake, cholesterol biosynthesis, and biliary secretion playing key roles (15).Gallstone disease (GD) can lead to acute cholecystitis or gallbladder inflammation, often resulting in severe abdominal pain and jaundice (16). Although GD is not fatal disease, it can cause significant gastrointestinal complications, including acute GD, pancreatitis, and gallbladder cancer (17; 18). Cholesterol super saturation is the primary risk factor for cholelithiasis, alongside nucleation and gallbladder hypo motility. Hypo motility is considered a critical factor in cholesterol gallstone disease (19).Additionally, conditions like cholecystitis, infections, narrowed biliary enteric anastomosis, post-traumatic biliary strictures and cholangitis are associated with the initial formation of stones in the biliary duct system (20). Among the molecular factors involved in gallstone formation, osteopontin (OPN) has gained attention for its multifaceted role in various pathological processes. OPN is implicated in the progression of atherosclerosis, cancer, metastasis, and hepatic inflammation or injury (21, 22). Recent experimental studies have demonstrated that OPN can modulate hepatic cholesterol metabolism, thereby contributing to the development of cholelithiasis (23). These findings suggest that OPN may serve as a key molecular link between inflammation, altered lipid homeostasis, and gallstone formation, further highlighting the complex interplay of metabolic and inflammatory pathways in the pathogenesis of gallstone disease. Vitamins are organic compounds containing carbon and hydrogen, essential for metabolic processes (24). Vitamins are organic compounds essential for numerous metabolic functions and play a critical role in maintaining cellular health by combating oxidative stress (24). Fat-soluble vitamins such as A, D3, and E act as potent antioxidants, yet the body cannot synthesize them, necessitating external supplementation to meet physiological needs (24). Vitamin A, available as retinoids from animal sources and provitamin A carotenoids from plants, supports immune function and epithelial integrity (4). Vitamin D, primarily acquired through sunlight and diet, exhibits antioxidant properties comparable to or exceeding those of Vitamin E (25, 26). Notably, Vitamins E, C, and β-carotene are among the most effective antioxidants for protecting tissues against oxidative damage (4, 27), highlighting their potential importance in mitigating oxidative stress-related disorders such as gallstone disease. The research aimed to induce gallstone formation in rabbits previously subjected to high oxidative stress, followed by lithogenic treatment with dihydrocholesterol and cholic acid. Additionally, it sought to examine the impact of oxidative stress and gallstone formation on the gallbladder mucosa in these animals.

Materials and Methods:

Ethical Approval

This study involved the use of thirty-six apparently healthy rabbits of both sexes. All experimental protocols were reviewed and approved by the Local Ethics Committee of the College of Veterinary Medicine, University of Duhok (Approval No. CVM20190910UD).

Animals

This study was conducted at the College of Veterinary Medicine, University of Duhok, in the Kurdistan Region of Iraq. Thirty-six healthy local rabbits, aged 10-14 months, with weights ranging from 900 to 1480 grams, were utilized. Prior to the experiment, all rabbits were acclimatized in the animal holding facility and maintained under standard laboratory conditions for a minimum of one week. They were housed in cages and provided with a standard locally prepared diet and unrestricted access to tap water.

Experimental Design

Thirty six rabbits were used and divided them into six groups (6 rabbits per group), each group consisting of three males and three females, and rabbit was housed in a stainless cage, and they distributed in the following groups:

Control Group: Rabbits in this group did not receive any medications; they were fed a standard rabbit diet and provided with water for six weeks.H2O2 Administered Group: Rabbits in this group were fed a standard rabbit diet and subjected to oxidative stress by adding 1% H2O2 to their drinking water for six weeks.

dihydrocholesterol + H2O2 Group: In addition to receiving 1% H2O2 in their drinking water, rabbits in this group were fed a lithogenic diet containing 1% dihydro cholesterol starting from the fourth week and continuing until the end of the sixth week to induce gallstone disease.

dihydrocholesterol+ Vitamin AD3E Group**:** Beginning in the fourth week of the trial and continuing until the conclusion, the rabbits in this group were fed 1% dihydrocholesterol. Additionally, they received twice

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weekly (every Sunday and Thursday) intramuscular injections of vitamin AD3E at a dose of 0.1 ml/ body weight as an antioxidant to help reduce oxidative stress and prevent the production of gallstones.Cholic acid +H2O2 group: The rabbits were given a lithogenic diet containing 0.5% cholic acid starting in the fourth week and continuing until the end of the sixth week to induce gallstones, in addition to 1% H2O2 in their drinking water.Cholic acid +vitamin AD3E group: Beginning in the fourth week of the experiment and continuing until the end, the rabbits were fed 0.5% cholic acid. In addition, twice a week, they received intramuscular injections of vitamin AD3E every Sunday and Thursday (0.1 ml/b.w.) as an antioxidant to help prevent gallstone development and balance the state of oxidative stress.

Sample Collection

After the six-week feeding period, all animals were allowed to fast for the entire night. All rabbits were then sacrificed, and each one underwent an immediate autopsy, a cholecystectomy, and a gallbladder wash with normal saline. Following the removal of the bile, each gallbladder was opened and fixed in 10% neutralized buffered formalin. The tissues were then dehydrated in progressively higher concentrations of alcohol, and the samples were cleared by placing them in xylene. Subsequently, tissues were embedded in pure white paraffin wax, which had a melting point of between 54 and 56 °C, in order to prepare paraffin blocks. To prepare the sections, the paraffin blocks were cut using a rotary microtome (Leica, Germany) at a thickness of 4-5 µm (28; 29). Ultimately, hematoxylin and eosin (H&E) stains were used to stain the sections. A field microscope was used to examine the prepared slides, and a digital computerized camera cannon (Leica, Germany) was used to take images. The histological alterations were examined and analyzed (30).

Result:

According to the current study's findings**, gallstones formed in 50% of rabbits in the DHC + vitamin AD_3E group and in 100% of rabbits in the DHC + H_2O_2 group**. Thus, the histopathological findings for all five treated groups were interrelated, but the severity and type of lesions varied according to the degree of lithogenic stimulation and stone formation. As seen in **figure 1A,** the **H_2O_2 group** displayed extensive destruction of the gallbladder epithelium with complete desquamation and a marked increase in connective tissue or fibrosis, indicating aggressive oxidative injury. In contrast, **figure 1B** illustrated the effects of a lithogenic diet combined with H_2O_2 in the **DHC + H_2O_2 group,** where complex papillary structures were evident due to prominent mucosal hyperplasia, along with a thickened epithelial layer suggestive of dysplasia .Additional findings included irregular gland-like structures, stromal thickening, and mild infiltration of inflammatory cells, all of which pointed to chronic inflammatory processes alongside proliferative changes. However, **Figure 2A**, representing the **DHC + vitamins AD_3E group,** revealed relatively less damage to the epithelial layer. There were projections of the epithelial lining forming fold-like structures, presence of goblet cells, and infiltration of inflammatory cells within the lumen of the villi . The observation of Rokitansky-Aschoff sinuses suggests early remodeling of the gallbladder wall. These changes indicate a partial protective effect of vitamin supplementation, consistent with the lower incidence of stone formation. On the other hand, **Figure 2B** (from the **cholic + H_2O_2 group)** showed similar mucosal changes to figure 2A, including hyper plastic mucosal folds, but lacked significant inflammatory cell infiltration. Instead, the muscular layer demonstrated notable fibrosis and interstitial edema, with overall mild damage to the gallbladder wall, suggesting a milder pathological outcome than the DHC + H_2O_2 group. The final group, represented in **figure 3A and 3B,** was the **cholic + vitamins AD_3E group,** which showed more pronounced histopathological alterations despite vitamin treatment. Severe infiltration of inflammatory cells was evident, along with adenomyomatous hyperplasia, prominent mucosal folds, and a thickened muscular layer. Rokitansky-Aschoff sinuses were also present. These findings indicate significant chronic inflammation and epithelial proliferation consistent with **severe cholecystitis,** possibly linked to persistent lithogenic stress despite antioxidant supplementation.

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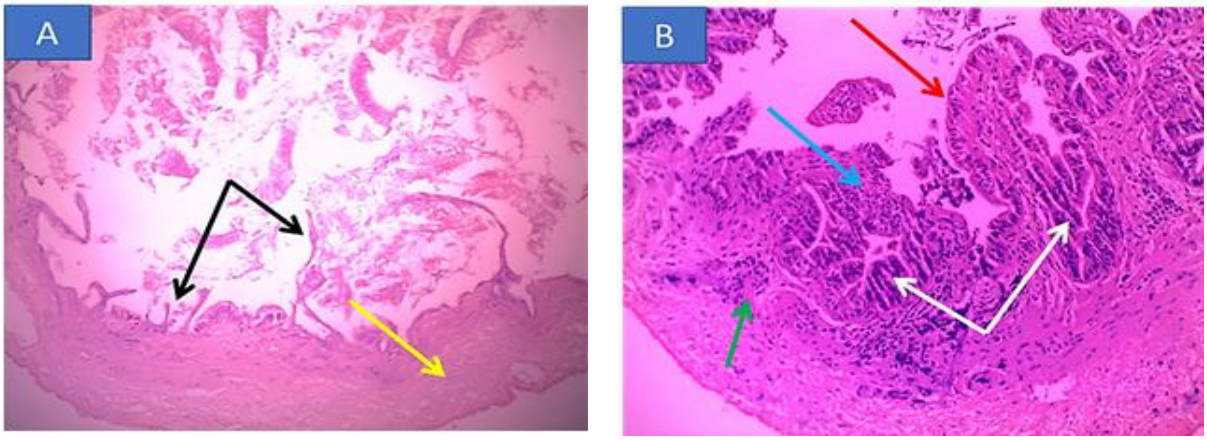
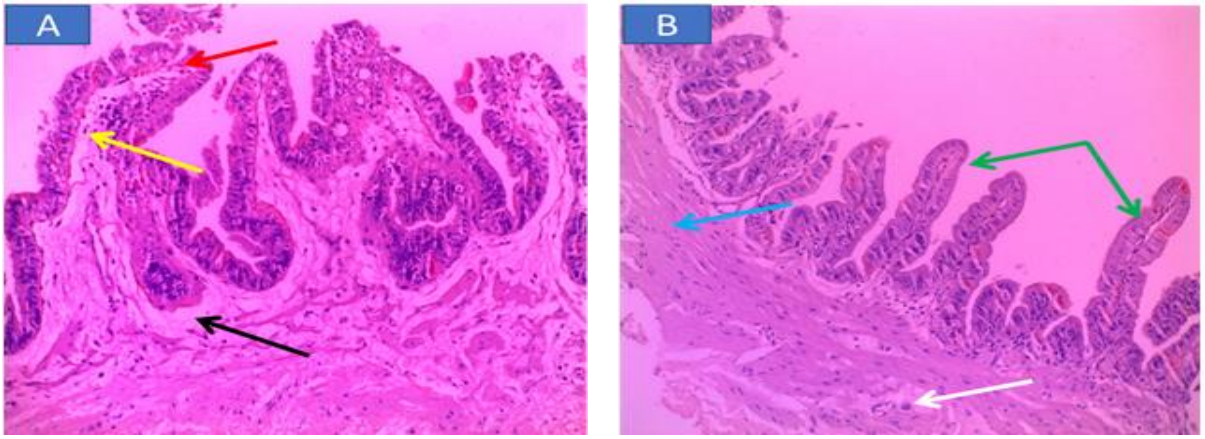


Fig;1. Histopathological examination of rabbit gallbladder exposed to H2O2 shows severe damage and desquamation of epithelial layers of gallbladder (black arrow) fibrosis (yellow arrow) (A). (B) rabbits of DHC + H2O2 group shows prominent mucosal hyperplasia with complex papillary structures (red arrow) and increased epithelial layers suggesting dysplasia (blue arrow). Irregular gland-like spaces, are present (white arrow). The thickened stroma and mild inflammatory infiltrate indicate chronic inflammation (green arrow). H&E 10x



Fig;2. Histopathological examination of gallbladder of rabbits of DHC + vitamins AD3E group shows mild destruction of epithelial layer with projection of epithelial layers like folds (red arrow) and presence of goblet cells as well as infiltration of inflammatory cells in the lumen of villi (yellow arrow) and Rokitansky-Aschoff sinuses (black arrow)(A).The (B) section was the cholic + H2O2 group, the gallbladder mucosa shows hyper plastic mucosal folds (green arrow) without significant inflammation but there is fibrosis in the muscular layer (blue arrow) and some edema (white arrow) . H,E 10 X

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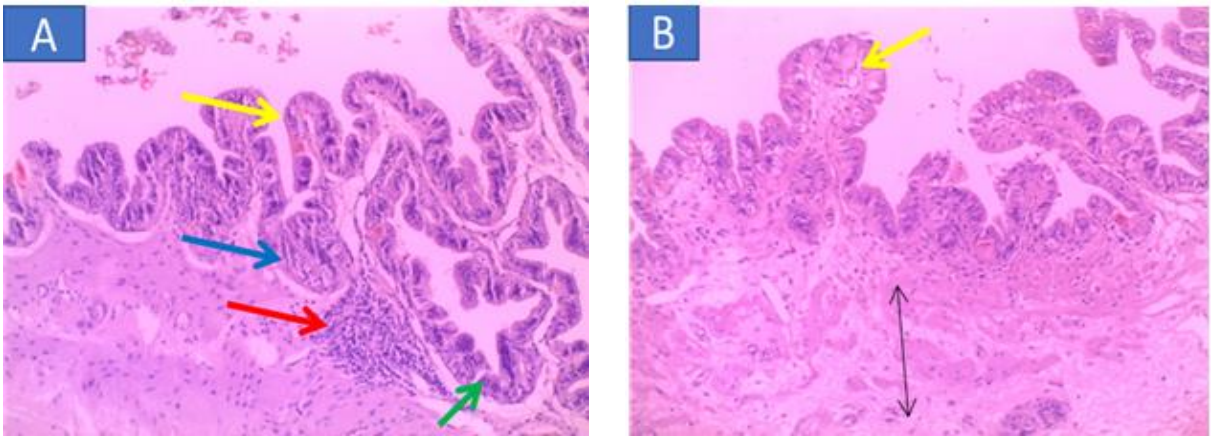

Fig;3. Histopathological examination of gallbladder of cholic + vit.AD3E group shows severe infiltration of inflammatory cells (red arrow) , adenomyomatous hyperplasia (blue arrow) with prominent mucosal folds (yellow arrow), thickened muscular layer (black arrow), and Rokitansky-Aschoff sinuses (green arrow) (A&B). H,E 10X.

Discussion

This study showed that there was a variation in the results between the experimental groups, particularly with respect to gallstone formation, tissue damage, and the protective role of vitamin AD3E.The group exposed to 1% hydrogen peroxide (H2O2) alone exhibited severe damage to the gallbladder, with complete desquamation of the epithelial layers. This finding aligns with (31) that have demonstrated the deleterious effects of oxidative stress on the biliary system. Oxidative stress, characterized by an overproduction of reactive oxygen species (ROS), leads to lipid peroxidation, protein damage, and apoptosis of gallbladder epithelial cells. In the H2O2 group, the ROS likely overwhelmed the cellular antioxidant defenses, resulting in significant tissue damage.The destruction of the epithelial layer is crucial as it disrupts the gallbladder's primary function of bile storage and concentration, which could further exacerbate gallstone formation. The lack of protective goblet cells in the H2O2 group, as observed histologically, could indicate that oxidative stress impairs the mucosal defenses of the gallbladder, contributing to the extensive damage observed (32). However; the combination of oxidative stress and a lithogenic diet (DHC + H2O2 group) resulted in 100% gallstone formation, indicating a strong synergistic effect between these factors. This finding supports earlier research that demonstrates the role of oxidative stress in exacerbating cholesterol crystallization and gallstone formation. The addition of H2O2 likely accelerated the oxidative modification of cholesterol and bile acids, leading to an environment conducive to gallstone nucleation and growth (32). Furthermore; (33) showed that oxidative stress has been shown to impair gallbladder motility, contributing to bile stasis, which is a key factor in the pathogenesis of gallstones. The histological evidence of epithelial damage and desquamation in this group suggests that the combined effect of oxidative stress and a lithogenic diet created a profoundly pathological environment within the gallbladder, making it highly susceptible to gallstone formation (33).In contrast, the group treated with vitamin AD3E (DHC + Vitamins AD3E) showed a significant reduction in gallstone formation (50%), as well as milder histopathological changes, such as only mild inflammatory cell infiltration and some preservation of the epithelial structure. These results emphasize the protective role of antioxidants in mitigating the effects of oxidative stress (34). Vitamin AD3E (a combination of vitamins A, D3, and E) is known for its strong antioxidant properties. Specifically, vitamin E is a lipid-soluble antioxidant that protects cell membranes from oxidative damage by neutralizing free radicals. Vitamin A has also been shown to enhance mucosal immunity, while vitamin D plays a role in modulating inflammation. In this study, the presence of goblet cells and mild damage in the DHC + Vitamins AD3E group suggests that the antioxidant properties of these vitamins helped preserve the epithelial integrity and mucosal defenses of the gallbladder (35). This result aligns with earlier findings (36) that demonstrated the protective effects of antioxidants in models of gallstone formation. However, it is important to note that while vitamin AD3E reduced the incidence of gallstones by half, it did not completely prevent their formation, indicating that oxidative stress and lithogenic diets still played a significant role in this process. The presence of mild inflammation and epithelial hyperplasia further supports this conclusion.On the other hand; the groups fed a cholic acid-rich diet also exhibited significant histopathological changes. Cholic acid, a bile acid, is known to increase cholesterol saturation in bile, promoting the formation of cholesterol gallstones. The

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Cholic + H2O2 group displayed marked edema in the muscular layer and elongated folds in the epithelial lining, both indicative of cholecystitis and bile stasis. These findings suggest that cholic acid exacerbates the effects of oxidative stress on the gallbladder, as seen in the structural changes and inflammation (37; 38). In the Cholic + Vitamins AD3E group, although antioxidants reduced some of the tissue damage, severe cholecystitis, hyperplasia, and mild ulceration were still observed. This suggests that while vitamin AD3E provided some protection, the aggressive lithogenic environment created by the cholic acid diet and oxidative stress was not completely mitigated (39). Previous studies have also noted that while antioxidants can reduce oxidative damage, they may not entirely prevent gallstone formation in the presence of strong lithogenic stimuli.

Pathophysiology of Gallstone Formation and Cholecystitis

The development of gallstones and cholecystitis observed in this study can be attributed to a combination of oxidative stress, cholesterol super saturation, and bile stasis. These factors disrupt the delicate balance of bile components, leading to crystallization and stone formation. Inflammation, as observed in multiple groups, is both a consequence of gallstone formation and a contributing factor, as it promotes further gallbladder dysfunction (38). While the presence of goblet cells and fold projections in some groups suggests a compensatory mechanism by the gallbladder in response to chronic irritation, possibly attempting to enhance mucus production and protect against bile acid-induced injury. However, in groups with severe oxidative stress, such as the H2O2 group, these protective mechanisms were overwhelmed (40).

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Conflict of interest

There is no conflict of interest in the present research as confirmed by the authors.

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Conclusion and Implications

This study highlights the critical role of oxidative stress in the pathogenesis of gallstone formation and gallbladder damage. The findings confirm that a lithogenic diet combined with oxidative stress leads to severe gallbladder injury and promotes gallstone formation. However, antioxidant treatment with vitamin AD3E offers significant protection by reducing oxidative damage, inflammation, and the incidence of gallstones. While the antioxidant effect is not absolute, it presents a promising therapeutic approach for preventing gallstone formation and mitigating gallbladder injury in the presence of oxidative stress.Future research should explore the dose-dependent effects of antioxidants and investigate other potential agents that could provide more comprehensive protection against gallstone formation, especially in individuals with high oxidative stress or dietary risk factors. Additionally, a longer-term study could help assess whether continuous antioxidant treatment might prevent gallstone recurrence after initial formation.

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