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ORIGINAL: Induction of Experimental Endometriosis in Rat: Evaluation of Systemic Inflammatory Response and Liver Tissue Changes

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ABSTRACT

Introduction: Nowadays non-alcoholic fatty liver disease (NAFAD) is considered as a serious problem in human societies. Recently, the possibility of an association between endometriosis and NAFAD has been considered. This study was designed to evaluate some general inflammation parameters and hepatic lesions during and after experimental endometriosis.

Material and Methods: In 20 female rats, the endometriosis model was induced by suturing parts of the uterine horn wall to the mesenteric gut. After four weeks, the rats in group I were euthanized and endometriosis cysts and some fragments of their liver were used for histopathological evaluation. At the same time, endometrial cysts were surgically removed in the rats of group II and they were kept for four weeks later. In addition, hematobiochemical evaluation was performed. In group II, similar evaluative investigations were performed 8 weeks after experimental surgery.

Results: Significant increase in triglyceride, LDL, AST, ALP, ALT and estrogen parameters was observed in this study ($P<0.05$), Whereas, total WBC count, lymphocyte, PCV and HDL level decreased significantly ($P<0.05$). In histopathological evaluation, induction of endometriosis was confirmed at the microscopic level, but no evidence of fatty liver or hepatic inflammation was found.

Conclusion: Despite notable changes in some hematobiochemical factors in rats with experimental endometriosis, there was no evidence of fatty liver and hepatic inflammation. Therefore, there may be no association between endometriosis and non-alcoholic fatty liver disease.

Introduction

Endometriosis is a disease specific to women that causes chronic abdominal pain and infertility at reproductive age (1). Primates' models are used to mimic endometriosis in women. The rodent model is currently preferred because of the high costs. Since endometriosis occurs only in humans and non-human primates, therefore, endometriosis must be induced in animals such as rodents to study its symptoms (2). Inflammatory response related to the endometriosis is due to the role of inflammatory cells including the infiltration and activation of peritoneal macrophages and their secreted

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cytokines (3,4). It is clear that the process of inflammation caused by this anatomical disorder affects ovarian function. Additionally, local inflammation can lead to adhesion, angiogenesis and endometrial cyst formation (5). Nowadays, non-alcoholic fatty liver disease (NAFAD) is considered a serious issue due to obesity and metabolic abnormalities. However, little is known about the association between symptoms of NAFAD and endometriosis. NAFAD is the accumulation of fat in the liver without inflammation or damage to the liver cells. People with NAFAD also have other metabolic abnormalities, such as obesity, diabetes mellitus, and abnormal blood lipids (triglycerides, cholesterol, and phosphorlipids). In addition to the symptoms of NAFAD, inflammation, injury, swelling and the presence or absence of fibrotic tissue can also be seen in the liver, which is called NASH (Nonalcoholic steatohepatitis, a type of non-alcoholic fatty liver disease) which characterized by inflammation and damage to the liver cells (6). Recently, it has been shown that the occurrence of endometriosis in women (about 8%) is related to NAFAD (about 24%) and venous thromboembolism (7). In addition, within 5 years, 35% of women undergoing endometriosis had been reported to have NAFAD (8). Estrogen level during endometriosis is high and liver is a vital organ of the body for hormone clearance and detoxification. Following endometriosis, the levels of estrogen-related hormones increase dramatically in the body (9). Estrogen plays a key role in lipid metabolism and with endometrial removal the risk of developing NAFAD in women is greatly increased (10). It is therefore hypothesized that following endometriosis, the probability of tissue changing in the liver increases due to abnormal accumulation of fats in the liver. However, some aspects of endometriosis have a great deal to do with the immune system and inflammation. The present study will also help us understand the process of inflammation and immune factors in the liver during and after endometriosis. Therefore, experimental endometriosis is induced in rats, and surgical treatment is considered as their main treatment, and in assessing the hemato-biochemical changes of the animals, tissue changes in the liver are assessed.

Methods

Ethical and executive aspects of this study were approved by the Research Council of the Department of Veterinary Clinical Sciences (170/1312).

Preparation of samples

A total of 29 healthy adult female rats with an approximate age of 8-12 weeks and weighing 180-230 g were assigned to the current investigation. Of these, 9 rats were sacrificed humanly (time 0) and 20 rats were randomly divided into two equal groups in order to induction of endometriosis. Animals in the first group were euthanized 4 weeks after induction of endometriosis and evaluated for histopathological and hematobiochemical analysis. In the second group, the induced endometrial cysts were removed surgically and then the animals were kept for another 4 weeks. Finally, the animals were euthanized to assess histopathological lesions and inflammatory process of the liver as well as evaluation of hepatic enzymes activity and some blood and biochemical parameters.

Surgery preparation and procedure

Experimental endometriosis was induced by implanting parts of the uterine horn at the mesenteric surface of the intestine (11). Following induction and maintenance of injectable anesthesia (intraperitoneally) by combination of ketamine (80 mg / kg) - xylazine (10 mg / kg) (12), and after aseptic celiotomy, longitudinal fragments harvested from the uterine horn and sutured to the mesenteric gut with 4.0 polyglactin 910 sutures to induce endometriosis and finally the abdomen closed routinely. Endometrial peritoneal cysts were developed for 4 weeks. At this stage, half of the rats were euthanized by overdose of anesthetics and considered as the first group. Subsequently, endometriosis cysts were removed after surgical celiotomy

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and rats were maintained again for 4 weeks and finally, after sacrificing the remaining rats, they were considered as the second group.

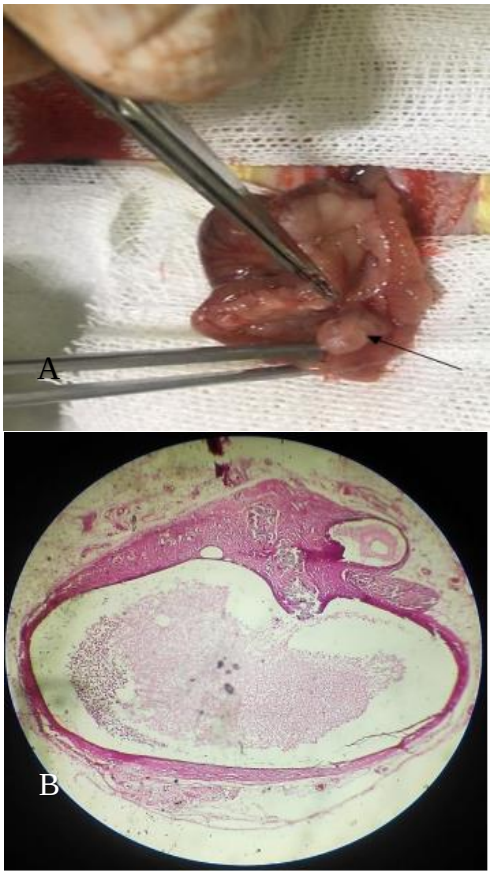

Figure 1. (A) Induced endometriosis cyst in rat mesentery (black arrow); (B) A microscopic cross section of the uterine mesenteric graft showing endometrial cyst induction

Sampling, histological examination, and blood works

Time 0, week 4 and week 8 after experimental induction of endometriosis were considered for hematobiochemical and histopathological evaluation. Autoanalytical apparatus was used to determine estrogen, AST, ALT and ALP in serum. In addition, mean and total count of white blood cells, neutrophil to lymphocyte ratio, hematocrit (PCV), total protein, albumin, globulin, glucose, cholesterol, triglyceride, LDL, HDL and estrogen levels were analyzed. For histopathological evaluation, appropriate tissue samples which collected from the livers and endometrial cysts, were then fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 µm thickness, and stained with Masson's trichrome and hematoxylin-eosin staining for light microscopic examination (13).

Statistical analysis

For hematological parameters analysis, descriptive statistics including the mean, standard deviation, median, minimum and maximum were calculated for all variables. The one-way ANOVA followed by Tukey's post hoc test were used for comparison of different parameters. The data were analyzed by SPSS software, version 22, and differences of $P<0.05$ were considered significant.

Results

The formation of peritoneal endometriosis cysts after 4 weeks was confirmed in both macroscopic and microscopic examinations (***Figure 1***).

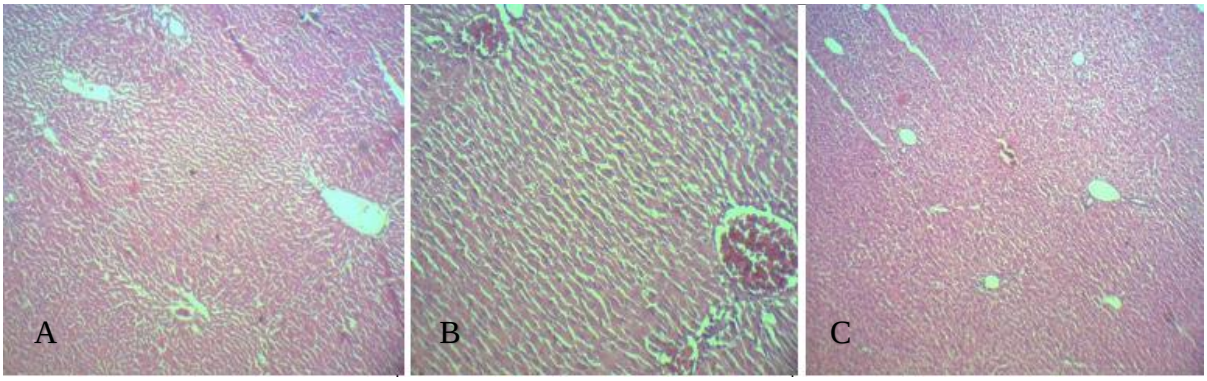

Figure 2. Hepatic sections; (A) normal tissue at time 0; (B) The fourth week when endometriosis was induced experimentally (group 1); (C) Eighth week of study when endometrial cysts were surgically removed (group 2)

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Microscopic examination of the animal's liver at the different times (Time 0 and weeks 4 and 8 after surgery) revealed no evidence of fatty liver (***Figure 2***). The presence of neutrophils, lymphocytes, and macrophages which are indicative of liver inflammation was not found in the histopathological sections. In addition, the ballooning hepatocytes as an independent factor in the diagnosis of NASH, cannot be seen on the histopathological examination.

Based on the ***Table 1*** and ***Table 2***, the results showed that the parameters of total WBCs, lymphocytes and PCV counts in the first and second groups were significantly decreased compared to the normal group at time 0 (baseline data) ($P<0.05$). The results also showed that the number neutrophils, monocytes and neutrophil to lymphocyte ratio in the fourth and eighth weeks (group 1 and group 2 respectively) were not significantly different when compared to the baseline data (time 0) ($P>0.05$). Total protein, albumin, globulin and glucose levels in the first and second groups were not significantly different from the time 0. It was also found that estrogen, LDL, ALP, AST and ALT levels increased significantly ($P<0.05$) in the groups 1 and 2 compared to the baseline data. The analyzed data showed that the cholesterol level in the first group was not significantly different from the baseline data ($P>0.05$), but it was significantly increased in the second group ($P<0.05$). Triglyceride level in the first group showed a marked increase ($P<0.05$) in comparison to the baseline data, while a significant decrease ($P<0.05$) was observed in the second group compared to the time 0. The results also showed that HDL level decreased significantly ($P<0.05$) in the groups 1 and 2 compared to time 0.

Table 1. Comparison of the mean of some blood parameters at different times

Blood parameters	Time 0 (N=9)	Week 4 (N=10)	Week 8 (N=10)
Total WBCs (10^9/L)	5133.33±331.45^a	3345±88.65^b	3735±191.64^b
Lymphocyte (10^9/L)	5133.33±331.45^a	2360±66.27^b	2652.1±201.67^b
Neutrophil (10^9/L)	1144.89±62.86^a	944.5±82.41^a	1029.1±68.52^a
Monocyte (10^9/L)	68.22±17.29^a	39.70±10.94^a	53.80±14.30^a
N/L ratio	0.31±0.03^a	0.40±0.04^a	0.41±0.04^a
PCV	0.48±0.01^a	0.39±0.01^b	0.43±0.02^b

Different superscript letters in each row indicates statistical significance.

Table 2. Comparison of the mean of some biochemical parameters at different times

Biochemical parameters	Time 0 (N=9)	Week 4 (N=10)	Week 8 (N=10)
Total protein (g/dl)	4.6±0.29	5.07±0.20	4.96±0.40
Albumin (g/dl)	2.81±0.21	3.41±0.24	3.87±0.46
Globulin (g/dl)	2.47±0.19	2.94±0.28	2.74±0.27
Glucose (mg/dl)	160.37±13.01	155.20±9.58	160.04±9.28
Cholesterol (mg/dl)	90.33± 12.89^a	161.50±13.95^a	136.80±15.77^{ba}
Triglyceride (mg/dl)	121.44±12.68^a	166.90±13.01^b	116±10.72^{ac}
LDL (mg/dl)	48.44±5.30^a	82.6±7.54^b	72.50±6.70^b
HDL (mg/dl)	45.55±2.79^a	32.20±2.98^b	32.00±3.27^b
AST (IU/l)	92.00±10.99^a	141.90±14.2^b	145.00±14.96^b
ALP (IU/l)	87.67±4.17^a	110.20±7.29^b	107.40±4.24^b
ALT (IU/l)	72.67±8.34^a	97.50±5.28^b	79.60±6.09^{ab}
Estrogen (pg/ml)	21.82±1.02^a	42.41±2.97^b	32.17±3.20^c

ALT (Alanine amino transferase); ALP (Alkaline phosphatase); AST (Aspartate amino transferase);
Different superscript letters in each row indicates statistical significance

Discussion

In the present study, notable changes in some hematobiochemical factors in rats with experimental endometriosis was observed, however there was no evidence of fatty liver

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and hepatic inflammation. One of the mechanisms that may contribute to the development of endometriotic lesions is the response between the scattered endometrial tissue and the immune system. Abnormal cellular and humoral immune function and numerous cellular mechanisms play an important role in the development and progression of endometriosis (14).

In one study, a significant decrease in the number of B lymphocytes was observed in women with endometriosis (15), and another study suggests a significant decrease in total T lymphocyte levels in women with endometriosis (16). This is in agreement with the present study because, in the present study, rats with experimental endometriosis showed a significant decrease in lymphocyte levels in time zero and a decrease in monocytes at no significant time at time zero. In another study, no changes in resting monocyte activity were observed in women with endometriosis, but monocytes increased in response to different stimuli in these women (17).

It has been reported that the levels of Colony-stimulating factor of granulocyte and macrophages, which are a growth factor in stimulating stem cells to produce granulocytes and lymphocytes, did not change in women with endometriosis compared to controls (18).

In the present study, we observed an increase in lymphocyte and monocyte levels compared to the fourth week after surgical treatment of experimental rats with endometriosis (Lymphocyte levels decreased significantly after surgery (week 8) compared to time zero and monocyte levels decreased after surgery (week 8) compared to time zero). In one study, the level of white blood cells, especially neutrophil levels, was increased in women with endometriosis (19). This is in contrast to the present investigation because in the current study, a decrease in the total white blood cell count in animals with endometriosis was observed in the fourth week. The level of total white blood cells also increased after surgery and in the eighth week. Another study reported no change in the absolute number of polymorphonuclear neutrophils, but found a slight but significant decrease in their chemotactic index. (Because polymorphonuclear neutrophils in women with endometriosis showed decreased chemotactic properties in response to a stimulus compared to the control group) (20). These findings are in agreement with our results, because in our study there was no significant difference in neutrophil levels in rats with endometriosis after 8 weeks of surgery.

Neutrophil to lymphocyte ratio has been reported to be one of the diagnostic tests with a sensitivity and specificity of 60% (19). It is reported that this index is not a good indicator for determining the severity of endometriosis in patients with moderate to severe degrees of endometriosis and is only dependent on the patient's age. It has been reported that there is no significant difference between neutrophil to lymphocyte ratio in patients with endometriosis (19), which is similar to the present study (21). Increased hemoglobin and hematocrit levels are usually dependent on blood concentrations. It has been reported that the percentage of hematocrit decreased significantly in patients with endometriosis, which is similar to the results of the present study. In the current study, a significant decrease in hematocrit percentage was observed in the fourth and eighth weeks compared to time zero (baseline data). It has been suggested that very severe endometriosis is associated with lower blood concentrations, with impaired RBC regulation and iron metabolism. Some evidence suggests that iron metabolism is potentially involved in the pathogenesis of endometriosis (22, 23). Iron can produce species of free radicals that are capable of inducing cellular damage and altering gene expression by enhancing the inhibition of transcription factors associated with endometriosis pathogens, such as the NF-κB that activates B cells (23). There is ample evidence that oxidative stress plays a role in the pathophysiology of endometriosis. Peroxigenase-1 (PON-1) is an HDL-dependent enzyme that prevents LDL oxidation. PON-1 activity

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levels were significantly decreased in patients with endometriosis (mild disease level) and lipid hydroperoxide (LOOH) levels were significantly higher than controls. There was a significant decrease between PON-1 activity and disease stage. It has been reported that women with endometriosis show a significant decrease in PON-1 activity and HDL levels and an increase in the levels of LOOH, total cholesterol, triglycerides, LDL, and lipoproxygenases (24), which is in agreement with the present study.

In rats with endometriosis, there was also a significant decrease in HDL levels, a significant increase in cholesterol and LDL levels and an increase in triglycerides. In the present study, LDL increased significantly in the eighth week (after surgery) compared to time zero (baseline data). The results showed that the cholesterol parameter increased significantly at week 8 compared to time zero. The results showed that the triglyceride parameter in the eighth week had a significant decrease compared to time zero. The results also showed that the HDL parameter at week 8 had a significant decrease compared to time zero. Oxidative stress has been reported to be a contributing factor in the pathophysiology of the disease (25).

It has been reported that levels of superoxide dismutase and glutathione peroxidase in the peritoneal fluid of women with endometriosis were significantly reduced compared to the control group, both of which play important roles in the formation of free radicals and reactive oxygen species (ROS) (25). Studies have also shown that ROS may increase the growth and adhesion of endometrial cells in the peritoneal cavity, stimulating endometriosis formation and infertility. Therefore, in women with endometriosis there is a significant decrease in antioxidant levels and a significant increase in LOOH (25). PON-1 is primarily responsible for degradation of lipid peroxides before accumulation in LDL (26) and increased LDL uptake (27). PON-1 directly inhibits macrophage oxidative stress and thus reduces the ability of macrophages to activate superoxidase anions and LDL oxidation (28).

In addition, PON-1 inhibits macrophage cholesterol biosynthesis and protects HDL against lipid peroxidation (29). Inhibition of HDL oxidation by PON-1 preserves the anti-atherogenic effects of HDL in reverse cholesterol transfer (30). The antioxidant activity of HDL is also mediated by PON-1 (31). Finally, possibly increased inflammation and oxidative stress in the progression of endometriosis may explain the decrease in PON-1 activity in moderate to severe endometriosis.

Endometriosis is a hormone-dependent inflammatory disease that is usually associated with high levels of estrogen and abnormal levels of cytokines, whose expression is regulated by GATA-3 in lymphocytes. GATA-3 is a specific transcription factor of Helper-2 T cells that is expressed in endometrial epithelial cells in patients with endometriosis (32). Therefore, GATA-3 may regulate the expression of cytokines in endometrial cells in patients with endometriosis. Estrogen regulates GATA-3 expression in a dose- and time-dependent manner. Estrogen induces translocation of GATA-3 from the cytoplasm to the nucleus and may be involved in the development and progression of endometrial disease by regulating the secretion of cytokines in ectopic endometrial cells in patients with endometriosis. Estrogen levels in women with endometriosis have been reported to be very high (33). This is similar to the present study because high estrogen levels were recorded in the rats with endometriosis at weeks 4 and 8 (after surgery) compared to time zero (baseline data). IL-6 is a Th2 cytokine that induces endometrial cell translocation, localization, and growth in patients with endometriosis (34). IL-6 has been reported to be less expressed in estrogen in endometriosis. In contrast to estrogen, GATA-3 induces IL-6 expression and therefore increased IL-6 expression in local lesions of patients with endometriosis may be due to the interaction of GATA-3 and estrogen effects (35, 36). Estrogen decreases the regulation of IL-8 expression and therefore increased IL-8 in local lesions of

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patients with endometriosis may be due to the coordination between GATA-3 activities and estrogen. IL-10 has been reported to increase in peritoneal fluids in patients with endometriosis (37). IL-10 reduces cellular immunity and contributes to the mechanism underlying the development and endometriosis. Estrogen increases IL-10 expression (38).

AST, ALT, ALP, and albumin can be used to evaluate patients with or suspected liver disease. ALT and AST are widely distributed throughout the body. AST is found primarily in the heart, liver, skeletal muscles and kidneys, whereas ALT is found primarily in the liver and kidneys and has lower levels in the heart and skeletal muscles (39). In the present study, we observed a significant increase in AST and ALT values in the fourth and eighth weeks compared to the control group (baseline data). ALT activity is higher than AST in many types of liver disease with the exception of alcoholic hepatitis. It seems that several causes lead to increased AST activity in alcoholic hepatitis.

Alcohol increases the activity of mitochondrial AST in plasma, whereas in other cases hepatitis does not (40). In most forms of liver injury, hepatocyte activity is decreased in both systolic and mitochondrial AST, but alcohol only causes a decrease in systolic AST activity (41). Pyridoxine deficiency (vitamin B6) is common in alcoholic hepatitis, which reduces hepatic ALT activity (42) and alcohol induces mitochondrial AST release from cells without cellular damage (43). AST and ALT are measured by catalytic activity (44), which requires pyridoxal-5-phosphate (P-5′-P) for its maximum activity, while the effect of P-5′-P reduction on ALT, more than its effect on AST (45). AST and ALT were significantly decreased in patients with renal failure compared to healthy subjects and may be due to P-5′-P serum bonds which increased overall P-5′-values. P and P-5′-P depletion is released which eventually reduces enzymatic activity (46). ALP is involved in the transport of metabolites through the cell membrane and is found in placenta, ileum mucosa, kidney, bone, and liver (47, 48). Bone, liver, and kidney isoforms of ALP have a common protein-like structure encoded by similar genes (49) but differ in carbohydrates. Hepatic isoenzyme half-life is about 3 days (50). Cholestasis stimulates ALP synthesis by hepatocytes and bile salts release ALP from the cell membrane (51). Diseases other than liver diseases that affect ALP levels include hemolysis, pregnancy, smoking, bone disease, tumors, severe enteritis (in neonates), hypophosphatase, and oral contraceptives (52, 53). The present study showed a significant increase in ALP values in the fourth and eighth weeks compared to the control group (baseline data). Because of the profound association between elevated ALP and its hepatic origin and increased activity of other canalicular enzymes (such as GGT (gamma glutathione transferase)), measurement of GGT activity is a good marker of liver ALP but is not a good test for the detection of ALP in bone diseases (54). Albumin is the most abundant plasma protein produced by liver cells. The level of albumin production depends on several factors, such as the presence of amino acids, plasma oncotic pressure, concentrations of inhibitory cytokines (especially interleukin-6) and some hepatocyte activities (55). It has been reported that antibodies to albumin have been rare in patients with endometriosis, which is consistent with the present study because we observed a (non-significant) increase in albumin in the fourth and eighth weeks compared to the control group (baseline). A significant increase in serum albumin levels to dehydration is the long-term use of tourniquet for blood sampling or evaporation. The main causes of albumin depletion include: decreased protein (nephrotic syndrome, burns, decreased protein due to intestinal damage), increased albumin circulation (catabolic states, glucocorticosteroids), reduced protein intake (malnutrition, diets with Very low protein) and liver disease (55). Plasma albumin rarely decreases in acute hepatitis because it has a long plasma half-life but gradually decreases in chronic hepatitis-progressing cirrhosis.

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Albumin is also a marker for the prognosis of liver cirrhosis (55).

Statistical analysis

Fatty liver disease includes all types of alcoholic fatty liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). NAFLD is associated with obesity, diabetes, and insulin resistance and is considered a manifestation of the liver in metabolic syndrome. Histopathology is the gold standard for evaluating the severity of liver injury in NAFLD and ALD. NAFLD has not always been associated with increased ALT or GGT (56). It should always be kept in mind that although overall fat intake is not associated with a risk of liver cirrhosis or liver cancer, it is associated with cholesterol (57). In patients with NAFLD, approximately 30% progress from non-alcoholic steatosis to non-alcoholic steatohepatitis (NASH). Of these patients with NAFLD, approximately 20% develop cirrhosis and 30-40% of those with cirrhosis have compensatory activity and die from liver injury over a 10-year period (58). On the other hand, the first and most common histological manifestations of ALD are steatosis. About 20-40% of patients with steatosis develop into alcoholic steato-hepatitis (ASH) (59). About 40% of patients with ASH develop cirrhosis (60). Histo-pathological analysis of liver biopsy is the gold standard and the only accurate method to evaluate the rate of steatosis, necrosis-inflammatory changes, and fibrosis in NASH, and thus distinguishes NASH from separate steatosis (61). Mostly lobular inflammatory infections are composed of neutrophils but usually lymphocytes and macrophages are also seen at histopathological stages. In practice, the presence of ballooned hepato-cytes is an independent issue in the diagnosis of NASH (not steatosis). No evidence of hepatitis and fatty liver was found in the present study and at histopathological sections of the liver at the fourth and eighth time points. In the present study, uterine implants were clearly seen in the cystic area after 4 weeks of surgery. Endometriosis cysts have been reported to be characterized by severe cell infiltration, edema, and persistent inflammatory lesions. Implants, proliferation, angiogenesis, and inflammation play key roles in the development and growth of endometrial lesions (62). In this study, an autologous rat model was used to induce endometrial surgery. Prostheses appear to play an important role in the progression of endometriosis (63), and in the implant tissue, proximal nerves have been found to be the best site for release of some mediators such as NGF, which in turn Participate in the development of pelvic pain caused by endometriosis (64). Drugs play an important role in the new angiogenesis process and other manifestations of endometriosis that guarantee oxygenation to the lesions (65). In endometrial lesions, both neutrophilic and eosinophilic myeloid cells are found at all stages of puberty, showing strong cytoplasmic responses to myeloperoxidase (66).

Conclusion

In the present study, there was a significant increase in parameters such as triglyceride, LDL, AST, ALP, ALT and estrogen following induction of experimental endo-metriosis in rats. Whereas, white blood cell count, lymphocyte, PCV and HDL counts decreased significantly. However, there was no evidence of fatty liver and liver inflammation in histopathologic evaluation, although endometriosis was confirmed at macroscopic and microscopic levels. In other words, despite significant fluctuations in some hematobiochemical factors in rats with experimental endometriosis, no evidence of fatty liver and hepatic inflammation was observed. Therefore, according to the data obtained from this study, there may be no association between endometriosis and non-alcoholic fatty liver disease, and the initial hypothesis of this study regarding the occurrence of liver inflammation and non-alcoholic fatty liver disease following endometriosis cannot be confirmed.

Ethical standards statement

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All investigational procedures used in this study were reviewed and approved by the Council of Department of Veterinary Clinical Sciences of the Shahrekord University (13990925; P/47/170).

Conflicts of interest

The authors declare no conflict of interest.

Authors' contributions

General design of study and performing of experiment: MJ, FS, ES, AB; Analysis of data and designing of graphs: MJ, MH; Interpretation of Histological section: MH; Writing primary draft: MJ; Manuscript critical revision: MJ, ES, AB.

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