Hepatoprotective Activity of Silymarin in Combination with Clorsulon Against Fasciola hepatica in Naturally Infected Sheep

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Abstract
Fasciolosis is a parasitic infection with socioeconomic implications that causes essential health problems in animals throughout the world. The current study intended to evaluate the hepatoprotective activity of silymarin alone and in combination with clorsulon in sheep naturally infected with Fasciola hepatica. For this purpose, a total of 40 sheep (Sangsari breed) were divided into five groups as follows: 1) the healthy sheep without treatment (the negative control), 2) the infected sheep without treatment (the positive control), 3) the infected sheep treated with silymarin (280 mg orally from the day 1 to 14), 4) the infected sheep treated with clorsulon (7.5 mg/kg orally on the days 5, 10, and 15), and 5) the infected sheep treated with silymarin + clorsulon. The assessed serum parameters included total bilirubin, total protein, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT). Additionally, histopathological changes in naturally Fasciola hepatica-infected sheep and controls were investigated using light microscopy. Biochemical analysis showed significantly lower levels of AST, ALT, ALP, GGT, bilirubin, protein, and albumin in infected animals than in the healthy group (P<0.05). Silymarin plus clorsulon treatment showed a remarkable improvement in both biochemical and histopathological parameters (P<0.05). In conclusion, the results of the biochemical analysis were in line with the pathological findings showing that silymarin, in combination with clorsulon, was superior to each compound alone in terms of hepatoprotective activity.

Keywords: Fasciola hepatica, Sheep, Silymarin, Hepatoprotective, Biochemical parameters, Clorsulon

Doğal Enfekte Koyunlarda Silymarinin Klorsulon ile Kombinasyonunun Fasciola hepatica’ya Karşı Hepatoprotektif Aktivitesi

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Fasciolosis, tüm dünyada hayvanlarda temel sağlık sorunlarına neden olan ve sosyoekonomik etkilere sahip paraziter bir enfeksiyondur. Bu çalışma, Fasciola hepatica ile doğa olarak enfekte olmuş koyunlarda silymarinin tek başına ve klorsulon ile kombinasyon halinde kullanımının hepatoprotetik aktivitesini değerlendirmeyi amaçlamıştır. Bu amaçla toplam 40 koyun (Sangsari ırkı) beş gruba ayrıldı: 1) tedavi görmeyen sağlıklı koyunlar (negatif kontrol), 2) tedavi görmemiş enfekte koyunlar (pozitif kontrol), 3) tedavi görmemiş enfekte koyunlar (1. günden 14. güne kadar oral yoldan 280 mg), 4) klorsulon ile tedavi edilen enfekte koyunlar (5, 10 ve 15. günlerde 7.5 mg/kg), ve 5) silymarin + klorsulon ile tedavi edilen enfekte koyunlar. Serum parametreleri analizi total bilirubin, total protein, albumin, alanin aminotransferaz (ALT), aspartat aminotransferaz (AST), alkaline fosfataz (ALP), ve gamma-glutamil transferaz (GGT) incelendi. Biochemik analiz, ast, alt, alp, bilirubin, protein ve albumin düzeylerinin enfekte hayvanlarda sağlıklı hayvanlardan az olduğunu gösterdi (P<0.05). Silymarin ve klorsulon kombinasyonu ile yapılan tedavi, hem biyokimyasal hem de histopatolojik parametrelerde kayda değer bir iyileşme gözeldon (P<0.05). Sonuç olarak, silymarinin hepatoprotetik aktivitesinin klorsulon ile kombinasyonu, diğer tedavi alternatiflerinden daha üstün olduğunu gösterdi.

Anahtar sözcükler: Fasciola hepatica, Koyun, Silymarin, Hepatoprotektif, Biyokimyasal parametreler, Klorsulon

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**INTRODUCTION**

Fasciolosis is a parasitic disease in domestic animals and humans mainly caused by a hepatic trematode parasite, *F. hepatica*. The parasite usually localized in the liver, gall-bladder, and bile ducts of the host. This infection has particular implications as it reduces meat and milk production, quantity and quality of wool, and fertility. One of the most effective flukicide compounds among anthelmintic drugs is clorsulon that belongs to sulphonamides. Based on fecal egg count or postmortem studies, clorsulon is mainly effective against mature flukes via subcutaneous injection or orally.

Although chemical compounds (clorsulon, triclabendazole, and albendazole) can facilitate parasite control, traces of hepatic injuries or pathological sequelae of the parasite remain in the liver of the host, making such drugs an inappropriate choice for medical application. The use of different compounds in combination is an excellent strategy to act against both parasite and its pathological sequelae. Due to its unique hepatoprotective, antioxidant, and anti-inflammatory activities, silymarin is currently at the core of many biological innovations in clinical medicine. It is a mixture of phenylpropanoid and flavonoid derived from the extract of *Silybum marianum* (Milk thistle). The use of this plant in liver disease and the gastrointestinal problem describes in ancient medical books. In folk medicine, in many countries, *S. marianum* is used for the treatment of gallbladder and liver disorders (jaundice, cirrhosis, and hepatitis) and protection against environmental and chemical liver poisoning. Silymarin is widely used as a nutritional supplement or therapeutic agent in liver disease without serious side-effects in both animals and humans.

The current study designed to evaluate the hepatoprotective activity of silymarin alone and in combination with clorsulon in sheep infected with *F. hepatica* by measuring some hepatic biochemical parameters (aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin, and total protein). Moreover, the hepatoprotective activity of the compounds was assessed using histopathological analysis.

**MATERIAL and METHODS**

**Drugs and Dosage**

Aqueous suspension of silymarin (Livergol; Goldaru, Iran) 280 mg was given from day 1 to 14 and clorsulon (Triveni Chemicals, India) 7.5 mg/kg/day administered orally on the days 5, 10, and 15.

**Experimental Design**

All experiments were directed in agreement with the Guide for the Care and Use of Laboratory Animals in Research, approved by the University.

Forty male and female Sangsari sheep, 3-5-year-old, weighing 30-55 kg, were assigned to five groups of eight sheep. Before starting the experiment, 100 sheep were inspected for the presence of *F. hepatica* based on the egg count of fecal samples. The fecal samples were collected directly from the rectum and preserved in a plastic bottle containing a 10% formalin solution. Thirty-two *F. hepatica*-infected sheep selected by the sedimentation technique allocated to four accidental groups. A healthy animal group was also used for better comparing the hepatoprotective effects of the compounds. Groups were designated as follows: group 1 (H), the healthy sheep without treatment (the negative control, n=8); group 2 (F), the infected sheep without treatment (the positive control, n=8); group 3 (F+S), the infected sheep with silymarin treatment (280 mg orally from the day 1 to 14, n=8); group 4 (F+CL) (the infected sheep with clorsulon treatment (7.5 mg/kg orally on the days 5, 10, and 15, n=8); and group 5 (F+S+CL), the infected sheep with clorsulon plus silymarin treatment (n = 8).

**Blood Samples and Biochemical Analysis**

Blood samples from the external jugular vein were collected on days 0, 7, 14, and 21 and kept at +4°C for further biochemical analysis. For serum separation, blood tubes centrifuged at 3000 rpm for 10 min. Due to the poor stability of enzymes and bilirubin in the serum, the samples analyzed within six h. Liver enzymatic activities of AST, GGT, ALP, and total bilirubin, and total protein measured according to standard procedures using a BT-1500 automatic analyzer.

**Histopathological Analysis**

Before the animals slaughtered, the transversal sections of all liver lobes were collected, fixed in buffered formaldehyde solution, embedded in paraffin, and sent to a histopathological laboratory. Five-micrometer sections were stained based on the hematoxylin-eosin (H & E) technique and examined under light microscopy. The levels of inflammation, bile duct hyperplasia, necrosis, and fibrosis were determined in 5 to 10 histological sections per animal, randomly chosen. Two blinded observers performed all evaluations. The score of lesions were distinguished as follows: Necrosis: 0, none; 1, focal necrosis less than 25%; 2, focal necrosis 25-50%; 3, zonal necrosis 50-75%; 4, massive necrosis. Inflammation: 0, none; 1, focal inflammation less than 25%; 2, focal inflammation between 25-50%; 3, zonal inflammation; 4, extensive inflammation. Fibrosis: 0, none; 1, perportal fibrosis; 2, portal expansion; 3, septal development; 4, global septal fibrosis. Hyperplasia of the bile duct: 0, none; 1, mild hyperplasia less than 25%; 2, moderate hyperplasia 25-50% lesions; 3, severe hyperplasia between 50-75% lesions; 4, extensive hyperplasia.
Statistical Analysis

Statistical comparisons and graphs performed with GraphPad Prism software using the one-way and two-way analysis of variance (ANOVA) tests. P-values of less than 0.05 were measured statistically significant.

RESULTS

Biochemical Parameters

In the present study, the mean serum levels of ALT, ALP, AST, and GGT were significantly higher in the infected sheep groups than in the healthy group before treatment. The mean levels of ALT, ALP, AST, and GGT in F. hepatica-infected sheep decreased following the administration of the combination treatment with silymarin and clorsulon after 21 days (Table 1). The hepatoprotective effect of silymarin was also remarkable on the level of these enzymes at different intervals, so that the mean levels of these enzymes decreased on the day 21 in the silymarin-treated group compared to the day 1 (P<0.05; Table 1).

As shown in Table 1, the total protein and albumin levels were significantly lower in the infected groups than in the healthy group on day 1 of the study. Lower levels of total protein and albumin are also other indicators of liver disorder or injury. As shown in Table 1, the primary levels of these proteins were significantly lower in the control group than in the healthy group. Comparison of before and after treatment (the day 1, 7, 14, 21) by silymarin with/without clorsulon significantly increased the total protein levels.

<table>
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<tr>
<th>Group</th>
<th>Time Interval (d)</th>
<th>Group 1 (H)</th>
<th>Group 2 (F)</th>
<th>Group 3 (F+S)</th>
<th>Group 4 (F+CL)</th>
<th>Group 5 (F+S+CL)</th>
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</table>

Data are expressed as mean ± standard error of the mean (SEM). Means with superscripts a, b, c, and d along a row differ significantly at P<0.05; Means with superscripts #, *, ^, and x along a column differ significantly at P<0.05. Group 1 (H), the healthy sheep without treatment; group 2 (F), the infected sheep without treatment; group 3 (F+S), the infected sheep with silymarin treatment (280 mg orally from the day 1 to 14); group 4 (F+CL) the infected sheep with clorsulon treatment (7.5 mg/kg orally on the days 5, 10, and 15); and group 5 (F+S+CL), the infected sheep with clorsulon plus silymarin treatment.
and albumin levels, which is a significant difference in the combination of these two drugs.

In the present study, the serum level of total bilirubin was remarkably higher in the infected groups than in the healthy group. The increased concentration of total bilirubin is another indicator of hepatic injury. While clorsulon showed a moderate effect on decreasing total bilirubin, treatment with a combination of silymarin and clorsulon resulted in a significant decrease in the serum level of total bilirubin at the end of the experiment (Table 1).

The total bilirubin level was also significantly lower in the group treated with silymarin alone than in the infected untreated group (P<0.01).

Effects of Silymarin, Clorsulon, and Their Combination on Histopathological Parameters

Liver histopathology disclosed more inflammatory cell infiltration, fibrosis, necrosis, and bile duct hyperplasia in F. hepatica-infected sheep on day one than in the healthy group (Fig. 1). As shown in Fig. 1A and B, the liver of group 1 (the healthy sheep with no treatment), had normal hepatocytes without any pathological lesions.

Fig. 2 shows the effects of F. hepatica parasites on the liver of the control group (without any treatment). The parasite caused massive necrosis, fibrosis, hepatitis, bile duct hyperplasia, and high rate of immune cell infiltration into

![Fig 1. Liver tissue samples of sheep in the group 1 (the healthy group) show no pathological changes. Arrowheads point to A, central vein (H&E, 40X) and B, healthy hepatocytes (H&E, 400X)](image)

![Fig 2. Liver tissue samples of sheep in the group 2 (no treatment) show pathological changes. Arrowheads point to immune cell infiltration, and arrow indicates fibrosis and bile duct hyperplasia (A, 400X & B, 100X)](image)

<table>
<thead>
<tr>
<th>Table 2. The pathological findings of sheep liver in all the groups</th>
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<tbody>
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<td><strong>Parameter</strong></td>
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<tr>
<td>Necrosis</td>
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<tr>
<td>Inflammation</td>
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<tr>
<td>Fibrosis</td>
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<tr>
<td>Bile duct hyperplasia</td>
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<tr>
<td>Total lesion</td>
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</tbody>
</table>

* P<0.05 compared with the healthy group; † P<0.0001 compared with the healthy group; ‡ P<0.0001 compared with the F. hepatica infected group.

F and P-value for within and between factors are as follows: Row factor, F (3, 140) = 1.018, P = 0.3867. Column factor, F (4, 140) = 381.2, P<0.0001. Data are expressed as mean ± SEM. Group 1 (H), the healthy sheep without treatment; group 2 (F), the infected sheep without treatment; group 3 (F+S), the infected sheep with silymarin treatment (280 mg orally from the day 1 to 14); group 4 (F+CL) the infected sheep with clorsulon treatment (7.5 mg/kg orally on the days 5, 10, and 15); and group 5 (F+S+CL), the infected sheep with clorsulon plus silymarin treatment. Lesion score: Necrosis: 0, none; 1, focal necrosis less than 25%; 2, focal necrosis between 25-50%; 3, zonal necrosis between 50-75%; 4, massive necrosis. Inflammation: 0, none; 1, focal inflammation less than 25%; 2, focal inflammation between 25-50%; 3, zonal inflammation; 4, extensive inflammation. Fibrosis: 0, none; 1, perportal fibrosis; 2, portal expansion; 3, septal development; 4, global septal fibrosis. Hyperplasia of the bile duct: 0, none; 1, mild hyperplasia less than 25%; 2, moderate hyperplasia between 25-50% lesions; 3, severe hyperplasia between 50-75%; 4, extensive hyperplasia
the liver of the infected sheep (Table 2, P<0.0001).

The therapeutic effects of silymarin and clorsulon on the liver of *F. hepatica*-infected sheep showed in Fig. 3 and Fig. 4, respectively. Although the bile duct hyperplasia reduced slightly in clorsulon-treated sheep (Table 2), there were no significant changes in histopathological parameters following treatment with silymarin (group 3) or clorsulon alone (group 4) compared with the control group. The high rates of necrosis, fibrosis, hepatitis, and immune cell infiltration were still significant in these groups even after 21 days of treatment (Table 2). The results indicated the hepatoprotective activity of silymarin when used in combination with clorsulon. In group 5 (clorsulon+silymarin treatment), the histopathological changes were considerable, as Fig. 5 shows mild fibrosis, mild hepatitis (Fig. 5A), mild bile duct hyperplasia, and less immune cell infiltration (Fig. 5B) after 21 days of combination treatment. Table 2 shows the histopathological findings of the liver in all groups. As can be seen, all the pathological parameters were significantly lower in sheep treated with the combination of silymarin and clorsulon than in other infected groups after 21 days (P<0.0001).

**DISCUSSION**

Many studies have been investigated to find a suitable drug to reduce the hepatotoxicity effects of antiparasitic drugs in parasitic diseases such as fasciolosis. *F. hepatica* migration to the liver causes damage to the hepatic cell wall and leads to hepatic tissue necrosis and Biliary obstruction [20]. These effects cause changes in hepatic biochemical serum parameters. It showed that some hepatic biochemical parameters such as AST, ALT, and GGT are the reliable indicators of fascioliasis in sheep and could be used to determine the effectiveness of anthelmintic therapy in infected animals [1,16,21,22]. It also indicated that partial recovery of the liver following some treatments is associated with lower activities of these enzymes in
F. hepatica-infected sheep (21). Although chemotherapy eliminates F. hepatica parasites significantly, less effective drugs may relieve the hepatic injuries and fibrosis due to parasites migration. Therefore, treatment targeting hepatic injuries caused by F. hepatica needs more investigations. Determination of plasma liver enzyme levels is a valuable method for assessing the efficacy of treatment in sheep infected with F. hepatica (1). Lower serum levels of ALT, glutamic-oxaloacetic transaminase (GOT), and GGT are considered normal in the blood of sheep, while the higher serum activities of ALT, ALP, AST, and GGT in the infected animals are mainly the consequence of hepatic lesion and cholestasis (11).

In the current study, we evaluated the hepatoprotective activity of silymarin alone and in combination with clorsulon against F. hepatica in naturally infected sheep at different intervals for the first time. The observed elevation of serum transaminases (AST and ALT) and GGT in the infected animals before treatment could be related to the degenerative changes and hepatocellular necrosis produced by the migration of flukes through the liver parenchyma (23). It has also been reported that the increased level of AST and GGT could be an indicator of chronic fascioliasis and liver cell damage (21,24,25), while using a hepatoprotective agent like silymarin could reduce the serum levels of AST and ALT (26). Besides, the GGT level reduction after treatment could be the consequence of partial recovery of the liver (21).

In the present study, the serum levels of GGT, AST, and ALT decreased significantly following treatment with a combination of silymarin and clorsulon at the end of the treatment course (day 21). The reduction of these enzymes between the days 14 and 21 (Table 1) could be attributed to eliminating the parasite and preventing liver damage through maintaining the integrity of the plasma membrane, thereby suppressing the leakage of enzymes. In the present study, the histopathological findings also confirmed the biochemical results in which the liver damage improved after combination treatment (Fig. 5). Another liver enzyme, ALP, also had a higher level in the infected groups than in healthy animals at the beginning of the study. The role of ALP is to transport metabolites across cell membranes. One of the most common reasons for hepatic disease is the pathological elevation of ALP levels (23). Although the combination treatment with silymarin and clorsulon resulted in a significant reduction of this enzyme (Table 1), it did not reach the baseline level at the end of the study, confirming that its level usually decreases slowly after resolution and thus, it needs more treatment time (27). Another reason for the increase in this enzyme in group 4 is due to the hepatotoxicity effect of sulphonamide drugs (28) and the sulphonamide structure of clorsulon (3), which was reduced by the administration of silymarin and removal of the parasite in group 5.

Moreover, the decreases in serum total protein and albumin in animals infected with F. hepatica are observed previously (29). Hypoalbuminemia causes by liver damage in acute and chronic fascioliasis and can cause biliary obstruction, cholangitis, anemia, destruction of liver tissue, and fibrosis (30). Clorsulon and its combination with silymarin were effective to some extent, even at the beginning of the treatment. The increased levels of total protein and albumin at the end of treatment (day 21) by silymarin with/without clorsulon could be related to the hepatoprotective and anthelmintic activities of silymarin and clorsulon, respectively. The considerable increase in the total protein and albumin levels occurred at the end of the treatment by the combination therapy suggesting improved liver damage and less bile duct hyperplasia and fibrosis (Fig. 5).

Furthermore, the biochemical analysis showed that total bilirubin was significantly higher (P<0.05) in the infected sheep than in the healthy group. This elevation may be attributed to the increased production of bilirubin because of hemolytic toxins produced by F. hepatica in the liver (23). Since the combination treatment could affect the parasites’ death and liver parenchyma at the same time, the lower levels of total bilirubin detected on day 21 (P<0.05). The improvement initiated on day seven and reached the average level similar to that of the healthy group on day 21 (Table 1). All the results of the biochemical analysis were consistent with pathological findings (Table 2).

The pathological results showed no significant changes following treatment with silymarin or clorsulon alone (Fig. 1, 2, 3, 4), while the decrease in immune cells infiltration, bile duct hyperplasia, and fibrosis were considerable following the combination therapy (Fig. 5). Silymarin and clorsulon alone were effective to some extent and reduced some of the biochemical parameters tested, but their combination was superior to each agent alone in terms of the histopathology of the damaged liver. Administration of silymarin, in addition to clorsulon, showed complete elimination of parasites. No visible eggs (data not shown) and healing of hepatic lesion injuries demonstrated that the use of silymarin along with clorsulon did not affect or interfere with the anthelmintic activity of clorsulon. The histopathological results of the present study confirmed biochemical findings of the current research, and measuring the studied parameters was useful to evaluate the effectiveness of hepatoprotective and anthelmintic agents.

The study, to the best of authors’ knowledge, is the first report showing the hepatoprotective and anthelmintic efficacy of a combination of clorsulon and silymarin against F. hepatica in naturally infected sheep (Sangsari breed). Silymarin showed significant hepatoprotective activity when used in combination with clorsulon rather than alone administration. The study results also confirmed that serum levels of AST, ALT, ALP, GGT, albumin, protein, and bilirubin (total) are reliable indicators of sheep fasciolosis.
and could be used to test the hepatoprotective effect of anthelmintic and hepatoprotective agents.

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**CONFLICT OF INTEREST**

All authors declare that there is no competing interest in the process of performing this manuscript.

**ETHICS APPROVAL**

The ethical committee approved the current work of the Science and Research Branch, Islamic Azad University. The number of approval: IR. IAU. SRB. REC. 1397. 12.

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