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Targeting *Sarcocystis fusiformis* cysteine protease as a possible approach to limit sarcocystosis infection

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In Loving Memory of Late Professor Doctor ""Mohamed Refaat Hussein Mahran""

Abstract
Worldwide, sarcocystosis infection in Egyptian buffaloes is common and lacks a particular therapy. A parasite cysteine protease (CP) is involved in several biological and pathogenetic activities, including the parasites' adhesion to host cells, tissue invasion, cytotoxicity, intake of nutrients, and immune evasion. We have previously reported the sequence of purified cysteine protease from *S. fusiforms* cysts. Herein, the purified *S. fusiformis* cysteine protease (SFCP) is used as an immunogenic for antisera production. Immunization with the SFCP-antigen promoted the immune response in rabbits and rats, exhibiting elevated specific antibodies. Thus, the therapeutic potential of the prepared antisera and SFCP vaccination was evaluated by employing *S. fusiformis*-infected rats. The result demonstrated that SFCP and the prepared antisera alleviate the oocyst shedding and intestinal damage in *S. fusiformis* infected animals. The serum levels of Th1 cytokines, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ and interleukin (IL)-12 as well as the Th2 cytokines (IL-4 and IL-6) were significantly increased in infected rats, with a maximum increase by ~1.75- 2.45 at week 2 post-infection compared to the control. Additionally, mRNA levels of nucleotide-binding oligomerization domain (NOD)1, NOD2, TNF-α, TLR4,TLR9, IFN-γ, IL-10,IL-6, transforming growth factor-beta (TGF-β) and IL-β were all up-regulated in the infected group compared to the control. However, the SFCP-vaccination and anti-SFCP sera modulate these elevations in serum and spleen. These findings demonstrated that the SFCP-vaccination and anti-SFCP sera could be novel therapeutic candidates for controlling *S. fusiformis* infection and its harmful effects.
Keywords: *Sarcocystis fusiformis*; Cysteine protease; Vaccination, Antisera; Cytokines.

1. Introduction

Sarcocystis spp. belongs to a group of parasites that are characterized by their complex life cycles (predator-prey). These parasites are part of the phylum Apicomplexa and they have a significant economic impact on the meat production of livestock [1]. They are a very diverse group of obligatory cyst-forming coccidian. The life cycle of Sarcocystis species requires two hosts; the definitive hosts show intestinal sarcocystosis, while the intermediate hosts show muscular sarcocystosis. The final hosts become infected after consuming sarcocysts. Bradyzoites are discharged from sarcocysts in the stomach and intestines, where they penetrate the intestinal epithelium and differentiate into microgametes (males) and macrogametes (females), which then unite to generate zygotes. These zygotes undergo encystation and sporulation, and oocysts (two sporocysts, each containing four sporozoites) are expelled in the stool. Oocysts or sporocysts can infect intermediate hosts via contaminated food. Motile sporozoites move through the intestinal epithelium and vascular endothelium, where they undergo asexual generations (schizogony or merogony), producing a large number of merozoites that enter the bloodstream and lodge in muscles to create sarcocysts[2-4].

Over a hundred types of Sarcocystis can infect wild and domesticated animals. Sarcocystis are harmful to animals including sheep and cattle, leading to financial damages [5]. Sarcocystis is a very common infection in cattle all over the world. It was first discovered in cattle muscle almost two hundred years ago [6]. *Sarcocystis fusiformis* species was recognized based on 98-99% identity with already recorded sequences of *S. fusiformis* from water buffaloes in Egypt [7, 8]. Research into mechanisms of pathogenesis in parasites and potential therapies

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often requires extensive in vivo experiments [6]. Since there is no known cure for Sarcocystis infection, prevention and exploring a specific therapy are the most important factors in controlling the disease [9].Understanding the mechanisms underlying host-parasitic interactions affecting various cells, signaling cytokines and molecules, hormones or metabolites is essential for developing effective anti-parasitic strategies and vaccines [10].

Proteases break down amide bonds in macromolecules and oligomeric peptides. Proteases are essential for numerous biological, physiological, and pathophysiological activities in most single-celled and multi-cellular parasites [11]. According to their hydrolysis mechanism of peptide, proteases are categorized into six groups: metalloproteases, aspartic, glutamic, cysteine, serine, and threonine proteases. Cysteine protease enzymes (CPs) are crucial in developing diseases caused by protozoan parasites. They are involved in the invasion of cells and tissues, the breakdown of proteins from either the host or the parasite, the process of self-digestion, and the *evasion*, which makes them promising targets for chemotherapy and vaccines [12, 13]. CPs play a crucial role during the oocyst phase of the parasite [14]. In *C. parvum* infected mice CP inhibitor K11777 can decreases or eradicates oocyst shedding [15]. CP inhibitors suppressed the hepatocytes invasion by sporozoites of *P. falciparum*[16], while interrupting a putative CP gene of *P. berghei* impeded sporozoite discharge from oocysts [17]. Moreover, Immunization of the cattle with *F. hepatica* CPs exhibited a notable level of protection against infection of the *F. hepatica* , as evidenced by a reduced rate of infection, a lower quantity of eggs present in the feces, and a decrease in the viability of the eggs when contrasted with unimmunized control groups [18, 19]. Thus, this study aimed to use the previously purified *S. fusiformis* cysteine protease (SFCP), the biochemical characterization of this protease suggested that it might be related to cysteine protease [8], for generating anti-SFCP sera and SFCP-vaccination to treat the sarcocystosis infection. Moreover, the cytokines profile as an immune activation marker in response to the prepared anti-SFCP-sera and SFCP vaccination and *S. fusiformis* infection was determined in Wistar rats.

2. Materials and methods

The experiments were carried out according to the applicable guidelines. The Medical Research Ethics Committee of the National Research Centre in Cairo, Egypt, gave their approval to this study (Subject N0). 1-3-1-5). The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org.

2.1. Production of antisera

2.1.1. Rabbit-antisera Preparation

Monovalent antiserum against the previously purified SFCP enzyme [8] was prepared in rabbits. Two rabbits (each 2.0±0.2 kg) were housed in the National Research Centre (Giza, Egypt). The animals were immunized by three separate subcutaneous injections of 50 µg of SFCP enzyme sample dissolved in 0.9% saline at three-week intervals. The first dose was emulsified in the complete Freund's adjuvant, whereas the subsequent doses were emulsified in the incomplete Freund's adjuvant. The animals were bled weekly; antisera were separated, pooled, and stored at -20 °C. The control rabbit was S.C. injected with 0.9% saline only three-week intervals.

2.1.2. Rat-antisera Preparation

Five rats (each 150.0±7.2 g) were injected with three S.C. injections of 10 µg of SFCP enzyme dissolved in 0.9% saline at three-week intervals. The first dose was emulsified in the complete Freund's adjuvant, whereas the subsequent doses were emulsified in the incomplete Freund's adjuvant. The Rats were bled every week; antisera were separated, pooled, and stored at −20 °C. The control rat group was S.C. injected with 0.9% saline only three-week intervals.

2.2. ELISA titers

The reactivity of the prepared anti-SFCP Abs was examined in 96-well microtiter plates using the ELISA test described by Ricoux et al. [20]. Briefly, each well was coated overnight with 100 µl of the purified enzyme (0.5 µg protein/well) dissolved in coating buffer (50 mM sodium carbonate buffer, pH 9.6). Non-specific sites were blocked for 1 h by the addition of 100 µl of the blocking solution (2% gelatin dissolved in coating buffer) at 37°C. Serial dilutions from the prepared anti-enzyme Abs in washing buffer (0.01 M PBS, pH 7.4 containing 0.05% Tween-20) were dispensed into duplicate wells and incubated for 1h at 37°C. After washing, anti-rabbit IgG-peroxidase conjugate diluted in PBS (1:4000) was added (100 µl/well) and incubated for 1h at 37°C. Finally, 100 µl of substrate buffer (0.33 mg OPD/ml dissolved in citrate buffer, pH 5.2, containing 0.04% H_2O_2) was added to each well, then the reaction was stopped after 20 min by the addition of 20 µl of a 1:20 dilution of sulfuric acid and the absorbance was determined at 490 nm with ELISA reader **(**BIO-TEK, South Korea**)**. All the values were recorded in duplicate. A standard curve between log conjugate dilution and log OD was plotted. The dilution gives 0.5 OD. at 490 nm was taken as the ELISA titer.

2.3. Sample preparation

Sarcocystis cyst collection: Samples from the esophageal muscles of buffalos were gathered during

the slaughtering process at Bassatin abattoir, Egypt, identified by a specialized veterinary doctor. These samples were then isolated. The cysts were carefully removed using fine forceps, and cleaned with saline solution. Two hundred cysts were partially homogenized in saline and 0.2 ml of cyst suspension was given orally to rat via capillary catheter.

2.4. Experimental design

Forty-eight male (Wistar) rats, weighing between 100 and 120 grams, were segregated into eight groups (G) and adapted to live for one week in standard enclosures and with a rodent diet. Rats were distributed into eight groups as follows: Four controls, Negative control/G1; vaccine/G3; rat antisera/G5 and rabbit antisera/G7 and experimental, infected/G2; infected-vaccinated/G4; infected rat antisera/G6 and infected rabbit antisera/G8 (Fig. 1). Three purified SFCP doses immunized the two groups (G3 and G4) before the cyst infection. The animals of groups G2, G4, G6, and G8 were infected with *S. fusiformis* cysts. G5 and G6 received two doses of the prepared rat-SFCP antisera. G7 and G8 were injected with 2 doses of the prepared rabbit-SFCP antisera. The antisera doses were injected i.p. at 2 and 8 dpi (days post-infection). One week from infection feces were gathered daily and checked for Sarcocystis sporocysts as outlined. The animals were sacrificed on day 30 after infection.

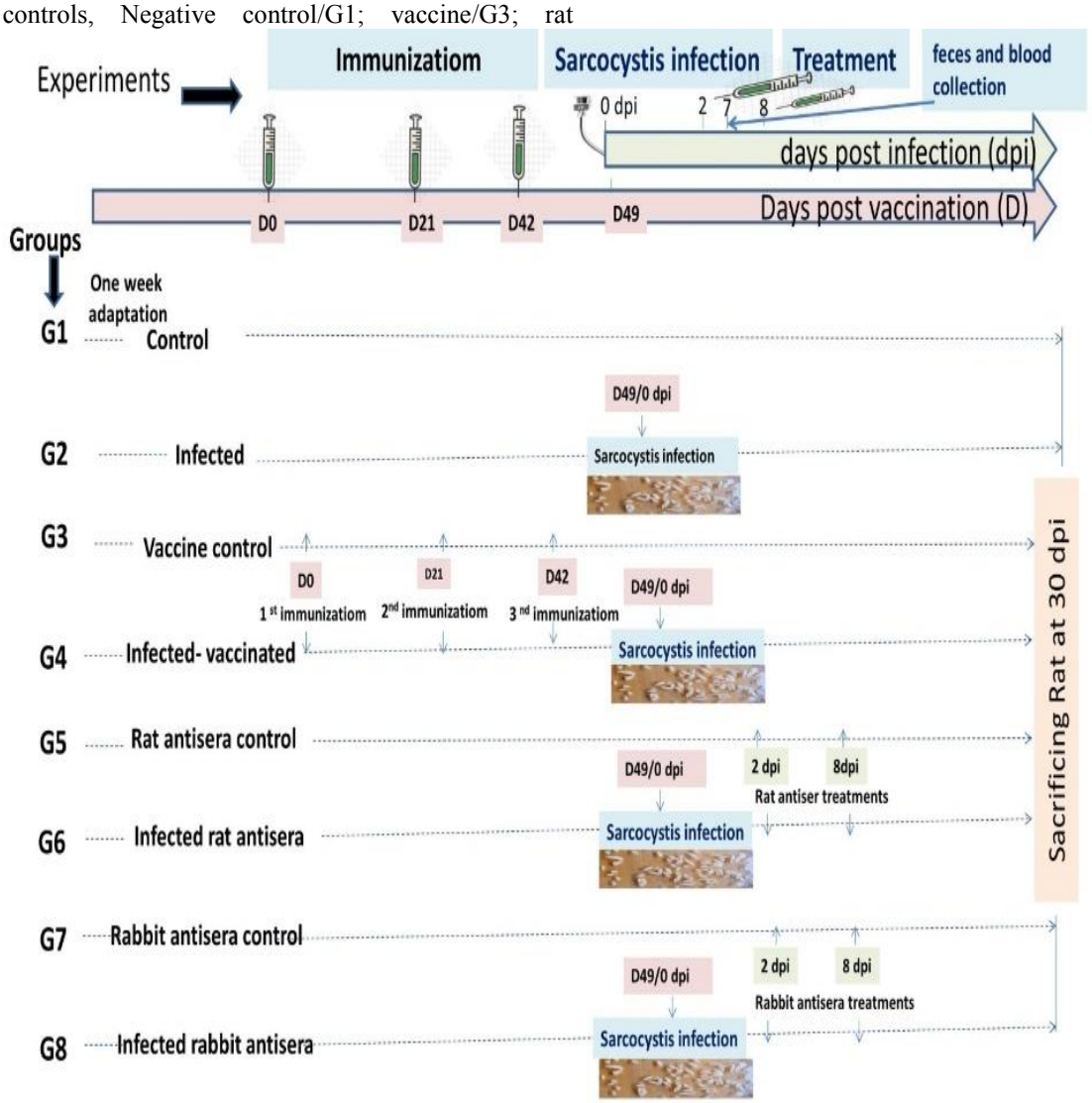


Fig. 1. A Schematic representation for experimental design. 48 male rats (6-8 weeks old) were separated into 8 groups (G1-G8); 6 rats/group. G1 served as control (without treatment and infection). G2 was the infected group without any treatment. The vaccination for G3-vaccine control and G4 infected-vaccinated were started after one-week acclimatization at days post-vaccination (D0) by purified *S. fusiformis* cysteine protease (SFCP) enzyme and repeated at D21 and D42. One week after vaccination all infected rats (infected/G2- infected vaccinated/G4; infected rat antisera /G6 and infected rabbit antisera/G8) were administered orally by Sarcocystis cysts at 0 day (day post-infection). Rat antisera and rabbit antisera treatments for rat antisera control/G5, G6, and rabbit antisera control/G7 and G8 were injected at 2- and 8-dayspost-infection(dpi). The collection of blood and feces was carried out during the experiment. Rats were sacrificed at 30 dpi.

2.5. Fecal flotation

Sheather's sucrose flotation method is used for the concentration of sarcocystis oocysts. The concentrated samples were examined for the presence of oocysts on slides using the 40X objective lens of a bright-field microscope and the presence of oocysts in the smear was confirmed under the oil immersion objective lens. The positive samples were then stored at 4°C for further molecular analysis [21]. The standard unit of measurement is the micron (μm = 0.001 mm) and about 10-15 cysts with the range in parenthesis were used for calculating the mean [22]. Histological preparations follow the previously outlined process [23].

2.6. Histological examination

In summary, colon tissues were cut into sections ranging from 3 to 4 millimeters thick, then preserved in a 4% Paraformaldehyde solution (PFA) in PBS, followed by a series of ethanol concentrations for dehydrating, a process of clearing with xylene, and finally, they were fixed in paraffin. These paraffin blocks were then cut into sections using a microtome, with each section being about 4 to 6 micrometers thick. The sections were then stained with Hematoxylin and Eosin (H&E) stain to examine the overall structure of the tissue. The stained sections were observed under a Leica microscope (CH9435 Hee56rbrugg) (Leica Microsystems, Switzerland) **[7]**.

2.7. Immunohistochemistry (IHC) test

2.7.1. Protocol of immunohistochemistry Staining: The unconjugated Rabbit Anti-active Caspase 3 Monoclonal Antibody (Abcam Cat# ab184787, Dilution: 1:1000) is currently being tested. After incubating sections from each group with the previously mentioned antibodies, the ABC-related chemicals (Vectastain ABC-HRP kit, Vector laboratories) were inserted. To distinguish an antigen-antibody complex, marker expression was identified with peroxidase and stained with diaminobenzidine (DAB, made by Sigma). Non-immune serum was used as a negative control instead of primary or secondary antibodies. Immuno-stained areas were checked and captured using a Leica magnifying instrument under various amplification abilities (CH9435 Hee56rbrugg) (Leica Microsystems, Switzerland) [10].

2.7.2. Numerical evaluation of immune-histochemical analysis (percentage of positive area): In each serial section of the studied groups, six high-power fields (x 400) with positive brown immunostaining were chosen for evaluation. Using a Leica QWin 500 image analyzer computer system (England), the area percentage of Caspase 3-stained sections was determined. This image analyzer consists of a Leica microscope, a colored video camera, a colored monitor, and a Leica IBM computer hard drive that is connected to the microscope and managed by Leica QWin 500 software. Each antibody's records were statistically described in terms of the area percent mean and standard deviation (mean ± SD).

2.8. Cytokines analysis

The cytokine levels of (IFN-γ, TNF-α, IL-4, IL-5, and IL-12) during immunization/vaccination weeks were examined to investigate the efficiency of the cellular immune response induced by purified CPSF. The cytokines levels were determined in serum using the ELISA kit (rat ELISA Sunlong, China), following the manufacturer's recommendations.

2.8.1. qPCR for cytokines

Twenty mg of frozen tissues of the spleen were used for RNA extraction using the protocol of Thermo Scientific GeneJET RNA Purification Kit. Reverse transcription was done by Thermo Scientific RevertAid First Strand cDNA Synthesis kit. qPCR was conducted using the QuantStudio 1 Real-Time PCR system and The HERA SYBR® Green qPCR Kit (QIAGEN). All reaction conditions and protocols were performed as recommended by the manufacturer. The following conditions were used for amplification: one cycle for 2 min at 95◦C, followed by 40 cycles of 10s a 95◦C and 30 s at 60◦C. Lastly, PCR was performed using the primers indicated in Table 1.

Table 1: Primer sequences used for qPCR analysis.

Primer	Forward (5′-3′)	Reverse (5′-3′)	Accession n°/ Reference
GAPDH	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA	NM_017008/[24]
IL-1β	TCCTCTGTGACTCGTGGGAT	TCAGACAGCACGAGGCATTT	NM_031512/[24]
IL-6	ACAAGTGGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC	NM_012589/[24]
TGF-β	GAAGCCATCCGTGGCCAGAT	TGACGTCAAAAGACAGCCACT	NM_021578.2/[24]
TLR4	CTACCTCGAGTGGGAGGACA	ATGGGTTTTAGGCGCAGAGTT	NM_019178/
TLR9	ATGGCCTGGTAGACTGCAACT	TTGGCGATCAAGGAAAGGCT	NM_198131/[24]
TNF-α	CATCTTCTCAAAACTCGAGTGACAA	TGGGAGTAGATAAGGTACAGCCC	NM_012675/[24]
NOD1	CTCAAAGGAGGACCTGCTGCTGGA	GAAGACAGTCTCGCCATGCTCGTTGA	[25]
NOD2	GGCAGCACAGGTGGACTCTGAGGATA	GCAGCAGCCTTAGCAGCAGTGAGTT	[25]
INF-γ	GTCATCGAATCGCACCTGA	GTGCTGGATCTGTGGGTTG	[26]
IL-10	GCTCTTACTGGCTGGAGTGAG	GCTCTTACTGGCTGGAGTGAG	[26]

Statistical analysis

The statistical analysis was carried out with GraphPad Prism 5 and Origin software. All measurements were reported as averages ± standard deviation (SD) or standard error (SE). Several sets of data were analyzed with one-way ANOVA with Tukey's test for several comparisons. For statistical analysis, a *P* value less than 0.05 was deemed to be statistically significant.All experimental procedures were carried out in compliance with relevant guidelines.

3. Results and discussion

Parasites engage with epithelial cells, leading to enhanced paracellular permeability and the death of enterocyte cells, which are the first signs of both normal and immune system problems. Among of molecules secreted by parasites that cause harmfulness, enzymes breaking proteins of which peptide hydrolases, particularly cysteine proteases (CPs), are abundant and have versatile lytic activities. Upon secretion, CPs can affect host tissues and immune responses beyond the site of parasite colonization, thereby increasing the pathogens' virulence [27]. The vaccine based on a cysteinyl protease is useful for controlling various infections including bacteria [28], virus parasites [29] and parasites [27]. Therefore, herein, we prepared antisera targeting SFCP in rats and rabbits and SFCP–vaccinated rats, and the efficiency of prepared antisera and SFCP-vaccine was evaluated in vivo using rats.

3.1. Preparation of anti-SFCP-Sera

The produced rabbit-SFCP antisera showed maximized ELIZA titer (1:60000) in the 7th week after the third SFCP injection (Fig. 2A).The produced rat-SFCP antisera exhibited maximized ELIZA titer (1:20000) in the 7th and 8th weeks after the third injection (Fig. 2B).The results indicate that the purified CPSF enzyme has great immunogenicity in both rat and rabbit. This strong immunogenicity of rat and rabbit antisera toward the purified SFCP led us to evaluate SFCP protection properties as a vaccine and antisera for Sarcocystis infection healing. The rabbit immunization with *S. mansoni* CP gave maximum antibody titer (1/250 dilution) after the second booster dose [30].

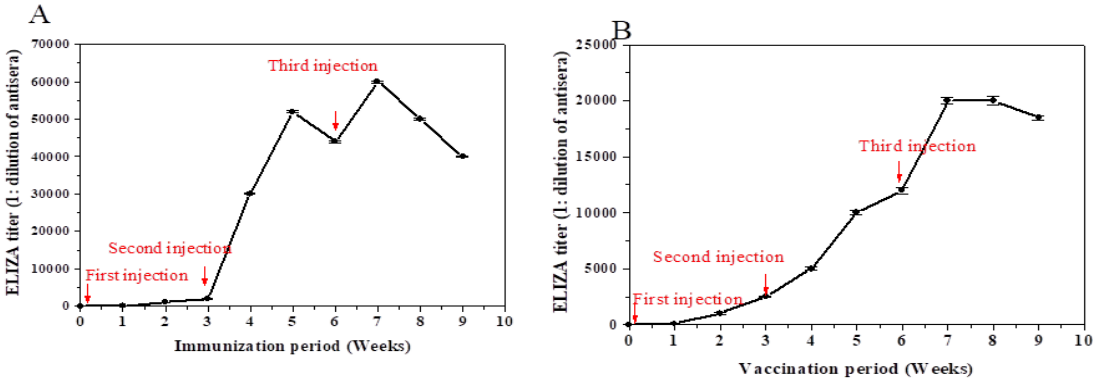


Fig. 2. Serum antibody responses/titers in rabbit (A) and rat (B) immunized groups with the purified CPSF enzyme. Sera of the immunized animals were collected weekly (0-9 weeks) and then analyzed using ELIZA assay period using the purified CPSF as a coating antigen.

3.2. Morphological feature and shedding of oocysts *in feces*

The therapeutic action of SFCP and its produced antisera against *S. fusiformis* infection were investigated after cyst administration. Rats shed oocysts after infection with *S. fusiformis* verifying that rats are considered a final host for *S. fusiformis*. The Morphological study of oocysts released in rats revealed that Sarcocystis oocysts are 15-20 µm in size with two sporocysts that each contains four sporozoites and a refractile residual body. They sporulated in the intestinal epithelium. Oocysts are delicate; therefore, excrement may contain both sporulated oocysts and single sporocysts (Fig. 3. A and B). Fayer et al (2004) have reported that oocysts of *S. suihominis* span from 12.3 to 14.6 µmin length and 18.5 to 20.0 µm in width. Inside these sporocysts are four sporozoites and a granular remaining body [3].

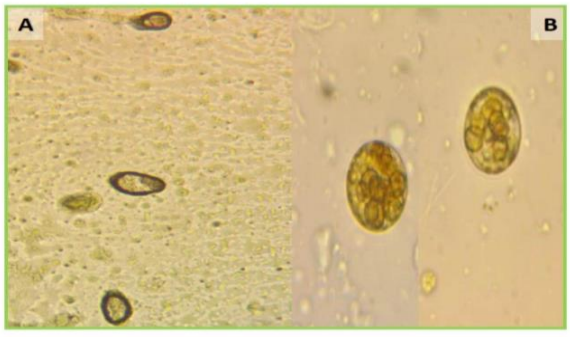


Fig.3. Fecal examination of rat infected with Sarcocystis fusiform cysts; unstained rat fecal smear showing Sarcocystis pp. oocysts X10 (A) and X40 (B).

The effectiveness of preventing oocyst release was measured by counting the number of oocysts excreted over 30 days post-infection (dpi) (Fig. 4). The excreta of non-infected groups didn't contain oocysts in all experimental periods. Further, the oocysts were not seen in infected and treated early. Oocysts were first observed in excreta of all infected rats at 10 dpi. The Sarcocystis oocysts shedding in all experimentally infected rats appeared on the 10th day post-infection and increased gradually to reach maximum (high peak) on the 18th day then begin decreased until about one-month post-infection. On this day (18^{th}) the percentage of reduction in the number of oocysts shedding was 71.7, 48, and 31.2 for infected-vaccinated, infected rabbit antisera, and infected rat antisera groups, respectively. In addition, in comparison with non-treated infected rats, the infected-vaccinated rats showed more improvement in the decline of oocysts shedding with a significant decrease from day 20 followed by infected rabbit-antisera and infected rat-antisera groups. Moreover, the shed oocysts numbers became zero on days 25, 29, and 29 for the infected-vaccinated group, infected-rabbit-antisera, and infected rat-antisera groups, respectively.

Similarly, a study that was carried out in dogs infected with *S. Cruzi* cysts began excretion of sporocysts, or occasionally oocysts, in the feces from days 10-12 after infection and lasted between 20-35 days [31]. However, in rats infected with *S. fusiformis* sporocysts were found at seven dpi and remained shed thirty days after infection [32].Many reports highlighted that blocking the action or function of cysteine proteases has a direct impact on the reduction of parasite infections. Cysteine Schistosoma gut-derived-cysteine proteases-based vaccine offers a strong defense against schistosome parasite infection. The vaccination protocol utilizing cysteine protease calpain (Sm-p80 subunit) provides 50-60% defense to *S. mansoni* challenge in mice [33].

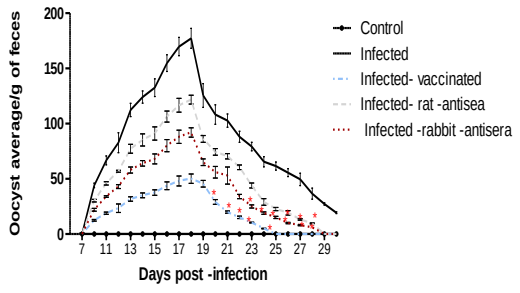


Fig. 4. The reduction of Oocysts shedding of Sarcocystis over time by SFCP. Values are presented as mean ± SD, n = 6 in all conditions. Asterisks (*) indicate a significant level at $p \leq 0.05$.

Additionally, homologous Sm-p80 specific serum provided 31.14% and 58.71% protection, respectively, based on the number of parasites and eggs per gram of tissues[34]. The vaccination of the cattle by *Fasciola hepatica* cathepsin L proteases showed protection against *Fasciola hepatica* and reduced number of eggs and viability of fluke eggs [35]. Reduced fecal egg counts, and stunting of adult worms, were results of a recombinant cysteine protease vaccination of dogs [18]. There was a notable 60% decrease in the number of fecal eggs in calves vaccinated with *O. ostertagi*cysteine proteinases [36] Inhibition of cysteine proteases showed a negative effect on oocyte shedding for many protozoa [15].Our findings and the above discussion, confirm that the SFCP vaccination and anti-SFCP sera could reduce the number of oocysts shedding for modulation of Sarcocystis infection.

3.3. The effect of Sarcocystis parasite and SFCP-treatments on large intestine morphology

3.3.1. Histopathological analysis of colon

Sections of the colon in the non-infected groups showed normal architecture (Fig. 5.a, c, e, and g). Meanwhile, the infected non-treated showed intense pathological changes including complete loss of the mucosal epithelium, marked by necrotic and degenerative cells in its wall, the shedding of epithelial cells, apoptosis in the intestinal gland cells, and a notable number of inflammatory cells in the lamina propria (Fig. 5b). Histopathological examination of Sarcocystis infected-vaccinated rats and infected-antisera rats treated displayed different levels of progress. Infected-vaccinated showed a remarkable improvement in the histopathological alterations illustrated by the fact that the intestinal mucosa was more regular and the largest part of Lamina propria seemed intact (Fig. 5d). The rat-antisera improved some lining epithelium of intestinal mucosa and intestinal glands but others still have an apoptotic appearance. Moreover, there was still some evidence of inflammatory cell infiltration in the lamina propria (Fig. 5f). The moderate improvement along the intestinal mucosa was detected in the colon of infected animals treated with rabbit antisera while some apoptotic lining epithelial cells can be seen. The vast majority of intestinal glands were undamaged (Fig. 5g). It can be concluded that the infected-vaccinated group displayed remarkable progress in the histopathological characteristics of the infected colon by *S. fusiformis* followed by the infected-rabbit antisera and infected-rat antisera groups.

3.4. SFCP treatments decrease the induced S. Fusiformis infection to colon apoptosis

The SFCP reduction effects of Sarcocystis-induced apoptosis detecting in the histological analysis led us to evaluate the apoptotic appearance in the colon sections by immunostaining apoptosis-related protein caspase-3. A very limited expression of caspase-3-immunopositive was observed in the colon section of the control groups (Fig. 6 a, c, e), whereas the most abundant accumulation of -caspase-3 immunoreactivity was in the colon of the infected group (Fig. 6b). The SFCP-vaccinated rat group showed little caspase-3 expression (Fig. 6d), whereas the infected anti-rabbit-treated group displayed moderate reactivity to caspase-3 antibody (Fig. 6f). To evaluate the apoptosis of epithelial cell, cells that showed positive staining for caspase-3 in the colon sections were tallied and the mean area percentage of labeled cells was calculated (Fig. 6B). The anti-caspase-3 immunostaining in the infected group was significantly increased (47.47±3.2%) when compared to the control rats ($p < 0.001$). However, the infected-vaccinated and infected-rabbit-antisera groups revealed a significant decrease in the mean area percentage (17.86 ±2.15 and 28.078 ± 3.16; respectively) versus the infected group ($p< 0.001$) but had not returned to the normal level when compared to the control group. This result indicates that SFCP-vaccination and the prepared SFCP-antisera significantly reduced the rate of cell death in the infected rats.

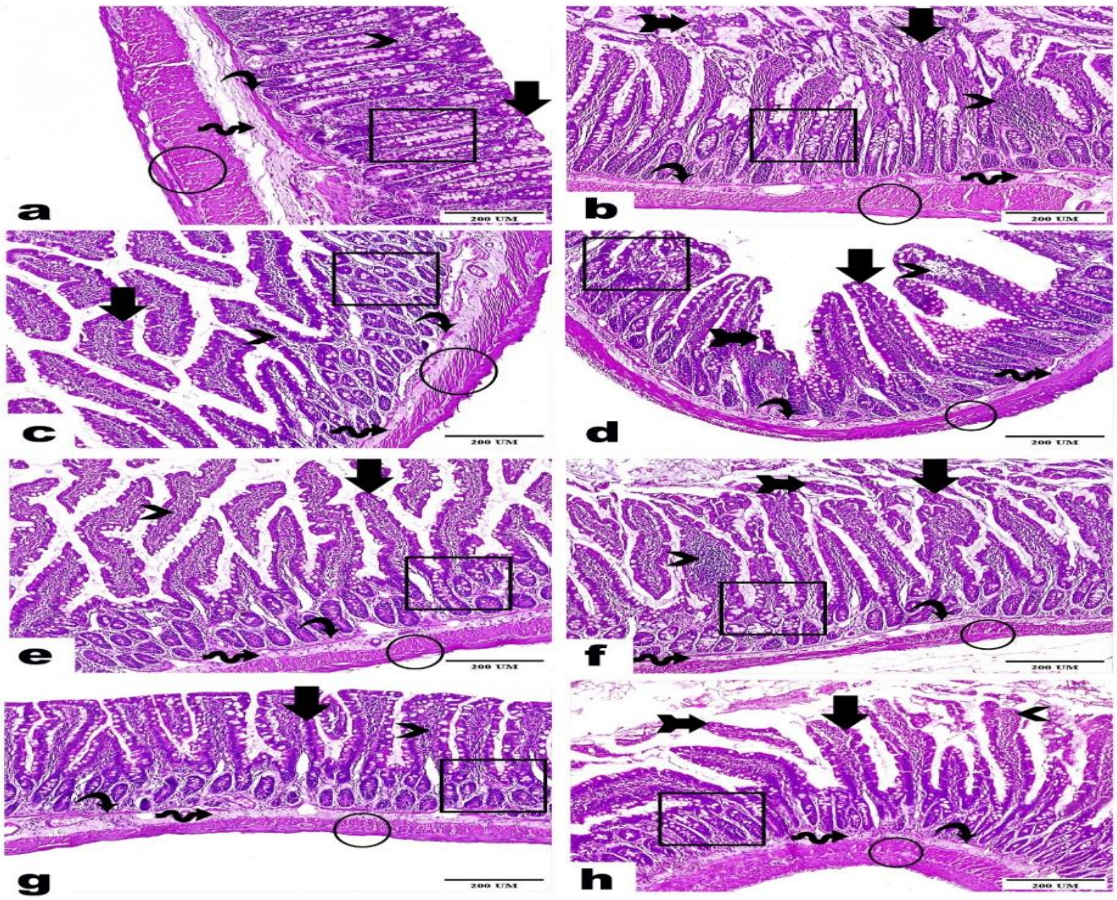


Fig. 5. Micrographs showed the differences in tissue structure among the groups being studied in the colon sections. Colon sections of a) negative, c)vaccine control, e) rat- antisera g) rabbit- antisera control animals showing a typical normal histological architecture of the colon, b) Colon of infected group emphasizing colitis with severe degenerative alterations, which are shown by; total disappearance of the mucosal epithelium with necrotic and degenerative cells in its lining (arrow), epithelial desquamation (arrow with tail), and cells lining intestinal glands that appear to be apoptotic (rectangle). Furthermore, there is a significant presence of inflammatory cells in the lamina propria (arrowhead). d)The colon section of the infected-vaccinated group revealed clear improvements along the intestinal mucosa, as evidenced by the fact that some of the mucosa had an intact structure (arrow), while a small number still had epithelial desquamation (arrow with a tail) and a degenerated intestinal gland (rectangle). The majority of the lamina propria appeared to be intact, except a few that appeared to have high vacuolations (arrowhead). f) Colon of infected-rat-antisera animal displaying limited progress in the intestinal mucosa, which was organized in some areas of the lining epithelium, and others, it was observed with cells undergoing apoptosis (arrow), and there was also evidence of cell shedding (arrow with tail) and a moderate number of inflammatory cells present in the lamina propria (arrowhead). The majority of intestinal glands appeared normal, while a few were observed with cells undergoing apoptosis (rectangle). h) Colon of infected rabbit antisera group exhibiting moderate improvements in the

intestinal mucosa that were organized in the majority of the lining epithelium, while some were noted with apoptotic lining epithelium (arrow), and there was also evidence of epithelial desquamation (arrow with tail). A limited number of inflammatory cells were observed along the lamina propria (arrowhead). Few intestinal glands had apoptotic lining epithelium (rectangle) spots, while the majority of intestinal glands were intact. The lamina muscularis mucosa (curvy arrow), submucosal connective tissue (wave arrow), and tunica muscularis (circle) are visible and intact in all groups. goblet cells (arrow), intestinal glands (rectangle), the lamina propria (arrowhead), the lamina muscularis mucosa (curvy arrow), submucosal connective tissue (wave arrow), and the tunica muscularis (circle). Magnification Power= x100; Scale bar = 200 µm.

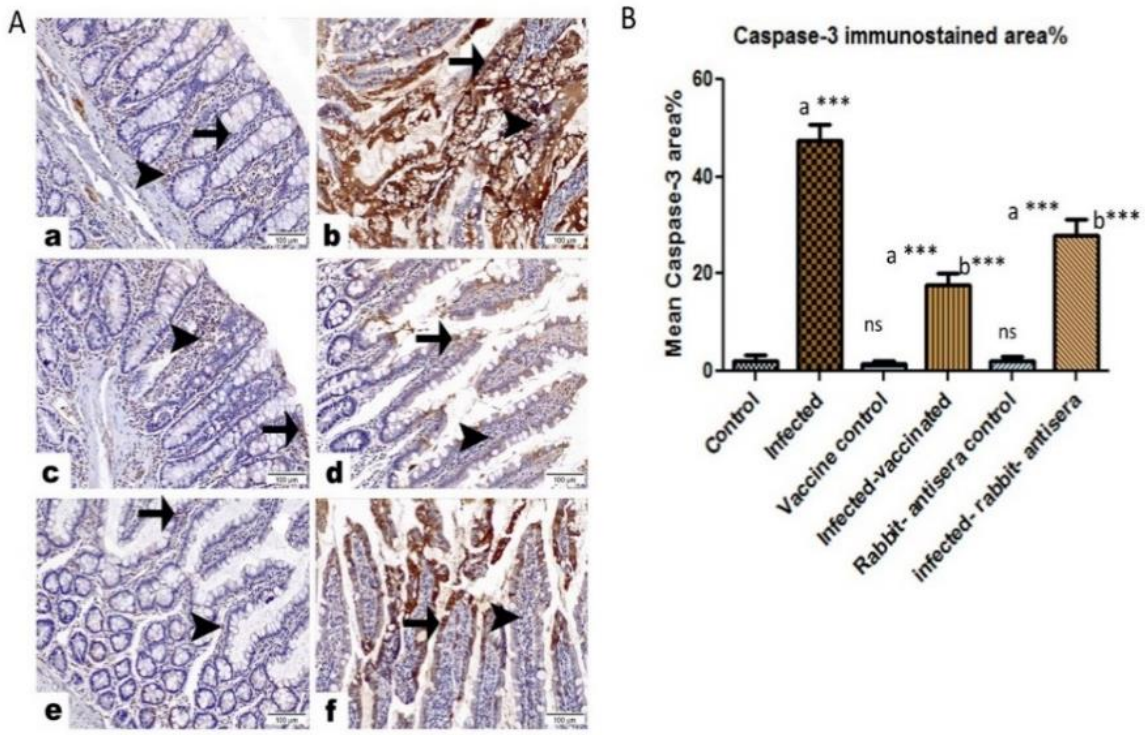


Fig. 6. SFCP-vaccination and rabbit antisera treatments diminish apoptosis-related protein in infected rats. A) Photomicrographs showing theFig. 6. SFCP-vaccination and rabbit antisera treatments diminish apoptosis-related protein in infected rats. A) Photomicrographs showing the immunohistochemical expression of Caspase- 3 along colon tissue sections between examined groups as follows: Controls including a) control; c) vaccine control; e) Rabbit antisera control rats. The positive reactivity of caspase-3 along the epithelial layer (arrow) as well as connective tissue of lamina propria (arrowhead) was scarcely exhibited in these controls. b) The infected group exhibits a significantly higher level of Caspase-3 reaction within the epithelial layer (arrow) and the connective tissue of the lamina propriacompared to the control group. d) Infected-vaccinated group showed few expressions of caspase-3 reaction along the epithelial layer (arrow) and connective tissue of the lamina propria (arrowhead) in comparison with its control. f) The infected-rabbit-antisera group revealed moderately expressed caspase- 3 reaction in the epithelial layer (arrow) and connective tissue of the lamina propria (arrowhead) compared to its control. B) Analysis of the immunostaining data for caspase-3 in colon tissue using quantitative scoring. Caspase-3 immuno-stained area% is presented as mean ±SD.The percentage of each chosen tissue region's Caspase-3 -positive stained area (n = 8). One-way ANOVA was conducted, followed by Turkey's post hoc test; ***$p < 0.001$, indicates the significant difference concerning the control group; b significant difference concerning the infected groups, no statistical significance for control. Magnification Power= x200 & Scale Bar= 100µm).

Parasitic CPs play a significant part in the disturbance of the connections between epithelial cells by breaking down mucin and villin, harming the proteins that hold cells together at the junctions, triggering cell death in epithelial cells, and breaking down immunoglobulins, cytokines, and defensins, ultimate induction of apoptosis. Similarly, Blastocystis cysteine proteases have active role in in

the disturbance of epithelial intercellular connections and, ultimately, in Blastocystis' induction of apoptosis [27]. Cryptosporidium infection results in a decrease of barrier function and elevation INF pathways[37]. The CPs emitted by Giardia had different protein focuses on have epithelial cells and in the long run disturb digestive homeostasis, thusly causing gastrointestinal harm [38].Various proteases upregulate in sexual stage (i.e., gametocytes and non-sporulatedoocysts) [39]. Deletion of protease in *C. parvum* results in reduction of oocyst shedding in vivo and macrogamont production in vitro [40]. It has been reported that parasitic CPs play a significant part in the disturbance of the connections between epithelial cells by breaking down mucin and villin, harming the proteins that hold cells together at the junctions, triggering cell death in epithelial cells, and break down immunoglobulins, cytokines, and defensins [41]. We previously exhibited that *S. fusiformis* protease efficiently degraded a number of mammalian proteins, involving fibrin collagen gelatin, albumin and hemoglobin [8]. In definitive hosts, parasite reproduces sexually in enterocytes and it requires protease for invasion, nutrient uptake and immune evasion and excystation of oocyst. The *S. fusiformis* protease (SFCP) may have a vital role during sexual stage development in the intestinal epithelium and this causes damage in the intestine. In the current study, anti-SFCP reduces this harm.

3.5. The impact of the Sarcocystis infection and SFCP on cytokines:

The host's innate immune system is triggered by parasitic infection, which starts a series of cytokine-mediated reactions. The immune response against parasites is determined in part by the complex balance between pro- and anti-inflammatory cytokines. The dysregulation of cytokines might cause immunological hyperactivation, which exacerbates symptoms [42]. The serum level of some cytokines (IL-4, IL-6, IL-12, IFN-γ, and TNF-α) was evaluated and presented in Fig. 7. A significant increase in the levels of the tested cytokines was observed after a week (7 days) of infection, which reflects the rapid severity of the Sarcocystis infection and the associated inflammatory activity. In the infected rat model, the most significant change in the levels of all tested cytokines was observed at week 2 after the infection by ~1.75- 2.45 increase-fold compared to the control group. However, at weeks 3 and 4, the levels of cytokines decreased but were still significantly higher than the control group. On the other hand, in the infected-vaccinated, infected-rat-antisera, and infected-rabbit-antisera rat models, it was obvious that the CPSF-vaccination, the prepared rat-antisera, and rabbit-antisera could significantly decrease the levels of examined cytokines in treated groups compared to the infected rat group during four weeks after infection. This decrease was observed obviously at week 2 after infection, and all cytokine concentrations approached the control group. No significant alterations were detected in the levels of cytokines after SFCP vaccination and the prepared antisera treated in control animals compared to negative control animals (data not shown).

The mRNA levels of TNF-α, IFN-γ, IL-10, IL-6, transforming growth factor-beta (TGF-β), and IL-β were significantly higher in the spleen of infected animals compared to the control group. Immune cells identify parasites through the interaction of key molecules present in their membranes with receptors found in innate immune cells, including Toll-like receptors (TLRs) and Nucleotide oligomerization domain (NOD)-like receptors (NLRs)[43]. qPCR analysis indicates significant up-regulation of the expression levels of NOD1, NOD2, TLR4, and TLR9 in the spleen of infected rats compared to negative control. SFCP attenuated the *S. fusiformis* -activated increase in NOD1, NOD2, TNF-α, TLR4, TLR9, IFN-γ, IL-12, IL-10, IL-6, TGF-β and IL-β mRNA in spleen. Therefore, it can be concluded that the purified SFCP as a vaccine, the prepared rat-antisera, and rabbit-antisera administration resulted in a fast decrease of blood levels and spleen of all examined cytokines in the infected rat model to the values close to normal (Fig. 8).

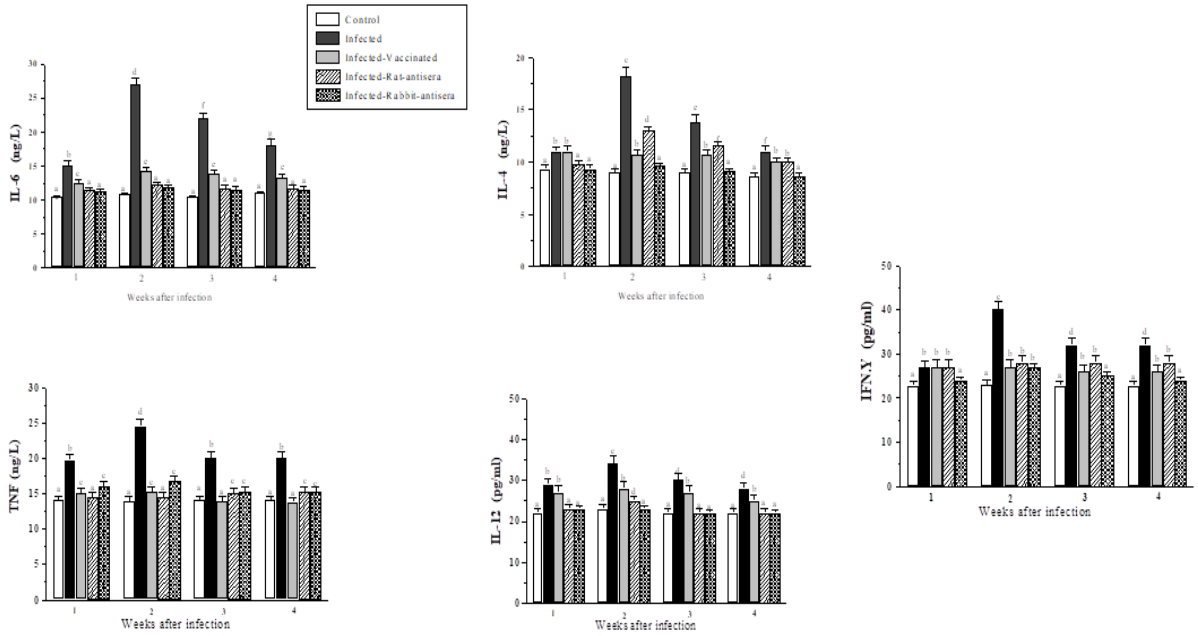
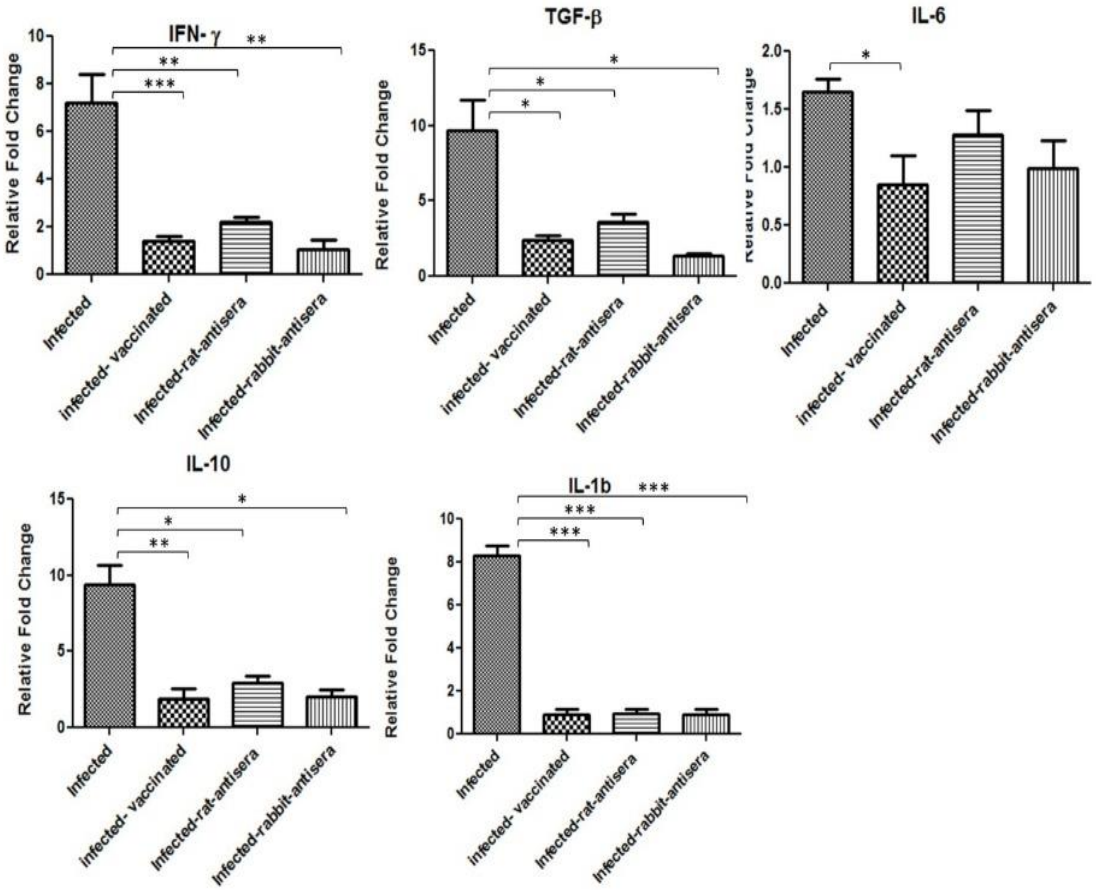


Fig. 7. Cytokine levels (IL-4, IL-6, IL-12, IFN-γ, and TNF-α) in blood serum of rats during four weeks after Sarcrocystis infection and under various treatments.



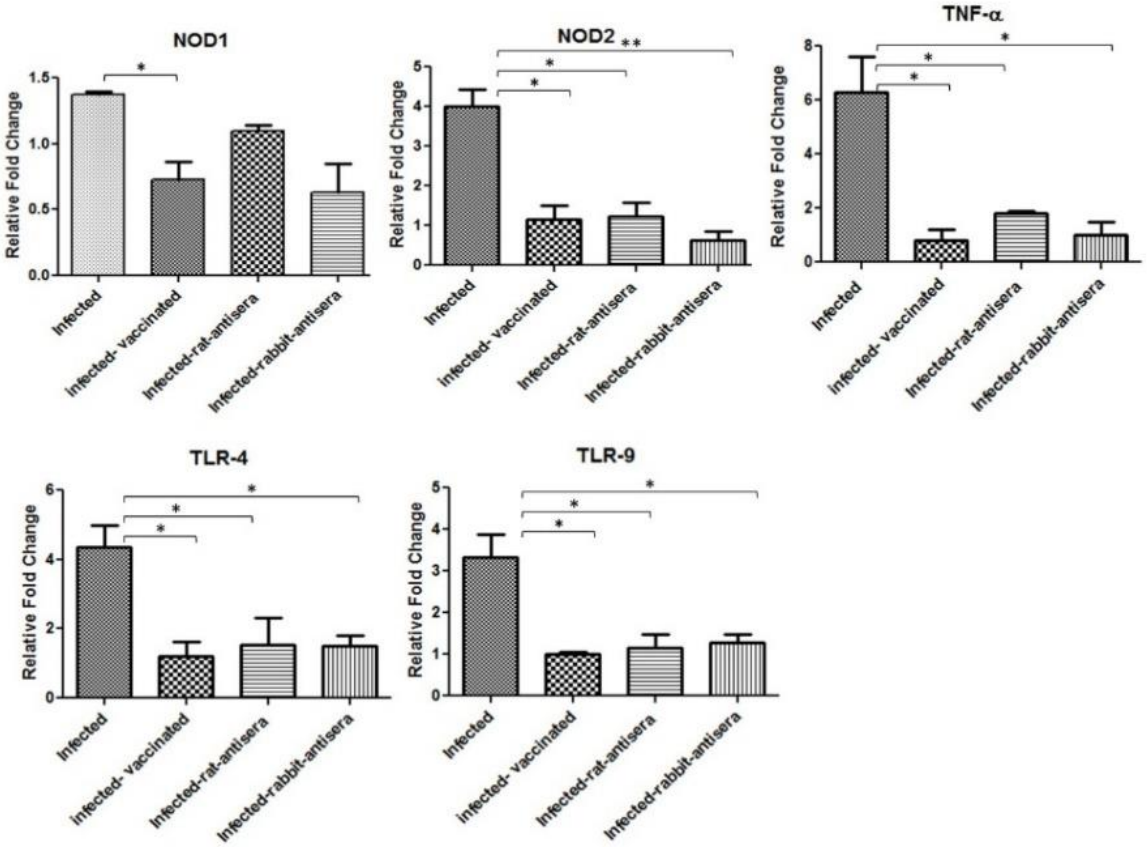


Fig. 8. Change in cytokine gene expression levels in the spleen compared to animals without the condition. The mRNA levels of cytokines NOD1, NOD2, TNF-α, TLR4, TLR9, INF-γ, TGF-β, IL-10, IL-β and IL-6 in the spleen at the end of the experiment were measured using quantitative PCR (qPCR). The results are presented as the ratio of the cytokine gene expression levels in the spleen to those in each (negative, vaccine, anti-rat and anti-rabbit) controls, which is set as 1. Values indicate the comparative level of the cytokine expressed in relation to the endogenous control of GAPDH. The data is displayed as averages ± the standard error of the mean (n = 3 separate experiments). Cytokine Ct values, obtained through qRT-PCR, were utilized to determine the relative fold change using the $2^{-\Delta\Delta Ct}$ method. Differences in cytokine gene expression levels between animals that received the treatments (SFCP, rat-antisera, and rabbit-antisera) and those that received a PBS but were still infected are indicated by asterisks (*) with p-values less than 0.05, double asterisks (**) with p-values less than 0.01 and three asterisks (***) with p-values less than 0.001.

Several studies reported the correlations between parasitic infection and cytokines induction. In serum; IL-1β IL-18, IL-15, IL-12, IL-10, and IL-13 and TNF-alpha levels in the blood were noticeably higher in rats that were given a fresh antigen of *S. fusiformis* on day 28 [44]. The blood and spleen responded to *S. neurona* infection by notable increases in B-cell percentages at day 28 dpi and in CD8 percentages at days 14 and 28 dpi [45]. Fresh extract of *S. Fusiformis* promoted the positive expression of CD4+ and CD8+ T cell numbers, so boosting their proliferation in the thymus and spleen showed adaptive immune responses [44].Under constant antigenic stimulation, the CD4 and CD8 lymphocytes can further develop into TCM cells—memory T cells secreting IL-2, TNF-α, and IFN-γ[46]. *S. fusiformis* whole cyst stimulated pro-inflammatory dendritic cells (DCs) that markedly released interleukin (IL)-12p40, tumor necrosis factor-alpha, IL-6, and IL-10. Additionally, these cells exhibited increased expression of TLR4, CD80, CD86, and major histocompatibility complex (MHC) class II on their surfaces, signifying complete maturation of the DCs [47]. Additionally, in camels, Sarcocystis infection increased inflammation by triggering IL-6 expression in various infected organs[48]. On the other hand, NOD proteins are triggered by parasites and contribute to controlling the inflammatory reactions of the host during malaria infection [49]. NOD1 and NOD2 give fundamental host guards against Trypanosoma cruzi [21] and Falciparum pythium.NOD1 and NOD2 assume a part in animating Th1 cell reactions expected for parasitic rescuing in mouse models of disease with Toxoplasma gondii [50], Trypanosoma cruzi[51], and Leishmania infantum [52]. To eradicate these infections, NOD1 and NOD2 receptors play a role in immune response induction and clearance[53]. TLR9

played a role in the induction of innate response and formation of an efficient Th1 immune response upon oral parasitic infection [54]. The careful balance between pro-inflammatory and anti-inflammatory cytokines guarantees efficient parasite removal [55]. Therefore, current work exhibited relieving the examined cytokines in blood and spleen by SFCP whether as a vaccine or antibodies may increase the efficiency of treatment and reduce the parasite.

4. Conclusion

In the current study, the SFCP has strong immunogenicity helps to provide good protection against *S. fusiformis* infection. The *S. fusiformis* infection can trigger both Th1 and Th2 immune reactions, as well as increase cytokine concentrations that could stimulate inflammation and apoptosis in theintestinal epithelial cells. Moreover, SFCP as a vaccine and antisera showed strong anti-Sarcocystis properties, with the greatest reduction in oocyst shedding and modulation of the Sarcocystis-induced apoptosis and cytokines elevation. The findings demonstrated that the SFCP-vaccination and prepared anti-SFCP sera could be novel therapeutic candidates for controlling *S. fusiformis* infection and its harmful effects.

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Author contribution statement

AZB, SAM and AMA designed the research; MKI, HAS, TBI and RMS conducted the research; AZB, SAM and AMA analyzed the data; AZB, SAM and AMA wrote the paper. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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