Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Research Article

Immunization of Rabbits Against Whole Crude Antigen of Sarcocystosis (*S. gigantea*) isolated from Macrocystis of Naturally Infected Sheep in Duhok Province, Iraq

Shivan N. Hussein, Assel A. Ibrahim and Mohammed S. Shukur

Department of Medicine and Surgery, College of Veterinary Medicine, University of Duhok, Kurdistan Region, Iraq.

Abstract

SARCOCYSTOSIS is an intracellular immunosuppressant parasitic disease caused by the genus *Sarcocystis* that infect various animal species and human. The current study was aimed to evaluate cellular and humoral immune responses of experimentally immunized rabbits to whole crude antigen isolated from *S. gigantea* of sheep. The haematological examination of immunized rabbits showed significant increase in the total number of white blood cells (18.8±2.0 x10^3/ µl) and differential leukocyte count including; neutrophils, eosinophils and basophils in comparison to non-immunized group. By evaluation of phagocytic activity, the number of phagocytes engulfing the pathogen was significantly increased in immunized rabbits (22.41±1.8) compared to control group (1.22±0.56). In delay type hypersensitivity of cutaneous reaction, the skin fold thickness approached its maximum (5.70 ±0.11 mm) after 48 hours of intradermal injection of the antigen. The total serum albumin and globulin levels were higher in post-immunized group (6.40±0.25 g/dl and 4.36±0.10 g/dl) when compared to control animals (4.82±0.14 g/dl and 2.04±0.11 g/dl), respectively. By biochemical analysis using both glutaraldehyde reaction test and sodium sulphite precipitation test, immunoglobulin level were higher in immunized animals compared to non-immunized animals. During serological analysis, significant difference (P<0.05) was observed in IgG level in immunized rabbits in comparison to control group. Histopathological examination of immunized rabbits showed increased infiltration with inflammatory cells, congestion, oedema and haemorrhage in different organs. In conclusion, the isolated whole crude antigen of *S. gigantea* has provided a fundamental insight about immune stimulation for activation of cellular and humoral antibodies in experimentally immunized rabbits. It's crucial to explore cross-reactivity between Sarcocystis and Toxoplasma infections during diagnosis and develop a sensitive test to differentiate and detect potential cross-reactions.

Keywords: Sarcocystosis, Rabbits, Cellular and Humoral immunity.

Introduction

Sarcocystis species are common protozoan parasite of the phylum apicomplexan, distributed globally among domestic and wild animals. These protozoa have two obligatory host life cycle including herbivorous or omnivorous as intermediate host in which they develop by asexual multiplication and carnivorous as definitive host in which sexual multiplication of protozoa develops [1]. There are more than 196 species of *Sarcocystis* distributed in all parts of the world [2]. *Sarcocystis* species are more specific to their final hosts than intermediate hosts. For instance, sheep can be infected with four common species of *Sarcocystis* including *Sarcocystis gigantean* (*S. ovifelis*) and *Sarcocystis medusiformis* that form macrosarcocystic species, while *Sarcocystsis tenella* (*S. ovicanis*) and *Sarcocystis arietcanis* which form microsarcocystic species [3]. In Iraq, the prevalence of macroscopic type of sarcocystosis in small ruminants was ranged from 1% to 98.9% [4,5]. The pathogenesis of sarcocystosis varies according to the species of *Sarcocysis* and species of host. Ovine can be heavily infested with *S. gigantea* which produce different sizes and shapes of embedded and bulged white creamy cysts. The infected parts of the body with macrocysts can only be identified by inspection after slaughtering of the infected animals at slaughterhouses [6]. The immune response to

*Corresponding authors: Mohammed S. Shukur, E-mail: mohammed.shukur@uod.ac Tel.: 009647504503309
(Received 16/02/2024, accepted 18/06/2024)
DOI: 10.21608/EJVS.2024.267435.1853

sarcocystosis is species specific in which the host protection against one species of the parasite cannot provide immunity against heterologous challenge [7]. Cell mediated and humoral immunity can be developed after immunization of the intermediate hosts against crude antigen isolated from cystizoites and merazoites of *Sarcocystis* species [8]. In cell mediated immunity, immune cells such as macrophages and lymphocytes migrate and disseminate through visceral and muscular tissues. Humoral immune response usually appears after 30 days of infection, but immunoglobulin M (IgM) antibodies appear earlier than immunoglobulin G (IgG) antibodies and has short duration of life and they disappear after maturation of the parasite [9,10]. Cell mediated immunity in Sarcocystosis is more significant than humoral immunity. Skin test or delayed type hypersensitivity test enable crude antigen stimulation of cell mediated immunity response to the specific antigen injected intradermally [11]. Lymphocyte transformation test (blastogenesis) has widely been used to measure the capability of lymphocytes to detect *Sarcocystis* antigen *in vitro* in order to evaluate immune response. Both lymphocytes transformation and delayed type hypersensitivity could be observed in the chronic infection after a month of infection in mice and man [12]. Furthermore, phagocytic cells activity test which measure the ability of phagocytes in ingestion of *Staphylococcus aureus* has previously been used to evaluate cell mediated immune response in immunized host against parasitic antigen [13]. In addition, for detection of anti-*Sarcocystis* antibodies, various serological and biochemical tests have been used. Direct agglutination test (DAT), immunofluorescent antibody test (IFAT) and dye test employ intact merozoites or cystizoites as antigen to detect antibody directed against the parasitic cell surface. Latex agglutination test (LAT) and indirect hemagglutination (IHAT) can be used for antibody detection with low sensitivity rate mainly in acute stage of infection [14]. ELISA for *Sarcocystis* antibodies improved for detection of sarcocystosis in ruminants through employing antigen prepared via ultrasonication or freeze-thawing of cystizoites, but it requires monoclonal secondary antibody for detection of specific type of the parasite [15]. The current study was aimed to evaluate the humoral and cellular immune response through immunization of rabbits against whole crude antigen isolated from cystizoites of ovine macrosarcocystosis (*S. gigantea*) and to determine the immuno-pathological reaction in different organs obtained from immunized rabbits after inoculation of the parasitic antigen.

Materials and Methods

Animals and ethical approval

Twenty local breed rabbits aged 10-12 weeks old were purchased from Duhok local markets (Duhok, Iraq) and kept in separate cages under specific pathogen free condition feeding with different types of vegetables at college of veterinary medicine, University of Duhok. This study was carried out in strict adherence to the regulations and under direct supervision and full approval from the Local Ethics Committee established at the College of Veterinary Medicine, University of Duhok (CVM2022120UD).

Parasitic isolation and antigen preparation

Fifty soft white-creamy colour macrosarcocystis weighed from 40 mg to 250 mg with the measurement size 6.5 ± 1.20 length x 3.5 ± 0.9 width were collected from oesophagus of naturally infected sheep from the local slaughterhouse (Duhok, Iraq).

The identification of the Sarcocystis gigantea species (accession number: ON533766; available at https://www.ncbi.nlm.nih.gov/nuccore/ON533766.1/) was validated through a preceding investigation, the details of which remain unpublished. The cysts were isolated and put in a clean glass petri-dish and washed several times with cold (4 °C) phosphate buffered saline (PBS) pH 7.2 to remove the attached muscle tissues. The cysts were raptured and softened by using sterile scalpel and thump forceps until a creamy colour suspension was formed. Then, the suspension was homogenized manually via a clean glass homogenizer followed by centrifugation at 2500 g for 5 minutes at 4 °C according to Morsy (38). The obtained supernatant of *S. gigantea* whole cyst antigen was collected as aliquots and stored at -20 °C until used. The total protein concentration of the prepared antigen was measured using NanoDrop (Thermo Scientific, USA).

Experimental Work

Immunization of rabbits

Twenty local breed rabbits (10 for control and 10 for immunization) were used for experimental immunization against whole crude antigen isolated from macrocystis of sheep to evaluate cell-mediated and humoral immunity. The back of the rabbit's neck was shaved and disinfected with ethanol 70%, then whipped with tincture iodine. Experimental rabbits were subjected to subcutaneous (SC) injection of 0.1 ml of suspended antigen mixed with 0.1 ml of adjuvant composed of black seed oil sourced from Vida Company (Duhok/Iraq). Additionally, 10 µl of penicillin-streptomycin (1000 I.U.-100 mg) were included to prevent bacterial infection during the immunization process. Control group rabbits received injections of 0.1 ml of phosphate buffered saline (PBS) at pH 7.2.

Haematological examination

Total leukocyte count

To assess the total leukocyte (white blood cell, WBC) count in both the immunized and control groups of rabbits, a hemacytometer (Hemacytometer Kit, India) was employed following the protocol outlined by Coles [34]. Blood samples were obtained from the saphenous vein of rabbits using heparinized tubes. These samples were drawn into the WBC pipette (white bulb) up to the 0.5 mark, then mixed with diluting fluid (Turke's solution) until reaching the 11 marks. The mixture was vigorously shaken for 2-3 minutes to ensure thorough mixing.

Subsequently, a cover slip was placed over the chamber, and a drop of the mixture was placed at the edge of the cover slip. After allowing the WBCs to settle for 2-3 minutes, the sample was examined under low (X10) power of a light microscope. WBCs in all four squares (chambers) were counted, and the total number was multiplied by 50 to determine the WBC count per microliter. This standardized procedure ensures accurate estimation of WBC count in the rabbit samples, facilitating comparison between the immunized and control groups.

Differential leukocyte count

To determine the differential leukocyte count, blood films were prepared from both the immunized and control group rabbits. These films were stained with 5% Giemsa stain following standard laboratory protocols. Subsequently, one hundred white blood cells (WBCs) of various types, including heterophils (neutrophils), lymphocytes, monocytes, basophils, and eosinophils, were enumerated to estimate the percentage rate (%) of leukocytes. This methodology, as outlined by (34), ensures the systematic evaluation of differential leukocyte populations, facilitating comparisons between the immunized and control groups of rabbits.

Total protein

Blood samples (1 ml) were obtained from the saphenous vein of each rabbit in both the control and immunized groups using disposable syringes (1 ml, 30G, Germany). These samples were collected for the evaluation of total serum protein, total serum albumin, and total serum globulins using a commercial kit (Biolabbo SAS, France). The assessment was performed using a spectrophotometer, with each component measured at its specific wavelength, as specified by the manufacturer's instructions. A Biochrom WPA Lightwave II UV/Visible spectrophotometer from the UK was utilized for this purpose.

Following collection, the serum samples from each group were separated and stored in Eppendorf tubes at -20°C until further analysis. This procedure ensured the preservation of sample integrity and stability for subsequent assays.

Quantitative evaluation of immunoglobulins

Glutaraldehyde coagulation test

This study utilized a modified method as initially outlined by Thrall [35]. About 250 µl of both control and immunized rabbit sera were introduced into a 10 ml U-shaped glass test tube, along with 25 µl of a 10% glutaraldehyde solution reagent. The test tubes were then left to incubate at room temperature and checked for coagulation every hour. The outcomes were classified as complete coagulation (>600 mg/dl of immunoglobulin), a semisolid clot (400-600 mg/dl of immunoglobulin), or no coagulation (<400 mg/dl of immunoglobulin).

Sodium sulphite precipitation test

Two millilitres of sodium sulphite solution at different concentrations 14%, 16% and 18% were put into three clean glass test tubes and one hundred microliters of rabbit's sera were added and mixed immediately and incubated at room temperature for one hour to determine the evidence of precipitation. The result was estimated to determine the precipitation (Table, 1). The cloudiness with visible flakes were considered positive while, the precipitation without observing flakes were considered negative [35].

Evaluation of cell-mediated immunity

Evaluation of delayed type hypersensitivity cutaneous reaction

The left side flank region of each rabbit in immunized group was carefully clipped by electric hair clipper (Fengz, 0.5 kg/ French) to provide a free hair marked circular area whipped with 70% of ethanol. They were injected intradermally with 0.05 ml (50 µl) of 6.5 mg/ml of the parasitic antigen using 1 ml syringe (30 G, Germany). The right flank regions were injected intradermally with 50 µl of PBS, pH 7.2. The hypersensitivity cutaneous reaction (swelling and redness of the skin) was assessed at different time points, 0 time point, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 36 hours, 48 hours, 72 hours, 96 hours, and 7 days post injection [12]. The mean thickness diameter of skin folds was measured by using a standard digital calibre (Vernier, Electronic digital calibre, 0-150 mm, Lezaco/ China).

Evaluation of Phagocytosis

Phagocytic cells activity test was used in this study for evaluation of phagocytosis as described by Oddera [36]. This test measures the ability of phagocytes of immunized rabbits for ingestion of *Staphylococcus aureus*. One ml of blood obtained

from each control and immunized rabbits were drawn from saphenous vein in heparinized tube for evaluation of activity of immune system (macrophages engulfing bacteria). Approximately, 250 µl of *S. aurous* stock (from thigh of chicken) were put into two test tubes containing 3 ml of peptone broth 1.5:100 (1.5 gm of peptone agar media diluted with 100 ml of distilled water) incubated at 37 °C for 24 hours. Then, 50 µl of bacterial broth was mixed with one ml of the freshly drowned blood in heparinized tube incubated for 1 hour at 37 °C followed by smear preparation on clean slides, air dried, fixed in methanol for 5 minutes and stained with 5% of Giemsa stain for 30 minutes. Finally, they examined under oil emulsion slide (100X) for estimation of the cell engulfing bacteria (*S. aureus*) and calculating the number of phagocytic cells in both groups by phagocytic index.

$$Phagocytic\ Index\ (PI) = \frac{Number\ of\ phagocytosed\ cells}{Total\ nimber\ of\ phagocytic\ cells} X\ 100$$

Evaluation of humoral-mediated immunity

For detection of IgG antibodies, a modified direct agglutination test of Hettiarachchi et al. (9) was used. Approximately, 25 µl of optimized parasitic antigen (diluted by double fold dilution 1:32) were mixed separately with the serum of control and immunized rabbits containing anti-*Sarcocystis* antibodies. For negative control, 25 ml of diluted intact antigen was mixed with PBS, pH 7.2 without adding the rabbit's sera. After agglutination (antibody-antigen reaction) in immunized rabbits, the suspension was mixed with 25 µl of two mercaptoehanol (2ME) incubated at 37 °C for 30 minutes for detection the type of antibodies IgG or IgM. The agglutination appearance determines the evidence of IgG protein, while in the absence of agglutination indicate of IgM protein.

Histopathological examination

After three weeks of subcutaneously injection of Sarcocystis antigen to rabbits, tissue samples from different organs including heart, brain, liver, lung and kidney of the immunized rabbits were collected and separately fixed in 10% of neutral buffer formalin in 50 ml of clean containers, followed by dehydration through different concentrations of alcohol. The organs were embedded in liquid paraffin and sectioned at 3 µm, then stained with Hematoxylin and Eosin (H and E) for detection of immuno-pathological reaction examined under microscope (40X) [37].

Statistical analysis

The data were expressed as mean ± Standard error (S.E). The statistical analysis was conducted by using SPSS statistical software (version 19/ IBM). $P< 0.05$ was considered statistically significant, while $P>0.05$ regarded as statistically non-significant.

Results

Evaluation of cell-mediated immune response

Total and differential leukocyte count

Total and differential leukocyte counts were estimated to determine the cellular activity in immunized rabbits. There was a significant increase ($P<0.05$) in the quantity of total leukocytes (18.8 ± 2.0 x10^3/µl) in the immunized group compared to those in control group (12 ± 2.7 x10^3/µl) (Table 2).

Phagocytic ingestion activity

The number of phagocytes ingesting bacteria (*S. aureus*) significantly increased ($P<0.05$) in the immunized group of rabbits, (22.41 ± 1.8) injected with whole crude antigen of the parasite in comparison to the control group (1.22 ± 0.56) (Table 3).

Different stages of phagocytic cells engulfing *S. aurous* were observed in the blood smear stained with 5% Giemsa in the immunized rabbits including phagocytic cells attacking *S. aurous*, phagocyte attachment with *S. aurous*, phagocytic cells starting engulfment of bacteria, and complete phagocytosis of bacteria by macrophages (Fig.1).

Delayed type hypersensitivity

There was a significant increase ($P< 0.05$) in the skin fold thickness after two hours of intradermal injection with the antigen, while the fold reached its maximum thickness within 36-48 hours (4.41 ± 0.1 mm to 5.70 ± 0.11 mm, respectively) (Fig. 2). Then, the thickness was subsided and reached its zero point (1.28 ± 0.03 mm) after 7 days of post-inoculation (Table 4).

Evaluation of humoral-mediated immunity

Total protein level (Albumin and Globulin)

The total serum protein, albumin, and globulin levels were found to be significantly increased (10.76 ± 0.26 gm/dl, 6.40 ± 0.25 gm/dl, and 4.37 ± 0.10 gm/dl) in the immunized group in comparison to the control group (6.86 ± 0.22 gm/dl, 4.82 ± 0.14 gm/dl, and 2.04 ± 0.11 gm/dl) (Table 5).

Quantitative values of Immunoglobulins

Biochemical reaction tests, including glutaraldehyde coagulation and sodium sulphate precipitation, were used to determine the quantitative values of non-specific immunoglobulins concentrations in the serum of immunized rabbits and the control group of rabbits. By the glutaraldehyde coagulation test, the concentration of immunoglobulin in immunized

rabbits was found more than 600 mg/dl, while in control it was less than 600 mg/dl (0.6 g/dl). By the sodium sulphate precipitation reaction test, the concentration of immunoglobulin in immunized rabbits was more than 1500 g/dl (1.5 g/dl) in comparison with the control group, which was between 500 and1500 g/dl (0.5-1.5g/dl).

Qualitative values of Immunoglobulins

There was a significant difference ($P<0.05$) in the detection of immunoglobulin G (IgG) antibodies in the sera of all immunized groups of rabbits compared to the non-immunized group of rabbits (Table 6).

Histopathological investigation

The histopathological reaction of the post-immunized rabbits with *Sarcocystis* antigen found significant infiltration of the inflammatory cells in addition to congestion, oedema, haemorrhage, and vasodilation of the heart, lung, kidney, liver, and brain due to a hypersensitive reaction (Fig.3).

Discussion

Sarcocystis species are ubiquitous intracellular protozoan parasites that have a great economic impact on livestock because of animal emaciation, decreased weight gain, decreased quality and quantity of meat, milk, and wool, transboundary restriction, condemnation of infected carcasses, abortion and even death [2]. Little information is known about the specific immune responses triggered by sarcocystosis incorporation with the other closely related apicomplexans such as *Neospora caninum* and *Toxoplasma gondii* [10]. Transferring antibodies via colostrum in passive immunity doesn't reveal that it protects from clinical sarcocystosis or provides obvious cross-protective immunity between different *Sarcocystis spp*. in the same host [14]. Although both humoral and cell-mediated immune response to unrelated antigens of *Sarcocystis* are suppressed during the primary infection, cell-mediated immunity responses are affected more intensely. It is known that merazoites and sporozoites are more immunogenic than bradizoites [8]. During the initial phase of infection, a T helper 1 cells response has been detected, while a T helper 2 response has been observed during the muscle stage of the parasitic infestation, which is correlated with hyper-eosinophilia [16] Generally, T helper 1 immune responses that are stimulated against the antigen of apicomplexan protozoan species are necessary for controlling the replication of parasites during infection [17]. However, the suppression of the immune response, especially the inflammatory cell response, leads to a prolongation of the life span and increases the ability of parasites to complete its life cycle [18].

In this study, whole crude antigens of macrocystis in sheep (*S. gigantea*) were used to immunize rabbits for evaluation of cellular and humoral immune responses. Similarly, a study [19] used the whole crude antigen of macrosarcocystosis in buffalo (*S. fusiformins*) to activate dendritic cells in a group of laboratory mice, which was assumed to be essential for activating cellular (adaptive) immunity. The immune status of post-immunized rabbits by hematological examination after 30 days of inoculation revealed as significant increase in the total value of heterophils, eosinophils, and basophils, while monocytes and lymphocytes were found to be lower in comparison with the control group. This might be related to the stimulation and migration of the granulated leukocytes to the site of injection, as well as the release of cytokines, histamine, and other enzymes that may lead to inflammation. Increased neutrophils with decreased lymphocytes could be an indication of immune responses and inflammation due to the pathogen [20]. The intensity of neutrophils in an immunized host could be related to an increase in the quantity of antigenic inoculation [4]. A significant increase in the absolute and relative values of neutrophils and basophils with a decreased number of lymphocytes has been reported in a study [21]. In contrast, they found non-significant differences in the values of total leukocytic count, eosinophils, and monocytes after 10 days of experimentally infected rabbits with coccidian antigen in comparison with the control group [21]. Furthermore, there was significant increase in the total leukocytic count and absolute number of neutrophils with a decrease in the number of lymphocytes, monocyte, and eosinophils during the infection of rats with *E. niesochulzi* after 7 days of inoculation [22]. In addition, a study found increased absolute values of lymphocytes, eosinophils, and basophils with decreased values of neutrophils and monocytes in immunized rabbits with *Cryptococcus neoformans* antigen in comparison with the control group [23].

In the current study, an evaluation of the total serum protein concentration showed an elevated concentration in immunized rabbits in comparison to non-immunized ones. These findings were consistent with those of some other researchers. Similar results have been found after inoculating guinea pigs with whole crude antigens of immature ticks [24]. Similarly, immunized calves with *Hyalomma anatolicum* antigen showed a significant increase in serum gamma globulin levels [25]. Likewise, another study reported similar findings after inoculating rabbits with *Hyalomma a. anatolicum* larval and nymphal extract antigens. These could be indications of the initial immune

response to the antigen inoculum by the inoculated animals [26].

This study clearly found an increase in the intensity rate of phagocytic cell activity for ingestion of *Staphylococcus aureus* in immunized rabbits. Similar results have been reported in a study following immunization of rabbits with antigen extracted from hard ticks (Ixodidae) of infected sheep [27]. It is known that phagocytosis is a critical biological process by which immunized rabbits can protect themselves from infectious and non-infectious diseases [28].

This research performed the cutaneous hypersensitivity reaction test in post-immunized rabbits to evaluate the host resistance against *Sarcocystis* antigen and to indicate cell-mediated immunity. A cutaneous reaction at the site of intradermal inoculation in post-immunized animals determined the acquired immune responses, including the infiltration of granulocytic cells such as eosinophils and basophils [27]. This type of reaction is known as cutaneous granulocytic (basophilic) hypersensitivity. It is a kind of delayed type hypersensitivity mediated via T helper 1 cells. The DHT response can be used as an indication of cell-mediated immune responses [10]. In a similar study, it was found that increased skin fold thickness reached its maximum level after 48 hours postimmunization in mice [29]. Also, in a consistent study, there was no significant difference in skin fold thickness between 24 hours (7.42±05 mm) and 48 hours (6.0±0.5mm) recorded with an antigen of concentration 15 mg/ml in rabbits by Al-Samarraae and colleagues [23]. The increased thickness of the skin fold at the injection site could be due to the aggregation of activated macrophages and other inflammatory cells in the host dermis and among muscle fibers. The inflammatory cell infiltration and increased permeability of blood vessels result in redness and swelling of the injected skin by antigen. The mixed antigen with adjuvants leads to prolongation, constant stimulation, and activation of macrophages and other non-specific inflammatory cells to attack and digest the injected antigen, which could lead to an increase in of skin fold thickness [13].

In the present study, the quantitative and semiquantitative values of non-specific immunoglobulins were estimated by different concentrations of sodium sulphite reactions and glutaraldehyde coagulation tests. An elevation of these values was found by this work in experimentally immunized hosts, in agreement with another research [27]. Similar to the investigation in this study for the determination the qualitative type of immunoglobulins, including IgG antibodies, Hettiarachchi and colleagues [9] performed direct agglutination in the microtiter plate after mixing the antigen of the parasite with the serum of the infected host. Furthermore, a direct agglutination test has been used to identify the humoral immune response after immunization of the host with *Sarcocystis* and *Toxoplasma gondii* intact antigens [14]. In addition, EL-Shanawany and colleagues [10] found an increase in the total level of IgM and IgG by using *Sarcocystis fusiformis* whole crude antigen to determine the infection rate in buffalos. Moreover, Nada and colleagues [15] investigated high infection rate of 93% in cattle by using the whole cyst's extract antigen of *S. fusiformis* in the diagnosis of sarcocystosis. It has been reported that IgG antibody levels were increased after 31 days of post inoculation in rabbits with the parasitic antigen, and the responses were also elevated after 38 days of inoculation [9].

Histopathological examination of immunized rabbits after inoculation with whole crude antigen revealed moderate infiltration of inflammatory cells, basophils, eosinophils, hemorrhage, oedema, vasculitis, and thickness of blood vessels in different organs, including the brain, heart, liver, kidney, and lung. These findings were consistent with those of other studies [30- 32]. Similarly, another study [29] reported histopathological changes including pneumonia with inflammatory foci, emphysema, congestion and thickness of blood vessels, thickness of the wall in the lung, alveoli and bronchi, as well as hyalin degeneration, inflammation, and oedema of heart muscle, contributed to the antigen isolated from *S. gigantea* cystzoites. In another study, multifocal necrosis infiltrated by macrophages, neutrophils, eosinophils, haemorrhage and vacuolar degeneration of myocytes were recorded in the skeletal and cardiac muscle of the intermediate host [33]. These pathological results could be due to over-concentration of the prepared intact antigen, which can cause over-stimulation of the immune responses, including activation of inflammatory cells, eosinophils, and basophils.

The isolated whole crude antigen from the oesophagus, diaphragm and abdominal muscle of ovine macrosarcocystis (*S. gigantea*) has provided a fundamental insight about immune stimulation for activation of cellular and humoral antibodies in experimentally immunized laboratory rabbits. The implied intact antigen has a great impact on the elevation of total white WBCs and differential leukocyte count, in addition to increasing the level of total proteins, including total serum albumin and total serum globulins, in the host after 30 days post inoculation. The internal organs of the immunized host developed mild to moderate histopathological

changes after antigen injection subcutaneously. The prepared antigen from this study could be used as a commercially developed vaccine after attenuation (attenuated vaccine) for the prevention and control of sarcocystosis in animals. It's important to investigate the cross-reactivity between *Sarcocystis* and toxoplasma infection during diagnosis, in addition to developing of a sensitive diagnostic test to differentiate and determine the cross reaction.

Authors' Contribution

Study concept and design: S. N. H.

Acquisition of data: S. N. H and M. S. S.

Analysis and interpretation of data: S. N. H. and A. A. I.

Drafting of the manuscript: S. N. H.

Critical revision of the manuscript for important intellectual content: M. S. S.

Statistical analysis: S. N. H.

Administrative, technical, and material support: A. A. I. and M. S. S.

Ethics

The ethical permission for the experimental use of the animals were approved by ethical committee of college of veterinary medicine, University of Duhok (CVM2022120UD).

Conflict of interest

The authors of study declare that they have no competing interest.

Authors' Contributions

Shivan N. Hussein, Assel A. Ibrahim, and Mohammed S. Shukur conceived and planned the experiments. Shivan N. Hussein carried out the experiments. Assel A. Ibrahim, and Mohammed S. Shukur planned and carried out the simulations. Shivan N. Hussein contributed to sample preparation. Assel A. Ibrahim, and Mohammed S. Shukur contributed to the interpretation of the results. Mohammed S. Shukur took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Acknowledgements
The authors of this study acknowledge the college of veterinary medicine, University of Duhok, for their support.

Competing Interests

The authors declare that there is no conflict of interest.

TABLE 1. Interpretation of sodium sulfite precipitation test in different concentrations.

Immunoglobulin concentration	Sodium sulphite concentration		
	14%	16%	18%
<500mg/ dl	Negative	Negative	Positive
500-1500 mg/ dl	Negative	Positive	Positive
>1500 mg/dl	Positive	Positive	Positive

TABLE 2. TLC and DLC of immunized and non-immunized groups of rabbits

Experimental Rabbits	WBCs x 10^3/ µl	Differential Leukocyte Count ± S.E				
	Mean ± S.E	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil
Control	12 + 2.7	47.4 + 0.82	36.6 ± 0.75	12.2± 0.43	3.0 ± 0.52	0.75 ± 0.18
Immunized rabbits	18.8 + 2.0	50.17 ± 0.92	25.5± 1.43	8.00 ± 0.36	12.33 ±1.00	4.00 ± 0.36

TABLE 3.Phagocytic index of whole crude antigen in control and immunized rabbits (Mean± Standard error).

Experimental rabbits	Phagocytic cells ± S.E
Control	1.22 ± 0.56
Immunized	22.41 ± 1.8

TABLE 4. Measurement of skin fold thickness per millimetre in post-immunized rabbits after intradermal injection of *Sarcocyctis* whole crude antigen.

No. of Immunized Rabbits	Skin fold thickness (mm) at 0 time	Post inoculation of immunized rabbits / Skin fold thickness (mm) ± S.E									
		2 hr.	4 hr.	6 hr.	12 hr.	24 hr.	36 hr.	48 hr.	72 hr.	96 hr.	7 days
10	1.24 ± 0.03	1.55 ± 0.03	1.83 ±0.02	1.97 ± 0.03	2.36 ± 0.04	3.33 ± 0.07	4.41 ± 0.1	5.70 ± 0.11	3.53 ± 0.1	2.42 ± 0.22	1.28 ± 0.03

TABLE 5. Total serum protein, albumin and globulin of control and post-immunized rabbits after injection with whole crude antigen.

Experimental Rabbits	Total serum protein gm/ dl	Total serum albumin gm/dl	Total serum globulin gm/dl
	Mean ± S. E	Mean ± S. E	Mean ± S. E
Control	6.86 ± 0.22	4.82 ± 0.14	2.04 ± 0.11
Immunized	10.76 ± 0.26	6.40 ± 0.25	4.36 ± 0.10

TABLE 6. Identification of IgG and IgM antibodies (agglutination reaction) in immunized and control group of rabbits.

Experimental Rabbits	Total Number	Immunoglobulin M	Immunoglobulin G
Control	10	Negative	Negative
Immunized	10	Negative	Positive

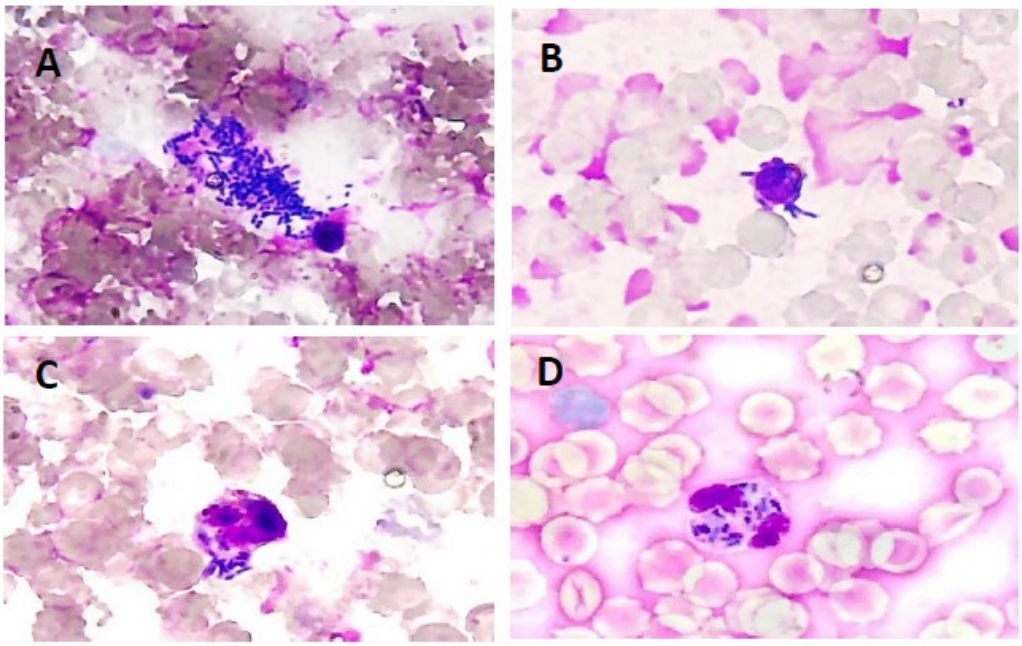

Fig.1. Stages of phagocytosis of post-immunized rabbits after staining by 5% of Giemsa stain (X 100). A/ Macrophage attacking S. aurous, B/ Macrophage attaching bacteria, C/ Macropgages begin engolfing the bacteria, D/ Phagocyosis of S. aurous by the macrophage.

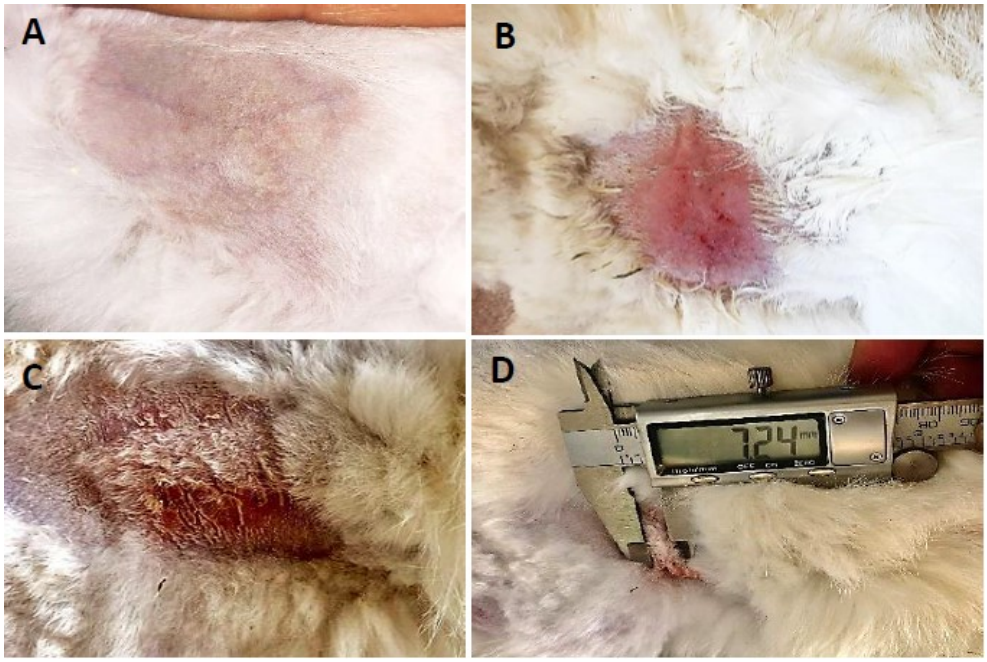

Fig.2. Intradermal injection of skin in post-immunized rabbits with whole crude antigen. A/skin fold thickness at zero time. B/skin inflammatory response after two hours of the antigen injection. C/skin fold thickness with edema, dandraf and cracks due to hypersensitivity reaction after 36-48 hours. D/skin fold thickness by electronic degital caliber (Vernier,mm).

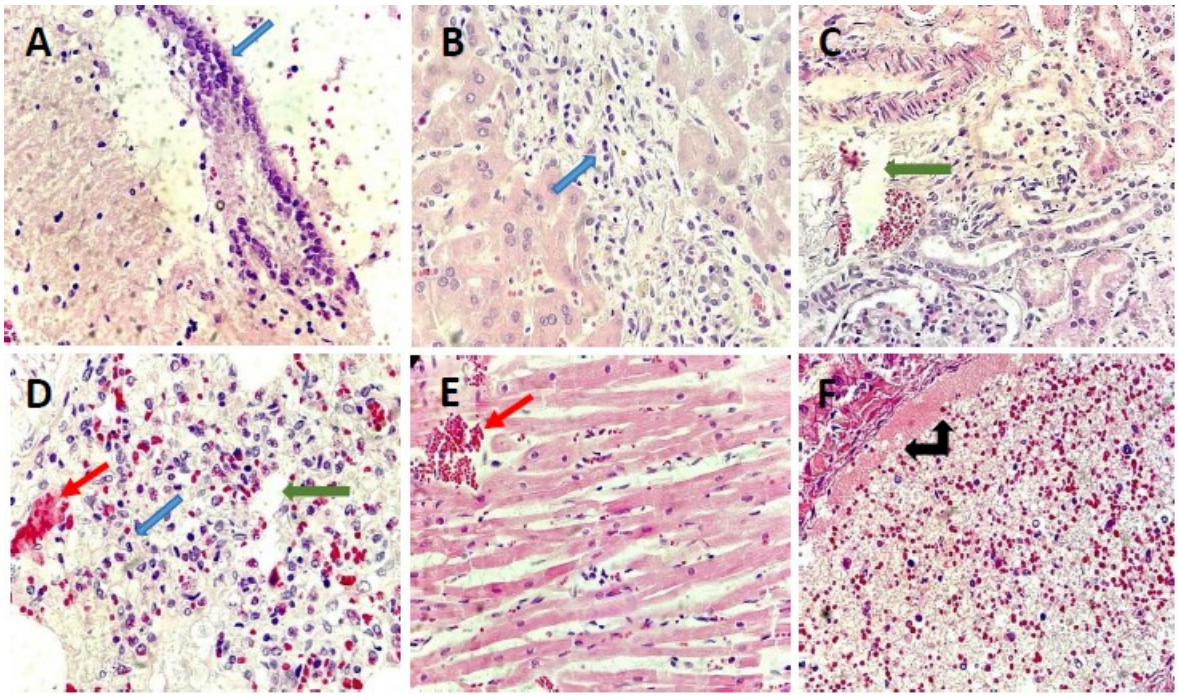

Fig.3. Histopathological images of cross sections in various organs of immunized rabbits. (A/brain, B/liver, C/kidney, D/lung, E/heart, F/blood vessels) showing sever infiltration of inflammatory cells (blue arrows), hemorrhage (red arrows), edema (green arrows) and congestion and thickness of blood vessels (black arrows), X40.

References

1. Buxton, D. Protozoan infections (*Toxoplasma gondii*, *Neospora caninum* and *Sarcocystis* pp.) in sheep and goats: Recent advances. *BMC Veterinary Research*, **29**, 289-310 (1998).
2. Dubey, J.P., Calero-Bernal, R., Rosenthal, B.M., Speer, C.A. and Fayer, R., Sarcocystosis of animals and humans. 2nd ed. Boca Raton: CRC Press, 350 (2015).
3. Dubey, J. and Lindsay, D.S. Neosporosis, toxoplasmosis, and sarcocystosis in ruminants. *Veterinary Clinics of North America: Food Animal Practice*, **22**(3),645-671 (2015).

4. Latif, B., Al-Delemi, J., Mohammed, B., Al-Bayati, S. and Al-Amiry, A. Prevalence of *Sarcocystis spp* in meat producing animals in Iraq. *Vet. J. Parasitol.,* **84**,85-90 (1999).

5. Barham, M., Stützer, H., Karanis, P., Latif. B. and Neiss, W. Seasonal variation in *Sarcocystis spp*. Infections in goats in northern Iraq. *Parasitology,* **130**(02),151-156 (2005).

6. Constable, P.D., Hinchcliff, K.W., Done, S. and Gruenberg, W. Diseases associated with protozoa. In: Veterinary Medicine: A Textbook of Diseases of cattle, horses, sheep, pigs, and goats. 11th ed., Saunders Elsevier; New York., 2134-2140 (2017).

7. Lindsay, D.S., Blagburn, B.L. and Braund, K.G., *Sarcocystis spp*. and Sarcocystosis. *Br. Med. J.,* **5**(3), 249-254 (1995).

8. Markus, M.B., Van Der Lugt, J.J. and Dubey, J.P. Sarcocystosis, In: Infectious Diseases of Livestock. Coetzer JAW, Tustin RC. (Eds.), 2nd Ed. vol. 1 Oxford University Press, Cape Town, Southern Africa., 360–375 (2004).

9. Hettiarachchi, D.C. and Rajapakse, R.P. Antigenic analysis of bovine *Sarcocystis spp*. in Sri Lanka. *Journal of the National Science Foundation of Sri Lanka.*, **36**(3),239-244 (2008).

10. El-Shanawany, E.E., Nassar, S.A. and Ata, E.B.,Detection of humoral and cellular immune responses in buffaloes naturally infected with sarcocystosis with risk factor assessment. *Acta Veterinaria.*, **69**(3),275-289 (2019).

11. Ananworanich, J. and Shearer, W.T. Delayed-type hypersensitivity skin testing. In: *Manual of Clinical Laboratory Immunology*, 5th ed. ASM Press. Rose, N.R., Hamilton, R.G., Detrick, B. USA., 212–219 (2002).

12. Hernández, A., Yager, J.A., Wilkie, B.N., Leslie, K.E. and Mallard B.A. Evaluation of bovine cutaneous delayed-type hypersensitivity (DTH) to various test antigens and a mitogen using several adjuvants. *Vet. Immunol. Immunopathol.,* **10**(104),45–58 (2005).

13. Tizard I.R. *Vaccincs and their production. Veterinary immunology an introduction*. 8th ed., Saunder Elsevier, 255-380 (2009).

14. Uggla, A. and Buxton, D. Immune responses against Toxoplasma and *Sarcocystis* infections in ruminants: diagnosis and prospects for vaccination. *Revue scientifique et technique (International Office of Epizootics),* **9**(2), 441-462 (1990).

15. Nada, M.S., Badawy, A.I. and Mona, A. Assessment of *Sarcocystis fusiformis*, Whole Cyst Extract Antigen from Buffaloes in Diagnosis of Cattle Sarcocystosis. *Zagazig Veterinary Journal*, **42**,131-136 (2014).

16. Tappe, D., Slesak, G., Perez-Giron, J.V., Schafer, J., Langeheinecke, A., Just-Nubling, G., Munoz-Fontela, C. and Pullmann, K. Human invasive muscular Sarcocystosis Induces Th2 cytokine polarization and biphasic cytokine changes, based on an investigation among travelers returning from Tioman Island, Malaysia. *Clin. Vaccine Immunol.,* **22**,674–677 (2015).

17. Hayashi, S., Chan, C.C., Gazzinelli, R. and Roberge, F.G. Contribution of nitric oxide to the host parasite equilibrium in toxoplasmosis. *J. Immunol.,* **156**,1476–1481 (1996).

18. Manicassamy, S. and Pulendran, B., Dendritic cell control of tolerogenic responses. *Immunol Rev.,* **241**,206–227 (2011).

19. Connick, K., Lalor, R., Murphy, A., O'Neill, S. and El Shanawany, E. *Sarcocystis fusiformis* whole cyst antigen activates pro-inflammatory dendritic cells. *Journal of Parasitic Diseases*, **44**,186-193 (2019).

20. Kulišić, Z., Tambur, Z., Maličević, Z., Aleksić-Bakrač, N. and Mišić, Z. White blood cell differential count in rabbits artificially infected with intestinal coccidia. *The Journal of Protozoology Research*, **16**(3-4), 42-50 (2006).

21. Thomas, M. and Schurr, K. The effect of Eimeria nieschulzi infection on leukocyte levels in the rat. *Journal of Eukaryotic Microbiology*, **25**,374-377 (2007).

22. Al-Samarraae, I.A, Al-Haddad, Z.A. and AL-Salmany, A.K. Study some immunological parameters in rabbits immunized with *cryptococcus neoformans*. *Iraqi Journal of Science*, **56**,3083-3087 (2015).

23. Rechav, Y., Magano, S. and Fielden, L. The effects of tick numbers and intervals between infestations on the resistance acquired by guinea-pigs to adults of Rhipicephalus evertsi evertsi (Acari: Ixodidae). *Experimental and applied Acarology,* **18**,735-745 (1994).

24. Banerjee, D.P., Momin, R.R. and Samantaray, S. Immunization of cattle (Bos indicus X Bos taurus) *against Hyalomma anatolicum anatolicum* using antigens derived from tick salivary gland extracts. *International journal for parasitology,* **20**,969-972 (1990).

25. Moshaveri-nia, A., Naiminik, M.H., Razmi, G. and Hashemitabar, G. Immunization of rabbits against *Hyalomma anatolicum anatolicum* using larval and nymphal extracts. *Iranian Journal of Veterinary Research*, **9**,208-212 (2008).

26. Salih, B.H. Study on some epidemiological factors of hard tick (Ixodidae) in sheep in Sulaimani province/Kurdistan region Iraq with trial to immunize rabbits against with larval extract of *Hyalomma a. anatolicum*. PhD thesis. UOS, College of agriculture. (2011).

27. Platt, N. and Fineran, P. Measuring the phagocytic activity of cells. *Methods in cell biology,* **126**,287-304 (2015).

28. Al-Hyali, N., Kennany, E. and Altaee, A. Effect of lysate of *Sarcocystis gigantea* in rats. *Iraqi Journal of Veterinary Sciences,* **25**,81-85 (2011).

29. Gozalom, A.S., Montall, R.J., Claire, M.S., Barr, B., Rejmanek, D., and Ward, J.M., Chronic polymyositis associated with disseminated Sarcocystosis in a Captive-born Rhesus Macaque. Vet Pathol., (44):695-699 (2007).

30. Origlia, J., Florencia, U., Piscopo, M. and Moré, G. Fatal sarcocystosis in psittacine birds from Argentina. *Parasitology Research,* **12**,1-7 (2021).

31. Zangana, I.K. and Hussein, S.N. Prevalence of Sarcocystis species (*Sarcocystis ovicanis* and *Sarcocystis capricanis*) in tongue muscle of sheep and goats in Duhok province, Kurdistan Region, North Iraq. *Sci. J. Koya Univ.,* **5**,36-40 (2017).

32. Daugschies, A., Hintz, J., Henning, M. and Rommel, M. Growth performance, meat quality and activites of glycolytic enzymes in the blood and muscle tissue of calves infected with *Sarcocystis cruzi. Vet. Parasitol.,* **88**,7-16 (2000).

33. Coles, E.H. Veterinary Clinical Pathology. 4th ed. Saunders Company, London, UK., 374-7 (1986).

34. Thrall, M.A., Weiser, G., Allison, R.W., and Campbell, T.W., Veterinary hematology and clinical chemistry. John Wiley and Sons, 463-472 (2012).

35. Oddera, S., Silvestri, M., Sacco, O., Eftimiadi, C. and Rossi, G.A. N-acetylcysteine enhances in vitro the intracellular killing of Staphylococcus aureus by human alveolar macrophages and blood polymorphonuclear leukocytes and partially protects phagocytes from self-killing. *The Journal of Laboratory and Clinical Medicine*, **124**(2), 293-301 (1994).

36. Bancroft, J.D. and Layton, C. The hematoxylins and eosin. In: Bancroft's theory and practice of histological techniques. 8th ed., Suvarna, K. John D. Bancroft, J.D., Stevens, A. Elsevier, 127-129 (2019).

37. Morsy, T., Abdel, M.M., Salama, M.M. and Hamdi, K.N. Assessment of intact Sarcocystis cystozoites as an ELISA antigen. *J. Egypt. Soc. Parasitol.,* 24,85-91 (1994).

التحصين التجريبي للأرانب ضد المستضد الخام المعزول من الحويصلات الكبيرة لداء الحويصلات الصنوبرية في الضأن

شيفان حسين، أسيل إبراهيم ومحمد شكر

قسم الطب والجراحة - كلية الطب البيطري - جامعة دهوك - إقليم كردستان - العراق.

المستخلص

يعد داء الحويصلات الصنوبرية من الامراض الطفيلية المثبطة للمناعة والتي يسببها طفيلي من جنس *Sarcocystis* ويصيب انواع مختلفة من الحيوانات والانسان. استهدفت الدراسة الحالية الى معرفة الاستجابة المناعية (الخلوية والمختلطة) للأرانب المحصنة تجريبيا ضد مستضد الحويصلات الكبيرة الضأني (*S. gigantea*). أظهرت نتائج الفحوصات الدموية للأرانب المحصنة زيادة معنوية ($P<0.05$) في العدد الكلي لكريات الدم البيض (18.8 ± 2.0 X10^3 / µl) ومن ضمنها العدلة والحمضة والقاعدية مقارنة بمجموعة الارانب غير المحصنة (مجموعة السيطرة). تم ايضا قياس الفاعلية البلعمية، اذ لوحظ زيادة معنوية ($P<0.05$) للخلايا البلعمية في التهام العامل المخمج في الارانب المحصنة (22.41 ± 1.8) مقارنة بمجموعة السيطرة (1.22 ± 0.56). وفي اختبار تفاعل الحساسية من النوع المتأخر للجلد لوحظ تثخن طية الجلد اذ وصلت الى اقصى سمك خلال 48 ساعة (5.70 ± 0.11 mm) عند حقن الجلد بالعامل المخمج، بعدها لوحظ التراجع في سمك الجلد (1.28 ± 0.03 mm) بعد 7 ايام من الحقن. كما لوحظ زيادة الالبومين الكلي والكلوبيولين في امصال الارانب المحصنة (4.36 ± 0.10 g/dl and 6.40 ± 0.25 g/dl) مقارنة بمجموعة السيطرة الغير محصنة (2.04 ± 0.11 g/dl & 4.82 ± 0.14 g/dl) على التوالي. ولوحظ ايضا ارتفاع القيمة الكمية للكلوبين المناعي في الحيوانات المحصنة باستخدام اختبار تفاعل كلوترالديهايد (>600mg/dl) واختبار ترسيب الصوديوم سلفيت اذ بلغت (>1500gm/dl). اما في الفحوصات المصلية لتحديد الكلوبيولين المناعي (IgG) وجد ارتفاعا معنويا (100%) في جميع الارانب المحصنة مقارنة بمجموعة السيطرة باستخدام اختبار التلازن المباشر. وعند اجراء الفحص النسيجي للأرانب المحصنة لوحظ ارتشاح الخلايا الالتهابية والقاعدية والحمضة مع وجود الاحتقان والوذمة والنزف على مختلف الاعضاء الداخلية.

مفاتيح الكلمات: الحويصلات الصنوبرية ، الارانب ، المناعة الخلوية والمختلطة.