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Immunological alterations of Lactoferrin, Silver Nanoparticles, and Nitazoxanide therapy in Immunocompromised Murine Cryptosporidiosis

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ABSTRACT

Worldwide, the protozoan parasite *Cryptosporidium* has emerged as a significant cause of diarrheal diseases, posing risks to both immunocompromised individuals and young children. Generally, cryptosporidiosis is not severe in healthy people. Still, it can result in life-threatening complications for those with weakened immune systems, including individuals with HIV/AIDS, cancer patients, organ transplant recipients on immunosuppressive therapies, and those with genetic immune disorders. Nitazoxanide, an antiparasitic medication, shortens the duration of diarrhea and decreases the release of *Cryptosporidium* oocysts. This study aimed to assess the impacts of silver nanoparticles (AgNPs) and lactoferrin (LF), both individually and in comparison, to nitazoxanide (NTZ), on immune changes in immunosuppressed mice. Seventy male Swiss albino mice were inoculated with $3x10^3$ oocysts each and were divided into seven main experimental and control groups. The experimental groups orally received NZ (200 mg/kg), LF (2g/Kg), silver nanoparticles (2 mg/Kg), and LF loaded with silver nanoparticles. Immunological alteration in immunocompromised mice was assessed by measuring IgG, IgM, and serum levels of IFN-γ, IL-4, IL-10, and IL-17. Infected mice treated with LF and AgNPs showed statistically significant reductions in mean serum levels of IgG and IgM when compared to those receiving NTZ. The LF group exhibited the most substantial decline in IL-4, IFNγ, and IL-17 serum concentrations. Levels of IL-10 were reduced in the group treated with LF, AgNPs, and NTZ.

1. Introduction

Numerous vertebrate hosts can develop gastroenteritis due to the protozoan parasite *Cryptosporidium*, which holds medical and veterinary significance. Both immunocompromised and immunocompetent individuals can contract the intestinal parasitic infection *Cryptosporidium parvum* (*C. parvum*). Various Cryptosporidium species are responsible for the global zoonotic disease known as cryptosporidiosis (Moawad et al., 2021). Today, cryptosporidiosis frequently causes human diarrhea, enterocolitis, and cholangiopathy (Checkley et al., 2015). This infection typically leads to a short illness characterized by watery diarrhea, malnutrition, and weight loss in healthy children, adults, and young animals. In immunocompromised humans and animals, however, the infection can be chronic and potentially fatal (Chalmers and Davies, 2010). One of the many antiviral medications being developed to treat flu and other viral respiratory illnesses is nitazoxanide, which is the only drug permitted by the FDA for the treatment of Cryptosporidium (Checkley et al., 2015).

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Numerous studies conducted in recent years have demonstrated the detrimental effects of silver nanoparticles on fungi and bacteria (Greulich et al., 2012; Johnston et al., 2010). This negative impact is frequently associated with oxidative stress and ion release (Johnston et al., 2010). Furthermore, recent findings have shown that lactoferrin possesses antifungal, antibacterial, antiviral, anticancer, antioxidant, and anti-inflammatory properties (Konuspayeva et al., 2005). It has been demonstrated to regulate numerous immunological processes, including cytokine release, natural killer (NK) cell activity, and lymphocyte stimulation and proliferation (Gauthier et al., 2006). Compared to other treatments studied for immunocompromised murine cryptosporidiosis, Ahmed B. Darwish et al. (2025) showed that the combination of NTZ, lactoferrin, and silver nanoparticles exhibited the highest rate of decrease in *Cryptosporidium* oocyst numbers. The current study evaluated the effectiveness of lactoferrin, silver nanoparticles, and their combination with NTZ on the immunological properties of artificially infected immunosuppressed mice.

Materials and Methods

1. Experimental animals:

The research was performed in Giza, Egypt, in the Parasitology Department of the Theodor Bilharz Research

regional geological knowledge but also has implications Institute (TBRI). Seventy male Swiss albino mice (CD1), ranging between eighteen and twenty grams, and six weeks of age, were used. In addition to receiving enough food and water, they were housed in boxes with pierced coverings that allowed for sufficient airflow and were cleaned daily. At 21 degrees Celsius, mice were housed in a room with ventilation for the duration of the research. Specialized containers for water and food with 24% proteins, 4% fats, and about 4-5% fibers were distributed. Furthermore, infections were stayed away from. To make sure the mice had acclimated to their new environment, they were housed in this habitat for seven days before the tests. All experimental procedures were approved by the Institutional Animal Care and Use Committee (SU-IACUC), Suez University, Egypt (261223), and performed under international guidelines for the care and use of research animals.

2. Materials:

a. Nitazoxanide (100 mg/5ml suspension) produced and supplied by [Medizen Pharmaceutical Industries for Utopia Pharmaceuticals, Egypt] was used. Using an esophageal tube, an oral dosage of nitazoxanide suspension (200 mg/kg body weight) was administered every day. The doses were administered in grams according to the mice's bodyweight (each of the mice averages from eighteen to twenty grams), starting 10 days post-infection and continued for five days (Abdou et al., 2013).

b. Dexamethasone was produced and supplied by [Kahira Pharmaceuticals and Chemical Industries Company, Shoubra-Cairo, Egypt]. For fourteen days, mice will be administered an oral dose of 0.25 mg/kg of dexamethasone. (Abdou et al., 2013).

c. Nano silver is made up of microscopic silver particles with sizes ranging in length from 10 to 30 nm (2 mg/kg mice) (October City, Nanotech Egypt). The most prevalent method for synthesizing stable, suspended colloidal particles of silver nanoparticles in organic or water-based solvents is chemical reduction. Citric acid, elemental hydrogen, and borohydride are common reducing agents. (Said et al., 2012).

d. Lactoferrin (1.8 ~ 2 g/kg): Lactoferrin was created from milk acid whey. In order to conduct hydrophobic interaction chromatography, proteins from ammonium sulfate precipitate were adsorbed to the medium. (Santos et al., 2011).

3. The parasite:

Feces samples were obtained from mice diseased with *Cryptosporidium Parvum*, which is obtained from the feces of diarrheal disease cows in Cairo University's Faculty of Veterinary Medicine, veterinarian clinics. Stool samples from infected cows were gathered in stool sampling jars. Maintaining the samples clean of urine and water has been considered. The stool samples were then sent to Theodor Bilharz Research Institute's (TBRI) parasitology division.

4. Immunosuppression:

Prior to infection with Cryptosporidium oocysts, the immune system underwent suppression for two weeks by oral administration of 0.25 mg/kg of the artificial corticosteroid dexamethasone (Dexazone) using an esophageal tube (Rehg et al., 1988; Abdou et al., 2013). Throughout this study, the identical dosage of dexamethasone was administered to each mouse.

Experimental design:

Seventy male Swiss albino mice (CD1), aged six weeks and weighing between 18 and 20 grams, were used. They were separated into 7 major groups, and the period of treatment for each experimental group is five successive days.

1) Ten immunosuppressed control mice were not infected with the parasite.
2) Ten immunosuppressed control mice were infected with the parasite.
3) Ten infected mice immunosuppressed received nitazoxanide (200 mg/Kg).
4) Ten immunosuppressed mice received lactoferrin (1.8 ~ 2 g/kg).
5) Ten immunosuppressed mice received silver nanoparticles (2 mg/Kg).
6) Ten infected immunosuppressed mice received lactoferrin loaded on nano silver.
7) Ten infected immunosuppressed mice received lactoferrin loaded with nano silver and also treated with nitazoxanide.

5. Mice inoculation:

To start the infection, every single mouse was given about 3×10^3 *Cryptosporidium* oocysts (Benamrouz *et al.,* 2012).

6. Animal scarification:

The anesthetic-anticoagulant solution (100 units/ml heparin and 500 mg/kg thiopental) was injected intraperitoneally into the mice. Then all mice were immediately beheaded to end their lives after 23 days of infection.

7. Immunological Assessment:

a. Determination of IgM and IgG antibodies

Sera were stored at room temperature after being removed from a freezer at -20 degrees Celsius. The current research used a sandwich enzyme-linked immunosorbent assay (ELISA) to measure the levels of mouse Cryptosporidiosis Antibody (IgM and IgG), C-IgM, and C-IgG in samples using the Qualitative Mouse Cryptosporidiosis Antibody (IgM and IgG) (C-IgM) and (C-IgG) kit (Cat. No: MBS9310461).

b. Determination of Cytokine Serum Levels

The serum was taken out of a freezer set at -20 degrees Celsius and left to melt at room temperature. Using a high-sensitivity sandwich ELISA kit (BOSTER BIOLOGICAL TECHNOLOGY Co., Ltd.), mouse serum levels of IFN-γ, IL-4, IL-10, and IL-17 were quantitatively determined. (Engvall and Perlmann, 1971).

8. Statistical Analysis:

The data was represented utilizing standard deviation (SD) and mean values in SPSS (version 16.0). Categorical variables are presented as percentages and frequency values, while continuous variables are represented. The two groups were compared using a student's t-test. Input from the associated tables was used to evaluate the extent of significance (P-value). The following is how the significance level was stated:

p>0.05 non-significant, p< 0.05 Significant, P<0.01 Highly significant, p< 0.001 Very highly significant.

Results

Assessment of IgM and IgG:

Table (1) illustrates the IgM levels in immunocompromised groups. In the infected (positive control) group, the IgM level was (0.72 ± 0.12), whereas in the immunocompromised (negative control) group, it was (0.22 ± 0.03). Significant increase (p<0.05) in the group that received lactoferrin was detected (0.8±0.09). IgM levels were higher in the group that received Lactoferrin, Silver Nano, and Nitazoxanide (0.94±0.04) than in the group that received Nitazoxanide (0.52±0.08).

Table (1): IgM and IgG levels in the treated immunosuppressed groups infected with *Cryptosporidium*.

Groups	IgM	IgG
Immunosuppressed control	0.22±0.03	0.43±0.09
Immunosuppressed infected control	0.72±0.12* (9.90)	1.5±0.2* (11.95)
Immunosuppressed (LF)	0.8±0.09* (14.98)	1.26±0.12* (13.55)
Immunosuppressed (AgNps)	0.67±0.08* (12.9)	1.07±0.12* (10.45)
Immunosuppressed infected (NTZ)	0.52±0.08* (8.60)	0.83±0.10* (7.28)
Immunosuppressed infected (LF+AgNps)	0.34±0.04* (5.88)	0.63±0.02* (5.31)
Immunosuppressed infected (LF +NTZ+AgNps)	0.94±0.04* (35.27)	1.18±0.06* (16.98)

Data was expressed as Mean ± standard deviation. P>0.05 non-significant, P< 0.05 Significant, P<0.01 highly significant, P < 0.001 very highly significant.
The unit of IgM and IgG is the Optical density unit (ODU).

Table (1) illustrates the IgG levels in various immunocompromised treated groups. The IgG level in the Immunosuppressed (control) group was (0.43±0.09), whereas the infected (positive control) group had a level of (1.5±0.2). The Lactoferrin-treated group had a very statistically significant (1.26±0.12). IgG levels were greater in the group that received Lactoferrin, Silver Nano, and Nitazoxanide (1.18±0.06) than in the group that received Nitazoxanide (0.83±0.10).

Table (2) illustrates the IL-4 levels in immunocompromised treated groups. IL-4 levels were (8.65±0.86) in the infected (positive control) group and (6.08±0.47) in the (Immunosuppressed control) group. The groups that received silver nanoparticles (16.9±0.36) and lactoferrin (5.97±0.33) had reductions in IL-4 levels than the group that received nitazoxanide (26±0.45).

IL-10 levels were (79.57±4.69) in the (Immunosuppressed control) group and (66.9±3.17) in the infected (positive control) group. The group that received Nitazoxanide, Silver Nanoparticles, and Lactoferrin showed a reduction in IL-10 levels (16.62±0.82) than the group that received Nitazoxanide (20.92±0.72).

The (Immunosuppressed control) group had an IFN-γ level of (12.71±1.22), whereas the infected (positive control) group had a level of (15.52±0.49). The IFN-γ level increased more in the Lactoferrin+ AgNPs group (30.8±0.98) than in the Nitazoxanide group (24.03±0.29).

The (Immunosuppressed control) group had an IL-17 level of (23.77±1.39), whereas the infected (positive control) group had a level of (33.95±1.76). The IL-17 levels increased in the Lactoferrin+AgNPs group (41.47±1.13) than in the Nitazoxanide group (39.55±0.74). During the infection, immunological evaluation of immunosuppressed mice groups revealed that all circulating cytokines (IFN-γ, IL-4, IL-10, and IL-17) increased in comparison to the control negative group. Mice treated with lactoferrin, Silver Nano, and Nitazoxanide showed highly significant statistical changes (p<0.05) in IL-4, IFN-γ & IL-17 in comparison to the as compared to NTZ treatment.

Table (2): IL-4, IL-10, INF-γ, and IL-17 levels in treated immunosuppressed groups infected with *Cryptosporidium*.

Groups	IL-4 (Pg/ml)	IL-10 (Pg/ml)	IFN-γ (Pg/ml)	IL-17 (mg/ml)
Immunosuppressed control	6.08±0.47	79.57±4.69	12.71±1.22	23.77±1.39
Immunosuppressed infected control	8.65±0.86* (6.31)	66.9±3.17* (5.48)	15.52±0.49* (5.24)	33.95±1.76* (11.12)
Immunosuppressed (LF)	5.97±0.33 (0.47)	73.87±1.63* (2.81)	15.22±1.43* (3.27)	30.75±1.29* (9.02)
Immunosuppressed (AgNps)	16.9±0.36* (44.77)	23.12±1.08* (28.73)	18.23±0.49* (10.28)	35.93±0.60* (19.67)
Immunosuppressed infected (NTZ)	26±0.45* (74.99)	20.92±0.72* (30.28)	24.03±0.29* (22.11)	39.55±0.74* (24.55)
Immunosuppressed infected (LF + AgNps)	19.18±0.66* (39.6)	40.42±3.18* (16.92)	30.8±0.98* (28.32)	41.47±1.13* (24.2)
Immunosuppressed infected (LF + NTZ+ AgNps)	30.6±0.88* (60.2)	16.62±0.82* (32.39)	41.28±0.92* (45.8)	50.52±0.88* (39.83)

Data was expressed as Mean ± standard deviation. P>0.05 non-significant, P< 0.05 Significant, P<0.01 highly significant, P < 0.001 very highly significant.

Discussion:

An obligatory intestinal parasite, Cryptosporidium attacks the epithelial lining of the luminal surfaces of the respiratory and gastrointestinal systems in a variety of hosts (Hailu *et al.*, 2020). Humans and a variety of domestic and wild animals can contract the parasitic protozoan *Cryptosporidium parvum* (*C. parvum*) (Ahmad *and* Karanis 2020). Treatments for *Cryptosporidium* have historically been insufficient and unpredictable; while some antiparasitic medications, like paromomycin and azithromycin, are occasionally employed, their effects are typically transient, and relapses can occasionally occur (Chen et *al.*, 2016).In the Nile Valley of Egypt, Rossignol et al. (2001) found that after four days of full-course nitazoxanide treatment, a greater percentage of non-immunodeficient children and adults with cryptosporidial diarrhea had decreased (80%).

In terms of the immunological response, after infection, serum IgM and IgG, which are specific antibodies of *Cryptosporidium* sp., are produced. But these antibodies alone cannot stop or manage a *Cryptosporidium* infection (Kassa et al., 1991), and they are not necessary for the parasites' recovery and removal (Mead, 2014). Gut-associated lymphoid tissue (GALT), on the other hand, is essential for the immunological mucosal response towards *Cryptosporidium* (Patricio et al., 2011). Numerous investigations have revealed that weaned and newborn mice (which have weakened immune systems) are particularly at risk for *Cryptosporidium* infection, which is indicated by a reduction in mucosal IFN-γ release (Costa et al., 2011). According to certain research, continuous exposure to the parasite's immunogenic antigens by inhalation previous to infection can raise IFN-γ production and hence lessen *Cryptosporidium* susceptibility (Roche et al., 2013; Manque et al., 2012).

This research has shown that mice that received lactoferrin loaded onto silver nanoparticles had lower IL-10 levels (40.42+3.18), with the reduction being greater than that of mice treated with a positive infected control. This result is consistent with the work of Halder et al. (2018), who discovered that lactoferrin-modified betulinic acid-loaded nanoparticles reduced IL-10 in *Leishmania donovani*-infected macrophages.

These findings also concurred with those of Semedo et al. (2022), who discovered that AgNp-Bio decreases IL-10 and increases pro-inflammatory cytokine production of TNF-α and IL-6 in TM3 cells infected with *T. gondii*.

These findings imply that AgNp-Bio might cause Leydig cells to produce a pro-inflammatory response, which would aid in the removal of intracellular parasites. A regulating cytokine called IL-10 inhibits the immunological and microbicidal reactions of phagocytic cells. It is crucial in controlling how susceptible cells are to different infections and how quickly such diseases develop (Mukherjee et al., 2012). Additionally, by inhibiting the local immune response, IL-10 promotes *T. gondii's* longevity in the brain (Sarciron and Gherardi, 2000).

Specific immune cells, including lymphocytes and NKs, respond when LF's immunomodulatory function is stimulated (Sienkiewicz et al., 2022). These interactions promote processes like migration, proliferation, and generation of cytokines. Therefore, in some viral infections, LF can influence immune systems by displaying pro- and anti-inflammatory actions (Bukowska-Ośko et al., 2021). In many experimental animals, especially mice, the antibacterial properties of LF have been shown in vivo when administered parenterally (Drago, 2007). Furthermore, research indicates that in normal human volunteers, bovine LF strengthens the antioxidant level and the immune function (Legrand and Mazurier, 2010). Additionally, because the glycosylation sites of the LF protein reveal its outermost surface and enable it to remain in interactions with microbes, many in vitro experiments have demonstrated that the structure of LF plays a crucial role in its antimicrobial effects (e.g., its interactions with viruses and bacterial toxins) (Latorre et al., 2010).

The processes behind LF's antiparasitic action are complicated (Kaufman, 2009). Immune status against *Plasmodium falciparum*, *Toxoplasma gondii*, *Eimeria stiedai*, *Trypanosoma cruzi*, *Trypanosoma brucei*, *Trichomonas faetus*, and *Entamoeba histolytica* could be due to the effects of LF.

When treating *C. parvum*, antibiotics like nitazoxanide are the initial stage of treatment; however, the results are unsatisfactory, and the medication's mechanism of action is also unclear (Rossignol et al., 2006). Antiparasitic action towards *C. parvum* sporozoites has been previously proven by LF protein and its peptides, specifically LF hydrolysate (LFH), with LFH achieving the greatest suppression rate. When combined with the *Cryptosporidium* monoclonal antibody (3E2 MAb), this peptide also demonstrated antiparasitic efficacy. The parasite's surface glycoprotein ligand (CSL) and apical complex, which aid in the parasite's attachment to the host cell, are the targets of the 3E2 Mab. Mab and the LF peptide worked together to reduce the vitality and infectious potential of sporozoites (Carryn et al., 2012). The function of LFH in the effectiveness of *Cryptosporidium* excystation was also investigated in earlier research. Although there was no impact on the excystation phase of the oocyst wall, the researchers demonstrated a potential decrease in sporozoite vitality and infectivity. These investigations revealed that although the LF protein or its peptides are resistant to the oocyst's robust wall, they can internalize through the endocytic route and prevent the parasite's free forms from surviving (Paredes et al., 2017). In comparison to the other treatments evaluated, Darwish et al. (2025) demonstrated that the combination of NTZ, Lactoferrin, and Silver Nanoparticles exhibited the highest rate of decrease in Cryptosporidium oocyst number. Also, Aly et al. (2025) found that LF or lactoferrin nanocapsule (LF-NC) was found to exert a potent immunomodulatory effect on infected mice as well as minimize pathological lesions.

Conclusion

In comparison to the other treatments studied, the current study showed that the combination of NTZ+Lactoferrin, loaded on Silver Nanoparticles, produced the best immunological outcomes in cases of *Cryptosporidium* infection. To treat immunocompromised mouse cryptosporidiosis, lactoferrin loaded on silver nanoparticles could be utilized as adjuvants in combination with other comparable drugs.

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