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Irradiated oocysts in combination with inulin adjuvant-induced potent immune responses against *Eimeria tenella* infection in broiler chickens

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Abstract

Coccidiosis is the leading parasitic disease in poultry. One of the most critical *Eimeria* species, *Eimeria tenella*, lives inside the cecal epithelial cells and induces bloody coccidiosis. The present study evaluated the effect of radiation-attenuated *E. tenella* oocytes mixed with inulin adjuvant on broiler chicken. Initially, the effect of irradiation on oocyst attenuation was confirmed. Then, one-day-old broilers (n = 90) were divided into nine groups on seven days of age as follow: Group 1 (400 attenuated oocysts + 1.00 mg of adjuvant), group 2 (400 attenuated oocysts + 0.50 mg adjuvant), group 3 (200 attenuated oocysts + 1.00 mg of adjuvant), group 4 (200 attenuated oocysts + 0.50 mg adjuvant), group 5 (1.00 mg adjuvant), group 6 (400 attenuated oocysts), group 7 (commercial vaccine), group 8 (negative control) and group 9 (blank). On day 21, we performed a challenge with *E. tenella* oocytes and investigated oocyst output and average weekly weight throughout the study. At the end of the study, we evaluated macroscopic lesion, histology, cytokine level and leukogram status. The results showed a statistically significant difference among groups. Furthermore, the optimal dose was 400 irradiated oocysts and 1.00 mg of inulin. Moreover, an X-ray could reduce the virulence of *E. tenella* oocytes. Inulin alone or combined with attenuated oocysts showed an acceptable effect on evaluated parameters.

Introduction

Parasitic diseases are the most critical factor preventing the expansion of agriculture in developing countries.[1] An extensive range of protozoan parasites infect the digestive tract of chickens, of which the protozoa of the *Eimeria* species are the most significant.[2] *Eimeria tenella* is the most prevalent highly pathogenic species[3] and is responsible for bloody coccidiosis[4] which results in substantial economic losses.[5]

Coccidiostat drugs, meticulous management measures and vaccination are among the control measures currently employed against this parasite.[6] To date, researchers have developed various vaccines against coccidiosis.[7] Live vaccines,[8] *Eimeria* precocious lines,[9,10] subunit vaccines,[10-12] and DNA vaccines[12] have been extensively researched. The only live vaccine that benefits from adjuvant addition is Evalon® (Hipra, Girona, Spain), which is extremely effective.[13] Since vaccination with live wild-type parasites or attenuated parasites has proven effective[14] and genetically engineered vaccines were ineffective, there is a desire for vaccine development and application of live parasite-based vaccines.[15]

Attenuated vaccines provide superior protection than other types of vaccines.[16] Generally, live parasites can be attenuated by irradiation, chemicals, repeated passage,[17] heat and genetic manipulation.[18] Radiation technology releases or transfers energy through waves (ionizing radiation) or electron particles (nonionizing radiation). This technology has been utilized in developing vaccines for humans and animals and as a new method to combat viral, bacterial, and parasitic diseases in research.[19] This method has advantages over other methods. Radiation attenuation does not destroy the pathogens protein structures, allowing it to preserve its multiplication capacity and metabolic activity.[20]

Adjuvants have been used over 70 years to enhance the immune response [21,22] against antigens.[22] Utilizing adjuvants[23] and immunostimulants[24] is a method for enhancing the efficacy[23] and duration of the protective

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effect.[25] The ineffectiveness of current vaccines is due to the lack of a suitable adjuvant to carry the vaccine. Inulin is a plant polysaccharide that positively affects poultry performance[26] and domestic animals' immunity.[27] As a plant product, inulin is biochemically neutral and nontoxic.[28] It is deemed a safe substance and increases both humoral and cellular immunity.[29]

Various studies have employed different types of nanoparticles[30] and microparticles[29] of inulin to deliver drugs to mucous membranes. Both microparticles and nanoparticles are very effective vaccine delivery systems,[31] and there is no clear evidence that one is preferable to the other.[32] When inulin combines with a liquid, it forms a submicron gel network of insoluble particles that can absorb large amounts of liquid like a sponge.[33] Inulin is an ideal drug delivery vehicle for the colon because it reaches the organ unreacted and releases the drug directly into the same organ.[28]

Only one study investigated the possibility of reducing the virulence of *E. tenella* oocysts via irradiation.[29] In this study, researchers used electron radiation to attenuate *E. tenella* oocysts. Electron beam-generating devices are unavailable on the market and are custom-made by manufacturers. Consequently, in the present study, we used X-ray irradiation to attenuate the oocytes and recorded the outcomes of clinical symptoms and oocyte counts. Positive results prompted us to evaluate the effectiveness of the X-ray method combined with inulin.

Materials and Methods

Oocysts preparation. Oocysts were collected from the caeca of naturally infected chickens and were identified (*E. tenella* oocysts) as previously described[34-36] and subsequently counted. For sporulation of oocysts, cotton soaked in a 2.00% potassium dichromate (Merck KGaA, Darmstadt, Germany) solution was placed next to the oocysts in a Petri dish with fresh air for 48 hr. Then, the oocysts were attenuated with X-rays (100 kV 5.00 mA per sec; Ecoray, Seoul, South Korea) according to the method described by Thabet *et al*.[29] Briefly, 230 µL of the pathogen suspension was placed in the center of a sterile 100 mm Petri dish covered with an oriented polypropylene (OPP) film, forming a liquid film with 100 µm. Afterward, the samples were irradiated using 1040HF X-ray machine (Ecoray), an X-ray device developed for veterinary medicine. The distance between the sample and the energy source was 50.00 cm at a dose of 0.30 mGy.

Attenuation evaluation. The attenuation of *E. tenella* was evaluated based on the number of excreted oocysts. To this end, we designed three groups of ten 21-day-old chickens. Then, we fed the first and second groups of 1,200 irradiated oocysts and 1,200 non-irradiated oocysts, respectively. The third group was considered the control group. Excretion of oocysts in all groups was examined from the sixth day post infection (PI) for five consequent days (up to 10th day PI), the severity of lesions in cecums was evaluated in all three groups. At this stage, according to the location of the lesions (cecums) and the severity of the lesions, the diagnosis of *E. tenella* oocysts was reconfirmed.[36-38]

Adjuvant preparation. To prepare the adjuvant, we dissolved 2.00 g of inulin (Beneo, Uzhhorod, Ukraine) in 40.00 mL ammonia solution (Merck KGaA) at 75.00 ˚C and incubated the mixture at – 20.00 ˚C for 24 hr. We then added 500 µL of chloroform (Merck KGaA) to the solution and incubated it at room temperature for 24 hr. The gamma-inulin precipitate was separated using a 3,200 *g* centrifuge for 10 min. Gamma-inulin is detected by the fact that it is insoluble at 37.00 ˚C.[30] At the second step chickens were immunized and challenged. Then the parameters were measured.

Chicken. All experimental procedures and chicken handling were carried out according to the standard animal experimentation protocols and this study by the Veterinary Ethnics Committee of Urmia University (IR-UU-AEC-3/7).

Immunization. We acquired day-old male and female Ross 308 broiler chickens from a reputable facility, prepared clean water and formulated feed for the experimental setup. On seven days of age, we divided them into nine groups as follow: Group 1: Inoculation of 400 attenuated oocysts + 1.00 mg adjuvant, Group 2: Inoculation of 400 attenuated oocysts + 0.50 mg adjuvant, Group 3: Inoculation of 200 attenuated oocysts + 1.00 mg of adjuvant, Group 4: Inoculation of 200 attenuated oocysts + 0.50 mg adjuvant, Group 5: Inoculation of 1.00 mg adjuvant, Group 6: Inoculation of 400 attenuated oocysts, Group 7: Inoculation of commercially available Livacox-T® (Biopharm, Prague, Czech Republic) vaccine as a positive control, Group 8: Negative control (no immunization - challenged), and Group 9: Blank (no immunization - no challenge).

Challenge. On day 21 after hatching (2 weeks after immunization), we challenged each chicken with 1,000 to 1,200 oocysts of *E. tenella* as previously described.[36]

Cytokine release. Cellular immunity was assessed 14 days after the challenge by measuring interferon gamma (IFN-γ) and inteulokin-4 (IL-4) by polymerase chain reaction (PCR) in chicken spleen cells. This activity was accomplished using a Rotor-Gene® device (Qiagen, St. Louis, USA) that employed the standard PCR technique. Briefly, we prepared the tissue sample and mixed it with phosphate buffer solution which was followed by centrifuge, precipitation and RNA extraction. Then master mix was added and the Rotor-Gene® was used to analyze the samples.

Leukocyte count. On day 35, two weeks after provocation, 1.00 - 2.00 mL of blood was drawn from the wing vein for leukocyte counting and the blood smear was prepared using the wedge method and stained via the Giemsa method.

Histological and macroscopic lesion scoring. Five chickens from each group were selected on day 35 for histological and macroscopic evaluation of lesions. Macroscopic lesions in the groups were scored based on the method described by Reid *et al.*[31]

Growth performance. Chicken performance and oocyst counts were recorded. Briefly, we measured the weight of chickens at the end of every week and checked clinical symptoms of illness. We also counted the oocyst output via the McMaster method.

Statistical analysis. All analyses were conducted using analysis of variance (ANOVA) followed by Tukey, least significant difference and Duncan tests using SPSS Software (version 26.0; IBM Corp., Armonk, USA). A $p < 0.05$ was considered statistically significant for the tests.

Results

Oocysts attenuation. Thirty chickens were used to evaluate oocyst excretion. No oocysts were produced in any of the three groups on day six. Table 1 displays the number of oocysts recovered on consecutive days.

Clinical symptoms. The study of clinical signs was performed through manual observation and without software. The chickens in the control group exhibited a healthy appearance and a good appetite. In the first group, fed with irradiated oocytes, the chickens showed mild disease symptoms, but their appetite was not changed significantly. In the second group, they showed symptoms of the disease. They were lethargic, with ruffled feathers and drooping wings. Bloody diarrhea was also observed in this group.

Cytokine production. The real-time polymerase chain reaction (RT-PCR) method was used to check the level of cytokine expression level. The data were then organized using ΔΔct, and bar graphs were created for each group to make the information more comprehensible (Fig. 1). Group 8 was considered the calibrator, and the level of cytokine expression in the other groups was expressed accordingly. The highest expression of interferon-gamma was observed in groups 5 and 7, at 17.83 times and 16.10 times that of group 8, respectively. The lowest expression in the immunized groups was recorded in group 4 at 2.62 times that of group 8. There was a statistically significant difference between the groups, per ANOVA and Duncan. In contrast, groups 5 and 7 showed no significant difference ($p \geq 0.05$). Comparing interleukin-4 to interferon-gamma, statistical differences among groups were less significant. According to the Duncan analysis test, there was no statistically significant difference among groups 1, 3 and 6 and between groups 5 and 7 ($p \geq 0.05$). The highest level of interleukin-4 among the immunized groups was observed in group 4 (9.38 times less than group 8), and the lowest level was recorded in groups 5 (7.16 times less than group 8) and 7 (8.75 times less than group 8).

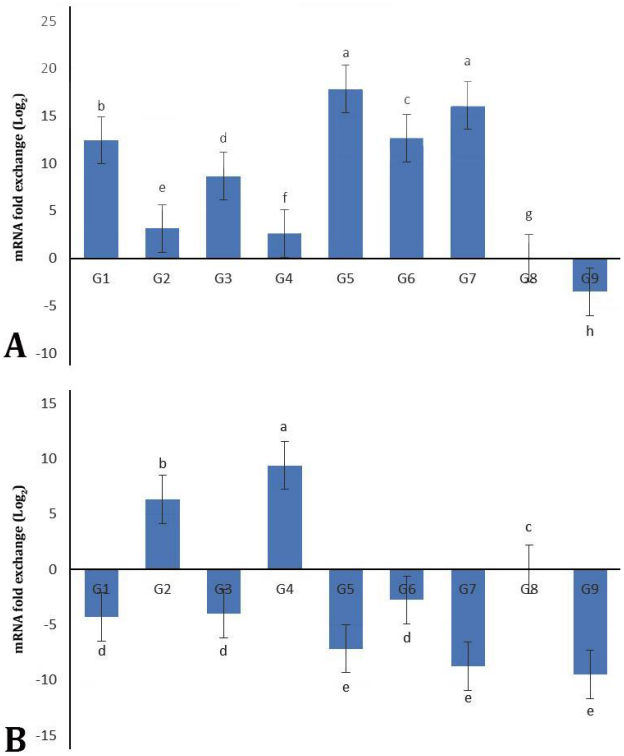

Fig. 1. Cytokine induction. **A)** IFN-γ and **B)** IL-4 induction was evaluated as representative of T helper 1 and T helper 2 type immune responses, respectively, by RT-PCR.

Table 1. Evaluation of oocysts per gram of feces oocyst (OPG) of the treatments after challenged.

Groups	Description	OPG count (%)
1	400 attenuated oocysts + 1.00 mg adjuvant + challenged	8.79[a]
2	400 attenuated oocysts + 0.50 mg adjuvant + challenged	13.10[b]
3	200 attenuated oocysts + 1.00 mg adjuvant + challenged	10.27[c]
4	200 attenuated oocysts + 0.50 mg adjuvant + challenged	14.39[d]
5	1.00 mg adjuvant + challenged	15.07[d]
6	400 attenuated oocysts + challenged	13.97[bd]
7	Livacox-T® + challenged	4.82[e]
8	Negative control + challenged	19.45[f]
9	Negative control + not challenged	0.00[g]

[a-g] Different superscript letters in column indicate significant ($p < 0.05$) differences between groups, challenge dose was 1,000 - 1,200 sporulated oocysts of *E. tenella*.

Leukocyte count. The microscopic observation of leukocyte count revealed the highest total white blood cell count and lymphocyte level in groups 1 and 7; They shared the lowest thrombocyte counts. Atypical lymphocytes were present in all groups in high numbers, sometimes reaching 50.00% of the total number of lymphocytes. One or two chickens from each group lacked platelets, while the rest had approximately 40,000 platelets. There was no significant correlation between granulocytes, monocytes and thrombocytes with lymphocytes. Moreover, no remarkable difference was observed between other cell populations in groups (Fig. 2).

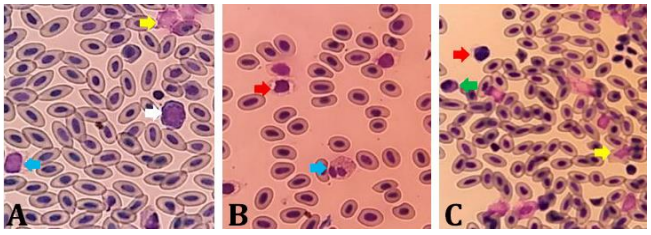

Fig. 2. Blood profile of treated animals. **A)** Atypical lymphocyte (yellow arrow), a monocyte (white arrow) and two granulocytes (blue arrow); **B)** thrombocytes (red arrow), and a granulocyte (blue arrow), **C)** thrombocyte (red arrow), small lymphocyte (green arrow) and atypical lymphocyte (yellow arrow), (Wright-Giemsa staining, 100×).

Histology and macroscopic lesions. The lesion score in group 9 chickens was 0.00. Chickens in groups 5 and 7 showed the lowest lesion score (0.20 and 0.50, respectively). The average lesions in groups 1, 3 and 6 were slightly higher (1.20, 1.40 and 1.00, respectively), however, significantly lower than in the control group, where a lesion score of 4 was recorded. Group 2 showed an average of 2.50, and group 4 was almost equal to the negative control group. We prepared and stained tissue sections of various parts of the intestine, including the cecum. The destruction of the intestinal epithelium and the presence of oocysts and other protozoan life stages were proportional to the visible damage (Fig. 3).

Growth performance. The average weight for each group was calculated separately on days 21, 28, 35 and 42, and the percentage change for every two consecutive weeks was recorded in Table 2. In the first week after the challenge (day 28), all groups showed a significant difference with group 7 (Livacox-T®; $p \leq 0.05$) except for groups 5 (1.00 mg adjuvant) and 9 (blank). We recorded the lowest weight gain in groups 2 (400 oocysts + 0.50 mg) and 4 (200 oocysts + 0.50 mg). Almost every group had a negative growth rate during the first week except for groups 5 and 7. In the second and third weeks after the challenge (days 35 and 42), group 5 exhibited a significant difference from other groups ($p \leq 0.05$), and we observed the highest average weight gain in this group. In general, mild coccidiosis symptoms were observed in all groups following the challenge, however, the symptoms were severe in groups 8 (negative control), 3 (200 oocysts + 1.00 mg), and 4. The symptoms included diarrhea, lethargy and fluffy feathers.

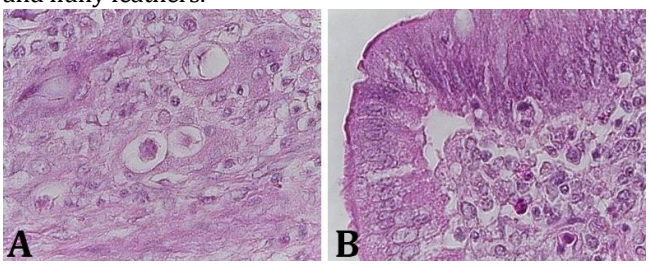

Fig. 3. Histological review of intestine epithelium. The presence of protozoa is shown in the epithelium of **A)** cecum and **B)** jejunum (Hematoxylin and Eosin staining, 100×).

Discussion

The analysis of the study's data revealed a significant difference among the groups inoculated with *E. tenella* under irradiation and combined with inulin adjuvant and the other groups. Irradiation is a method that can be utilized to reduce the virulence of vaccines.[32] This technique has previously been applied to oocysts.[9,33] The ionizing radiation slows down the cell cycle. Where the radiation dose is high, the cell cycle stops completely.

The X-rays attenuating *Pseudomonas aeruginosa* have induced broad immune responses by inhibiting proliferation and maintaining antigenicity.[17] In addition, the inactivation of the virus by X-ray has induced favorable

Table 2. Growth performance (mean ± SD) of the treatment groups during the experimental period.

Groups	Day 1 - 7	Day 8 - 14	Day 15 - 21	Day 22 - 28	Day 29 - 35	Day 36 - 42
1	150.40 ± 0.92^{a}	250.40 ± 1.03^{a}	449.80 ± 1.24^{a}	384.40 ± 5.40^{a}	612.00 ± 3.74^{a}	917.00 ± 1.22^{a}
2	151.60 ± 1.36^{a}	250.00 ± 1.00^{a}	450.60 ± 1.56^{a}	265.20 ± 6.09^{b}	313.00 ± 3.74^{b}	451.00 ± 1.87^{b}
3	149.20 ± 0.86^{a}	250.20 ± 1.06^{a}	449.40 ± 1.74^{a}	371.80 ± 2.81^{a}	608.00 ± 3.39^{a}	886.00 ± 6.00^{c}
4	151.40 ± 1.20^{a}	250.20 ± 1.35^{a}	449.60 ± 0.92^{a}	267.40 ± 5.96^{b}	314.00 ± 9.79^{b}	446.00 ± 1.87^{b}
5	149.80 ± 1.35^{a}	250.20 ± 1.49^{a}	450.80 ± 1.42^{a}	416.80 ± 6.09^{d}	717.40 ± 9.36^{d}	947.20 ± 5.91^{e}
6	150.60 ± 1.03^{a}	250.20 ± 1.06^{a}	449.60 ± 0.92^{a}	835.40 ± 9.20^{c}	1,058 ± 20.03^{c}	1,334.80 ± 7.07^{d}
7	150.40 ± 1.20^{a}	252.00 ± 1.51^{a}	450.20 ± 1.56^{a}	863.00 ± 8.60^{c}	1,000 ± 0.00^{e}	1,314 ± 3.67^{f}
8	150.00 ± 1.22^{a}	250.20 ± 1.51^{a}	449.60 ± 1.20^{a}	126.40 ± 7.23^{e}	261.00 ± 4.58^{f}	348.00 ± 2.55^{g}
9	151.00 ± 1.00^{a}	250.40 ± 0.92^{a}	450.00 ± 1.14^{a}	852.00 ± 7.17^{c}	1,100 ± 0.00^{e}	1,352 ± 1.22^{d}

$^{a-g}$ Different superscript letters in column indicate significant ($p < 0.05$) differences between groups, challenge dose was 1,000 - 1,200 sporulated oocysts of *E. tenella* on day 21 of age.

immunological characteristics.[39] X-rays inactivate pathogens primarily by destroying nucleic acids and preserving structural components like proteins.[40]

It has been reported that irradiated *Toxoplasma gondii* maintains its morphology, metabolism and cell invasion properties. The use of X-rays in reducing bacterial virulence has induced broader cross-cellular and humoral immune responses.[17] In the polio vaccine study, radiation was used to reduce virulence and this technique has been recommended for use in other viral vaccines.[41] This method has also been employed successfully on bacteria,[40] malaria,[42] *T. gondii,*[43] scorpion toxin,[44] tetanus toxin,[45] *Salmonella typhimurium,*[46] and numerous other studies.

Several critical factors influence the pathogenicity of *E. tenella* infection such as the number of ingested oocysts, oocyst strains and environmental factors.[47]

The number of oocysts in the immunization groups was 200 and 400. Coccidiosis may be acute or fatal depending on the amount of infectious oocysts ingested. In infections with fewer than 150 oocysts, the cecal mucosa will have bleeding points and a slight discoloration of the cecal wall. Oocysts between 150 - 500 may cause minor bleeding in the mucosa and damage and thickening of the cecal wall. In the meantime, 1,000 to 3,000 oocysts are responsible for high mortality, bleeding, some blood clots and abnormal cecal contents. In addition, 5,000 oocysts or more will cause the highest mortality and bleeding as well as blood clots that are frozen and calcified in the cecum.[48]

Inulin has been used as an adjuvant in various studies. It is hypothesized to work by modulating the function of antigen-presenting cells in a non-inflammatory manner, despite its mechanism of action is the subject of ongoing research. As a result, it stimulates and activates antigen-specific helper T cells which in turn allows the expansion of memory B cells and CD8 T cells.[49]

Several studies have demonstrated the effect of gamma-inulin on complement system activation in various animals. Gamma-inulin has been successfully used in *in vitro* designs of influenza, hepatitis B, malaria and papillomavirus vaccines. It can stimulate both humoral and cellular immunity,[50] however, the effect of inulin on the population of T lymphocytes is controversial.

In groups containing inulin and oocysts, those with more oocysts performed better.

Since Livacox-T® was commercially available at the time of the study and no vaccine contained the *E. tenella* strain alone, we used it as the positive control.

This study conclusively demonstrated that X-rays could deactivate *E. tenella* oocysts and that combining this technique with inulin adjuvant yielded satisfactory results. The variation in inulin concentration affected the number of excreted oocysts. In addition, the optimal dose consisted of 400 irradiated oocysts and 1.00 mg of inulin.

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Conflict of interest

The authors declare that they have no financial or other conflicts of interest that could inappropriately influence this study.

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