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Evaluation of the effects of diclazuril and toltrszuril on hematological changes, antioxidant status immune response and cecal histoarchitecture of chickens experimentally infected with *Eimeria tenella*

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ABSTRACT

Hundred Cobb chicks of one day old were used in this study. The chicks were divided into 4 groups (n = 25 / group). Group 1 was used as a control group; groups 2, 3, and 4 were experimentally infected with *Eimeria tenella* (*E. tenella*) at the 14th day of age. G2 received no treatment; G3 and G4 were treated with diclazuril 1ml/4 L in drinking water and toltrazuril 7 mg/k.g B.wt for 2 successive days respectively at 5 days post infection. The obtained results revealed significant decreases in hemoglobin concentration, red blood cells counts and PCV% in the infected group compared with the control group. The levels of SOD were significantly decreased while MDA level was significantly increased and the levels of IL -2 and IL- 6 were increased in *E. tenella* infected group. Histopathological results revealed heavy infestation of the intestinal crypts with different developmental stages in *Eimeria tenella* infected group compared with control group. The treatment with diclazuril and toltrazuril improved the deterioration caused by E.tenella, particularly the diclazuril treated group.

1. INTRODUCTION

One of the finest methods for delivering high-quality animal protein for human consumption is chicken industry. According to Bootwalla (2005), broilers are the quickest, most cost-effective, and most efficient converter of plant material into food with a high biological value.

The development of the poultry industry is still being hampered by numerous enteric diseases as coccidiosis (Saima et al., 2010). According to Verheyen et al. (1988), there are 7 recognized Eimeria species that can infect chickens: *Eimeria acervulina, E. maxima, E. tenella, E. brunetti, E. necatrix, E. mitis,* and *E. praecox.E. tenella* predominantly invades and lives in the caecal lining epithelium of exposed hens, causing hemorrhagic feces, reduced body weight increase, decreased feed efficiency, and ultimately mortality with major economic consequences El-Abasy et al. (2003). It is well known that several bodily systems in chickens are compromised by the pathogenic effects of *E. tenella* species. The effects on blood cells have been examined in a variety of research (Fetterer and Allen, 2001).

Avian coccidiosis, is known to induce significant alterations in hematology and serves as a concrete evidence of the physiologic changes and immunological responses brought on by disease (Anas et al. 2018). The severity of anemia in cases of coccidiosis is highlighted by the blood loss in the digestive tract caused by *E. tenella* (Hauck, 2017). *E. tenella* infection affects the antioxidant status of the bird, especially MDA, which increased in response to a decrease in SOD activity (Abd El-Maksoud et al., 2014). Cytokines have a significant role in regulating the host immunological response to Eimeria infection in poultry Min et al. (2001). Interleukin-2 (IL-2) is thought to be essential for chickens' immune defense Miyamoto et al .(2002) .

The convenience of administration is one benefit of chemoprophylactic coccidiosis control. The majority of anticoccidial medications are added to milled feed or infused into drinking water, allowing for direct and efficient administration without the need for additional labor costs. Where chemo-prophylactics are successfully applied, treated birds do not need to compete with the parasite for energy (Attree, 2021).

In this regard, *E. tenella* infection was induced expiremental in chicken broilers and treated with diclazuril and toltrazuril. For the differential diagnosis, treatment, and management of coccidiosis in chickens, it will be helpful to understand the hematological, biochemical, immunological, and histological alterations that occur throughout Eimeria tenella's life cycle. This study looked into the therapeutic effects of the two anticoccidial medications, diclazuril and toltrazuril, as well as studying hematological, biochemical, immuno-

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logical, and histological changes brought on by *Eimeria tenella* infection in chicken broilers.

2. MATERIAL AND METHODS

All experiments using birds were conducted according to the guidelines for the use of animals provided by the ethical committee at Benha University with ethical approval number BUFVTM 41-09-23

2.1. Birds:

One hundred Cobb mixed breed broiler chicks at one day old were purchased from Al-Kahira Poultry Company. Four groups were created at random from the chicks. Group 1 was regarded as the control negative group, uninfected and untreated, while Group 2 was infected with 100000 sporulated *E. tenella* oocysts using the intra-crop route at 14 days of age Dalloul et al., 2003, Group 3 was infected with the same dose of sporulated *E. tenella* oocysts on the 14th day old chicks and then treated with diclazuril 1 ml/4 liter drinking water Assis et al., 2012 at the fifth day P.I. for two consecutive days. Group 4 received the same dose of sporulated *E. tenella* oocysts and was then given toltrazuril 7 mg/kg b.wt El khouly et al. (2016) at the fifth day P.I.

2.2. Diclazuril

Pharma Swede Company in Egypt provided the Diclazuril Diclosol® suspension, 10 mg/ml. It was supplied in drinking water at a concentration of 2.5 ppm (1 ml/4 liters of water).

2.3. Toltrazuril

A therapeutic dose of 7 mg/kg body weight of toltrazuril solution (Toltrasol 2.5% ARABCOMED Company) was given orally.

2.4. Sampling:

Following the collection of blood samples at the age of 21 and 28 days, five birds from each group were sacrificed. Two blood samples were drawn from the wing vein, one for hematological analysis and the other for serum separation and biochemical analysis. The first blood sample was drawn with an anticoagulant. Caecal tissue specimens for histological analysis were taken at the same time.

2.5. Clinicopathological assays
2.5.1. Hematological assay

Red blood cell (RBC) count, hemoglobin (Hb) concentration, PCV% were determined according to the routine hematological procedures as described by Feldeman et al., 2000 Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Dacieand Lewis (2002).

2.5.2. Oxidative stress biomarkers

Superoxide dismutase (SOD) activity was assessed in sera samples using ELISA kits from Quantichrom, BioAssay Systems, and Canyman Chemical Company, USA. K). The serum concentrations of MDA (lipid peroxidation), were assessed by using the obarbituric acid reactive substances (TBARS) produced during oxidative stress (Ohkawa et al., 1979) (ELISA Kit: Quanti ChromTM, Bioassay Systems, USA, Catalog No. DTBA-100), following to Ohkawa et al. (1979). The assays were conducted according to the manufacturers' protocols

2.5.3. Inflammatory markers:

Using ELISA kits from BG Biotechnology Co., Ltd., the inflammatory markers interleukin-2 (IL-2) and interleukin 6 (IL-6) were assessed in accordance with the manufacturer's guidelines and previous report (Abdel Maksoud et al., 2019).

2.5.4. Pathological examination

Caecal tissue specimens from sacrificed birds were fixed in 10 %formalin solution then dehydrated in ascending grades of alcohol, cleared in xylene, embedded in paraffin wax. Tissue paraffin sections were cut at 5 um thickness and stained with Hematoxylin and Eosin (Bancroft et al., 2008).

2.6. Statistical analysis

The statistical software program SPSS for Windows (Norusis 2008) was used to do a one-way ANOVA analysis on the collected data followed by Tukey's multiple comparisons at $p<0.05$ as a post –hoc test . The data were shown as mean ± standard deviation (S.D)

3. RESULTS

3.1. Hematological findings

The hematological results at days 21 and 28 days of age revealed significant decrease in RBCs count, hemoglobin concentration, and PCV% in *E. tenella* infected group (G2) compared to control group (G1) However, compared to (G2) with a considerable increase in these parameters was seen in groups 3 and 4, diclazuril and toltrazuril treated groups respectively (Tables 1, 2). A significant increase in MCV and MCH values in (G2) with an improvement in diclazuril treated group (G3) and in toltrazuril treated one (G4). The improvement was more pronounced in (G3).

Table (1): Effect of *E. tenella* infection on hematological parameters in chickens at 21 days old

Item	control	*E. tenella*	Diclazuril	Toltrazuril
Hemoglobin (g/dl)	7.1 ± 0.27^{a}	5.34 ± 0.43^{c}	6.04 ± 0.53^{b}	5.74 ± 0.27^{bc}
RBCs ($x10^6$/µl)	2.84 ± 0.21^{a}	1.93 ± 0.2^{c}	2.32 ± 0.13^{b}	2.16 ± 0.14^{bc}
PCV (%)	37.44 ± 0.88^{a}	32.04 ± 1.03^{c}	33.74 ± 1.09^{b}	32.82 ± 0.93^{bc}
MCV (fl)	132.22 ± 6.67^{c}	166.82 ± 13.21^{a}	145.34 ± 4.03^{b}	152.48 ± 6.26^{b}
MCH (pg)	25.08 ± 1.61^{b}	27.76 ± 2.20^{a}	$25.94 \pm 1.15a^{b}$	$26.60 \pm 1.1a^{b}$
MCHC (g/dl)	18.94 ± 0.75^{a}	16.62 ± 1.07^{c}	$17.88 \pm 1.0a^{b}$	17.50 ± 0.38^{bc}

Table (2): Effect of *E. tenella* infection on hematological parameters in chickens at 28 days old

Item \ Group	Control	*E. tenella*	Diclazuril	Toltrazuril
Hemoglobin (g/dl)	7.48 ± 0.19^{a}	5.48 ± 0.36^{d}	6.70 ± 0.42^{b}	6.16 ± 0.39^{c}
RBCs (x10^{6}/µl)	3.03 ± 0.15^{a}	1.97 ± 0 .15^{d}	2.68 ± 0.09^{b}	2.39 ± 0.19^{c}
PCV (%)	38.12 ± 1.14^{a}	32.4 ± 1.48^{c}	35.16 ± 0.86^{b}	34.48 ± 1.29^{b}
MCV (fl)	126.02 ± 3.45^{c}	165.06 ± 7.97^{a}	131.02 ± 3.65^{c}	144.62 ± 7.52^{b}
MCH (pg)	24.58 ± 0.83^{b}	27.88 ± 1.74^{a}	24.92 ± 1.24^{b}	25.82± 1.41^{b}
MCHC (g/dl)	19.62 ± 0.39^{a}	16.86 ± 0.47^{c}	19.04 ± 0.84^{a}	17.86 ± 0.77^{b}

3.2. Inflammatory and oxidative stress biomarkers

The infected group's serum IL-2 and IL-6 levels significantly increased at 21 and 28 days of age, respectively. When compared to group 2, the diclazuril and toltrazuril-treated groups showed a considerable decline. At 21th and 28th day of age to the conclusion of the expiration, there was a substantial decline in the level of SOD and a large increase in the concentration of MDA with regard to the antioxidant biomarkers. These parameters were presented in tables (3,4) and revealed a significant improvement in both the treatment groups (G3) diclazuril treated group and (G4) toltrazuril treated one. It notes worthy to mention that diclazuril showed much improving in the hematological and immunological tested parameters compared with toltrazuril although not significant.

Table (3): Effect of *E. tenella* infection on some immunological and antioxidant parameters in chickens of different groups at 21 days old

Item \ Group	Control	*E. tenella*	Diclazuril	Toltrazuril
IL-2 (pg/ml)	6.3 ± 0.9^{c}	10.2 ± 0.5^{a}	8.2 ± 0.7^{b}	8.7 ± 0.4^{b}
IL-6 (pg/ml)	29.5 ± 1.1^{b}	34.0 ± 1.3^{a}	29.6 ± 0.9^{b}	29.5 ± 0.7^{b}
MDA (nmol/ml)	10.2 ± 0.9^{c}	17.8 ± 1.1^{a}	12.1 ± 1.0^{b}	12.4 ± 1.3^{b}
SOD (n/m)l	42.8 ± 1.3^{a}	28.1 ± 1.3^{c}	40.4 ± 1.1^{b}	37.8 ± 1.8^{b}

Table (4): Effect of *E. tenella* infection on some immunological and antioxidant parameters in chickens of different groups at 28 days old

Item \ Group	Control	*E. tenella*	Diclazuril	Toltrazuril
IL-2 (pg/ml)	6.3 ± 1.2^{c}	10.5 ± 0.4^{a}	7 .2 ± 0.8^{b}	7.8 ± 0.7^{b}
IL-6 (pg/ml)	29.36 ± 1.6^{b}	34.96 ± 2.0^{a}	30.86 ± 1.9^{b}	30.46 ± 2.2^{b}
MDA (nmol/ml)	10.4 ± 1.0^{c}	18.0 ± 1.5^{a}	12.3 ± 1.5^{b}	12.7 ± 1.5^{b}
SOD (n/ml)	43.1 ± 1.9^{a}	23.3 ± 2.1^{d}	38.2 ± 0.8^{c}	35.1 ± 2.7^{b}

3.3. Pathological findings:

Macroscopically, the initial sacrifice (at the 21th day) showed the caecum of the infected chickens showed severe bleeding and severe of congestion (Fig. 1A and 1B), while both treated groups (G 3 and G 4) showed improvement in the previously stated lesion. Microscopically, the cecum of the infected non-treated group demonstrated mucinous degeneration with large clusters of developmental stages of *Eimeria tenella* (Fig. 3A), in contrast to the normal caecal structure of the control group (Figs. 2A and 1B).Submucosal blood vessel congestion and perivascular necrosis were seen (Fig. 3B). The presence of eosinophils cells was linked to hemorrhage in the intestinal villi (Fig. 3C).The expression of living coccidial stages within the caecal epithelial cells was significantly reduced in the *E. tenella*-infected group when diclazuril was administered (G. 3; Fig. 3D). Similar to this, there was a moderate lesion in the group caecum of chickens infected with *E. tenella* and treated withtoltrazuril(Fig. 3E and 3F) .

On the 28th day, the second sacrifice was conducted. Macroscopically, the cecum of the infected group showed lesions that were remarkably comparable to those that were present on day 21 (G. 3&4), but there were none in either of the treated groups. The *E. tenella*-infected non-treated group (G.2) showed evident *E. tenella* infection at various developmental stages in the intestinal crypts and in the lamina propria, along with different coccidial stages linked to the infiltration of esinophilic cells (Fig. 4A). Figure 4B shows mucinous degeneration along with eosinophil-dominated leukocytic cell infiltration. Figure 4C shows lymphoid depletion along with some mucosal gland degradation. There was a noticeable improvement in the *E. tenella* group (G.3) that had been infected and given diclazuril treatment (Fig. 4D). Only a few modest lesions could be seen in *E. tenella* that was infected and treated with toltrazuril group (G.4) (Fig. 4E).

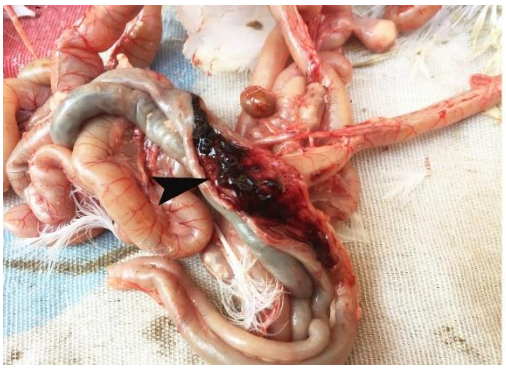

Figure 1: Caecum of infected group at 21th day showing A) Open caecum with contents tinged with blood.

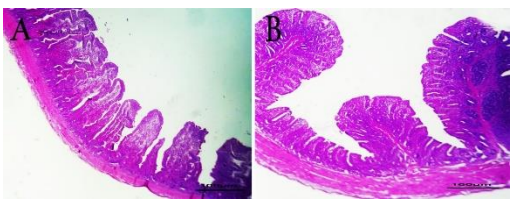

Figure 2: Photomicrograph of H&E stained section of caecum of control group showing (A) & (B) normal structure of both mucosa and submucosa.

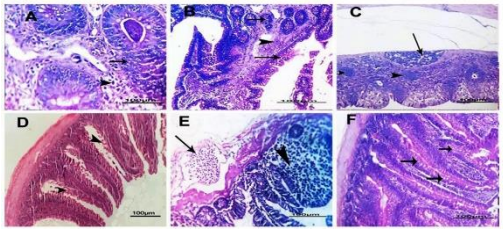

Figure 3: Photomicrographs of H&E stained sections of cecum of G.2 (infected group) (A-C), G.3 (D) treated group with diclazuril and G.4 (E&F) treated group with toltrazuril at 21th day showing. A) Mucinous degeneration (arrow) with developmental stages of *Eimeria tenella* (arrow head), B) congestion of submucosal blood vessels (arrows head) with perivascular necrosis (arrows), C) hemorrhage in the intestinal villi (arrows), D) mild increase in muscularis thickness, E) showing lymphoid depletion (arrow) with desquamation of villus tips (arrow head) with absence of developmental stages of *Eimeria tenella*, F) lymphoid depletion (arrow head). Scale bar = 100 µm.

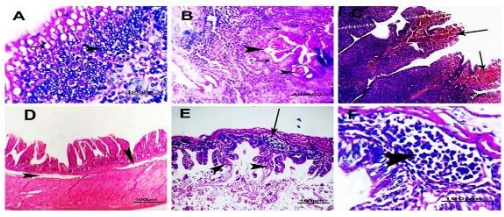

Figure 4: photomicrographs of H&E stained sections of cecum of G.2 (infected group) (A-C), G. 3 (D) treated group with diclazuril and G.4 (E&F) treated group with toltrazuril at 28th day showing. A) A few developmental stage of *Eimeria tenella* (arrow) with few eosinophils infiltration (arrow head). B) Mucinous degeneration (arrows) with leukocytic cells infiltration of mainly eosinophils (arrow head). C) Lymphoid depletion (arrow) with degeneration of some mucosal gland (arrow head). D) Mild desquamation of some villi (arrows head). E) Congestion of submucosal blood vessels (arrow) with lymphoid depletion (arrow head). F) Mucinous degeneration of some villi (arrows). Scale bar = 100 µm.

4. DISCUSSION

One of the most significant parasitic diseases of chicken is poultry coccidiosis, and *E. cerulean, E. tenella, and E. maxima* are the major species that frequently detected in the field (Lillehoj et al., 2004). Of the three species, *E. tenella* is the most pathogenic. In addition to the pathognomonic caecal lesions, the present study assessed how the *E. tenella* infection affected hematological, antioxidant, and immunological markers. The hematological results of this study indicated macrocytic hypochromic anemia in the birds infected with *E. tenella* at the 21st and 28th days of the experiment evidenced by significantly decreases in RBCs, Hb, PCV%, and MCHC values and significantly increases in MCV values. These results agree with Youssef et al. (2015).Histamine is generated during tissue injury induced by *E. tenella*, increasing the permeability of blood capillaries and venules, which allows for the escaping of significant amounts of blood, which could be the cause of anemia (Muhammad et al., 2020). In comparison to the infected group, the diclazuril-treated group showed a significantly higher Hb concentration, PCV%, and RBC count as well as significantly higher MCV values. These findings agree with those of El-Maddawy et al.(2022).

Additionally, cocktail birds infected with *Eimeria aratinga* and then treated with diclazuril demonstrated improvement in the fore mentioned parameters. Salem et al., 2022. Hematological indicators improved in chickens infected with coccidiosis and treated with toltrazuril Hagag et al. (2020).The fact that diclazuril and toltrazuril both suppressed coccidial proliferation and, as a result, stopped bleeding, may be the cause of the improvement in hematological parameters in both treatment groups.

According to Allen et al. (1997), increased immune cell activity that results in an excess of free radicals and an increase in ROS production that leads to lipid peroxidation may be to blame for the rise in MDA concentration in broiler chickens infected with *E. tenella*, or it may be because malonaldehyde is produced during the process of lipid peroxidation due to the influence of ROS on the polyunsaturated lipids. Its levels in blood and tissues are inversely correlated with the cellular damage brought on by ROS (Pajic et al., 2018).Our findings (the decrease of SOD level and the increase of MDA concentration) concur with those published by Toah et al. in 2020, who showed a substantial rise in the amount of MDA in the serum of *E. tenella*-infected chicks. According to Cam et al. (2008), the decreased SOD may be caused by the release of reactive oxygen species (ROS) as a result of the chicken coccidiosis-related tissue damage. SOD, an antioxidant enzyme, was therefore heavily ingested to help the diseased bird's combat oxidative stress. Abd El-Maksoud et al., (2014) and Pajic et al., (2018) both came to similar conclusions.The present investigation demonstrated the antioxidant and anticoccidial activities of diclazuril and toltrazuril. Treatment with the anticoccidial medications might restore the activity of the measured antioxidant enzyme (SOD) towards the normal values. Diclazuril's antioxidant effectiveness was reported by ELmahallawy et al. in 2022, who claimed that by lowering the caecal and hepatic tissues and lipid peroxidation in hens infected with *E. tenella*, diclazuril could restore the activities of the antioxidant enzymes to normal levels. According to Bozkurt et al. (2016), a different hypothesis clarified that the anticoccidials affected the reduction in ROS production, which in turn resulted in lessened lipid peroxidation. Likely, Eraslan et al. (2004) reported on the effects of toltrazuril treatment, stating that it could reduce the oxidative damage brought on by coccidiosis in chickens that had been exposed to *E. tenella*. Similar outcomes in Japanese quail were also observed (Nasr El Deen et al., 2021). In the present work when compared to control group, the inflammatory cytokines IL-2 and IL-6 showed a considerable rise in *E. tenella* infected group. To help with the avian Eimeria infection, inflammatory cytokines IL-2 and IL-6 were raised (Jiao et al., 2018). IL-2 is a biologically active cytokine with a broad spectrum that primarily stimulates the immune system to generate cellular immunological responses, encourages CD4 and CD8 cell differentiation and proliferation, and is crucial in the battle against viral, bacterial, and parasite infection. Our findings are consistent with Miyamoto et al.'s (2002) discovery that serum IL-2 levels significantly increased in *E. tenella*-infected chickens. As IL-2 functions as a growth, survival, and differentiation factor for T-lymphocyte and NK cells (Abbas et al., 2010). Prior

study also noted a significant rise in the production of IL-2 mRNA in Eimeria infection in chickens (Hong et al., 2006).Additionally, Kotenko et al. (2003) demonstrated that the recombinant IL-2 protein can minimize cecum lesions, boost IgA antibody secretion, limit coccidia replication, and increase relative weight gain. Endothelial cells, macrophages, and T lymphocytes are the main producers of IL-6. It is in charge of B-cell proliferation and maturation into antibody-producing cells (Abbas et al., 2010). Our previous results concur with those of Lynagh et al. (2000), who noted that the activity of IL-6 in the serum of chickens infected with *E. tenella* suggested a potential function for this cytokine in acquired immunity. Additionally, Hong et al. (2006) found that chickens infected with *E. acervulina*, *E. maxima,* and *E. tenella* had significantly higher levels of IL-6 gene expression, which is similar with the current study. Diclazuril and toltrazuril treatment after *E. tenella* infection in chickens resulted in a considerable reduction in IL-2 and IL-6 levels as compared to the infected non-treated group. These outcomes demonstrated the potency of both medications in managing Eimeria infection. According to Abdelhady et al. (2021), supported our findings, diclazuril reduced the high jejunal gene transcripts for IL-6. On the other hand, Nasr El Deen et al., (2021) found a substantial rise in IL-2 mRNA expression in the caecal tissue of quails that had been infected with Eimeria species and subsequently given toltrazuril. Adamu et al. (2013) reported that most pathogenic second generation schizonts of *E. tenella* induce tissue damage, hemorrhages, and degenerative changes in the caecal mucosa and muscularis. Our results in infected non treated group were consistent with their findings. Additionally, those findings were first reported by Bould et al. (2009) who showed that the severe destruction in the caecal mucosal layer, penetrating villus epithelial cells, extensive desquamation of the caecal epithelium, and hemorrhagic feces caused by the initial adhesion and invasion of *E. tenella* to the intestinal epithelium of the host cells, which must occur across the mucus interface. The current research found that the cecum tissue of the *E. tenella*-infected group displayed distinct glandular and epithelial hyperplasia, as well as evidence of mucosal lining deterioration and necrosis. The mucosa of the groups treated with diclazuril and toltrazuril showed modest degenerative and hyperplastic alterations, which is consistent with the findings of Tian et al. (2014) who reported that the damage in birds with *E. tenella* infection treated with diclazuril was obviously lessened. Likely, Ashraf et al. (2011) and Hagag et al. (2020) investigated the anticoccidial activity of toltrazuril and found that treated chickens with toltrazuril developed moderate lesions in the caecum.

5. CONCLUSIONS

Toltrazuril and diclazuril, which are both known to belong to the chemical class of triazines, were found to have both lessened the deterioration in hematology, biochemistry, immunology, and histopathology caused by *E. tenella* infection in chicken broilers. In certain ways, diclazuril is more effective at treating *E. tenella*.

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