The effect of tropical fasciolosis on the adrenal gland dysfunction was investigated in male Murrah buffalo yearlings. Eight animals were randomly assigned to two groups (Groups 1 and 2) of four animals each. Animals in Group 1 were administered per os with the primary infection dose of 800 viable $F$. gigantica metacercariae, whereas Groups 2 animals served as healthy controls. The Group 1 animals sequentially developed the characteristic clinical manifestations of tropical fasciolosis and had prepatent period of 92-95 days. A significantly higher serum cortisol concentration ($p<0.05$) was persistently observed from week-4 post-infection (PI) onwards in Group 1 animals. The highest cortisol level (31.2±4.2 nmol/L) suggestive of $F$. gigantica induced persistent stress, declined from week-10 PI onwards yet it remained higher in the disease hosts than the healthy controls. Further it was also associated with eosinophilia (14.0±1.8%) and leucocytosis (9.5±0.2x10³/cmm). Interestingly, hypercortisolemia in Group 1 animals was the highest (82.97%) during early prepatency followed by 38.6% in late prepatency and 6.2% in the patency phase of the disease. Evidently, animals in Group 1 persistently suffered from variable degrees of stress in comparison with the healthy controls. On necropsy, the histopathological picture of zona fasciculata revealed hypertrophic corticocytes with altered shape, arrangement and vacuolation in the infected animals (Group 1), confirming hyperactivity of the glands throughout the period of investigation. The infected animals had adult fluke recovery of 331.8±19.5 (41.47% of the infection dose) on the Day 112 PI, with a significant increase ($p<0.01$) in the hepatic tissue mass (35.14%). The complex pathophysiological events occurring during the different stages of bubaline fasciolosis vis-à-vis growth and development of the distome and impact of persistent disease stress on overall health have been discussed.

**Keywords:** $F$. gigantica, buffalo, adrenal glands, cortisol, stress

### INTRODUCTION

Tropical fasciolosis, caused by $Fasciola$ gigantica, has been recognized as a major constraint on the animal productivity in the South Asian countries, including India (Gupta and Singh, 2002; Garg et al., 2009). The distome exerts a variety of deleterious effects on the host, including anaemia, feed conversion and weight gain efficiency, lipolysis, persistent hypoxemia,
oxidative stress, besides elevated activities of certain enzymes suggestive of hepato-biliary trauma and cholestasis (Mehra et al., 1999; Yadav et al., 1999; Ganga et al., 2004; Edith et al., 2010). Recently, elevated serum cortisol concentration and depressed erythrocytic indices during the acute course of experimental bubaline fasciolosis have also appeared in the literature (Ganga et al., 2007). The overall deterioration in the body conditions and lack of energy in the *F. gigantica* infected host could be synergistic consequence of multiple factors, including progressive altered physiological activities of the adrenals. This study is a continued effort in the above direction. The findings reported herein on adrenal gland dysfunction are of pathophysiological interest. The report correlates the persistent cortisolemia with *F. gigantica* induced stress during different stages of acute fasciolosis and attempts to interpret the development of pathognomonic manifestations in the diseased host.

**MATERIALS AND METHODS**

**The animals**

Eight male buffalo yearlings of the Murrah breed, weighing between 200-250 kg, were procured from the field. The animals were stall fed on a balanced diet. They had access to cultivated fodder ad libitum and received concentrate mixture. They were maintained under a standard intensive system of management. Fresh drinking water was offered 2-3 times daily. Absence of parasitic infections was ensured through regular coprological examination for a period of at least 13 weeks. At the age of 12-15 months, they were randomly assigned to two groups of four animals each. On Day 0 of the experiment, each animal in Group 1 was administered per os the primary infection dose of 800 viable *F. gigantica* metacercariae (bubaline origin), as an electuary made of molasses and wheat flour. The animals in Groups 2 were administered electuary devoid of *F. gigantica* metacercariae and maintained as healthy controls. The health status of the experimental animals was critically monitored twice a day for the development of symptoms suggestive of illness and was on spot recorded along with their severity, frequency and duration, until necropsy of the animals at the end of the experiment.

**The Parasite**

Adult *F. gigantica* flukes were recovered at necropsy from the hepato-biliary system and gall bladder of the bubaline livers obtained from a local abattoir. The eggs discharged by the flukes in phosphate buffer saline (PBS) were used for in vitro culture, infection of *Lymnaea auricularia* snails and *F. gigantica* metacercariae were harvested on 4 cm² polysheets. The *Lymnaea auricularia* snails, collected from the endemic areas, were screened, in vitro acclimatized and bred. Each snail, aged 10-15 weeks, measuring 14-15 mm in length, was in vitro infected with 8-10 *F. gigantica* miracidia, and the *F. gigantica* strain was maintained in the author’s laboratory for the production of bubaline origin metacercariae for the experimental purpose. The *F. gigantica* metacercariae were stored at 4°C in sterile distilled water until use (Gupta and Yadav, 1994). These were orally administered to the target animals after a viability test. The viability of metacercariae was between 95-98% on the day of infection.

**Techniques**

The weekly blood samples were aseptically collected by jugular vein puncture from individual animals under the experiment for sixteen weeks.
The serum was separated from the blood for the estimation of cortisol, properly labeled and store at -20°C. Standard haematological techniques were used to monitor weekly alterations in haemoglobin (Hb), packed cell volume (PCV), total erythrocyte counts (TEC), erythrocyte sedimentation rate (ESR), total leucocyte counts (TLC) and eosinophils (Jain, 1986). Copro egg counts for each animal were estimated weekly following dilution technique (Sharma et al., 1989).

Cortisol determination

The serum cortisol concentration was determined via immunotech radio immuno assay (RIA) with a commercially available kit (M/s Beckman Company, France). The RIA of cortisol is a competition assay. A standard curve was plotted, while incubating the serum of the healthy controls and standard in monoclonal antibody coated tubes with I\textsuperscript{125} labeled cortisol tracer. Thereafter, the liquid contents of the tube were aspirated and bound radio activity was measured. A calibration curve was established and unknown values were interpolated from the standard curve.

On necropsy, the liver from each experimental animal was removed, thoroughly washed, blotted, weighed and measured. Tissues showing gross lesions were cut to appropriate size, fixed in warm 10% formal saline and processed for histopathological examination following standard protocol. Sections were cut into 5 μm thickness and stained with Mayer’s alum haematoxylin and alcoholic eosin prior to microscopic examination and recording pathological changes in the respective groups.

Statistical analysis

In order to facilitate precise interpretation of data, the weekly fluctuations data on haematology and cortisol profile during the course of investigation were clubbed together and the mean values were derived for four clinical stages of the disease viz. (a) preinfection values on week-0, (b) early prepatency levels from week-1 to 6 PI, (c) late prepatency values from week-7 to 13 PI, and (d) the mean values during patency from week-14 onwards, depending upon the growth, developmental stages of *F. gigantica* adolescercariae and their location in the hepato-biliary system of the host. Research data so generated were subjected to the student’s ‘t’ test (Snedecor and Cochran, 1989).

RESULTS

Clinico-parasitological profile

No untoward clinical signs were observed until the fourth week PI. Thereafter, the infected animals (Gr-I) sequentially exhibited pathognomonic signs of the disease described elsewhere (Yadav et al., 1999). The signs were of variable duration and were intermittent in nature. One out of the four animals died on Day 110 PI. Loss of subcutaneous fat (lipolysis), emaciation, marked muscular weakness and straight legged-
skeletal braced posture constituted terminal signs of the disease. Healthy controls did not develop any sign and were maintained perfect health status during the course of investigation.

The infected animals discharged *F. gigantica* eggs from Day 92 PI onwards with the mean EPG of 88.8±13.0 during the week. In these animals, the pre-patent period varied from 92-95 days. A progressive increase in faecal egg counts was evidenced during the patency; the highest mean EPG of 192.5±12.6 was recorded during week-16 PI. None of the healthy controls passed out *F. gigantica* eggs in their faeces during the course of study.

On necropsy, the carcass of the infected animals (Gr-I) was emaciated, icteric appearance and there was extensive gelatinization of subcutaneous fat. The liver was grossly enlarged and had exemplary appearance of *F. gigantica* infested liver. There was significant increase (p<0.01) in the hepatic tissue mass (35.14%) in the infected animals (Group 1) in comparison with the healthy controls. The abdominal cavity contained 0.75-1.5 litre of sero-sanguineous fluid, suggestive of ascites. The healthy controls (Groups 2) did not reveal any grossly visible pathological picture and were devoid of *F. gigantica*, suggestive of perfect health status during the course of investigation.

The numbers, mean length and width and weight of the flukes recovered from each infected animal have been tabulated in Table 1. On necropsy, 41.47% of the primary dose of infection was recovered as adult fluke population from hepatic parenchyma, bile ducts and gall bladder of the infected animals (Group 1). The in situ fluke population recovered from the infected animals had normal growth and development, and their length and width were within normal range for adult *F. gigantica*.  

**Blood profile**  
The weekly fluctuations in the serum cortisol levels during the course of infection in the respective groups of animals are shown in Figure 1. The pre-infection hormone levels in all the experimental animals were within normal range (12.9±1.6 to 15.3±1.9 nmol/L) and were comparable until week-2 PI. Thereafter, with the invasion of the developing adolescercariae in the hepatic tissues, the hormone levels started rising from the third week PI and remained significantly elevated (p<0.05-0.01) during the fourth through eighth week PI in the Group 1 animals than the healthy controls (Groups 2). The hormone concentrations were further analyzed in the respective groups during different stages of the infection and are presented in Figure 2. The cortisol levels were 82.97% higher in Group 1 animals during early prepatency (weeks 1-6 PI). Thereafter, the serum levels progressively declined to 38.6% during late prepatency (weeks 7-12 PI). The levels declined further during patency (weeks 13-16 PI), and were just 6.2% higher in Group 1 animals. The highest concentration (31.2±4.2 nmol/L) of the cortisol was observed during week-4 PI in the infected group of animals (Group 1). In healthy controls (Groups 2), the cortisol concentration fluctuated within normal range (12.9±1.6 to 14.4±1.8 nmol/L) and did not alter significantly.

The weekly data on erythrocytic indices in the respective groups have been summarized in Table 2. The pre-infection Hb, PCV and TEC in the infected group of animals (Group 1) were within normal range and were comparable with the healthy controls (Groups 2). Thereafter, these erythrocytic indices progressively declined from the fourth week PI onwards and touched the critical levels in Group 1 animals during week 8 PI. During early prepatency (weeks 1-6 PI), the falls were
Table 1. Dose of infection, fluke recovery and biometry in the infected animals.

<table>
<thead>
<tr>
<th>Group (n=4)</th>
<th>Dose of infection (mc)</th>
<th>No. of fluke recovered</th>
<th>Fluke recovery (%)</th>
<th>Mean length of fluke (mm)</th>
<th>Mean width of fluke (mm)</th>
<th>Mean weight of fluke (g)</th>
<th>Weight of liver (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>800</td>
<td>320</td>
<td>40.00</td>
<td>51.4</td>
<td>11.3</td>
<td>1.32</td>
<td>5.59</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>312</td>
<td>39.00</td>
<td>50.3</td>
<td>11.2</td>
<td>1.28</td>
<td>5.43</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>355*</td>
<td>44.38</td>
<td>53.1</td>
<td>12.0</td>
<td>1.35</td>
<td>5.85</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>340</td>
<td>42.50</td>
<td>54.0</td>
<td>11.8</td>
<td>1.41</td>
<td>5.50</td>
</tr>
<tr>
<td>Mean</td>
<td>800</td>
<td>331.8</td>
<td>41.5</td>
<td>52.2</td>
<td>11.6</td>
<td>1.3</td>
<td>5.6</td>
</tr>
</tbody>
</table>

mc: metacercariae

*One animal out of four died on Day 110 post-infection.

Table 2. The altered haematological indices (Mean ± S.E.) in the respective groups.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Groups</th>
<th>Hb (g/dL)</th>
<th>PCV (%)</th>
<th>TEC (x10⁶/cmm)</th>
<th>ESR (mm/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Gr-I</td>
<td>12.97±0.9</td>
<td>39.88±1.0</td>
<td>6.32±0.5</td>
<td>34.0±8.5</td>
</tr>
<tr>
<td></td>
<td>Gr-II</td>
<td>13.03±0.8</td>
<td>40.13±1.8</td>
<td>6.35±0.5</td>
<td>35.2±7.8</td>
</tr>
<tr>
<td>2</td>
<td>Gr-I</td>
<td>12.25±0.2</td>
<td>39.3±0.1</td>
<td>6.11±0.2</td>
<td>34.75±4.9</td>
</tr>
<tr>
<td></td>
<td>Gr-II</td>
<td>12.75±0.3</td>
<td>40.2±0.3</td>
<td>6.54±0.2</td>
<td>36.0±2.8</td>
</tr>
<tr>
<td>4</td>
<td>Gr-I</td>
<td>9.2±0.4</td>
<td>28.75±1.4</td>
<td>4.8±0.2</td>
<td>34.0±4.3</td>
</tr>
<tr>
<td></td>
<td>Gr-II</td>
<td>12.9±0.3</td>
<td>39.9±0.3</td>
<td>6.5±0.2</td>
<td>34.25±5.0</td>
</tr>
<tr>
<td>6</td>
<td>Gr-I</td>
<td>5.65±0.2a</td>
<td>17.08±0.8a</td>
<td>2.8±0.1</td>
<td>59.25±7.0a</td>
</tr>
<tr>
<td></td>
<td>Gr-II</td>
<td>12.95±0.2a</td>
<td>39.8±0.4a</td>
<td>6.54±0.1a</td>
<td>34.75±2.8a</td>
</tr>
<tr>
<td>8</td>
<td>Gr-I</td>
<td>5.43±0.2b</td>
<td>16.85±0.3b</td>
<td>2.8±0.1b</td>
<td>55.0±8.17b</td>
</tr>
<tr>
<td></td>
<td>Gr-II</td>
<td>13.05±0.2b</td>
<td>40.25±1.0b</td>
<td>6.76±0.1b</td>
<td>37.0±1.8b</td>
</tr>
<tr>
<td>10</td>
<td>Gr-I</td>
<td>5.53±0.1b</td>
<td>18.25±0.1b</td>
<td>2.74±0.1b</td>
<td>64.5±4.9b</td>
</tr>
<tr>
<td></td>
<td>Gr-II</td>
<td>13.1±0.3</td>
<td>40.25±0.8</td>
<td>6.72±0.2</td>
<td>37.5±3.4a</td>
</tr>
<tr>
<td>12</td>
<td>Gr-I</td>
<td>5.73±0.2</td>
<td>19.5±0.1b</td>
<td>3.1±0.1b</td>
<td>42.25±9.2</td>
</tr>
<tr>
<td></td>
<td>Gr-II</td>
<td>13.15±0.3</td>
<td>40.25±0.4</td>
<td>6.7±0.1</td>
<td>33.75±7.0</td>
</tr>
<tr>
<td>14</td>
<td>Gr-I</td>
<td>6.05±0.2a</td>
<td>21.25±0.7a</td>
<td>3.21±0.1a</td>
<td>45.75±6.4</td>
</tr>
<tr>
<td></td>
<td>Gr-II</td>
<td>13.2±0.4a</td>
<td>39.95±0.6a</td>
<td>6.57±0.1a</td>
<td>34.25±3.0</td>
</tr>
<tr>
<td>16</td>
<td>Gr-I</td>
<td>6.28±0.2a</td>
<td>22.25±0.8a</td>
<td>3.24±0.1a</td>
<td>41.0±6.2</td>
</tr>
<tr>
<td></td>
<td>Gr-II</td>
<td>13.13±0.2a</td>
<td>39.7±0.5a</td>
<td>6.54±0.1a</td>
<td>36.25±2.3</td>
</tr>
</tbody>
</table>

The values bearing different alphabet as superscripts, differ significantly (p<0.05-0.01).
Figure 1. Serum cortisol levels (Mean ± SE) in the respective groups.

Figure 2. Serum cortisol levels (Mean ± SE) during different stages of the disease.
Figure 3. Total leucocyte counts (Mean ± SE) in the respective groups.

Figure 4. Eosinophils (Mean ± SE) in the respective groups.
30.4%, 28.86% and 27.7%, respectively, for Hb, PCV and TEC. However, during late prepaternity (weeks 7-12 PI), the falls were comparatively more: 57.1%, 54.31% and 54.4%. The altered levels of these haematological indices exhibited slight improvement during patency (week 12 PI onwards), but persistently remained depressed and were significantly different (p<0.05-0.01) from the preinfection values. Overall falls of 57.8%, 57.09% and 56.4%, respectively, for Hb, PCV and TEC were recorded in Group 1 animals by the end of the experiment. However, the erythrocyte sedimentation rate (ESR) showed an increasing trend during the corresponding weeks, but the values were significantly (p<0.05-0.01) higher during weeks 6-12 PI. In the healthy controls (Groups 2), these values fluctuated within normal range and were not significantly different (Table 2).

The infected animals (Group 1) revealed significant (p<0.05) leucocytosis with predominantly neutrophilia from the fourth week PI onwards and attained the highest level of 9.5±0.2x10^3/cmm by the end of the experiment (Figure 3). Likewise, eosinophils markedly increased in the infected group of animals (Group 1) and attained the highest level of 14.0±1.8% in the Group 1 animals by the end of the experiment (Figure 4).

The adrenal glands of the infected animals (Group 1) were grossly enlarged. There was an increase in the thickness of the cortex due to increased width of zona fasciculata. The corticocytes were hypertrophied, lost their
normal ovoidal/polyhydral shape and became elongated and columnar shaped. Their typical cord like arrangement pattern was disturbed. The cortical sinusoids were also comparatively dilated and engorged (Figure 5). The cytoplasm of the corticocytes evidenced single and/or multiple vacuoles. However, the outermost zona gromerulosa and the innermost zona reticularis of the cortex and the adrenal medulla did not reveal any appreciable histological changes. The adrenal glands of the healthy controls had normal shape, size and histological appearance.

**DISCUSSION**

The Group 1 animals receiving a primary infection dose of 800 *F. gigantica* metacercariae, sequentially exhibited characteristic signs of the tropical fasciolosis in buffaloes, higher faecal egg counts, depressed erythrocytic indices, leucocytosis and eosinophilia, pathognomonic hepatic lesions and in situ 41.47% fluke population of the dose of infection, analogous to earlier description (Yadav et al., 1999). The emaciated carcasses with gelatinization of subcutaneous fat on necropsy of Group 1 animals, strongly indicated the effect of elevated cortisol concentration on the overall metabolism operational in Group 1 animals and the magnitude of disease stress incidental to the distome, to meet extra energy requirements of the hosts having apyrexic inappetence with normal digestibility of nutrients (Mehra et al., 1999; Yadav et al., 1999). Besides, the periodical depressed erythrocytic indices, increased erythrocyte sedimentation rate (ESR) and leucocytosis with predominantly neutrophilia and eosinophilia in the Group 1 animals are in consonance with earlier reports on these aspects (Yadav et al., 1999; Ganga et al., 2007; Edith et al., 2010). The clinical manifestations and progress of the disease were synchronous with the growth, maturation of the infection dose and in situ fluke establishment in the infected animals, as elaborately discussed elsewhere (Yadav et al., 1999).

The adrenals are the most sensitive and versatile steroid hormone producing endocrine glands. They spontaneous interact with an altered in situ environment or a stimuli, and refluxed glucocorticosteroids, especially cortisol, into the host circulation to encounter the stimuli. An analysis of post-infection serum cortisol activity in the infected animals (Group 1) revealed that hypercortisolemia was the highest (82.97%) during early prepatency followed by 38.6% in late prepatency and 6.2% in the patency phase of the disease. These animals persistently had elevated hormone activity in the circulation in comparison with healthy controls, and this indicated variable degree and magnitude of adrenocortical response to the in situ distome population (stressor). The response was, however, variable depending upon clinical phase of the disease, in situ activities and location of the *F. gigantica* adolescercariae or adult fluke population.

The highest concentration of the hormone in Group 1 animals during the fourth week PI was suggestive of the highest degree of *F. gigantica* induced stress. Subsequent, progressive fall in the hormone activity synchronized well with the arrival and establishment of the causative organism in the hepatobiliary network, caessation of traumatic activity of the distome and partial resolution of the lesions in the hepatocytes and/or partial recovery of the animals from disease stress. On the contrary, the healthy controls (Groups 2) maintained along with the infected animals in the same environment did not suffer from any hypercortisolemia as
deduced from non-significant, all time within range fluctuations of the cortisol concentrations during the course of investigation. This rules out remotest possibility of the external/environmental/managemental factor(s) contributing towards the increased cortisol activity in Group 1 animals.

Further histopathological changes in zona fasciculata reported herein were also suggestive of hyperactivity of the corticocytes, persistently discharging cortisol into the host circulation, in proportion to nature and magnitude of the stimuli originating from the in situ host parasite interaction in Group 1 animals during different stages of the disease. The lesions did not completely resolve by the end of the experimental period and the animals continued to suffer subclinical stress incidental to the in situ flukes. Analogous hypercortisolemia and the histological changes in the zona fasciculata were also ascribed to Trypanosoma congolense infection in cattle (Ogwu et al., 1992). It is therefore speculated that the long-term secretion of cortisol (for 112 days) in Group 1 animals seems to have influenced the metabolism, and immune response of the host and consequently resulted in hepatomegaly, loss of collagen and elastin in the dermis, skeletal muscle weakness of the extremities and abdomen, lipolysis, etc., often evidence on necropsy in bubaline fasciolosis (Aiello and Mays, 1998). These findings on adrenal dysfunction during the course of investigation seems interesting to further explore to investigate and understand the impact of F. gigantica on the host as a syndrome rather than a disease confined to the liver.

The erythrocytic indices of Group 1 animals progressively decreased from week-4 PI onwards and significantly fell (p<0.05-0.01) during prepatency. It was, however, the highest during late prepatency, whereas erythrocytic sedimentation rates showed an increasing trend during the corresponding weeks. The events were synchronous with in situ migration, development of F. gigantica adolescercariae inflicting traumatic lesions/haemorrhagic tracts in the liver parenchyma. Partial improvement in the erythrocytic indices from week 12 PI onwards (during patency) indicated caessation of traumatic activities of the distome and its final establishment in the bile ducts. Analogous fluctuations were also documented earlier (Yadav et al., 1999). The altered erythrocytic indices during the acute course of bubaline fasciolosis seems to be a remarkably complex event in its origin and pathogenesis governed by several factors (Isseroff et al., 1979). The injurious effects of the fluke metabolites on circulating erythrocytes were recently confirmed by intraperitoneal inoculation of in vitro released F. gigantica metabolites for seven days in fluke free rats. A progressive and significant fall in erythrocytic indices was witnessed causing normocytic normochromic anaemia in the rats (Ganga et al., 2004a). Besides, it has also been documented that the in situ developing adolescercariae, at times accidentally feed on blood oozed out from the traumatic lesions in the liver (Radostits et al., 1994). Evidently, the ultimate consequence of the above on going events was a significant fall in oxygenated erythrocytes in circulation for a prolonged period of the experiment, causing generalized hypoxemia in the diseased host. The persistent eosinophilia reported herein, despite hypercortisolemia in the infected host, seems to be incidental to host-parasite interaction. The observed leucocytosis in Group 1 animals was response of the host defense mechanism against the invading distome.

In conclusion, the pathogenesis of tropical fasciolosis in buffaloes is a complex subject. More critically planned experiments with the main focus on F. gigantica induced hypercortisolemia
(persistent stress); physiological dysfunctions of the adrenal cortex through hypothalamus-pituitary-adrenal gland axis during the various stages of tropical fasciolosis are needed to precisely elucidate the persistent stress in large ruminant populations at risk of the disease. It would be interesting to investigate: (a) whether the *F. gigantica* induced dysfunction of adrenal cortex is a primary or secondary hyper adrenocorticism, (b) whether proliferation of corticocytes in zona fasciculata is induced and modified by the adreno corticotropic hormone or some other factors produced by corticotropic cells in the pituitary gland of the host, and (c) whether *F. gigantica* induced anaemia is consequential to injurious effects of the fluke metabolites on erythrocyte membrane and/or depressed bone marrow activity coupled with prolonged hypoxemia. Investigations should also be conducted to appreciate the overall impact of the aetiological agent on altered metabolism vis-à-vis health status, growth and development, and productivity of the buffaloes in the endemic areas.

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