

Acute neurotoxicity of acetaminophen in chicks

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ABSTRACT

Acetaminophen is a non-steroidal drug used as an anti-inflammatory, analgesic and antipyretic in humans and animals. In chicks, neurotoxicity associated with acetaminophen has not been fully elucidated. The aim of this study was to identify the neurobehavioral, biochemical and histopathological effects of acetaminophen in 7 day-old broiler chicks. The acute LD₅₀ of acetaminophen was estimated by the up- and- down method, and then the influence of acetaminophen on the open field activity and tonic immobility test was recorded. The behavioral signs and toxicity scores were recorded. The liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were estimated. Histopathology of the brain and liver were performed. The acetaminophen LD₅₀ value in chicks was 1077 mg/kg, intramuscularly. Acetaminophen reduced the general locomotive activity of the chicks, measured in the open- field arena, as a result of a significant rise in latency in moving from the central square, and a reduction in the numbers of lines crossed as well as reduction in the vocalization score compared to the control. Intramuscular injections of acetaminophen at doses of 500 and 1000 mg/kg induced signs of toxicosis, such as head dropping, closed eyelids, immobility, loss of vocalization, and recumbency, followed by death. Histopathological examination of the brain showed the presence of congestion of blood vessels, vasogenic edema and necrosis of Purkinje cells. Degenerative changes and liver enzyme function showed liver dysfunction. Our results show behavioral, biochemical and histopathological data demonstrating that acetaminophen at high doses produced acute neurotoxicity in chicks.

Key words: acetaminophen; neurotoxicity; open-field; chicks; LD50

Introduction

Acetaminophen is usually used as an antipyretic and analgesic agent (TRUMPER et al, 2005). The drug has also been used in broilers to induce certain models of toxicosis, which needs further delineation (MARMAT and RATHORE, 2015; JOULIDEH POUR, 2016). In spite of the limited information about acetaminophen in birds, it has limited therapeutic applications in chickens (HAWKINS and PAUL MURPHY, 2011; MARMAT and RATHORE, 2015 and MARMAT

and RATHORE, 2017). Several reports have shown the pharmacokinetics and toxicity of acetaminophen in experimental animals (JAMES et al, 2003), as well as in humans (INSEL, 1996). However, in chickens the data are rather scarce (NEIRINCKX et al, 2010; MOHAMMAD et al, 2012). Some reports have documented the therapeutic use of acetaminophen in the poultry industry, which calls for some concern among veterinarians regarding its extensive application in that business in the near

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future (JAYAKUMAR et al, 2010). Acetaminophen is mainly metabolized in the hepatic tissue through the combination with sulfate and glucuronic acid, and then excreted in the urine (HENDERSON et al, 2000; WALUBO et al, 2004). The metabolism of acetaminophen produces an extremely reactive cytochrome P450-dependent metabolite known as N-acetyl-p-benzoquinone imine (NAPQI) (HENDERSON et al, 2000). This metabolite reacts with glutathione to form a non-toxic conjugate that can be excreted in urine (WALUBO et al, 2004). Critically, acetaminophen overdose saturates these metabolic pathways, thus forming excess NAPQI, which binds to cellular protein, including the mitochondria, leading to liver injury as a result of, most probably, oxidative stress (HENDERSON et al, 2000; OJO et al, 2006). Importantly, the isoform CYP2E1 is expressed in the brain, indicating that acetaminophen could be metabolized by nerve cells, generating a toxic metabolite that causes neurotoxicity (JOSHI and TYNDALE, 2006; POSADAS et al, 2007). Acetaminophen is able to cross the blood - brain barrier, and it decreases the reactivity of glutathione and promotes oxidative stress in brain tissues (DA SILVA et al, 2012). While other reports emphasize the contrary, the antioxidant characteristics of this drug could be the basis for considering it in the neuroprotective therapy for some neurodegenerative disorders (MAHARAJ et al, 2004). Despite the widespread scientific investigations regarding the toxic impacts of acetaminophen on various organs, especially hepatic and renal tissues, the available findings about its pathological effects on neural tissue is still lacking in young chicks. For this reason, the goal of this study was to investigate the neurotoxic and neurobehavioral impact of acetaminophen in a model of young chicks.

Materials and methods

Animals. The experiment was conducted on sixty 7-14 day-old broiler chicks (Ross) of both sexes, with an average weight of 85-190 g obtained from a local hatchery. The chicks were kept under constant illumination at a room temperature between 32-35 °C. Wood shavings were used as litter. Water and diet were provided *ad libitum*.

Acetaminophen pure powder (Hebel -JIHENG, Group China) was dissolved in propylene glycol (99%, Sigma Chemicals, USA) at 5 ml/kg (volume of injection). All experiments complied with university regulations dealing with the humane and appropriate use of laboratory animals in experimental research.

The research protocol and experimental design were reviewed and approved by the Scientific Committee of the Department of Physiology, Biochemistry, and Pharmacology of the College of Veterinary Medicine under reference No. 3rd meeting in 5-11/11/2018.

Acute median lethal dose of acetaminophen in chicks. The acute (24-hour) median lethal dose (LD₅₀) of acetaminophen was determined for the first time by using the up-and-down method (DIXON, 1980). The injected chicks were observed individually for one hour to check for any signs of toxicity, and then the mortality within 24 hours was recorded.

Acetaminophen effects on open-field activity and tonic immobility test in chicks. Eighteen chicks were randomly divided into three groups (6/group). The chicks were injected with either 5 ml/kg propylene glycol (control group) or 100 and 200 mg/kg acetaminophen, intramuscularly. Following the preliminary experiments on chicks, the doses of acetaminophen were selected on the basis of the absence of any apparent manifestation of toxicity within one hour of injection. Thirty minutes after propylene glycol or acetaminophen injection, the chicks were examined during the open field activity test for 5 minutes (AL-ZUBAIDY and MOHAMMAD, 2013; MOUSA et al, 2021).

Each chick was placed separately in the middle of an open field arena (60 x 60 x 30 cm) which was divided into 16 equal squares. Fifty grams of wheat grain were dispersed on the surface. The number of squares entered by both their feet in 5 minutes was recorded. All the chicks were used for the tonic immobility behavioral paradigm (HENNIG et al, 1984) following the open-field activity test. By holding the chick in both hands and putting it on a wooden table for 15 seconds, then removing the hands, the time it took the chick to stand upright

was measured (MOUSA et al, 2021; Al-ZUBAIDY and MOHAMMAD, 2013).

Signs of acetaminophen toxicosis. Eighteen chicks were randomly divided into three groups (6/group). The dosages of acetaminophen (0 propylene glycol-vehicle, 500, 1000 mg / kg, i.m), were selected following determination of acute LD₅₀ for acetaminophen. Within 2 hours, latencies for displaying any sign of poisoning were registered. In each treated group, the severity of toxicosis was scored as reported earlier (Al-BAGGOU and MOHAMMAD, 1999). The following grades were allocated to the proportion of incidence of indication of toxicity (head drooping, closing eyelids, immobility, loss of vocalization, and recumbency), including 24-h lethality: Grade 1 (1~25%), Grade 2 (26~50%), Grade 3 (51~75%) and Grade 4 (76~100%). The score for toxicity would be 20 (5 signs, occurring 100 percent,) in a group displaying all the signs of poisoning (head drooping, immobility, loss of vocalization, closing eyelids and recumbency) (Al-BAGGOU and MOHAMMAD, 1999).

Biochemistry assessment. After 2 h from the acetaminophen injection (0 propylene glycol-vehicle, 500 and 1000 mg/kg, i.m.), blood was obtained from the jugular vein of chicks from every group. Geno TEK. Chemistry Analyzer Smart-150, U.S.A. was used to determine serum AST and ALT activity levels (expressed in U/L). Chicks treated with acetaminophen at 1000mg/kg were dissected, and the brain and liver samples were taken for histopathological examination.

Histological examination. The chicks treated with acetaminophen 1000 mg/kg were dissected to collect 1cm³ from both brain and liver tissues for histopathological testing. Then, these specimens were fixed in 10% neutral buffer formalin for 72 hours. Following fixation, the specimens were processed by routine histological processing to obtain 5-6 micrometer (µm) thick sections using a rotary microtome. Samples were stained with Harris Hematoxylin and Eosin (SUVARNA et al, 2013). The histological sections were photographed using a tube microscope camera, HDCM-5, provided by picture analysis software.

Statistical analysis. Continuous data were evaluated statistically by one-way analysis of variance, followed by the least significant difference test, whereas the frequency data were subjected to Fisher's exact probability test (KUSS et al, 2014). The significance level was P< 0.05.

Results

LD50 experiment. The acute (24-hour) LD₅₀ of acetaminophen in chicks injected intramuscularly was (1077mg/kg) (Table1). The signs of toxicity that emerged within 2-12minutes following i.m. injection included head drooping, closing of the eyelids, immobility, loss of vocalization and recumbency, followed by death (Table 1).

Acetaminophen effects on open-field activity and the tonic immobility test. The results from the 5-minute open-field activity and tonic immobility test of acetaminophen-treated chicks (100 and 200) mg/kg i.m. are shown in Table 2.

Acetaminophen reduced the general locomotive activity of the chicks in the open-field arena, as a result of a significant increase in the time taken to move from the central square, and a reduction in the number of lines crossed, as well as a reduction in the vocalization score compared to the control values (Table 2). Acetaminophen also prolonged the duration of the tonic immobility reaction of the chicks in comparison with controls.

Acute toxicity signs of acetaminophen. Acute toxicity signs of acetaminophen and toxicity scores are shown in Table 3. Further, acetaminophen at 500 and 1000 mg/kg, i.m. produced signs of poisoning in the boiler chicks, which was manifested as head drooping, closing of the eyelids, immobility, loss of vocalization and recumbency.

Biochemistry assessment. The serum (ALT and AST) activities of 1000 mg/kg of acetaminophen increased significantly compared with control group (Table 4).

Table 1. The median lethal dose of acetaminophen (LD50) in chicks over 24 hours

Value	Results
LD50 mg/kg	1077 i.m
Range of doses mg/kg	2500-1000 = 1500
Initial dose mg/kg	2500
Last dose mg/kg	1000
Increase or decrease in the dose mg/kg	500
Number of chicks	6 (XXOXXX) *
Signs of poisoning	Closed eyelids, head dropping, immobility, loss of vocalization, recumbency, followed by death.
Range of latency to the onset of poisoning (min)	2-12

*X = died; O = survived

Table 2. Effect of acetaminophen on five minute open -field activity and tonic immobility test

Variable	Control (propylene glycol)	Acetaminophen - mg/ kg, intramuscularly	
		100	200
Latency to move / s	89.80 ± 54.08	180.00 ± 0.00*	180.00 ± 0.00*
Lines crossed	2.00 ± 1.4	0.16 ± 0.16	0.00 ± 0.00*
Escape jumps	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Distress calls (scores)	2.33 ± 0.17	1.4 ± 0.42	0.00 ± 0.00 ^a
Pecking (scores)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Defecations	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Tonic immobility / s	12 ± 6.35	97.83 ± 14.39*	143 ± 32.81 *

Data was the Mean ± S.E. For 6 chicks / group. Significantly different from the control group (propylene glycol) at $P < 0.05$; a Significantly different from the second group (100 mg/kg of acetaminophen) at $P < 0.05$; Behavioral measurements were taken 30 min after the acetaminophen injection.

Table 3. Signs of acetaminophen toxicity with toxicity scores

Groups	Onset of Toxicity signs (min.) +	Toxicity signs %					Toxicity scores
		Immobility	Loss of vocalization	Close the eyelids	Head dropping	Recumbency	
Control (propylene glycol)		0.00	0.00	0.00	0.00	0.00	
Acetaminophen (500 mg/kg) i.m.	4.33 ± 0.49	100*	100*	100*	83*	83*	20
Acetaminophen (1000 mg/kg i.m.)	2.83 ± 0.31*	100*	100*	100*	100*	100*	20

Data are the Mean ± SE. For 6 chicks / group. Significantly different from the control group (propylene glycol) at $P < 0.05$.

Table 4. Serum AST and ALT levels in the chicks treated with acetaminophen

Groups	AST (U/L)	ALT (U/L)
Propylene glycol (control)	140.33 ± 10.13	8.11 ± 1.74
Acetaminophen (500 mg/kg)	198.67 ± 10.18*	10.66 ± 2.37
Acetaminophen (1000 mg/kg)	298.33 ± 5.23*a	19.33 ± 2.59*a

The values represent mean ± SE for 6 chicks/ group. Blood samples were obtained after 2 h of acetaminophen injection (500 and 1000mg/kg, i.m). * Significantly different from the control group (propylene glycol) at P< 0.05. a Significantly different from the second group (500 mg/kg of acetaminophen) at P< 0.05

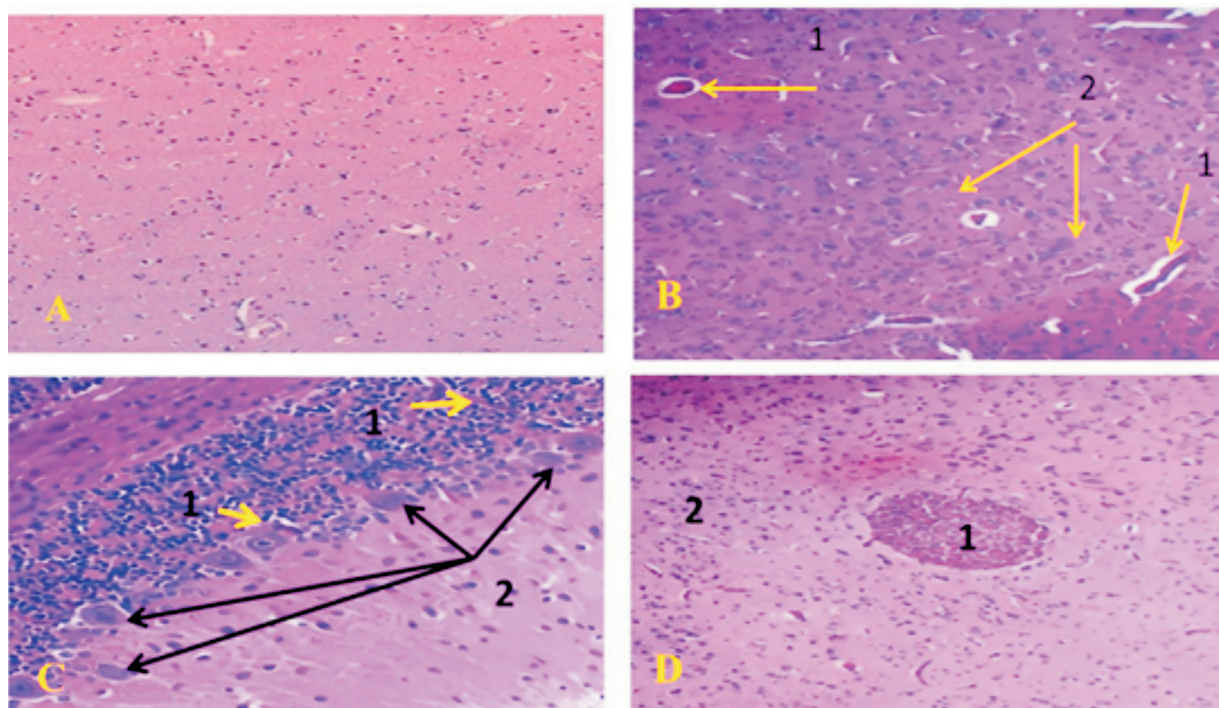


Fig. 1. Microphotograph of a chick's brain treated with paracetamol 1000 mg/kg i.m. (A) cerebral section from the control group (H&E, ×100). (B) Cerebrum, congested blood vessels with vasogenic edema (1), neuronal necrosis (2) (H&E, ×300). (C) Cerebellum, congested blood vessels (1), purkinji cell necrosis (2) (H&E, ×300). (D) Cerebrum infarction foci (1), infiltration of mononuclear cells (2) (H&E, ×100).

Histopathological findings. Fig. 1A, brain: microscopic pathological examination of a brain specimen of chicks treated with acetaminophen 1000 mg/kg b.w i.m. showed congestion of blood vessels, neuronal necrosis in the cerebrum, with vasogenic edema, compared with the control group. Fig. 1B - severe congestion of the cerebellum, necrosis of purkinje cells. Fig. 1C - the cerebrum appears multiple foci of infarction with infiltration of mononuclear cells Fig. 1D.

Fig. 2, liver: microscopic pathological test of a liver specimen of chicks injected acetaminophen 1000 mg/kg b.w i.m indicated necrosis of hepatocytes, the central vein shows congestion and dilatation compared with the control group Fig. 2B, another section shows hemorrhage in the hepatic parenchyma Fig. 2 C, infiltration of inflammatory cells (lymphocytes, macrophage and mononuclear). Fig. 2 D, deposition of fibrous tissue with infiltration of mononuclear cells in the portal area also reported Fig. 2 E, hyperplasia of epithelial cells lining the bile duct, Fig. 2 F.

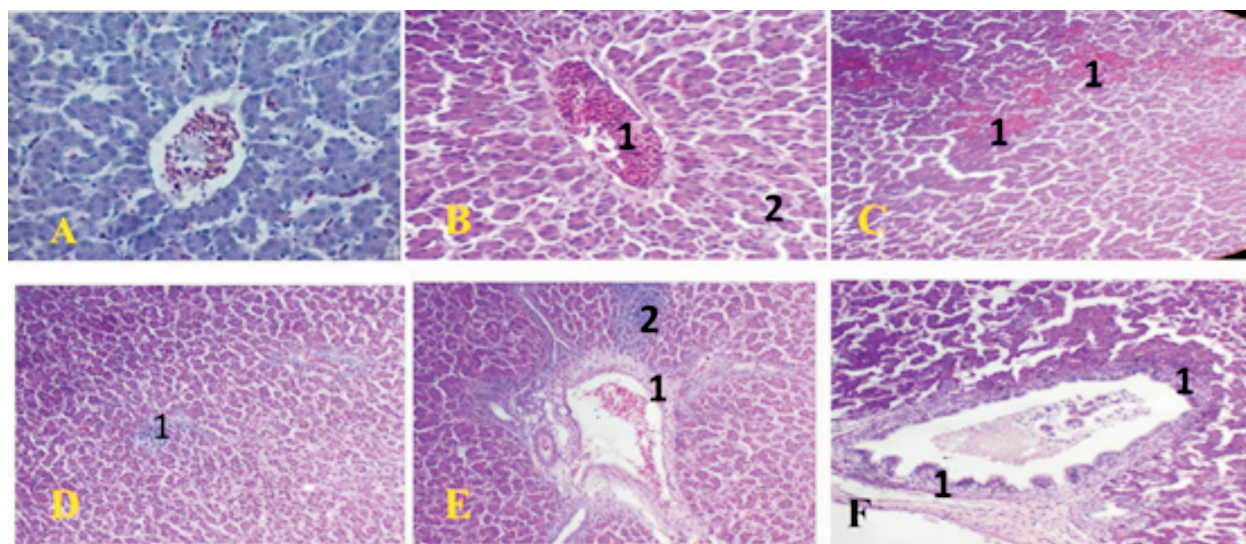


Fig. 2. Microphotograph of a chick's liver, treated with paracetamol 1000 mg/kg i.m. (A) normal architecture of liver (H&E, $\times 150x$). (B) Dilation and congestion of the central vein (1), necrosis of hepatocytes (2) (H&E, $\times 150$). (C) Hemorrhage in the hepatic parenchyma (1) (H&E, $\times 150$). (D) Infiltration of mononuclear inflammatory cells (1) (H&E, $\times 100$). (E) Deposition of fibrous tissue (1) with infiltration of mononuclear cells (2) in the portal area. ($\times 150$). (F) hyperplasia of epithelial cells lining bile duct (1) (H&E, $\times 200$).

Discussion

To our knowledge, the toxicological aspect of acetaminophen in avians lacks information. Thus, our study aimed to clarify the acute neurotoxic effect of acetaminophen in broiler chicks. In this study, acute LD_{50} values of acetaminophen were found by the up and down method, which might be the first attempt, in this context, to investigate LD_{50} in 7-day old broiler chicks using the above strategy. The acetaminophen signs of toxicity observed in this study included: depressive effects, such as closing the eyes, immobility, loss of vocalization, dropping the head, and recumbency. These observations expand the range of signs in this kind of toxicity over the findings of MARMAT et al (2015), which were restricted to dullness and unconsciousness in chicks treated with acetaminophen at 1800 mg/kg. Our additional findings may highlight the importance of using the up-and-down method as a practical tool to investigate toxicity in avians.

Acetaminophen is considered as probably one of predisposing factors of neurobehavioral or neurodegenerative disorders (SHARPE, 2008). These disorders can be confirmed using open-field and/or tonic immobility tests (HAYES,

2007; AL-ZUBAIDY and MOHAMMAD, 2013). Acetaminophen in an acute single dose (100 - 200 mg/ kg) i.m. produces an open-field test behavior pattern characterized by a decrease in general locomotive activity. Furthermore, the tonic immobility test demonstrates the depressing action of acetaminophen on the brain. The resulting depression may be due to the ability of acetaminophen to increase the level of GABA, concomitantly reducing the availability of glutamic acid, which is responsible for excitatory neurotransmission by affecting the brain N-methyl-D-aspartate receptors, and thereby altering cognitive function and neurotoxicity (BLECHARZ-KLIN et al, 2014). It is well established that drugs depressing brain activity may decrease the general locomotion of chicks and rodents in the open-field behavioral pattern, and vice versa (HAYES, 2007; TSUNEYOSHI et al, 2007). Our histological findings showed degenerative changes in neuronal tissues as a result of acetaminophen treatment (Fig. 1), and these results agree with previous reports in rats (POSADAS et al, 2007; ESSAWY et al, 2017).

In addition, acetaminophen is a potential activator of the neuronal CYP2E1, therefore toxic metabolites, such as NAPOI, is expected. An increased level of NAPOI inhibits glutathione, hence promoting oxidative stress and concomitant neurotoxicity (JOSHI and TYNDALE, 2006). Excess amounts of acetaminophen negatively affect antioxidant agents such as vitamin C and superoxide dismutase (NENCINI et al, 2007). This may disturb the oxidants-antioxidant balance in the brain, leading to pathological changes, such as tissue degeneration (HALLIWELL, 2006).

In the brain, due to elevated unsaturated fatty acid content, high oxygen consumption, as well as poorly designed oxidative defense mechanisms, the brain tissue is extremely exposed to oxidative stress (FLOYD, 1999).

Several studies have confirmed that acetaminophen administration induces liver damage in humans and rodents, through mitochondrial injury and oxidative stress, as well as activation of c-Jun N-terminal kinase (JNK), with fragmentation of nuclear DNA (HEARD et al, 2014). While in rats the injury and cell death mechanisms, though rare, occur as a result of apoptosis (HEARD et al, 2014; JAESCHKE et al, 2014). Our study agrees with previous research confirming that an overdose of acetaminophen in mice, rats and chicks may lead to serious widespread cell necrosis in the hepatic centrilobular region, elevating the levels of serum ALT and AST (MARMAT and RATHORE, 2015; MARMAT and RATHORE, 2017; DU et al, 2017). All these expected toxic effects cause shock to the chicks, treated with acetaminophen, leading to progressive dullness, unconscious and death (MARMAT and RATHORE, 2015). Acetaminophen-induced hepatotoxicity may result in hepatic encephalopathy, through accumulation of ammonia, in addition to the formation of ROS that damages brain tissues (TRUMPER et al, 1996).

Conclusion

The data from this study showed that acetaminophen at high doses causes acute neurotoxicity in chicks, in the form of reduced open-field activity, tonic immobility testing, and brain histopathology. Future studies are essential

to explain the precise neurotoxic mechanism of acetaminophen when it is used at toxic doses.

Conflict of interest

The author declares no conflict of interest.

Author's contribution

All the work was done by the author, except histopathological reading which was done by a specialist in pathology.

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SAŽETAK

Acetaminofen je nesteroidni lijek koji se upotrebljava kao protuupalno, analgetičko i antipiretičko sredstvo u ljudi i životinja. U pilića nije potpuno poznata povezanost neurotoksičnosti i acetaminofena. Cilj ovog istraživanja bio je identificirati neurobiheviornalne, biokemijske i histopatološke učinke acetaminofena u sedmodnevnih brojlera. Akutni LD50 acetaminofena procijenjen je metodom "up and down" nakon čega je zabilježen utjecaj acetaminofena na aktivnost na otvorenom i toničnu nepokretnost. Zabilježeni su biheviornalni znakovi i razina toksičnosti. Izmjerene su vrijednosti jetrenih enzima, aktivnost aspartat-aminotransferaze (AST) i alanin-aminotransferaze (ALT). Učinjena je histopatološka pretraga mozga i jetre. Vrijednost LD50 acetaminofena u pilića bila je 1077 mg/kg, intramuskularno. Acetaminofen je smanjio opću lokomotornu aktivnost i stupanj glasovnog izražavanja pilića na otvorenom prostoru, kao posljedica znakovitog porasta latencije u odnosu na piliće kontrolne skupine. Intramuskularna injekcija acetaminofena u dozama od 500 i 1000 mg/kg izazvala je znakove toksikoze kao što su padanje glave, spuštene kapci, nepokretnost, gubitak glasa, ležeći položaj te smrt. Histopatološka pretraga mozga pokazala je kongestiju krvnih žila, vazogeni edem i nekrozu Purkinjeovih stanica. Došlo je do degenerativnih promjena i disfunkcije jetre što su pokazali jetreni enzimi. Biheviornalni, biokemijski i histopatološki podaci iz ovog istraživanja pokazali su da acetaminofen u visokim dozama uzrokuje akutnu neurotoksičnost u pilića.

Ključne riječi: acetaminofen; neurotoksičnost; otvoreni prostor; pilići; LD50
