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The Effect of Paracetamol on The Development of Chicken Embryos

Hendry Tri Sakti Surya Gunawan Saragih*, Alfian Primahesa, Shintia Oktaviana, Agnessia Asian Budi Setyo, Alifia Anisya, Hayu Swari Allimi, Laili Mufli Zusrina, Ruth Liananda Citra Dolok Saribu, Ardaning Nuriliani, Nur Indah Septriani
Faculty of Biology, Universitas Gadjah Mada,
Jl. Teknika Selatan, Sleman, Daerah Istimewa Yogyakarta 55281, Indonesia.

Corresponding author*
saragihendry@ugm.ac.id

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Abstract

Paracetamol (N-Acetylaminophenol) is the most widely used over-the-counter drug in the world. Although considered safe for pregnant mothers, there is a concern regarding the teratogenic effect of paracetamol. This study aims to observe the teratogenic effect of paracetamol developing embryos. Using chicken embryos, the embryos were incubated for 48 hours and then injected with paracetamol in 3 concentrations, namely 10 ppm, 15 ppm, and 20 ppm. The control group and treatment group consisted of 3 replicates of fertile embryos. Then, the embryos were incubated again for 48 hours in the incubator. Data analysis was carried out descriptively by observing any developmental alterations. Results showed that paracetamol caused enlargement of the head and heart edema. Exposure to paracetamol at concentrations of 15 ppm and 20 ppm affects the morphology of chicken embryos, especially the formation of the head and disrupts the process of angiogenesis and normal heart formation, causing bleeding and edema at higher concentrations.

Keywords: abnormality; chicken embryo; development; paracetamol.

INTRODUCTION

Paracetamol (para-hydroxyacetanilide), the common name of acetaminophen (N-acetyl aminophenol), is an over-the-counter (OTC) drug used as an analgesic and antipyretic (Sharma and Mehta, 2014). Aside from pain and fever, it's also commonly used to ease headaches, both by itself or in conjunction with other migraine medications such as caffeine and non-steroidal anti-inflammatory drugs (NSAIDs) (Pini et al. 2008), and it's used to treat patent ductus arteriosus in newborns (Ohlsson and Shah, 2020). It is one of the most commonly used medications worldwide (Moore and Moore, 2016; Becker, 2015). Paracetamol is used by over 50% of pregnant women during their pregnancy (Lupattelli et al. 2014; Werler et al. 2005). Existing guidelines recommend the use of paracetamol during pregnancy at the lowest possible dose in the shortest possible time, with health professional consultation recommended if prolonged use is necessary (European Medicines Agency, 2019; Food and Drug Administration, 2019).

Over the last few decades, there has been a concern over the increasing prevalence of urogenital, neurodevelopmental, and reproductive disorders in children and adults alike (Bauer et al., 2021). Because paracetamol readily crosses the placental barrier Rayburn et al. 1986), paracetamol was indicated as one cause of the rising incidence. Thus, several researchers urge caution when administering paracetamol to pregnant mothers (Nilsen et al., 2023; Bauer et al., 2021), while some disagreed over the conclusion of the review due to faulty methodology and failure to consider confounding factors in studies used to support the notion that paracetamol is the leading cause of said birth disorders (Alwan et al., 2022; Damkier et al., 2022).

Even though several observational studies on paracetamol's effect on human fetuses have been made (Bauer et al., 2018; Andrade, 2016), studies of the teratogenic effects of paracetamol in animal models are rather scarce. Several studies conducted on zebrafish larvae have shown that paracetamol induces heart defects and affects the hatching rate of its larvae, as well as its motor function (Rosas-Ramírez et al., 2022; Xia and Zhou, 2017). However, an *ex vivo* study on rat embryos shows no prominent defect (Moungmaithong et al., 2022). There is considerable difficulty in investigating the teratogenicity of paracetamol because, in mammals, maternal prostaglandin is often able to compensate for the disruption of fetal prostaglandin synthesis, thus rendering it difficult to assess the importance of prostaglandin and cyclooxygenase in embryogenesis (Cha et al. 2006). Chick embryo, with its similarity to the mammalian embryo and ease of labeling, is a great

alternative for embryology studies (Ribatti and Annese, 2023). Research on the effect of paracetamol on chicken embryo has not been widely conducted. Thus, in this study, we expose chick embryos to different doses of paracetamol to study its effect on embryo growth.

MATERIALS AND METHODS

Procedures

Incubation the eggs

The eggs that were obtained are placed in an incubator at a temperature of 37 ± 1ºC. Incubation was carried out for 48 hours. Total 12 eggs incubated and then divided into 4 groups (control/0 ppm, 10 ppm, 15 ppm and 20 ppm paracetamol).

Preparation paracetamol solution

Paracetamol solution was prepared by dissolving a paracetamol tablet and then diluting into 10 ppm, 15 ppm and 20 ppm.

Exposure of chicken embryo to paracetamol

The eggs were injected with paracetamol in 3 concentration groups; 10 ppm, 15 ppm and 20 ppm. The eggs were hollowed out to inject 0.1 ml of paracetamol. After that, the eggs is closed with hypafix and returned to the incubator. The Injection of the eggs were conducted in LAF (Laminar Air Flow) to minimize contamination. The eggs were incubated again for 48 hours.

Embryo collection and making whole mount preparations

Eggs that have been incubated for 96 hours are taken from the incubator. The eggshell was opened in a warm 0.9% NaCl solution. The embryo was washed with 0.9% NaCl solution. Embryos were fixed using Bouin's solution for 24 hours. After fixation, the embryo was washed using 70% alcohol and stained using Eosin for 30 seconds. The Embryo was dehydrated using graded alcohol for 5 minutes each. After that, the embryo was cleared using Xylol for 30 minutes.

Data analysis

Embryos were observed using a Leica microscope and then documented. Data analysis used a descriptive method by observing and explaining the visible abnormalities.

RESULTS AND DISCUSSION

To check the possibility of abnormalities in chicken embryo growth caused by paracetamol, 3 treatments with different doses are needed, namely 10 ppm, 15 ppm, 20 ppm, and a control as a comparison. Based on the results of microscopic observations, it can be seen that abnormalities can be seen in embryos treated with 10 ppm. The most visible abnormalities are the head (mesencephalon and telencephalon) and heart (Figure 3). Embryos treated with 15 ppm and 20 ppm experienced abnormal growth, and their size was huge, apart from that, embryos treated with 15 and 20 ppm experienced a very dark color change so that it was difficult to distinguish between the head and body (Figure 4 and Figure 5). Untreated embryo embryos (control) did not experience abnormalities (Figure 2).

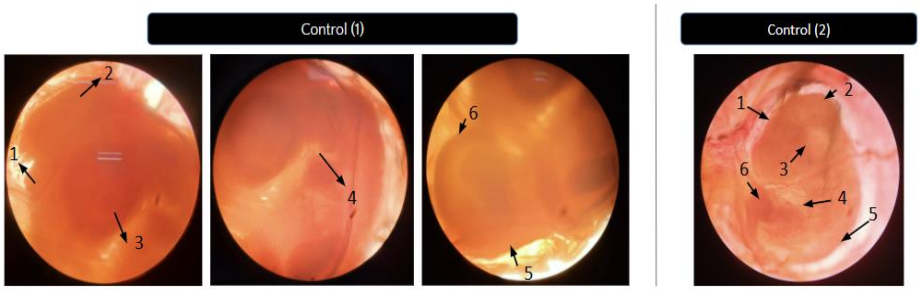

Figure 1. Control Group. No abnormalities were seen. The sizes of (1) Diencephalon, (2) Mesencephalon, (3) Eye, (4) Conus arteriosus, (5) Somite and (6) Tail normal. Magnification: x40.

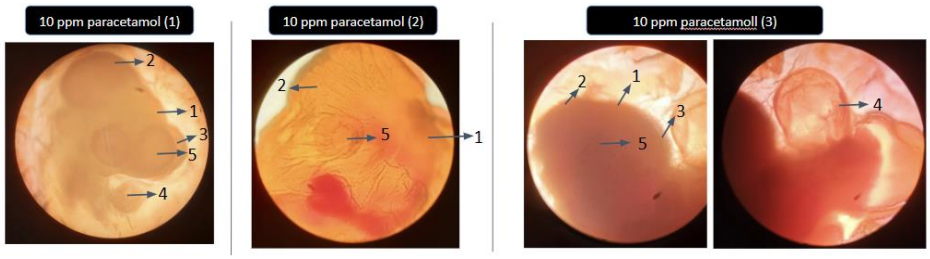

Figure 2. Embryos treated with 10 ppm Paracetamol. Abnormalities can be seen in the head and heart. (1) Diencephalon, (2) Mesencephalon, (3) Telencephalon and (4) Conus arteriosus look enlarged/swollen. Magnification: x40.

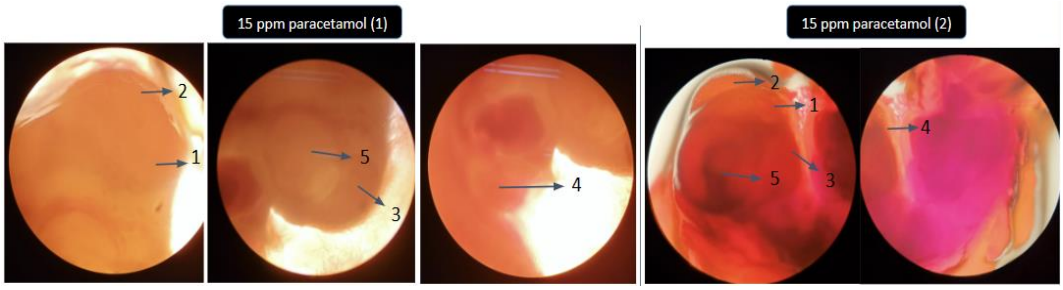

Figure 3. Embryos treated with 15 ppm Paracetamol. Abnormalities can be seen in the head and heart. Parts of the head such as (1) Diencephalon, (2) Mesencephalon, and (3) Telencephalon look enlarged/swollen. Magnification: x40.

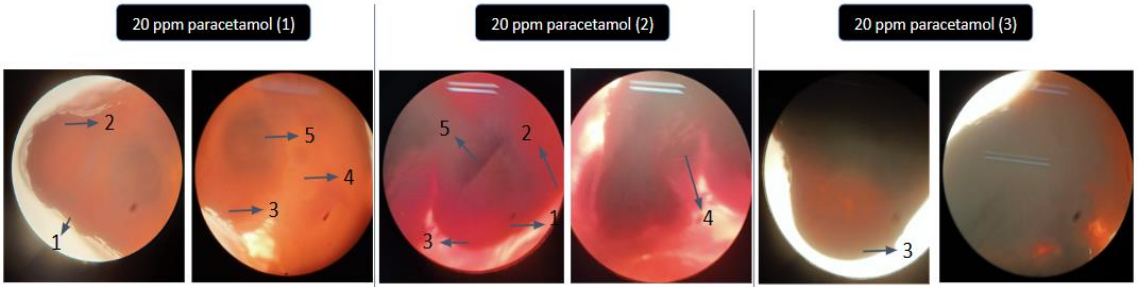

Figure 4. Embryos treated with 20 ppm Paracetamol. Abnormalities can still be seen in the head and heart. In some preparations, parts of the head such as (1) Diencephalon, (2) Mesencephalon, (3) Telencephalon and (4) Heart look enlarged/swollen. Magnification: x40.

Discussion

The results showed that several abnormalities were observed in embryos exposed to paracetamol. Several abnormalities were larger head size and cardiac edema. The enlargement of the head is due to astrocyte swelling in the brain. NAPQI (N-Acetyl-p-benzoquinone imine), a paracetamol metabolite, is known to be hepatotoxic (Athersuch et al., 2018). Damage to the liver thus impaired its capability of maintaining blood ammonia homeostasis, forcing other organ systems to compensate, including the nervous system via astrocytes (Aldrige et al., 2015). This caused astrocyte swelling, which in turn caused fluid accumulation (edema) in the brain. However, the molecular mechanism of the swelling of astrocyte cells due to ammonia is still unknown (Seperhrinezhad et al., 2020). Traditionally, glutamine - the product of ammonia detoxification - accumulation in astrocytes was blamed as the sole cause of edema due to its nature as an osmolyte; however the emerging "Trojan Horse" hypothesis posits that excess glutamine converted by astrocytes was transported to astrocyte's mitochondria and would later be converted to ammonia and glutamate, causing oxidative stress and mitochondrial damage that leads to swelling (Scott et al., 2013). Cardiac edema is caused by a buildup of fluid in the heart's ventricles (edema). NAPQI is known to irreversibly bind to glutathione synthetase (GS) *in vitro* and cause glutathione depletion (Walker et al. 2016). As glutathione plays a central role in antioxidant defense, depletion of glutathione causes oxidative stress (Sekhar et al., 2011). ROS and oxidative stress can act as signaling molecules that can stimulate 2 things: cardiomyocyte cell hypertrophy or apoptosis. Cardiomyocyte apoptosis can cause cardiac dysfunction and congenital heart failure (Chung and Lin, 2018). One of the manifestations of heart failure is cardiac edema (Abassi et al., 2022).

Mechanism of action of paracetamol in the body.

Paracetamol is mainly converted to soluble glucuronide and sulfonate by phase II metabolizing enzymes, with a fraction of it converted to NAPQI in the cytochrome 450 system (Roy et al., 2013). In therapeutic doses, approximately 10% of paracetamol is metabolized by CYPs to form NAPQI, which is then conjugated by intracellular glutamate and ultimately excreted as conjugates with cysteine and mercapturic acid (Mazaleuskaya et al., 2015). Paracetamol has more potency when prostaglandins are synthesized from added arachidonic acid in low concentrations or after activating delayed pathways via cytokines (Graham and Scott, 2003). Paracetamol has a major pharmacological effect as an inhibitor of prostaglandin synthesis in cells under low arachidonic acid conditions and is selective against COX-2, one of the cyclooxygenase (COX) enzymes (Graham et al., 2013). COX enzymes, also known as Prostaglandin H Synthases (PGHS), catalyze the first step of prostanoid synthesis by converting arachidonic acid to prostaglandins (PGs), including PGH2, PGE2, 15-keto-PGE2, PGD2, PGF2α, PGI2, and thromboxane A2 (TXA2) (Seo and Oh, 2017). This enzyme has functions namely fatty acid cyclooxygenase by catalyzing the change of arachidonic acid to PGG2 and prostaglandin hydroperoxidase by catalyzing the change of PGG2 to PGH (McCrae et al., 2018). Peroxidase function is the basis of COX-1 and COX-2 activity, but

they can function independently and allow them to oxidize various organic substances using hydrogen peroxide or other peroxides (Yang et al., 2020). Paracetamol is one of the COX-1 substrates that can be oxidized, and it is thought that the peroxidase function of COX-2 can also oxidize paracetamol (Graham et al., 2013). COX-1 is constitutively expressed and present in the majority of healthy human tissues, also plays a role in normal physiological processes, while COX-2 is often upregulated by infection and inflammation, but can be constitutive although it is still upregulated by peripheral inflammation in the spinal cord (Radi et al., 2010). COX-1 is down-regulated in the oral mucosa after oral surgery, and it has been suggested that paracetamol may inhibit COX-1 in the early stages of inflammation, but with continued inflammation, COX-2 is induced (Lee et al. 2007), and then subjected to inhibition by paracetamol. Then, the following mechanism is COX-2 selective oxygenation inhibition of endocannabinoids, 2-arachidonoylglycerol, and anandamide (arachidonoyl ethanolamide) resulting in active Prostaglandin products (Alhouayek and Muccioli, 2014). Selective inhibitors of paracetamol and COX-2 reduce the amount of endocannabinoids that are oxidized and cause inhibition of endocannabinoid metabolism so that it can establish interactions between endocannabinoids with paracetamol and some NSAIDs (Roy et al., 2013). Interestingly, R-isomers of ibuprofen and other related drugs also inhibit endocannabinoid-mediated oxidation of endocannabinoids (Duggan et al., 2011).

Teratogenic effects of paracetamol

The teratogenic effects of paracetamol can be caused by several mechanisms, namely the metabolic products of paracetamol - N-APAP (N-acetyl-P-Aminophenol); NAPAP will interfere with cell signaling pathways by increasing oxidative stress in the embryo which can then interfere with the organogenesis process (Gutiérrez - Noya et al. 2021; Rosas- Ramírez et al. 2022). Oxidative stress refers to increased levels of intracellular reactive oxygen species (ROS) that cause damage to lipids, proteins, and DNA (Schieber and Chandel, 2014). Paracetamol will also inhibit the production of PGE2 (Prostaglandin) such as VEGF (Vascular Endothelial Growth Factor) and bFGF (Basic Fibroblast Growth Factor) which play a role in the process of angiogenesis (Cha et al. 2006). Disruption of the angiogenic balance can increase vascular dysfunction, which is responsible for several systemic effects on blood pressure and organ function (Waller et al. 2021). In addition to playing a role in the angiogenesis process, bFGF will also regulate muscle growth (Pawlikowski et al. 2017), and regulate neuroepithelial proliferation, migration, and differentiation so that the combination of several mechanisms can interfere with the process of organogenesis by chicken embryos resulting in embryonic morphological abnormalities.

CONCLUSIONS

Paracetamol can cause abnormalities in chicken embryos. Embryos that were not treated did not experience abnormalities, while embryos that were given 10 ppm paracetamol experienced abnormalities in the form of enlarged heads and hearts. Exposure to paracetamol at concentrations of 15 ppm and 20 ppm affects the morphology of chicken embryos, especially the formation of the head and disrupts the process of angiogenesis and normal heart formation, causing bleeding and edema at higher concentrations.

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