[Vetmed Journals] Article submission

actavet@vfu.cz Tue, Jul 9, 2019, 6:55 PM

to epymuhammadl, me, widjiati1962, widjiati, ekamirta, eka-p-h, bencthp, benjamin-c-t-09, lita-r-y, lita.yustin

Dear Dr Epy Muhammad Luqman, M.Sc,
Thank you for your interest to publish in Acta Veterinaria Brno. Your manuscript **Interaction between Autophagy, Apoptosis and Necrosis of Infant Mice (Mus musculus)'s Brain Cells from Its Carbofuran Exposed Mothers during Lactation Periods** written by Muhammad Luqman Epy, Widjiati Widjiati, Pramyrtha Hestianah Eka , Christoffel Tehupuring Benyamin and Rakhma Yustinasari Lita has been registered as 65/2019-ACTA and submitted to reviewers.
If the reviews are not available within **4** months after submission **(but not incorporating time needed to amend the article at the author's site, if instructed so by the editorial office)**, authors are free to submit the manuscript to another journal. However, their announcement of cancelling the assessment procedure has to be sent to the editorial office to e-mail address actavet@vfu.cz
Yours sincerely,
Assoc. Prof. MVDr. Josef Kameník, CSc., MBA
Editor-in-Chief
Acta Veterinaria Brno
Palackeho tř. 1/3
612 42, Brno
CZECH REPUBLIC
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[Vetmed Journals] Corrected paper submission

actavet@vfu.cz Sat, Jul 27, 2019, 1:53 PM

to epymuhammadl, me, widjiati1962, widjiati, ekamirta, eka-p-h, bencthp, benjamin-c-t-09, lita-r-y, lita.yustin

Dear Dr Epy Muhammad Luqman, M.Sc., DVM,
Many thanks for the corrected paper submission. Your paper has been sent to reviewers who will evaluate corrections made according to their recommendations.

Yours sincerely,
Assoc. Prof. MVDr. Josef Kameník, CSc., MBA
Editor-in-Chief
Acta Veterinaria Brno
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[Vetmed Journals] Corrected paper submission

External
Inbox

actavet@vfu.cz Mon, Jul 29, 2019, 3:42 PM

to epymuhammadl, me, widjiati1962, widjiati, ekamirta, eka-p-h, bencthp, benjamin-c-t-09, lita-r-y, lita.yustin

Dear Dr Epy Muhammad Luqman, M.Sc., DVM,
Many thanks for the corrected paper submission. Your paper has been sent to reviewers who will evaluate corrections made according to their recommendations.

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Editor-in-Chief
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evaluation response my paper

Inbox

epy muhammad luqman <epy-m-l@fkh.unair.ac.id> Thu, Jan 23, 2020, 4:36 PM

to actavet, Epy

Dear Assoc. Prof. MVDr. Josef Kameník, CSc., MBA
Editor-in-Chief
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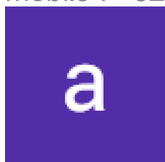
I have submitted my paper correction on July 29 2019 and my paper has been sent to reviewers who will evaluate corrections made according to their recommendations. But until now I have not received the evaluation response.

Please inform me about the results of the evaluation of my paper. Thank you.

--
Dr. Epy Muhammad Luqman
Sekretaris LPPM Unair
Urusan Pengembangan Masyarakat
mobile : +628123090594



actavet <actavet@vfu.cz> Fri, Jan 24, 2020, 1:53 PM

to me

Dear Dr. Luqman,

your manuscript is in "under review" status. Unfortunately, the peer-review of the manuscript was delayed due to difficulties in finding suitable reviewers. Even the experts/reviewers you designed and recommended did not accept the possibility to review your text.

As soon as we have reports from the opponents, I will contact you immediately.

Best regards

Josef Kamenik

Acta Veterinaria Brno - manuscript 065-2019

Inbox

actavet <actavet@vfu.cz>

Fri, Apr 3, 2020, 12:53 PM

to me

Dear Dr Epy Muhammad Luqman,

I would like to inform you that our Editorial Office has already received two positive reviews on your article ID 065/2019-ACTA entitled „Interaction between Autophagy, Apoptosis and Necrosis of Infant Mice (*Mus musculus*)'s Brain Cells from Its Carbofuran Exposed Mothers during Lactation Periods".

As attached files you can find the reviewers´ comments.
Please, don´t forget to send me (via e-mail) together with corrected manuscript also "Reply Letter" where is necessary to give all the changes performed in the text of the manuscript according the reviewers´ comments. The manuscript should have line numbering and all corrections done should be colour marked.
In a case you don´t agree with some of comments you should to explain it in "Reply Letter", too.

Best regards

Assoc. Prof. Dr. Josef Kamenik, CSc., MBA
Editor-in-chief

Manuscript 065/2019 ACTA VETERINARIA

Reviewer 1:
Publish as Original Paper after major revisions and a second review
Completeness(good), Scientific merit(good), Citations(good), International relevance(good), Importance to field(good), Language quality(fair), Conciseness(fair), Technical quality(fair), Clarity of presentation(fair), Originality(good),
Amount of samples is not satisfactory

Comment:

The manuscript titled "Interaction between Autophagy, Apoptosis and Necrosis of Infant Mice (Mus musculus)'s Brain Cells from Its Carbofuran Exposed Mothers during Lactation Periods" informs about the reaction of neural system/cells in milk sucking infant mice to carbofuran from milk of carbofuran exposed mothers.

Generally, the study is interesting and brings an information that carbofuran in the milk has an effect on neural system in sucking mice. However, the manuscript should be improved.

Introduction: The study of Qiao et al. 2002 is not the human study; avoid using the word "child" in the sentence line 36.

Methods:

The methodology is missing some values. How many mice (mothers) used in the study is mentioned in the abstract only. Thereafter is no mention, how many infant mice were euthanized from each mother (1 or all, how many) and how many brains from each group were used in the study and if all sections were processed by both techniques.

Provide more detailed description how the calculating the expression of Akt and mTORC1/necrosis/apoptosis was performed (in one section from each brain or more sections/whole brain). Was this calculation performed in whole brain or it was focused to cerebellum only?

Not to all chemicals the manufacturer is described.

Correct: "horseradish" line 85, "distilled" line 88, 90, "phosphate" line 87

Results:

Unify the using the present and past tense

Discussion:

It is too long, some parts more fit to introduction, and should be rewritten (e.g. first paragraph). This section should put the results in appropriate context regarding relevant literature and the importance of new observations should be highlighted.

Line 213: what is reference No. 2?

Reviewer 2

Publish as Original Paper after minor revisions

Completeness(fair), Scientific merit(good), Citations(good), International relevance(fair), Importance to field(fair), Language quality(good), Conciseness(good), Technical quality(fair), Clarity of presentation(good), Originality(good),

Amount of samples is not satisfactory

Comment:

In Materials and Methods there is characterisation of male and female mice but there is little infromation about infant mice (just 10-days-old mice). It is not clear how many of infant mice were used in the experiment and how many of them come from mothers exposed to ¼ LD50, 1/8 LD50 and 1/16 LD50. That is the reason why I can not assess if the number of samples is sufficient. According to authors there were 5 mice in experimental group but it is not clear how many of them were exposed to ¼ LD50, 1/8 LD50 and 1/16 LD50 of carbofuran.

epy muhammad luqman <epy-m-l@fkh.unair.ac.id> Apr 6, 2020, 6:42 PM

to actavet

Dear

Assoc. Prof. Dr. Josef Kamenik, CSc., MBA

Editor-in-chief

The following attachment i send a revision of my article entitled „Interaction between Autophagy, Apoptosis and Necrosis of Infant Mice (*Mus musculus*)'s Brain Cells from Its

Carbofuran Exposed Mothers during Lactation Periods“ ID 065/2019-ACTA and a reply according the reviewers´ comments. Thank you
--
Dr. Epy Muhammad Luqman
Sekretaris LPPM Unair
Urusan Pengembangan Masyarakat
mobile : +628123090594

Reply Letter

Titled: Interaction between Autophagy, Apoptosis and Necrosis of Infant Mice (*Mus musculus*)'s Brain Cells from Its Carbofuran Exposed Mothers during Lactation Periods.
ID number: ID 065/2019-ACTA

The reviewers´ comments	**Line**	**Description of changes** (colour marked text)
Reviewer 1:		
The study of Qiao et al. 2002 is not the human study; avoid using the word “child” in the sentence	37	I have revised this comment
The methodology is missing some values. How many mice (mothers) used in the study is mentioned in the abstract only. Thereafter is no mention, how many infant mice were euthanized from each mother (1 or all, how many) and how many brains from each group were used in the study and if all sections were processed by both techniques.	69, 70, 76	I have added the information
Provide more detailed description how the calculating the expression of Akt and mTORC1/necrosis/apoptosis was performed (in one section from each brain or more sections/whole brain). Was this calculation performed in whole brain or it was focused to cerebellum only?	80, 81, 82, 103, 104, 106	I have added the information
Not to all chemicals the manufacturer is described	77, 78, 86, 91, 101, 102, 104	I have added the information
Correct: “horseradish” line 85, “distilled” line 88, 90, “phosphate” line 87	91, 93, 96	I have revised this comment
Results: Unify the using the present and past tense	117, 118, 128, 129, 133, 138, 139, 141, 144, 149, 151, 153, 157	I have revised this comment

Discussion: It is too long, some parts more fit to introduction, and should be rewritten (e.g. first paragraph).	163	The sentence of the first paragraph has been deleted
This section should put the results in appropriate context regarding relevant literature and the importance of new observations should be highlighted.	260, 261, 262, 264, 265, 266	
Line 213: what is reference No. 2?	206	I have revised this comment
Reviewer 2		
In Materials and Methods there is characterisation of male and female mice but there is little infromation about infant mice (just 10-days-old mice). It is not clear how many of infant mice were used in the experiment and how many of them come from mothers exposed to ¼ LD50, 1/8 LD50 and 1/16 LD50. That is the reason why I can not assess if the number of samples is sufficient.	69	I have added the information
According to authors there were 5 mice in experimental group but it is not clear how many of them were exposed to ¼ LD50, 1/8 LD50 and 1/16 LD50 of carbofuran.	81, 82	I have added the information

Interaction between Autophagy, Apoptosis and Necrosis of Infant Mice *(Mus musculus)*'s Brain Cells from Its Carbofuran Exposed Mothers during Lactation Periods

Abstract

This study aimed to determine the mechanism of autophagy brain neuron cell death, apoptosis and necrosis in infant mice *(Mus musculus)* from its carbofuran exposed mothers during lactation period. This laboratory experimental study used 20 mice and carbofuran was exposed using LD_{50} fractions gavage in mice with the doses of 1.25 mg/kg Body Weight (BW) (1/4 LD_{50}), 0.625 mg/kg BW (1/8 LD_{50}) and 0.3125 mg/kg BW (1/16 LD_{50}). Mothers were exposed to carbofuran during lactation from Day 1–9. In Day 10, infant mice were sacrificed to count the number of neuron cells expressing p*rotein kinase B* (PKB or *Akt*) and mammalian target of rapamycin complex 1 (mTORC1) as autophagy pathway using immunohistochemistry, apoptosis using Tunel Assay and necrosis using hematoxylin and eosin staining. The measurement results of Akt, mTORC1, apoptosis, and cell necrosis were analyzed by analysis of variance (ANOVA) and Duncan tests. The results of the study showed that carbofuran exposure to the mothers during lactation causes an increase in necrosis and apoptosis of neuronal cells but does not cause autophagy in neuron cells via the Akt/mTOR pathway of infant mice. The increasing apoptotic neuron cells open the opportunities for prevention and handling the effects of reactive oxygen species activities due to carbofuran exposure during lactation periods.

Keywords: Neuron cell death, *Akt*, mTORC1

Carbamate insecticides are widely used throughout the world due to their low toxicity and shorter half-life which causes carbamates to be widely used to replace organophosphates. Research in Blora - Central Java, Indonesia found that carbofuran residues in beef exceeded the maximum residual limit (MRL) set by Food and Agriculture Organization (FAO), which was 169.17 ppb/0.17 mg/kg Body Weight (BW) (FAO standard of 50 ppb/0.05 mg / kg) (Indraningsih 2008). Oral administration of carbofuran has been proven to stimulate reactive

oxygen species (ROS) in mice brains (Kamboj et al. 2008). Intraperitoneal administration of sub-acute carbofuran has been confirmed to increase brain oxidative stress as the dose increases. An increased uncontrolled ROS will in turn lead to injury and neuron cell death (Gupta et al. 2007).

Carbofuran can increase the mortality of embryonal neuronal necrosis and apoptosis, but no apoptosis of neuron cell death was found from the carbofuran exposed mothers within four days of lactation (Luqman et al. 2019; Luqman et al. 2018). In the brain development stage during the embryonal period, the cerebrum neuron cells develop early and experience the peak development in mid-pregnancy. The cerebellar neuron cells develop in the mid-pregnancy until a few days after the infant is born and experiences a developmental peak at the end of the lactation period (Qiao et al. 2002). The duration and period of carbofuran exposure to brain development (fetus and born children) provide varying results. The type of brain cell death during development due to carbofuran exposure (necrosis and apoptosis) is caused by ROS activity (Luqman et al. 2019; Luqman et al. 2018).

Oxidative stress triggers cell death in all types of cell death such as autophagy, apoptosis and necrosis. Oxidative stress can stimulate death initiators, effector molecules and signaling pathways to determine the route of death. Autophagy is a cytoprotective process associated with apoptosis and necrosis to stimulate pro-survival or pro-death functions. Mammalian target rapamycin (mTOR) are cascade signals contributing to various processes such as cell proliferation, growth, and nutrient uptake. Mammalian target of rapamycin complex 1 (mTORC1) is the hub of a major pathway for cell growth. By phosphorylating several targets, mTORC1 promotes anabolism, including mRNA translation, synthesis of lipids, purines, and pyrimidines, while inhibiting autophagy (Ben-Sahra and Manning 2017). The Akt-mTOR signaling pathway is very important in the development of cerebral neocortical nerves that play an important role in cognition, emotional, language, and behavioral abilities (Wang et al. 2017). P*rotein kinase B* (PKB or *Akt*) is known to inhibit the death of apoptotic neuron cells, and the mechanical target rapamycin (mTOR) is the downstream effect of Akt to control protein synthesis. Akt and mTOR multiple barriers reduce the acute cell death and improve the long-term cognitive deficiency after cortex effects in mice (Liu et al. 2014). Akt-mTOR inhibition reduces the necrotic cell death in the cornu amonis 3 and 1 (CA3 and CA1) regions of hippocampus and improves cortical function in mice (Park et al. 2012).

An understanding of the cell death mechanism and barriers to autophagy brain cell death (Akt/mTOR), apoptosis and necrosis of mice due to carbofuran exposure during lactation period is very necessary to obtain the basis for handling and preventing carbofuran exposure during lactation period. This is important, because understanding the mechanism will find out the most sensitive period and brain cells which become a target due to carbofuran exposure during lactation period. If we can know the mechanism, prevention efforts can be made, thus, the reduction in reflexes and motoric skills in infants can be avoided.

Materials and methods

Experimental Animals

This study used mice infant (n=20) from 20 breeding mice (Mus musculus), 20 female mice 10 weeks old with the body weights between 25-35 grams and 20 male mice 12 weeks old obtained from Veterinaria Farma Surabaya. The mice were adapted to the environment for 7 days and on Day 8, they were injected with *pregnant mare serum gonadotropin* (PMSG) (Folligon™, Intervet, Boxmeer, Holland), with a dose of 5 IU/mouse. On Day 10, they were injected with human chorionic gonadotropin (hCG) (Chorulon™, Intervet, Boxmeer, Holland) with a dose of 5 IU/mouse and then mated with male mice (1:1). The mice were then kept in cages and fed with *ad-libitum*. On Day 11, a pregnancy examination was conducted, if a vaginal plug was seen then it was declared as Day 0 pregnancy. The pregnant mothers are then grouped for 5 mice in each cage to give birth. Mothers with carbofuran/2,3-Dihydro-2,2-dimethyl-7-benzofuranol N-methylcarbamate 98% (Aldrich Chemistry USA-426008-5G) exposure with fraction doses of 1.25 mg/kg Body Weight (BW) (1/4 LD_{50}), 0.625 mg/kg BW (1/8 LD_{50}) and 0.3125 mg/kg BW (1/16 LD_{50}) during the Day 1 to Day 9 lactation period given gavage. At the 10-days-old mice were sacrificed to collect infants' cerebellum, then histopathological

preparations were made. One cerebellum of infants' mice was used of infant groups (litter) from mother who exposed to carbofuran (5 infant/group).

Microscopic examination was carried out by calculating the expression of Akt and mTORC1 using immunohistochemistry. Brain tissue of 10-days-old mice was fixed in buffer 10% formalin and paraffin 5 µm sections were prepared placed on object glass using polylysine. Object glasses were cooled down and blocked with 1% bovine serum albumin/BSA (Sigma Aldrich - **B2064**) for an hour and washed trice in PBS (pH 7.4) for 5 min. Slides were labelled with primary antibodies Akt monoclonal antibody (Santa Cruz Biotechnology, USA) and mTORC1 monoclonal antibody (Santa Cruz Biotechnology) in 1% BSA overnight at 4 °C. The object glasses were labelled with biotin secondary antibody label (DakoCytomation, Denmark) in composition 1:500 for an hour at room temperature and washed using PBS (pH 7.4). The object glasses were supplemented with strepovidin horseradish peroxidase (Chemicon-AP342P), 1:500, for 40 min and washed with PBS (pH 7.4). Then the glasses were covered with diaminobenzidine tetrahydrochloride (DAB) chromogen substrate for 20 min and washed with phospate buffered saline (PBS) followed by washing in distillated water for 5 min in three repetitions and counterstained.

Cell apoptosis examination was conducted by Tunel assay. A total of 25 µg/mL of proteinase K were applied to object glasses for 25 min and the glasses were washed in distillated water. Internal peroxidase activity was blocked by incubating object glasses in 3% H_2O_2 in absolute methanol for 5 min. The glasses were washed in PBS and incubated for an hour at 37 °C in digoxigenin-containing solution labelled with deoxy-UTP and terminal deoxynucleotidyl transferase. Then the glasses were washed in PBS and incubated for 30 min in solution containing anti-digoxigenin peroxidase. The glasses were washed in PBS for 5 min, incubated in diaminobenzidine solution (3, 3 '- Diaminobenzidine tetrahydrochloride, Sigma-Aldrich, Saint Louis, Missouri, USA), and counterstained with methyl green. Necrosis cells examination was conducted by hematoxylin and eosin (HE) staining (Hematoxylin Staining for Millicell®-HA, Merck, Germany) the brain cells of infant mice. Three slices of each sample were observed and examined by microscope (Olympus® CX-41)

Data analysis

The average number of cerebellum neuron cells expressing Akt and mTORC1 under a light microscope with 400 magnification. The expression of Akt, mTORC1 and apoptosis were identified by the color reactions that arise which was yellowish brown. Neuron cells that experienced necrosis were characterized by neuron cells that were pyknosis and karyorrhexis. Expression data of Akt and mTORC1, apoptosis and necrosis from brain cells in infant mice were analyzed using analysis of variance (ANOVA) test, if the results were significantly different from control group followed by Duncan test. $P < 0.05$ was considered to be statistically significant. In facilitating statistical calculations, Statistical Product and Service Solution (SPSS) version 17.0 is used.

Results

The observation of infant mice's brain from the carbofuran exposed mother for nine days showed an increase in apoptosis and necrosis ($P<0.05$), while the Akt and mTORC1 expressions do not show any significant differences (Table 1).

The results of immunohistochemical staining showed that Akt expression is a black-colored cytoplasm of neurons. In the Figure 1, there was no any significant difference and the carbofuran exposed group with the dose of 1.25 mg/kg BW presents a decrease in Akt expression as low as 12.03% (Table 1 and Figure 1).

Fig 1. Figure of Akt expression in the brain. The arrow indicates Akt expression, which is indicated by the presence of brown chromogen (arrow). IHC 400X. K: Control, A = 1/16 LD_{50} = 0.3125 mg/kg BW, B = 1/8 LD_{50} = 0.625 mg/kg BW, C = 1/4 LD_{50} = 1.25 mg/kg BW. Akt: P*rotein kinase B* (PKB or *Akt*); mTORC1: Mammalian target of rapamycin complex 1; LD_{50}: Lethal dose, 50%; BW: Body Weight.

The results of immunohistochemical staining presented the mTORC1 expression in the black-colored cytoplasm of neurons. In the Figure 2, there was no any significant difference and the carbofuran exposure group with the dose of 1.25 mg/kg BW expressed a decrease in the lowest expression of mTORC1 protein by 22.68% (Table 1 and Figure 2).

Fig 2. Figure of mTORC1 expression in the brain. The arrow showed the presence of mTORC1 expression, which is indicated by the presence of brown chromogen (arrow). IHC 400X. K: Control, A = 1/16 LD_{50} = 0.3125 mg/kg BW, B = 1/8 LD_{50} = 0.625 mg/kg BW, C =

1/4 LD_{50} = 1.25 mg/kg BW. Akt: P*rotein kinase B* (PKB or *Akt*); mTORC1: Mammalian target of rapamycin complex 1; LD_{50}: Lethal dose, 50%; BW: Body Weight.

In the description of apoptotic cells, it showed cytoplasm in blackish brown cells, while the healthy cells appear in green. In Figure 3, there was a significant difference between treatment and control groups. In the treatment group of carbofuran exposure with the dose of 1.25 mg/kg BW, it showed the highest apoptosis compared to control group. The increase of apoptosis in the treatment group reached 23.06% to 31.97% (Table 1 and Figure 3).

Fig 3. Description of apoptotic expression in the brain. The arrows showed apoptotic expression, which is characterized by the presence of brown chromogen (arrow). Tunnel Assay 400X. K: Control, A = 1/16 LD_{50} = 0.3125 mg/kg BW, B = 1/8 LD_{50} = 0.625 mg/kg BW, C = 1/4 LD_{50} = 1.25 mg/kg BW. Akt: P*rotein kinase B* (PKB or *Akt*); mTORC1: Mammalian target of rapamycin complex 1; LD_{50}: Lethal dose, 50%; BW: Body Weight.

The image of hematoxylin eosin (HE) staining indicated neuronal necrosis. It appeared that neuron cells experience pyknosis and karyorrhexis. In the treatment group of carbofuran exposure with a dose of 1/8 LD_{50} = 0.625 mg/kg BW, it only showed necrosis and was not significantly different from the group 1/4 LD_{50} = 1.25 mg/kg BW. The highest increase of necrosis was in the treatment group with 1/4 LD_{50} = 1.25 mg/kg BB which reaches 86.36% (Table 1 and Figure 4).

Fig 4. Description of active neuron cells in the brain in each representative group. The red arrow indicated the presence of neuron cells that appeared to be active and still normal, and the yellow arrow shows the images of necrotizing neuron cells (HE Enlargement 400x). K: Control, A = 1/16 LD_{50} = 0.3125 mg/kg BW, B = 1/8 LD_{50} = 0.625 mg/kg BW, C = 1/4 LD_{50} = 1.25 mg/kg BW. Akt: P*rotein kinase B* (PKB or *Akt*); mTORC1: Mammalian target of rapamycin complex 1; LD_{50}: Lethal dose, 50%; BW: Body Weight.

Discussion

Carbofuran could increase the death of necrosis and apoptosis in neuron cells during embryonal period. The duration and period of carbofuran exposure to brain development in fetus and born child gave varying results. The type of brain cell death during development due to the exposure of carbofuran was caused by ROS activity (Luqman et al. 2019; Luqman et al. 2018). Death of necrosis and apoptosis due to ROS could be triggered by autophagy through the PI3K-Akt-mTOR signal activity. Oxidative stress was able to cause severe damage to DNA, RNA, protein, and trigger the autophagy and apoptosis (Deng et al. 2013). The PI3K/Akt/mTOR pathway played an important role in autophagic cell modulation (Lin et al. 2016). The mechanical target rapamycin (mTOR) was one of the main modulators of autophagy which could be regulated by various signaling pathways (Sage et al. 2016).

Furthermore, Akt was known to inhibit the death of neuronal apoptosis. The double inhibition of Akt and mTOR reduces acute cell death and increases the long-term cognitive deficits in infant mice. Inhibition of Akt/mTOR did not affect the assembly of necrosome complex but inhibits the oxidative stress and cell death. Although activation of Akt was limited as antiapoptotic, the ongoing Akt activity can increase cell death by different mechanisms (Li et al. 2011).

In this study, the administration of carbofuran did not significantly increase the expression of Akt and mTORC1 in the brain of infant mice along with the increasing dosage given to mothers during Day 1 until Day 9 lactation period (Table 1). Akt and mTORC1 concentrations could change due to extracellular and intracellular stimuli. Carbofuran did not increase the expression of Akt and mTORC1 neurons which reveals that although carbofuran exposure during the lactation period causes an increased ROS activity (Luqman et al. 2018). However, it did not affect the number of cerebellum neurons expressing Akt and mTORC1. It

tells that the PI3K/Akt /mTOR signaling pathway did not participate in autophagy regulation caused by carbofuran exposure. Administering carbofuran during lactation period caused a significant increase in necrotic and apoptotic cells. This describes that the carbofuran exposure did not cause autophagy through ROS activity and ROS activity could cause the process of necrosis and apoptosis (Table 1).

ROS is a by-product of biological reactions from energy generation, mainly produced in mitochondria through oxidative metabolism (Zorov et al. 2014). It is estimated that ROS produced by mitochondria is around 1-2% of the total level of oxygen consumption in normal cells. ROS is a very small molecule, such as superoxide anion ($O_2\bullet^-$), hydroxyl radical ($^\bullet OH$) and hydrogen peroxide (H_2O_2) which are produced in cell organelles, especially in mitochondria (Zhang et al. 2009). ROS acts as the second messenger in various signal transduction. The increased ROS couldnot induce autophagy by increasing the expression of Akt, mTORC1 and apoptosis simultaneously or separately due to the high reactivity and damages to proteins, lipids and DNA (Chen et al. 2008).

Oral and intraperitoneal carbofuran administrations have been proven to stimulate ROS characterized by an increase in malondialdehyde (MDA) in adult mice's brain. This increase in ROS can reduce enzyme antioxidant activity such as superoxide dismutase (SOD) and catalase (CAT) in the brain. The decreasing catalase activity in responding to carbofuran induction can reduce protection against free radicals. A simultaneous decrease in SOD and catalase activity causes the brain to be more susceptible to the oxidative induction of carbofuran (Rai and Sharma, 2007). DNA or chromosome damage due to hydroxyl radical ($^\bullet OH$) will activate p-53 and p-53 activation which trigger Bax activity (Kamboj et al. 2008). Bax protein will suppress BCl-2 activity in the mitochondrial membrane, resulting in changes in the permeability of mitochondrial membrane. This change in the permeability of mitochondrial membrane will lead to the release of cytochrome C into the cytosol. Cytochrome C will activate Apaf-1 and then activate the caspase cascade, starting from pro-caspase 9 to caspase 3. The active Caspase 3 will activate DNA-se and penetrate the core membrane then damage the DNA, thus, the cell undergoes apoptosis (Dutordoir et al. 2016).

The interaction between autophagy and apoptosis was complex and controversial, depending on cell type and stress (Levine and Yuan 2005). Autophagy can inhibit or delay the occurrence of apoptosis, or promote apoptosis, or induce autophagic cell death regardless the apoptosis (Fimia and Piacentini 2010). This study revealed that carbofuran did not induce PI3K/Akt/mTORC1 which is a representation of autophagic pathway and induced apoptosis and necrosis through ROS activity. The insignificance of expression in PI3K/Akt/mTORC1 did not reduce apoptosis and necrosis of neuron cells due to the oxidative stress. These results could help improve the understanding in the mechanisms of neuronal cell death due to oxidative stress, and the intricate relationship between PI3K/Akt/mTORC1 which represent the pathways of autophagy, apoptosis and necrosis. It was necessary to modulate the types of neuron cell death due to oxidative stress which may be the direction of new therapies to reduce cell apoptosis and prevent the degeneration due to carbofuran exposure.

Apoptosis and necrosis are the two main modes of cell death which requires the balanced molecular interactions between these two cellular death modes. Some death initiators, effector molecules and signaling pathways have been identified as the primary mediators by acting as switches to decide the route of cell death to be taken depending on the specific situation (Nikoletopoulou 2013). Using the conventional histology, it is not easy to distinguish apoptosis from necrosis, because these two processes can occur simultaneously depending on the intensity and duration of injury, Adenosine triphosphate (ATP) depletion rates and availability of caspase (Zeiss 2003). In some cases, the type of injury or degree of injury determines the cell death by apoptosis or necrosis. Various lesions such as heat, hypoxia, radiation, and low-dose anticancer drugs, it can induce apoptosis but the same

lesions with the higher doses can result in necrosis (Elmore 2007). Low stimulation causes apoptosis, while conditions for energy reduction or caspase activation lead to necrotic (Zeiss 2003). Some of the main morphological changes in necrosis include cell swelling, cytoplasmic vacuole formation, infiltration of endoplasmic reticulum, formation of cytoplasmic blebs, mitochondrial freezing, disaggregation of ribosomes, disturbed organelle membranes, swollen and ruptured lysosomes, and cell membranes' disruption. Cell death caused by cytoplasmic freezing, karyolysis nuclei and lysis are classified as necrosis (Martin 2010).

In this study, carbofuran could increase the neuronal apoptosis compared to controls but did not show any significant difference in the higher doses (Table 1 and Figure 2). This suggests that small doses could cause apoptosis, because only very low doses could induce moderate apoptosis, while in larger doses it leads to necrosis (Burniston et al. 2005). At low doses, carbofuran couldnot initiate necrosis, an increase in new necrosis occurs at a dose of 1/8 LD_{50} = 0.625 mg/kg BW and did not show any significant difference in neurons which experience necrosis at the higher doses (Table 1 and figure 3). At the higher doses, carbofuran did not present any significant differences in apoptosis and necrosis because the body's ability to respond to carbofuran metabolites in breast milk has reached maximum ability. The effects of chemical compounds in the body were proportional to the receptors that bind them. As Kenakin (2014) states that the maximum effect was obtained from an injury when all receptors have been bound by the chemical materials.

The number of neurons that experience necrosis was relatively the same as many cells that experience apoptosis due to carbofuran exposure. In contrast to embryonal exposure, the number of neuron cells that experience necrosis was not as much as cells that experience apoptosis due to carbofuran exposure. This was due to embryonal neurogenesis; many neuron cells undergo the physiological apoptosis as an effort to homeostasis (Widjiati and Luqman 2012). This finding implies that besides not causing any autophagy cell death, carbofuran caused necrosis and apoptosis and opens the opportunities for cell survival. These findings indicate that ROS plays an important role in the process of necrosis and apoptosis caused by carbofuran exposure. However, any detailed mechanisms for the process of necrosis and apoptosis must be further investigated. This study reveals that carbofuran did not induce autophagy through the axis of ROS-AKT-mTOR, but the other ROS mechanisms that could cause neuronal cell death by necrosis and brain apoptosis in the infant mice. Thus, two or three types of cell death can be induced simultaneously or consecutively when cells are exposed to certain stimuli. If all three types of cell death are placed on the axis according to the superiority of cell survival, autophagy and necrosis would be placed at the opposite end, while apoptosis will be placed in the center (Chen et al. 2018).

The conclusions from this study showed that there was no any significant increase in the expression of Akt and mTORC1, the increased apoptotic death and necrotic cells. Carbofuran exposure to the mothers during the lactation period caused the increase in neuronal cell death by apoptosis and necrosis and the decrease in the ability of neuron cells to develop, especially expression of Akt and mTORC1.

Conflict Of Interest

Authors declare that they have no conflict of interest.

Acknowledgments

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actavet <actavet@vfu.cz> Apr 7, 2020, 1:52 PM

to me

Dear Dr Epy Muhammad Luqman,
thank you for the revised manuscript. I sent it to a reviewer for the second review. As soon as I receive the reviewer's opinion, I will inform you immediately.

Best regards

Josef Kamenik

actavet <actavet@vfu.cz> Apr 24, 2020, 1:31 PM

to me

Dear Dr Epy Muhammad Luqman,
I received the opponent's comments after the second revision of the manuscript. He/she has the following notes:
I have one reservation, the discussion is quite long and there is generally known information that does not need to be developed so much (eg the usability of ROS in the cell, etc.). I am sending commented / highlighted errors in the attachment.

I would like to ask you to modify the manuscript according to his/her notes.

Best regards

Josef Kamenik

Reply Letter

Titled: Interaction between Autophagy, Apoptosis and Necrosis of Infant Mice (*Mus musculus*)'s Brain Cells from Its Carbofuran Exposed Mothers during Lactation Periods.
ID number: ID 065/2019-ACTA

The reviewers´ comments	**Line**	**Description of changes** (colour marked text)
Could not	196	I have revised this comment
Present study or previously cited?	216-224	The sentences in this discussion represent the opinion of the authors of (Present study)
I have one reservation, the discussion is quite long and there is generally known information that does not need to be developed so much (eg the usability of ROS in the cell, etc.).	199-212 226-242	I had deleted line 226-242 and did not reduce the meaning of the text.
Could not	247	I have revised this comment

This was already mentioned above 263-265 I had deleted line 263-265

Interaction between Autophagy, Apoptosis and Necrosis of Infant Mice *(Mus musculus)*'s Brain Cells from Its Carbofuran Exposed Mothers during Lactation Periods

Abstract

This study aimed to determine the mechanism of autophagy brain neuron cell death, apoptosis and necrosis in infant mice *(Mus musculus)* from its carbofuran exposed mothers during lactation period. This laboratory experimental study used 20 mice and carbofuran was exposed using LD_{50} fractions gavage in mice with the doses of 1.25 mg/kg Body Weight (BW) (1/4 LD_{50}), 0.625 mg/kg BW (1/8 LD_{50}) and 0.3125 mg/kg BW (1/16 LD_{50}). Mothers were exposed to carbofuran during lactation from Day 1–9. In Day 10, infant mice were sacrificed to count the number of neuron cells expressing p*rotein kinase B* (PKB or *Akt*) and mammalian target of rapamycin complex 1 (mTORC1) as autophagy pathway using immunohistochemistry, apoptosis using Tunel Assay and necrosis using hematoxylin and eosin staining. The measurement results of Akt, mTORC1, apoptosis, and cell necrosis were analyzed by analysis of variance (ANOVA) and Duncan tests. The results of the study showed that carbofuran exposure to the mothers during lactation causes an increase in necrosis and apoptosis of neuronal cells but does not cause autophagy in neuron cells via the Akt/mTOR pathway of infant mice. The increasing apoptotic neuron cells open the opportunities for prevention and handling the effects of reactive oxygen species activities due to carbofuran exposure during lactation periods.

Keywords: Neuron cell death, *Akt*, mTORC1

Carbamate insecticides are widely used throughout the world due to their low toxicity and shorter half-life which causes carbamates to be widely used to replace organophosphates. Research in Blora-Central Java, Indonesia found that carbofuran residues in beef exceeded the maximum residual limit (MRL) set by Food and Agriculture Organization (FAO), which was 169.17 ppb/0.17 mg/kg Body Weight (BW) (FAO standard of 50 ppb/0.05 mg / kg) (Indraningsih 2008). Oral administration of carbofuran has been proven to stimulate reactive oxygen species (ROS) in mice brains (Kamboj et al. 2008). Intraperitoneal administration of sub-acute carbofuran has been confirmed to increase brain oxidative stress as the dose increases. An increased uncontrolled ROS will in turn lead to injury and neuron cell death (Gupta et al. 2007).

Carbofuran can increase the mortality of embryonal neuronal necrosis and apoptosis, but no apoptosis of neuron cell death was found from the carbofuran exposed mothers within four days of lactation (Luqman et al. 2019; Luqman et al. 2018). In the brain development stage during the embryonal period, the cerebrum neuron cells develop early and experience the peak development in mid-pregnancy. The cerebellar neuron cells develop in the mid-pregnancy until a few days after the infant is born and experiences a developmental peak at the end of the lactation period (Qiao et al. 2002). The duration and period of carbofuran exposure to brain development (fetus and born children) provide varying results. The type of brain cell death during development due to carbofuran exposure (necrosis and apoptosis) is caused by ROS activity (Luqman et al. 2019; Luqman et al. 2018).

Oxidative stress triggers cell death in all types of cell death such as autophagy, apoptosis and necrosis. Oxidative stress can stimulate death initiators, effector molecules and signaling pathways to determine the route of death. Autophagy is a cytoprotective process associated with apoptosis and necrosis to stimulate pro-survival or pro-death functions. Mammalian target rapamycin (mTOR) are cascade signals contributing to various processes such as cell proliferation, growth, and nutrient uptake. Mammalian target of rapamycin complex 1 (mTORC1) is the hub of a major pathway for cell growth. By phosphorylating several targets, mTORC1 promotes anabolism, including mRNA translation, synthesis of lipids, purines, and

pyrimidines, while inhibiting autophagy (Ben-Sahra and Manning 2017). The Akt-mTOR signaling pathway is very important in the development of cerebral neocortical nerves that play an important role in cognition, emotional, language, and behavioral abilities (Wang et al. 2017). P*rotein kinase B* (PKB or *Akt*) is known to inhibit the death of apoptotic neuron cells, and the mechanical target rapamycin (mTOR) is the downstream effect of Akt to control protein synthesis. Akt and mTOR multiple barriers reduce the acute cell death and improve the long-term cognitive deficiency after cortex effects in mice (Liu et al. 2014). Akt-mTOR inhibition reduces the necrotic cell death in the cornu amonis 3 and 1 (CA3 and CA1) regions of hippocampus and improves cortical function in mice (Park et al. 2012).

An understanding of the cell death mechanism and barriers to autophagy brain cell death (Akt/mTOR), apoptosis and necrosis of mice due to carbofuran exposure during lactation period is very necessary to obtain the basis for handling and preventing carbofuran exposure during lactation period. This is important, because understanding the mechanism will find out the most sensitive period and brain cells which become a target due to carbofuran exposure during lactation period. If we can know the mechanism, prevention efforts can be made, thus, the reduction in reflexes and motoric skills in infants can be avoided.

Materials and methods

Experimental Animals

This study used mice infant (n=20) from 20 breeding mice (Mus musculus), 20 female mice 10 weeks old with the body weights between 25-35 grams and 20 male mice 12 weeks old obtained from Veterinaria Farma Surabaya. The mice were adapted to the environment for 7 days and on Day 8, they were injected with *pregnant mare serum gonadotropin* (PMSG) (Folligon™, Intervet, Boxmeer, Holland), with a dose of 5 IU/mouse. On Day 10, they were injected with human chorionic gonadotropin (hCG) (Chorulon™, Intervet, Boxmeer, Holland) with a dose of 5 IU/mouse and then mated with male mice (1:1). The mice were then kept in cages and fed with *ad-libitum*. On Day 11, a pregnancy examination was conducted, if a vaginal plug was seen then it was declared as Day 0 pregnancy. The pregnant mothers are then grouped for 5 mice in each cage to give birth. Mothers with carbofuran/2,3-Dihydro-2,2-dimethyl-7-benzofuranol N-methylcarbamate 98% (Aldrich Chemistry USA-426008-5G) exposure with fraction doses of 1.25 mg/kg Body Weight (BW) (1/4 LD_{50}), 0.625 mg/kg BW (1/8 LD_{50}) and 0.3125 mg/kg BW (1/16 LD_{50}) during the Day 1 to Day 9 lactation period given gavage. At the 10-days-old mice were sacrificed to collect infants' cerebellum, then histopathological preparations were made. One cerebellum of infants' mice was used of infant groups (litter) from mother who exposed to carbofuran (5 infant/group).

Microscopic examination was carried out by calculating the expression of Akt and mTORC1 using immunohistochemistry. Brain tissue of 10-days-old mice was fixed in buffer 10% formalin and paraffin 5 μm sections were prepared placed on object glass using polylysine. Object glasses were cooled down and blocked with 1% bovine serum albumin/BSA (Sigma Aldrich - **B2064**) for an hour and washed trice in PBS (pH 7.4) for 5 min. Slides were labelled with primary antibodies Akt monoclonal antibody (Santa Cruz Biotechnology, USA) and mTORC1 monoclonal antibody (Santa Cruz Biotechnology) in 1% BSA overnight at 4 °C. The object glasses were labelled with biotin secondary antibody label (DakoCytomation, Denmark) in composition 1:500 for an hour at room temperature and washed using PBS (pH 7.4). The object glasses were supplemented with strepovidin horseradish peroxidase (Chemicon-AP342P), 1:500, for 40 min and washed with PBS (pH 7.4). Then the glasses were covered with diaminobenzidine tetrahydrochloride (DAB) chromogen substrate for 20 min and washed with phospate buffered saline (PBS) followed by washing in distillated water for 5 min in three repetitions and counterstained.

Cell apoptosis examination was conducted by Tunel assay. A total of 25 μg/mL of proteinase K were applied to object glasses for 25 min and the glasses were washed in distillated water. Internal peroxidase activity was blocked by incubating object glasses in 3% H_2O_2 in absolute methanol for 5 min. The glasses were washed in PBS and incubated for an hour at 37 °C in digoxigenin-containing solution labelled with deoxy-UTP and terminal deoxynucleotidyl transferase. Then the glasses were washed in PBS and incubated for 30 min in solution containing anti-digoxigenin peroxidase. The glasses were washed in PBS for 5 min, incubated in diaminobenzidine solution (3, 3 '- Diaminobenzidine tetrahydrochloride, Sigma-Aldrich, Saint Louis, Missouri, USA), and counterstained with methyl green. Necrosis cells examination was conducted by hematoxylin and eosin (HE) staining (Hematoxylin Staining for Millicell®-HA, Merck, Germany) the brain cells of infant mice. Three slices of each sample were observed and examined by microscope (Olympus® CX-41)

Data analysis

The average number of cerebellum neuron cells expressing Akt and mTORC1 under a light microscope with 400 magnification. The expression of Akt, mTORC1 and apoptosis were identified by the color reactions that arise which was yellowish brown. Neuron cells that experienced necrosis were characterized by neuron cells that were pyknosis and karyorrhexis. Expression data of Akt and mTORC1, apoptosis and necrosis from brain cells in infant mice were analyzed using analysis of variance (ANOVA) test, if the results were significantly different from control group followed by Duncan test. $P < 0.05$ was considered to be statistically significant. In facilitating statistical calculations, Statistical Product and Service Solution (SPSS) version 17.0 is used.

Results

The observation of infant mice's brain from the carbofuran exposed mother for nine days showed an increase in apoptosis and necrosis ($P<0.05$), while the Akt and mTORC1 expressions do not show any significant differences (Table 1).

The results of immunohistochemical staining showed that Akt expression is a black-colored cytoplasm of neurons. In the Figure 1, there was no any significant difference and the carbofuran exposed group with the dose of 1.25 mg/kg BW presents a decrease in Akt expression as low as 12.03% (Table 1 and Figure 1).

Fig 1. Figure of Akt expression in the brain. The arrow indicates Akt expression, which is indicated by the presence of brown chromogen (arrow). IHC 400X. K: Control, A = 1/16 LD_{50} = 0.3125 mg/kg BW, B = 1/8 LD_{50} = 0.625 mg/kg BW, C = 1/4 LD_{50} = 1.25 mg/kg BW. Akt: P*rotein kinase B* (PKB or *Akt*); mTORC1: Mammalian target of rapamycin complex 1; LD_{50}: Lethal dose, 50%; BW: Body Weight.

The results of immunohistochemical staining presented the mTORC1 expression in the black-colored cytoplasm of neurons. In the Figure 2, there was no any significant difference and the carbofuran exposure group with the dose of 1.25 mg/kg BW expressed a decrease in the lowest expression of mTORC1 protein by 22.68% (Table 1 and Figure 2).

Fig 2. Figure of mTORC1 expression in the brain. The arrow showed the presence of mTORC1 expression, which is indicated by the presence of brown chromogen (arrow). IHC 400X. K: Control, A = 1/16 LD_{50} = 0.3125 mg/kg BW, B = 1/8 LD_{50} = 0.625 mg/kg BW, C = 1/4 LD_{50} = 1.25 mg/kg BW. Akt: P*rotein kinase B* (PKB or *Akt*); mTORC1: Mammalian target of rapamycin complex 1; LD_{50}: Lethal dose, 50%; BW: Body Weight.

In the description of apoptotic cells, it showed cytoplasm in blackish brown cells, while the healthy cells appear in green. In Figure 3, there was a significant difference between treatment and control groups. In the treatment group of carbofuran exposure with the dose of 1.25 mg/kg BW, it showed the highest apoptosis compared to control group. The increase of apoptosis in the treatment group reached 23.06% to 31.97% (Table 1 and Figure 3).

Fig 3. Description of apoptotic expression in the brain. The arrows showed apoptotic expression, which is characterized by the presence of brown chromogen (arrow). Tunnel Assay 400X. K: Control, A = 1/16 LD_{50} = 0.3125 mg/kg BW, B = 1/8 LD_{50} = 0.625 mg/kg BW, C = 1/4 LD_{50} = 1.25 mg/kg BW. Akt: P*rotein kinase B* (PKB or *Akt*); mTORC1: Mammalian target of rapamycin complex 1; LD_{50}: Lethal dose, 50%; BW: Body Weight.

The image of hematoxylin eosin (HE) staining indicated neuronal necrosis. It appeared that neuron cells experience pyknosis and karyorrhexis. In the treatment group of carbofuran exposure with a dose of 1/8 LD_{50} = 0.625 mg/kg BW, it only showed necrosis and was not significantly different from the group 1/4 LD_{50} = 1.25 mg/kg BW. The highest increase of necrosis was in the treatment group with 1/4 LD_{50} = 1.25 mg/kg BB which reaches 86.36% (Table 1 and Figure 4).

Fig 4. Description of active neuron cells in the brain in each representative group. The red arrow indicated the presence of neuron cells that appeared to be active and still normal, and the yellow arrow shows the images of necrotizing neuron cells (HE Enlargement 400x). K: Control, A = 1/16 LD_{50} = 0.3125 mg/kg BW, B = 1/8 LD_{50} = 0.625 mg/kg BW, C = 1/4 LD_{50} = 1.25 mg/kg BW. Akt: P*rotein kinase B* (PKB or *Akt*); mTORC1: Mammalian target of rapamycin complex 1; LD_{50}: Lethal dose, 50%; BW: Body Weight.

Discussion

Carbofuran could increase the death of necrosis and apoptosis in neuron cells during embryonal period. The duration and period of carbofuran exposure to brain development in fetus and born child gave varying results. The type of brain cell death during development due to the exposure of carbofuran was caused by ROS activity (Luqman et al. 2019; Luqman et al. 2018). Death of necrosis and apoptosis due to ROS could be triggered by autophagy through the PI3K-Akt-mTOR signal activity. Oxidative stress was able to cause severe damage to DNA, RNA, protein, and trigger the autophagy and apoptosis (Deng et al. 2013). The PI3K/Akt/mTOR pathway played an important role in autophagic cell modulation (Lin et al. 2016). The mechanical target rapamycin (mTOR) was one of the main modulators of autophagy which could be regulated by various signaling pathways (Sage et al. 2016).

Furthermore, Akt was known to inhibit the death of neuronal apoptosis. The double inhibition of Akt and mTOR reduces acute cell death and increases the long-term cognitive deficits in infant mice. Inhibition of Akt/mTOR did not affect the assembly of necrosome complex but inhibits the oxidative stress and cell death. Although activation of Akt was limited as antiapoptotic, the ongoing Akt activity can increase cell death by different mechanisms (Li et al. 2011).

In this study, the administration of carbofuran did not significantly increase the expression of Akt and mTORC1 in the brain of infant mice along with the increasing dosage given to mothers during Day 1 until Day 9 lactation period (Table 1). Akt and mTORC1 concentrations could change due to extracellular and intracellular stimuli. Carbofuran did not increase the expression of Akt and mTORC1 neurons which reveals that although carbofuran exposure during the lactation period causes an increased ROS activity (Luqman et al. 2018). However, it did not affect the number of cerebellum neurons expressing Akt and mTORC1. It tells that the PI3K/Akt /mTOR signaling pathway did not participate in autophagy regulation caused by carbofuran exposure. Administering carbofuran during lactation period caused a significant increase in necrotic and apoptotic cells. This describes that the carbofuran exposure did not cause autophagy through ROS activity and ROS activity could cause the process of necrosis and apoptosis (Table 1).

ROS is a by-product of biological reactions from energy generation, mainly produced in mitochondria through oxidative metabolism (Zorov et al. 2014). It is estimated that ROS produced by mitochondria is around 1-2% of the total level of oxygen consumption in normal cells. ROS is a very small molecule, such as superoxide anion ($O_2{\bullet}^-$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) which are produced in cell organelles, especially in mitochondria (Zhang et al. 2009). ROS acts as the second messenger in various signal transduction. The increased ROS couldn't induce autophagy by increasing the expression of Akt, mTORC1 and apoptosis simultaneously or separately due to the high reactivity and damages to proteins, lipids and DNA (Chen et al. 2008).

The interaction between autophagy and apoptosis was complex and controversial, depending on cell type and stress (Levine and Yuan 2005). Autophagy can inhibit or delay the occurrence of apoptosis, or promote apoptosis, or induce autophagic cell death regardless the apoptosis (Fimia and Piacentini 2010). This study revealed that carbofuran did not induce PI3K/Akt/mTORC1 which is a representation of autophagic pathway and induced apoptosis and necrosis through ROS activity. The insignificance of expression in PI3K/Akt/mTORC1

did not reduce apoptosis and necrosis of neuron cells due to the oxidative stress. These results could help improve the understanding in the mechanisms of neuronal cell death due to oxidative stress, and the intricate relationship between PI3K/Akt/mTORC1 which represent the pathways of autophagy, apoptosis and necrosis. It was necessary to modulate the types of neuron cell death due to oxidative stress which may be the direction of new therapies to reduce cell apoptosis and prevent the degeneration due to carbofuran exposure.

In this study, carbofuran could increase the neuronal apoptosis compared to controls but did not show any significant difference in the higher doses (Table 1 and Figure 2). This suggests that small doses could cause apoptosis, because only very low doses could induce moderate apoptosis, while in larger doses it leads to necrosis (Burniston et al. 2005). At low doses, carbofuran couldn't initiate necrosis, an increase in new necrosis occurs at a dose of 1/8 LD_{50} = 0.625 mg/kg BW and did not show any significant difference in neurons which experience necrosis at the higher doses (Table 1 and figure 3). At the higher doses, carbofuran did not present any significant differences in apoptosis and necrosis because the body's ability to respond to carbofuran metabolites in breast milk has reached maximum ability. The effects of chemical compounds in the body were proportional to the receptors that bind them. As Kenakin (2014) states that the maximum effect was obtained from an injury when all receptors have been bound by the chemical materials.

The number of neurons that experience necrosis was relatively the same as many cells that experience apoptosis due to carbofuran exposure. In contrast to embryonal exposure, the number of neuron cells that experience necrosis was not as much as cells that experience apoptosis due to carbofuran exposure. This was due to embryonal neurogenesis; many neuron cells undergo the physiological apoptosis as an effort to homeostasis (Widjiati and Luqman 2012). This finding implies that besides not causing any autophagy cell death, carbofuran caused necrosis and apoptosis and opens the opportunities for cell survival. These findings indicate that ROS plays an important role in the process of necrosis and apoptosis caused by carbofuran exposure. However, any detailed mechanisms for the process of necrosis and apoptosis must be further investigated. Thus, two or three types of cell death can be induced simultaneously or consecutively when cells are exposed to certain stimuli. If all three types of cell death are placed on the axis according to the superiority of cell survival, autophagy and necrosis would be placed at the opposite end, while apoptosis will be placed in the center (Chen et al. 2018).

The conclusions from this study showed that there was no any significant increase in the expression of Akt and mTORC1, the increased apoptotic death and necrotic cells. Carbofuran exposure to the mothers during the lactation period caused the increase in neuronal cell death by apoptosis and necrosis and the decrease in the ability of neuron cells to develop, especially expression of Akt and mTORC1.

Conflict Of Interest

Authors declare that they have no conflict of interest.

Acknowledgments

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May 5, 2020, 1:06 PM

to me

Dear Dr. Epy Muhammad Luqman,

Thank you for the last revision of your manuscript. It will be published in 2/2020 Acta Veterinaria Brno journal.

Best regards

Josef Kamenik

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Dear Dr. Epy Muhammad Luqman,
thank you very much! This week you will receive from the copy-editor the typed text of the article for final checking before printing.

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