



SURAT KETERANGAN

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No	Judul Karya Ilmiah	Tahun Pelaksanaan Penelitian
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3	Human Chorionic Gonadotropin (hCG) from Urine of Pregnant Women to Manipulate in vivo Ovulation and Pregnancy of Madura Cows	2019
4	Anti Early Embryonic Protein (EEP) for Pregnancy Test by Microtiter Strip in Dairy Cows	2019
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7	Diagnosis of Single and Twin Pregnancy, and Early Embryo Mortality Through Progesterone Level Test on Local Does.	2019
8	Improvement of Pregnancy Rate in Bali Cows with the Combination of Equine Chorionic Gonadotropine (eCG) from Local Pregnant Mare with PGF _{2α} .	2019
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Adapun penelitian tersebut layak dilakukan, meskipun belum ada **Ethical Clearence** karena menggunakan hewan coba yang minimal dan menghasilkan output yang sangat baik.

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Surabaya, 3 April 2023

Wakil Dekan III,

Prof. Dr. Mustofa Helmi Effendi, drh., DTAPH

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Potency of mycotoxin binders on MDA level, expressions of caspase 9 and caspase 3 in the uterus of mice exposed to zearalenone

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Abstract

This study examined effect of mycotoxin binder administered to female mice exposed to zearalenon on apoptosis incidence by observe at MDA, Caspase 9 and 3 levels in mice uterus. Negative control group (K-) was not exposed to zearalenon and without the administration of mycotoxin binders, positive control group (K+) exposed to zearalenon of 0.1 mg/head/day and without the provision of mycotoxin binders; and treatment groups (P1, P2, P3) were exposed to zearalenon 0.1 mg/head/day by providing mycotoxin binders each 0.5; 1; 2 mg/head/day. Zearalenon and mycotoxinbinders administration was conducted for 10 days. Results on MDA level were as follows 15.48 ± 0.50 (K-), 45.59 ± 0.50 (K+), 34.92 ± 3.38 (P1), 27.72 ± 1.25 and 23.89 ± 3.74 . Caspase 9 levels showed the following results: 0.3 ± 0.60 (K), 8.3 ± 0.90 (K+), 3.6 ± 0.41 (P1), 3.3 ± 0.34 (P2) and 2.8 ± 0.28 (P3), while the levels of Caspase3 were as follows: 3.35 ± 0.44 (K-), 12.5 ± 0.66 (K+), 3.6 ± 0.41 (P1), 4.80 ± 0.43 (P2) and 3.85 ± 0.50 (P3). In conclusion, mycotoxin binders may lower malondialdehyde (MDA) level and the expression of caspase 9 and caspase3 in the uterus of mice exposed to zearalenon.

Keywords: Zearalenone, mycotoxin binders, MDA level, caspase 9, caspase 3

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قوة رابطات السموم الفطرية على مستوى المالوندايالديهيد وتعبيرات انزيمي الكاسبيس 9 و 3 في رحم الفئران المعرضة للزيرالينون

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الخلاصة

بحثت هذه الدراسة تأثير رابطات السموم الفطرية المعطاة لاناث الفئران المعرضة للزيرالينون على حدوث موت الخلايا المبرمج من خلال مراقبة مستويات المالوندايالديهيد وانزيم كاسبيس 9 و 3 في رحم الفئران. لم يتم تعريض فئران مجموعة السيطرة السالبة (K-) للزيرالينون ولم تعامل برابطات السموم الفطرية ايضا. تم تعريض فئران مجموعة السيطرة الموجبة (K+) للزيرالينون بجرعة 0.1 ملغم /راس/يوم ومن دون اعطاء رابطات السموم الفطرية ايضا. عرضت مجاميع الفئران المعالجة (p1,p2,p3) بالزيرالينون بجرعة 0.1 ملغم /راس/يوم مع اعطاء رابطات السموم الفطرية بالجرع 0.5 و 1 و 2 ملغم/راس/يوم على التوالي. تم اعطاء الزيرالينون والسموم الفطرية لمدة 10 ايام. كانت نتائج قياس مستوى المالوندايالديهيد كانت كالاتي: مجموعة السيطرة السالبة (K-) (15.48 ± 0.50) ومجموعة السيطرة الموجبة (45.59 ± 0.50) وكانت نتائج المجاميع المعالجة (p1,p2,p3) (34.92 ± 3.38) و (27.72 ± 1.25) و (23.89 ± 3.74) و (0.3 ± 0.60) و (8.3 ± 0.90) و (3.6 ± 0.41) و (3.3 ± 0.34) و (2.8 ± 0.28) و (3.35 ± 0.44) و (12.5 ± 0.66) و (3.6 ± 0.41) و (4.80 ± 0.43) و (3.85 ± 0.50) و (3.35 ± 0.44) و (12.5 ± 0.66) و (3.6 ± 0.41) و (4.80 ± 0.43) و (3.85 ± 0.50) كالاتي: مجموعة السيطرة السالبة (K-) و مجموعة السيطرة الموجبة (K+) في حين كانت نتائج المجاميع المعالجة (p1,p2,p3) (3.35 ± 0.44) و (12.5 ± 0.66) و (3.6 ± 0.41) و (4.80 ± 0.43) و (3.85 ± 0.50) كالاتي: مجموعة السيطرة السالبة (K-) و مجموعة السيطرة الموجبة (K+)

الفطرية قد تعمل على تخفيف مستوى المألوندايالديهايد و تعبيرات انزيم الكاسبيس 9 و 3 في رحم الفئران المعرضة للزيرالينون. ونتائج المجاميع المعالجة (p1,p2,p3) و (0.43 ± 0.80) على التوالي. نخلص في النهاية الى كون رابطات السموم

Introduction

Feed composed of a single or a mixture of food ingredients, whether processed or unprocessed, which is given for animal's survival, production, and breed. Corn is one of the agricultural commodities very important for humans and livestock. Corn is a high-energy feed ingredients, but contain relatively low protein compared to other grain feed. Corn contains a protein content of about 8.6 to 9.0%. Corn protein is not fermented or degraded by rumen microorganisms, but digested and absorbed in small intestine.

The fungus *Fusariumgraminearum* is a pathogenic flora that may attack corn and capable of producing zearalenon (ZEN). ZEN may increase estrogen levels that trigger estrus cycle disorders (1). The ZEN is a natural estrogen mycotoxins that may induce changes in metabolic profile and organ cell activity of the uterus, liver, spinal cord, brain, mammary gland, and intestinal epithelial cells (2).

The objective of this study was to demonstrate the potential of mycotoxin binders on MDA level, cytochrome c, the expression of caspase 9, caspase 3 in female mice exposed to ZEN. The benefits of this study was to provide knowledge in the field of veterinary medicine about mycotoxin binders in preventing potential negative effects on livestock reproduction caused by ZEN. In the field of animal health, it is expected that this study on the potential of mycotoxin binders may be solution to address reproductive health problems posed by ZEN and can improve livestock productivity through control of mycotoxins from the feed.

Materials and methods

Treatment for experimental animals

This study used 20 female mice (*Musmusculus*) with 4 mice in each groups as experimental animals with the following criteria: healthy, aged 8 to 9 weeks with body weight ranging from 20 to 40 grams and sexually mature. This study was an experimental laboratory study with five treatments as follows: negative control (K-) without being administered with zearalenon (Biotech Co., Ltd., hangzhou, Zhejiang, China) and mycotoxin binders (Impextraco Ltd, Bangkok, Thailand), positive control (K+) given with zearalenon dose of 0.1 mg/head/day without mycotoxin binders (dilution of zearalenon with aquadest), treatment 1 (P1) given with zearalenon in a dose of 0.1 mg/head/day with mycotoxin binders of 0.5 mg/head/day, treatment 2 (P2) was given with zearalenon in a dose of 0.1 mg/head/day with addition of mycotoxin binders 1

mg/head/day, and treatment 3 (P3) received zearalenon in a dose of 0.1 mg/head/day with administration of mycotoxin binders 2 mg/head/day. Zearalenon and mycotoxin binders administration was done orally for ten days. Variables observed in this study were MDA level and the expression of caspase 9 and caspase3.

Animal surgical procedures and sampling

Surgery was performed on day 15 after (Prior to treatment the mice are adapted for 4 days, giving zearalenone and mycotoxin binders for 10 days starting day 5 to day 14 and day 15 surgery) carrying out cervical os dislocation. Disinfection was done with 70% alcohol, then surgery was done quickly to remove the uterus and stored in buoin solution with composition of 75 ml saturated picric acid, 25 ml of 40% formalin and 5 ml glacial acetic acid for making immunohistochemical preparations (3).

MDA measurement procedure

MDA examination procedure is initiated by weighing 0.3 gram uterus added with 4.5 ml of cold PBS and then manual crushed, and then centrifuged at $320 \times g$ for 15 minutes. The supernatant was taken as many as 4 ml, and then added with 1 ml TCA 15% and followed by addition of 1 ml of 0.37% TBA solution in 0.25N HCl. Once mixed evenly, it was heated in a water bath with a temperature of $80^\circ C$ for 15 minutes, cooled at room temperature for 60 minutes, then centrifuged at $320 \times g$ for 15 minutes. Thereafter, the absorbance of uterus MDA supernatant can be measured using a spectrophotometer ($\lambda = 532 \text{ nm}$) and calculated using standard curve regression line of MDA solution (4).

Identification of caspase 9 and caspase 3 expression by immunohistochemistry test

Uterus preparations, which had been made on object glass, were dipped in xylol twice, alcohol-rise (100%, 90%, 80%, 70% and 30%) and distilled water, respectively. Furthermore, preparations were washed in PBS in pH 7.4 for 3 times each for 5 minutes and soaked in 3% hydrogen peroxide (H_2O_2) for 5 - 10 minutes, soaked in 1% BSA in PBS for 10 - 30 minutes at room temperature, added with Biotin labeled anti-caspase 9 IgG primary antibody for 1 hour at room temperature and washed in PBS pH 7.4 for 3×5 minutes. Then, Streptavidin-Horse Radish Peroxidase (SA-HRP) was added for 30×60 minutes in room temperature, washed in PBS pH 7.4 for 3×5 minutes, added with 3,3-Diaminbenzidine tetrahydrochloridechromogen (DAB) for 10 - 20 minutes,

washed with distilled water for 3 × 5 minutes at room temperature and then added with counterstain (Methylen blue/Methylen green) for 3 minutes. Mounting was then carried out with entellan. Observations were made with Olympus® CX-41 microscope in magnification 40, 100, 400, 1000 times. Determination of the amount of expression of caspase 9 and caspase3 can be seen from the number of brownish discoloration on the uterine organs compared to controls (5).

Data analysis

MDA content data were tested for homogeneity with the Kolmogorov-Smirnov test. If the data were homogeneous, the test was followed with parametric analysis using one-way ANOVA and if significantly different (P<0.05) it was followed by Fisher's LSD test. If the data was not homogeneous the analysis used non-parametric Kruskal-Wallis test and if it was significantly different (P<0.05) it was followed by Mann-Whitney test. Data on caspase 9 and 3 expression were tested with nonparametric comparative Kruskal-Wallis test followed by Mann-Whitney test.

Results

The results of calculations and scoring of MDA, Caspase 9 and Caspase 3 in uterine cells of mice exposed to zearalenon can be seen in Table 1.

Table 1 shows that MDA level, the expression of caspase 9 and 3 decreased. MDA levels in the treatment groups K+ (45.59^d±0.50) was significantly different from all treatments P1 (34.92^c±3.38), P2 (27.72^b±1.25), P3 (23.89^b±3.74) and K- (15.48^a±0.50). Caspase 9 expression was decreased in P1 (3.6^b±0.41), P2 (3.3^b±0.34) and P3 (2.8^b±0.28) were significantly different from K+ (8.3^c±0.90), while K- (0.3^a±0.60) was significantly different from all treatments. Caspase3 expression was decreased in P1 (7.70^c±0.73), P2 (4.80^d±0.43), P3 (3.85^a±0.50) and K- (3.35^a±0.44) were significantly different from K+ (12.5^b±0.66), while P3 (3.85^a±0.50) and K- (3.35^a±0.44) were not significantly different.

Discussion

Results of statistical analysis showed that zearalenon was able to modulate or trigger ROS. It is characterized by the appearance of MDA in treatment group receiving zearalenon added with mycotoxin binders and negative control (K-) in the uterus. Effects of mycotoxin binders has been prove to become antagonist against zearalenon as indicated by decreased MDA levels.

Free radicals can be produced by a variety of physiological processes and has an important role as a signaling molecule such as electron transport. In

physiological conditions, free radicals may be mitigated efficiently by antioxidants to prevent cell damage. In pathological conditions, excessive production of free radicals that followed the decrease in antioxidants and can not dampen the action of free radicals, resulting in oxidative stress (6). Oxidative stress can be harmful because it can damage macromolecules in cells such as carbohydrates, proteins and DNA. Damaged macromolecules may then lead to cell death (7).

Table 1: MDA, Caspase 9 and Caspase 3 Levels in Mice Uterus

Treatment	MDA level ± sd	Caspase 9 expression ± sd	Caspase 3 expression ± sd
K -	15.48 ^a ±0.50	0.3 ^a ±0.60	3.35 ^a ±0.44
K +	45.59 ^d ±0.50	8.3 ^c ±0.90	12.5 ^b ±0.66
P1	34.92 ^c ±3.38	3.6 ^b ±0.41	7.70 ^c ±0.73
P2	27.72 ^b ±1.25	3.3 ^b ±0.34	4.80 ^d ±0.43
P3	23.89 ^b ±3.74	2.8 ^b ±0.28	3.85 ^a ±0.50

Different superscripts in the same column show significant differences among treatments (P<0.05), Description: K- = group of mice not exposed to zearalenon and without the provision of mycotoxin binders; K+ = group of mice exposed to zearalenonof 0.1 mg/head/day and without the provision of mycotoxin binders; P1, P2, P3 = zearalenon group of mice exposed to 0.1 mg/head/day with administration of mycotoxin binders 0.5; 1; 2 mg/head/day. MDA: Malondialdehyde.

Zearalenon lipophilic nature makes it able penetrate cell membranes and interact with the mitochondria. During interaction phase with mitochondria zearalenon inhibits mitochondrial respiratory pathway, causing ATP depletion, thereby increasing the amount of reactive oxygen species (ROS) that may damage mitochondrial DNA or insert themselves in replicating process, or directly caused mitochondrial permeability transition (MPT) to make a hole in MPT pore that is located on the inside of the membrane. The opening of this MPT pore is the onset of cell death, often called the process of apoptosis (8,9).

Mycotoxin binders containing aluminosilicate compounds have proven to be the most effective to bind mycotoxinszearalenon. Action mechanism of mycotoxin binders is by eliminating methyl group on zearalenon chemical structure (10). Silicate structure consists of neosilicate, sorosilicate, inosilicate, sicosilicate, filosilicate, and tectosilicate. The structure will be transformed into Hydrated Sodium Calcium Aluminosilicate which is easily metabolized by the body. Mycotoxin binders are also equipped with a decontaminant material useful in improving animals' condition and

eliminating mycotoxins negative effects during exposure (11,12).

Research (7,8) concluded that the molecular toxic effects caused by zearalenon may cause oxidative stress and induces apoptosis intrinsic pathway in mitochondria. In many apoptotic process, especially in the liver and gonads, excessive production of ROS is associated with the apoptosis pathways of mitochondria. A study by (13) showed that ROS resulting from zearalenon exposure and mitochondrial changes is due to increased ROS levels. This indicates that zearalenon induces oxidative stress that causes apoptosis.

The existence of free radicals may break DNA chains or cause changes in DNA nucleotides composition. DNA damage activates p-53 and the latter's activation may trigger Bax activity, suppressing Bcl-2 in mitochondrial membrane, resulting in mitochondrial membrane permeability changes, leading to the release of cytochrome C to cytosol. Cytochrome C activates Apaf-1 and subsequently activates caspase cascade and activates the DNA-se, and then damages the DNA so that the cells undergo apoptosis (14).

There are two important components that cause cytochrome C release, the mitochondria permeability transition pore (MPTP) and the proapoptotic protein Bax. The opening of MPTP is influenced by several factors, such as calcium accumulation, oxidants and low mitochondrial transmembrane potential. The merging of Bax with MPTP may form a pore in mitochondrial outer membrane, so as to facilitate the release of determinants of apoptosis, the cytochrome C, AIF and Smac/Diablo, which will then be translocated into the nucleus and causes of DNA fragmentation (8,9).

Caspase 9 expression of in K- group was significantly different from all treatments. So was K+. However, among groups P1, P2 and P3 there was no significant difference. Meanwhile, between treatments P1, P2 and P3 there was significant difference.

A study by (15) mentions that zearalenon may initiate apoptotic process. Apoptotic molecular process occurs through intrinsic pathway in mitochondria with caspase 9 as the initiator and caspase 3 as the executor. Zearalenon is designated as a cytotoxic agent that triggers hyperestrogenism in reproductive system as it is able to induce massive apoptosis.

Hyperestrogenism would disturb Transmissible Permeability Pore (PTP) consistency on mitochondrial membrane. PTP damage may result in megachannel activation that will affect electron transport failure. Active megachannel may also be followed by the influx of extracellular Ca²⁺ ion and accumulates with intracellular Ca²⁺ ions containing in the mitochondria and endoplasmic

reticulum. High Ca²⁺ level which may increase Bax expression and decrease Bcl-2 (2).

Caspase 9, which acts as apoptosis initiator, may be dimerized, triggering a feedback by inhibiting Bcl-2 release of and binding procaspase3 to activate caspase3. Caspase3, which act as executor, may assist cytoplasmic endonucleases and proteases activation that may fragment nuclear DNA and degrade cytosol protein. The final result in fragmentation process is the formation of apoptotic bodies containing intracellular organelles and expresses phosphatidylserine that may trigger phagocytosis (16,17). The expression of caspase3 in K- group was significantly different from that in other treatment groups. However, group K+ was significantly different from groups P1 and P2, but not significantly different from P3.

Apoptotic molecular process occurs through the intrinsic pathway in the mitochondria and caspase3 as executor. Zearalenon is expressed as cytotoxic agents in reproductive system as it can induce massive apoptosis. A study by (18) also explains that zearalenon exposure may lead to cell death, largely by apoptosis and in part by necrosis fraction. Zearalenon exposure leads to loss of Mitochondrial Membrane Potential (MMP), mitochondrial change in the amount of protein Bcl-2 and Bax, and cytoplasm cytochrome c release. Cytochrome c release may trigger procaspase 9 and Apaf-1 merges into complex apoptosome. It is this apoptosome that will modulate the formation of the executioner caspases, one of which is caspase3 (19).

Caspase3, that acts as executor, may help endonucleases activation and cytoplasmic proteases that may lead to nuclear DNA fragmentation and degrade cytosol protein. Final result in fragmentation process is the formation of apoptotic bodies containing intracellular organelles and expresses phosphatidylserine that will trigger phagocytosis (16,17).

In conclusion, in this study, mycotoxin binders may decrease malondialdehyde (MDA) level, caspase 9 and caspase3 expressions in mice (*Mus musculus*) uterus exposed to zearalenon. Mycotoxin binders can be given simultaneously with the feed to prevent the effects that arise when cattle consume feed contaminated with *Fusarium graminearum* fungi that produces zearalenon. Storage of animal feed should be reliable to avoid mold growth that is harmful to livestock.

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