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PREFACE

In this edition, Volume 26 No 3, we proudly present articles from animal and veterinary sciences including genetics; reproduction, feed technology, and veterinary. The articles published in this edition are:

"Fluctuating Asymmetry Increases with Heat Stress Disruptions on Bali Cattle (Bos javanicus) at Different Altitude"; "Effect of Protection of Soybean Meal Using Mahogany Leaf Extract in Ruminant Diet on Rumen Fermentation Products"; "Amantadine resistance of clade 2.3.2 H5N1 Avian Influenza Virus from Waterfowl in Indonesia"; "Interaction Effect of Vitamin E-selenium Supplementation and Metabolic Energy on Reproductive Performance of Joper Breeders"; and "Studying the Liver Function in Male Neonates of Rats Born to Sertraline-Treated Mothers".

We extent high appreciation to all peer reviewers who make this journal academically high value. Hopefully, these articles would offer any benefit to readers and the end-users of technological innovation, and attract interests from scientists to contribute their papers to the Indonesian Journal of Animal and Veterinary Sciences.

Chief Editor

Bogor, September 2021

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Fluctuating Asymmetry Increases with Heat Stress Disruptions on Bali Cattle (*Bos javanicus)* at Different Altitude

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ABSTRAK

Suhendro I, Jakaria J, Priyanto R, Manalu W, Noor RR. 2021. Fluktuasi asimetris meningkat seiring dengan gangguan stres panas pada Sapi Bali *(Bos javanicus*) di ketinggian yang berbeda. JITV 26(3):89-95 DOI: http://dx.doi.org/10.14334/jitv.v26i3.2758.

Sapi Bali (*Bos javanicus*) merupakan spesies yang umum tersebar di seluruh Indonesia dan dapat bertahan hidup di iklim tropis yang panas dan lembap. Fluktuasi asimetris (FA) adalah penyimpangan acak dari simetri bilateral sempurna. FA sering digunakan untuk mengukur stabilitas perkembangan pada individu dan dapat digunakan sebagai penanda dampak stres lingkungan dan genetik. Penelitian ini bertujuan untuk mengetahui pola ketidakstabilan perkembangan sapi Bali akibat cekaman lingkungan pada berbagai ketinggian tempat dengan menggunakan indeks FA. Indeks FA dalam penelitian ini digunakan untuk membandingkan asimetris ciri fisik sapi Bali di dataran tinggi Sembalun dan dataran rendah Serading. Enam puluh lima ekor sapi Bali yang digunakan dalam penelitian ini dipelihara pada ketinggian yang berbeda, yaitu Serading, Pulau Sumbawa (50 m dpl), dan Sembalun, Pulau Lombok (1.186 m dpl). Sifat fisik sapi Bali yang diukur adalah lingkar tanduk (tanduk), jarak pinggul ke tulang belakang (panggul), lingkar metatarsal (metatarsal), dan lingkar metakarpal (metakarpal). Indeks FA1 dan FA5 menunjukkan perbedaan metakarpal yang signifikan antara sapi Bali yang dipelihara di populasi Sembalun dan Serading (P<0,05). Indeks sifat ganda FA11 sebagai kombinasi dari semua sifat juga menunjukkan perbedaan yang nyata (P<0,01). Dapat disimpulkan bahwa variasi cekaman panas menurut ketinggian mempengaruhi asimetris sapi bali.

Kata Kunci: Altitud, Fluktuasi asimetris, Sapi Bali, Stres panas

ABSTRACT

Suhendro I, Jakaria J, Priyanto R, Manalu W, Noor RR. 2021. Fluctuating asymmetry increases with heat stress disruptions on Bali cattle *(Bos javanicus*) at different altitude. JITV 26(3): 89-95 DOI: http://dx.doi.org/10.14334/jitv.v26i3.2758.

Bali cattle (*Bos javanicus*) are common species distributed throughout Indonesia to survive in tropical hot, and humid climates. Fluctuating asymmetry (FA) is a random deviation from perfect bilateral symmetry. FA is often used to measure developmental stability in individuals and can be used as a marker for the impact of environmental and genetic stress. This study aims to investigate the pattern of developmental instability in Bali cattle as caused by environmental stress at various altitudes using the FA index. FA indexes in this study were used to compare asymmetrical physical traits of Bali cattle in Sembalun high altitude and Serading low altitude. Sixty-five Bali cattle were used in this study reared at different altitudes, namely Serading, Sumbawa Island (50 m above sea level), and Sembalun, Lombok Island (1,186 m above sea level). The physical traits of Bali cattle measured were horn circumference (horn), a distance of hip to spine bone (pelvic), metatarsal circumference (metatarsal), and metacarpal circumference (metacarpal). The FA1 and FA5 indexes showed significant metacarpal differences between Bali cattle reared at Sembalun and Serading population (P<0.05). FA11 multiple trait index as a composite of all traits revealed a significant difference as well. (P<001). It can be concluded that various heat stress by altitude impacts the asymmetry of Bali cattle.

Key Words: Altitude, Bali cattle, Fluctuating asymmetry, Heat stress

INTRODUCTION

Habitat alteration due to geographical location, latitude, altitude, or human perturbation causes stress, leading directly or indirectly to the animal population's different developmental, growth, and fitness. An animal living in such a habitat should allocate a significant amount of energy to overcome environmental stress while allocating less energy to growth and reproduction (Palmer 1994). Animals can adapt to unfavorable environments through behavioral, physiological, or morphological changes (Niyas et al. 2015; Berihulay et al. 2019). One of the adaptation processes was morphological changes, in which the variations in the level of environmental stress can contribute to the instability development of morphology (Kriegman et al. 2018).

Environmental disturbances cause developmental noise in animals and disrupt the stability of their morphological development, leading to fluctuations

asymmetry (FA), especially in bilateral organisms (Breed & Moore 2016). FA is defined as random deviations from perfect symmetry with a normal distribution and a zero mean (Palmer 1994). Its distribution varies amongst populations and is influenced by genetic and environmental variables (de Coster et al. 2013). FA also reflected the organism's inability to buffer its development against random perturbation (Stevenson 2000). Therefore, high-quality individuals (i.e., good genes) can develop more symmetrically than their less well-adapted counterparts, either because they receive more energy or use energy more efficiently (Stevenson 2000; Holló 2017).

FA is practical enough to be widely used and applied across biological systems since it relates to life history and fitness characteristics (van Dongen & Gangestad 2011; Lajus et al. 2019). FA can be used as a biomarker since it is relatively easy to measure compared to other biomarkers that require more expensive equipment (Coda et al. 2016; Simbula et al. 2021). Some researchers have suggested FA as a marker to estimate how well a population is coping with changes in environmental conditions, especially stressful conditions such as heat shock, radiological, oil spills, and metal contaminants that are expected to persevere for prolonged periods in the environment (Beasley et al. 2013). FA can also be emphasized to estimate fitness since collecting a direct fitness measure from the population in the field is difficult (Baucom & de Roode 2011; Lajus et al. 2019).

Animals will have different proportions of asymmetry between populations living in disturbed habitats than in natural habitats (Alroy 2017; Teresa Capucchio et al. 2019). Several studies have shown that stressful conditions can cause FA in some populations; the study of *Calliphora vicina* in various temperature regimes (Limsopatham et al. 2018); bumblebee under various stress conditions (Gerard et al. 2018); several stressors (i.e. natural toxin, parasite, thermic and inbreeding stress) on wing shape and size of bumblebee (Gerard et al. 2018); Caspian whipsnake in diverse stressful habitats due to human disturbance (Bellaagh et al. 2010).

This microclimate created by altitude makes a specific habitat variation from the valley to the top of the mountain for animals living (Wang et al. 2011). The altitude difference directly affects animals' environmental habitat, such as a difference in temperature, humidity, wind speed, and solar radiation. Bali cattle is Indonesia's most adaptive cattle (Martojo 2012) since it can be bred in various environments on Indonesia's humid tropical island. Bali cattle could survive in various environments, such as in Sumatera and Kalimantan oil palm farms, a breeding village in Java and Bali, the steppe and savannah in NTT, and hill and mountain Mt Rinjani. This study aims to use the FA index to investigate the pattern of developmental instability in Bali cattle due to environmental stress due to altitude differences.

MATERIALS AND METHODS

Sample collection

Morphological data were collected from the Bali cattle population in October – November 2019, reared at different altitudes to indicate heat stress induction. The first population was chosen at a low altitude of 50 meters above sea level (MSL) at the Center for Livestock Breeding and Forage (BPT-HMT) Serading, Sumbawa Island. The second population was chosen from a high altitude of 1,186 MSL in the village breeding center of Mount Rinjani valley, Sembalun, Lombok Island. A total of 65 cattle was used in this study, with 40 heads in Serading and 25 in Sembalun. Relative humidity (Rh) and ambient temperature (Ta) were measured using a Thermo-Hygrometer which was then calculated into the temperature-humidity index (THI). The condition information of the research location is presented in Table 1.

Recording of physical parameters

Physical parameters measured in the study were circumference of horn (horn), hip to spine bone distance (pelvic), metatarsal circumference (metatarsal), and metacarpal circumference (metacarpal). Scales were measured both on the right (R) and left (L) sides. The fluctuations asymmetry of Bali cattle was calculated by comparing their physical parameters using the FA index. The FA index used was FA1, FA5, and FA11. FA for every single trait was compared using FA1 (mean absolute asymmetry: mean |R-L|) and FA5 ($0.798\sqrt{(R-L)2/N}$); N = number of subjects in the sample). Meanwhile, FA for multiple traits was compared using FA11 [(Ai)/N, (Ai) = Σ|R-L| for all traits] (Palmer & Strobeck 2003). The FA1 is simple and common to use, and it can be compared to the other FA. FA5 is effective in detecting small FA differences between populations and is effective in small sample sizes. The FA11 offered a combined comparison of multiple features.

Statistical analysis

Outlier data for the FA values were excluded by Grubb's test aberrant (de Muth 2019). The Chi-square test calculated independent data on several morphological characters used to develop the FA index. Skew and kurtosis were used to test the normal

Table 1. The natural conditions of the sampling location

Site	Latitude	Longitude	Altitude	Ta	Rh	THI
Serading	8°34'04" S	117°29'48" E	50 MSL	30.70±4.68[a]	51.57±17.42[a]	78.79±3.67[a]
Sembalun	8°21'48" S	116°31'49 E	1186 MSL	23.64±3.36[b]	58.57±11.22[b]	70.51±4.31[b]

Means in the same column with different superscripts differ significantly (P<0.05). S= south, E = east, MSL = meters above sea level, Ta = temperature ambient; Rh = relative humidity; THI = temperature-humidity index

Table 2. FA-index representative parameters in two cattle populations

Site	Trait	$\frac{(R+L)}{2}$	R-L	R-L		
				Mean	Skew	Kurtosis
Sembalun (24)	Horn (cm)	13.55	0.31	0.06	1.95201	4.21027
	Pelvis (cm)	14.69	0.13	-0.04	2.42186	4.21027
	Metacarpal (cm)	15.32	0.23	0.15	1.37435	-0.01901
	Metatarsal (cm)	19.65	0.46	0.04	2.56753	7.07699
Serading (37)	Horn (cm)	15.29	0.53	0.18	1.24883	1.02443
	Pelvis (cm)	14.70	0.15	0.01	3.43039	11.12044
	Metacarpal (cm)	14.93	0.78	-0.11	1.40628	1.58457
	Metatarsal (cm)	20.20	0.81	-0.08	1.96479	5.60927

L: the value of the left side; R: the value of the right side

distribution (Palmer & Strobeck 2003), and a t-test was used to check the expected mean zero (mean absolute FA compared to zero) (Palmer 1994). The FA indexes were analyzed using R Studio (R Studio, BC, Boston, MA, 2020) with Mann Whitney U test (Neuhäuser 2011). Correlations were calculated for each pairwise combination of the four traits across all observations. Correlations between absolute R-L (right-left) cattle size measurements were evaluated using Pearson correlations and FA measurements using Spearman rank correlations (Schober et al. 2018).

RESULT AND DISCUSSION

There was little available cattle study on phenotypic changes during ontogenetic development, and this study only used morphological data from adult specimens that showed no visible signs of abnormalities. The samples were collected at intermediate age because FA levels were generally lowest at intermediate ages and higher early and late in life (Graham & Özener 2016). It is because the declining relative growth rate's allometric effects may have been more important at an early stage of life. Later in life, growth may have become less precise due to the accumulation of accidents at a level lower than morphogenesis (Miller & Zachary 2017).

The use of physical traits such as horn, pelvic, metacarpal, and metatarsal is easily identifiable and quantifiable. These traits refer to previous studies on FA. Several FA studies have used tarsal as part of the calculation due to its relationship to forest landscape connectivity and phenotypic adaptation (Callens et al. 2011; de Coster et al. 2013). The Kruuk et al. (2003) study used antlers (horns) to reference the FA because of their effect on secondary sexual traits and their relationship to growth traits. Pelvic in several studies related to animal carcass size (Van Rooyen et al. 2012; Agamy et al. 2015).

The representative value of physical traits on the right and left sides are presented in Table 2. Bali cattle's mean traits ((R+L)/2) in Sembalun high altitude are almost lower than in Serading. The absolute difference between right and left values for each trait reflects the degree of asymmetry relative to the ordinary form; the Serading Bali cattle population has a more significant value deviation than the Sembalun Bali cattle population. Trubyanov & Glotov (2010) defined skewness and kurtosis as the indication of normal distribution. Skewness is commonly defined as a measure of symmetry or asymmetry datasets with a perfectly symmetrical data set is skewness close to 0. Kurtosis is mainly about the tails of the distribution and how heavy the tails are. A normal distribution has a kurtosis of 3. The population of Bali cattle in each location is quite asymmetrical since the dataset deviates significantly from the normal distribution as determined by skewness kurtosis analysis.

Only 37 Bali cattle in Serading and 24 in Sembalun were included for the study after eliminating outlier data and outlier asymmetry values by Grubb's test

Table 3. Correlation of size and FA in all traits with a correlation between trait mean size is above the diagonal, and the absolute FA is below the diagonal.

	Horn	Pelvic	Metacarpal	Metatarsal
Horn		0.443***	0.338**	0.343**
Pelvic	-0.215		0.568***	0.451***
Metacarpal	0.243	0.02		0.642***
Metatarsal	-0.113	0.014	-0.293	

*** indicates P < 0.001. There were no significant correlations below the diagonal.

Table 4. Comparisons of FA1, FA5, and FA11 indexes (Mann Whitney-test)

FA index	Traits	Sembalun	Serading	Combined
FA1	Horn	0.31	0.53	0.44
	Pelvis	0.13	0.15	0.14
	Metacarpal	0.23[a]	0.78[b]	0.57
	Metatarsal	0.46	0.81	0.67
FA5	Horn	0.25	0.42	0.35
	Pelvis	0.10	0.12	0.11
	Metacarpal	0.18[a]	0.63[b]	0.45
	Metatarsal	0.37	0.64	0.54
FA11		1.13[a]	2.27[b]	1.82

Superscript shows a significant difference (P<0.05)

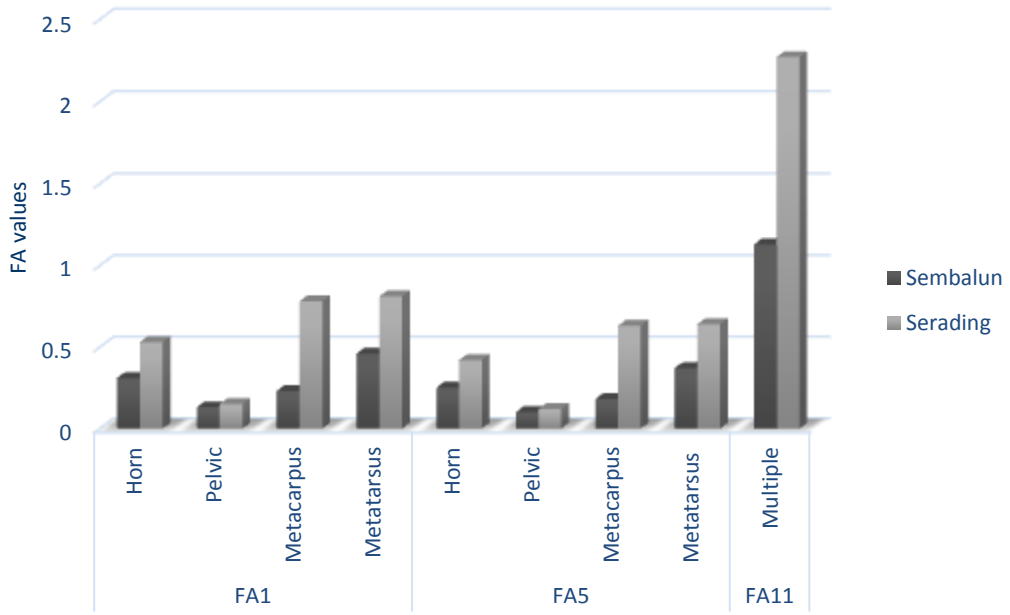

Figure 1. Comparison of FA values of the two cattle populations

aberrant. According to the Chi-Square test, the horn, pelvic, metacarpal, and metatarsal traits are independent (P = 0.993). The average size of each cattle trait was closely correlated with all other traits, with the highest correlation (0.642) between metacarpal and metatarsal diameter (Table 3). Even after a sequential Bonferroni correction (Sedgwick 2012) for multiple testing, all six pairwise correlations were meaningful at the 5% level. On the other hand, the absolute FA did not significantly correlate with the four traits (Table 3).

The physical traits were calculated into fluctuating asymmetry to assess the index of development instability of Bali cattle. The FA index of physical trait at different altitude are presented in Table 4. The simple indexes of FA1 and FA5 compared each trait to the altitude. However, the multiple indexes of FA11 combined all traits to compare with the altitude. The multiple traits (FA11) were significantly different between the two populations (P<0.01). While the altitude was compared in each parameter, there was a significant difference in metacarpal trait by using simple indexes of FA1 and FA5. The difference between the other FA scales (horn, pelvic, metatarsal) was insignificant, but the Metatarsal scale shows a near-significant differentiation (P=0.053). The physical traits of the metatarsal and metacarpal diameter could be used to compare the Bali cattle due to different stress habitats. This is in line with the study from Bellaagh et al. (2010) about the Caspian whipsnake population's FA value having significant differences in the FA11 index and simple sublabial index due to differences in stress habitats.

The Mann-Whitney test results from the FA11, $FA1_{metacarpal}$, and $FA5_{metacarpal}$ indexes showed a significant difference between the Bali cattle population's high-altitude Sembalun and low-altitude Serading. Bali cattle population at low altitude shows a more asymmetrical variation as a phenotypic variation in the metacarpals scale and whole scale as one in FA11. In contrast, the FA1 and FA5 indexes scale in horn, pelvic, and metatarsal showed no significance. The horn and pelvic scale have a high degree of phenotypical stability, not asymmetrical at different stress conditions.

Animals living in uncomfortable environments should allocate a large amount of energy to cope with environmental challenges while less energy is spent on development and reproduction (Palmer 1994). This low energy allocation contributes to low growth (Madhusoodan et al. 2019) or fluctuating asymmetry of the animal (Stevenson 2000). Tuyttens et al. (2005) also discovered a negative relationship between FA and body weight growth in fattening rabbits. Bali cattle acclimated raised in harsh environments had a smaller body size than commercial cattle (Depison et al. 2020) and have lower average daily gain (Marsetyo et al. 2006; Baco et al. 2019). More stressful environments, such as increasing heat stress levels at low altitudes, altered the development stability of Bali cattle, causing them to grow more asymmetrically. This low altitude has higher fluctuation asymmetrical at all traits and all indexes (Figure 1). This continuous situation causes cattle to require more energy to their bodies in order to survive rather than gaining weight.

CONCLUSION

The metacarpal and metatarsal scales were useful for Bali cattle fluctuating asymmetry studies based on their variance and distribution of asymmetry at the population level. On the other hand, horn and pelvic were less effective in describing the FA in Bali cattle. All four physical traits are easily measured and would be ideal for FA studies. It is concluded that a higher stress level affecting the Bali cattle population might raise the FA.

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Effect of Protection of Soybean Meal Using Mahogany Leaf Extract in Ruminant Diet on Rumen Fermentation Products

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ABSTRAK

Ifani M, Suhartati FM, Rimbawanto EA. 2021. Proteksi bungkil kedelai menggunakan ekstrak daun mahoni pada ransum ruminansia: pengaruhnya terhadap produk fermentasi rumen. JITV 26(3): 96-107. DOI: http://dx.doi.org/10.14334/jitv.v26i3.2829.

Penelitian bertujuan mengkaji pengaruh proteksi bungkil kedelai menggunakan ekstrak daun mahoni terhadap produk fermentasi rumen secara *in vitro*. Materi yang digunakan yaitu cairan rumen sapi, ransum basal terdiri dari konsentrat dan rumput gajah dengan rasio 60:40%, dan daun mahoni. Penelitian dilaksanakan dalam tiga tahap yaitu ekstraksi mahoni, proteksi protein menggunakan ekstrak mahoni, dan tahap *in vitro*. Pengujian dilakukan secara *in vitro* dan mengunakan rancangan acak lengkap (RAL). Perlakuan terdiri 4 macam proteksi bungkil kedelai dengan konsentrasi tanin 0% (P0); 1,5% (P1); 3% (P2); dan 4,5% (P3). Data yang diperoleh dianalisis dengan analisis variansi dan diuji menggunakan orthogonal polinomial. Hasil penelitian menunjukan bahwa penambahan bungkil kedelai terproteksi ekstrak daun mahoni berpengaruh secara kubik terhadap VFA parsial, gas metan dan protein terlarut pasca rumen, berpengaruh secara kuadratik terhadap protozoa, serta berpengaruh secara linear terhadap N-NH_3, SPM, dan RUDP. Pemberian ekstra daun mahoni taraf 1,5% menghasilkan produk fermentasi yang tidak berbeda dengan kontrol, sedangakan pemberian taraf 3% mendapatkan produk fermentasi tertinggi. Pemberian ekstak daun mahoni taraf 4,5% menghasilkan SPM, dan RUDP tertinggi namun terjadi penununurunan pada protein terlarut yang menunjukan terjadinya *over proteksi*. Penambahan ekstrak daun mahoni taraf 3% secara efektif mampu meningkatan produk fermentasi rumen, RUDP, dan protein terlarut tanpa mengganggu aktivitas bakteri rumen.

Kata Kunci: Mikroba, Protein, Rumen, Tanin

ABSTRACT

Ifani M, Suhartati FM, Rimbawanto EA. 2021 Protection of soybean meal using mahogany leaf extract in ruminant ration against rumen fermentation products. JITV 26(3): 96-107. DOI: http://dx.doi.org/10.14334/jitv.v26i3.2829.

The study was aimed to examine effect of protecting soybean meal using mahogany leaf extract on rumen fermentation products in vitro. The material used was cow rumen fluid, basal ration consisting of concentrate and elephant grass with a ratio of 60:40%, and mahogany leaves. The research was carried out in three stages: mahogany extraction, protein protection using mahogany extract, and in vitro stages. The test was conducted in vitro based on a completely randomized design (CRD). The treatments consisted of 4 kinds of soybean meal protection with 0% tannin concentration (T0); 1.5% (T1); 3% (T2); and 4.5% (T3). Data obtained were analyzed by analysis of variance and tested using orthogonal polynomials. Results showed that addition of protected soybean meal with mahogany leaf extract had a cubical effect on partial VFA, methane gas and post-rumen dissolved protein, a quadratic effect on protozoa, and a linear effect on N-NH_3, SPM, and RUDP. Giving extra mahogany leaves at a level of 1.5% produced a fermented product that was not different from the control while giving a level of 3% got the highest fermentation product. Giving mahogany leaf extract at a level of 4.5% resulted in the highest SPM, and RUDP but there was a decrease in soluble protein, which indicated the occurrence of overprotection. The addition of 3% mahogany leaf extract effectively increased rumen fermentation products, RUDP, and soluble protein without disturbing the activity of rumen bacteria.

Key Words: Microbes, Protein, Rumen, Tannins

INTRODUCTION

Soybean meal is a source of high-quality feed protein with a crude protein content of 49%. Waldi et al. (2017) showed that soybean meal has a high in vitro solubility (60-80%) so that it is quickly degraded in the rumen and within 48 hours it's protein degradation reached 24,149 gN/hour and was indicated by high levels of ammonia (12.97-14.42 mM). Soybean meal protein that is resistant to degradation in the rumen ranges from 22-53%, while the digestibility of the rumen-resistent protein in the small intestine reaches 86-100% (Arisya et al. 2019). The high level of protein degradation of soybean meal in the rumen causes soybean meal ~~to~~ need to be protected to reduce its degradability so that it can be used more efficiently.

Protein protection is commonly done by heating, adding formaldehyde, and using polyphenol compounds

in plants such as tannins. Soybean meal protection using formaldehyde has a weakness. It can inhibit microbial activity in the rumen because formaldehyde dissolves and enters the rumen fluid (Suhartanto et al. 2014), while protein protection by heating makes protein difficult to be digested (Mayangsari et al., 2013). The digestibility of crude protein of soybean meal without protection, protection by heating, and protection with formaldehyde resulted in different digestibility, namely the highest protein digestibility of soybean meal protected by heating which was 96.9%, followed by digestibility of soybean meal. Without protection was 96.4%, and the lowest protein digestibility was protection with formaldehyde at 66.3% (Stern, et al., 2006).

Protein protection using condensed tannins is an alternative that can be used as a protein protection agent because it has the best efficiency level compared to protein protection using heating or formaldehyde. Condensed tannins have the potential to protect proteins from microbial degradation in the rumen (Prayitno et al., 2018) so that they can increase the amount of protein available to the host, while hydrolyzed tannins in excess amounts have the potential to poison livestock. The binding of the tannin-protein complex occurs at pH 3.5-7.0 and will separate at pH <3.5 (Min and Solaiman 2018). The pH value corresponds to the pH in the abomasum. Separated tannin-protein complex bonds in the abomasum can increase protein in the intestine. This has been proven by Cahyani et al. (2012) who reported that the protein protection treatment of soybean flour using mangrove leaf tannins was able to reduce fermentability due to the formation of a tannin-protein complex. (Rimbawanto et al. 2015) stated that the use of calliandra condensed tannins in trash fish increased total protein digestibility in vitro.

The process of protein digestion depends on the separation of the tannin-protein complex in the abomasum because the bonds of the tannin-protein complex cannot wholly release each other, it is related to the different tannins and protein structures (Díaz Carrasco et al. 2017). Yusiati et al., (2018) found that mahogany leaves had a total tannin content of 11.9 mg/100 mg DM, consisting of condensed tannins 9.241±0.02mg/100mg DM and hydrolyzed tannins of 2.707±0.06 mg/100 mg DM. The ability of the tannin to bind protein reaches 1,217 mg BSA/mg leaf DM, so that mahogany leaves have the potential to be used as a source of condensed tannins in protein protection for ruminant feed.

The high tannin content in mahogany leaves is expected to increase the effectiveness of soybean meal protein protection. In addition, when viewed from the availability, mahogany leaves are still widely available in the surrounding environment, but not many have been used. The use of mahogany leaves as a protein feed protection agent can increase the usefulness of this plant. Research on mahogany leaves as a source of tannins has not been done much, and the research is only limited to knowing the potential of mahogany leaves as goat feed ingredients. Therefore, this study aimed to examine the effect of soybean meal protected by mahogany leaf extract on the production of partial VFA (acetate, propionate, and butyrate), methane gas, protozoa, N-NH3, microbial protein synthesis, RUDP, and post-rumen protein solubility in vitro.

MATERIALS AND METHODS

The material used in this in vitro experiment was rumen fluid from three beef cattle taken from the Bantaruni Slaughterhouse (RPH), Kembaran District, Banyumas Regency, immediately after the cattle were slaughtered. The rumen was opened using scissors, then the contents was taken and put in two pieces of cheesecloth, then squeezed. The squeezed liquid was then accommodated through a funnel into an empty thermos previously filled with hot water to maintain the temperature in the thermos between 39-40 ^{o}C. The tested rations was concentrate and forage with a ratio of 60:40 DM. The protected soybean meal was given as additional feed at 2% DM of the total concentrate. The concentrate comprised of 20% DM coconut cake, 50% DM cassava, and 30% DM polar. The composition of the feed is shown in Table 1. Proximate analysis of feed ingredients was carried out using the AOAC (2005) method to determine the nutrient content of each. Dry matter levels were obtained by heating 2 g DM of samples at 105 ^{o}C for 8 hours or until the sample weight was stable. Ash levels were obtained by heating 2 g DM of samples at 600 ^{o}C for 12 hours. The protein content of the feed ingredients was obtained by distilling 0.1 g DM of sample, and the result of the distillation was then titrated with HCl solution. The fat content of feed ingredients was obtained by extracting 2 g DM of legume samples in soxhlet with ether as solvent. The crude fiber content of feed ingredients was obtained by washing 1 g DM of legume samples with several chemical solutions such as H2SO4, NaOH, acetone, and aquadest.

The crude extract of mahogany leaves comes from old mahogany leaves from a seven-year-old mahogany tree planted in the Faculty campus environment; General Sudirman University Livestock, with andosol type soil media and an average plant environmental temperature of 27 ^{o}C and humidity of 92-93%. Mahogany leaves before extraction were heated at a temperature of 60oC, finely ground with a 200 mesh sieve. The ability of condensed tannins to bind BSA was used as the basis for determining the level of condensed tannins in the treatment. The level of condensed tannins given is below the optimum,

Table 1. Nutrient Composition of Feed Ingredients

Feed	Dry Matter (%)	Ash (%)	Crude Protein(%)	Crude Fat (%)	Crude Fiber (%)
Elephant grass	23.74	15.73	6.62	4.2	12.44
Coconut meal	86.00	13.01	21.52	24.58	14.46
Onggok	85.34	12.93	2.23	16.10	15.89
Pollard	85.55	13.37	12.51	8.13	14.71
Soybean meal	87.34	11.20	38.92	9.79	19.53
Nutrient Compositions of Concentrate					
Concentrate	85.54	12.37	13.34	6.2	21.65
Nutrient Composition of Diet					
Diet	69.78	13.34	11.01	5.4	17.9

optimum, and above optimum levels. The mahogany leaves used contained a total phenol of 15,32 mg/100 mg BP, a total non-tannin phenol 2.05 mg/100 mg BP and a total tannin content of 13.27 mg/100 mg BP consisting of condensed tannins 10.03 mg/100 mg DM and hydrolyzed tannins 3.24 mg/100 mg DM. The ability of mahogany tannins to bind BSA optimally was 5.93 mg BSA/100 mg DM mahogany leaves.

In vitro research was carried out at the Laboratory of Nutrition and Animal Feed Sciences, Fapet, Unsoed. Partial VFA analysis (acetic, propionic, and butyric acids) and methane gas was analysed using a set of gas chromatography. N-NH_3 and microbial protein synthesis was measured by a spectrophotometer with an oxidizing solution, $HClO_4$ 70%, $NH_4H_2PO_4$ 0.0285 M pH 2, $AgNO_3$ 0.4, HCl 0.5 N. Dissolved protein and RUDPwas determined using oven, digestion flask and concentrated sulfuric acid scale, selenium catalyst, 40% NaOH, 3% H_3BO_3, indicator methyl red and 0.1 N HCl.

The treatment consisted of 4 of tannins concentration levels to protect soybean meal namely of 0; 1.5; 3; and 4.5% (Table 2). The experimental method applied was completely randomized design with one-way classification. Each treatment was replicated six times so that there were 24 experimental units. The data obtained were tabulated and then analyzed using analysis of variance. If the treatment has a significant effect on the response variable, it is further tested using orthogonal polynomials.

This study was carried out in three stages: the preparation stage, the rumen fluid incubation stage, and the measurement stage. The preparation stage includes the extraction of mahogany leaves. Extraction of mahogany leaves was carried out according to Rimbawanto et al. (2015) method and determination of total tannin in mahogany leaves based on Makkar (2003). The addition of tannins into soybean meal was done by spraying mahogany leaf extract using a sprayer into soybean meal until homogeneously mixed and then dried at room temperature. The second stage is the incubation stage**.** The incubation stage consists of anaerobic incubation and aerobic incubation, according to Tilley & Terry (1963). The third stage was measurement phase consisted of measuring the concentration of partial VFA (acetate, propionate, butyrate), methane gas, concentration of N-NH3, protozoa, and protein solubility.

The variables measured in this study were partial VFA concentrations (acetate, propionate, butyrate) which were measured using a gas chromatography technique following the method of Filipek and Dvorax (2009); Methane gas is calculated using the formula of Orskov (1996); Concentration of N-NH3 using the hypochlorite staining method (Weatherburn 1967); Microbial protein synthesis was measured using the method proposed by Zinn & Owens, (1986); Protozoa population using methyl green formaline staining method which was calculated using a microscope according to Ogimoto & Imai (1981); RUDP was calculated as % RUDP = 100 - % CP degradation (Chumpawadee et al., 2005); and Post-rumen crude protein digestibility or protein solubility (in vitro) was tested by looking at the sensitivity of the feed to the pepsin enzyme in an acidic environment (Calsamiglia and Stern 1995).

Mahogany leaves contain 10,03 mg/100 mg DM condensed tannins, and the ability of mahogany to bind protein reaches 1.217 mg BSA/mg DM (Yusiati et al. 2018). Crude protein of soybean meal reached 38,90% or equivalent to 389 mg of CF, so it was found that every 1 mg of condensed tannins in mahogany leaves could bind 336.89 mg of protein or every 1gram DM of soybean meal could be bound with condensed tannins of 3%. The provision of condensed tannins was calculated based on the ability of mahogany leaf condensed tannins to bind to pure protein, the varying

levels of soybean meal protein degradation became the basis for giving mahogany leaf condensed tannins in several different levels.

This study was aimed to evaluate the condensed tannins of mahogany leaves in binding soybean meal protein, so as to determine the optimal level of protein protection. Soybean meal has various levels of degradation in the rumen reaching 50-80% so that the level of tannin protection is varied, namely 0; 1.5; 3; and 4.5% were expected to determine the optimal concentration to protect soybean meal without disturbing rumen microbial activity and increasing post-rumen total protein availability.

RESULTS AND DISCUSSION

Average yield of rumen fermentation products of rations added with the protected soybean meal is shown in Table 2. The average concentration of acetic acid in the study ranged from 28.01±2.1 mM to 32.22±6.59 mM. Results of the analysis of variance (Appendix 3) showed that the addition of mahogany leaf extract with different levels had a very significant effect ($P<0.01$) on the production of acetic acid.

The orthogonal polynomial test (Appendix 4) shows that the addition of Mintai extract has a cubic effect on the concentration of acetic acid with the equation $Y= 39.63 – 13.19 X + 9.89 X2 – 1.51 X3$ (Figure 1) with a coefficient of determination (r^2) = 0.51 and the correlation coefficient (r) = 0.71. Based on the resulting coefficient of determination, the equation can predict the effect of adding protected soybean meal with mahogany leaf extract to acetate with an accuracy of 51%. Then 49% is influenced by factors outside the treatment.

Figure 1 shows that the addition of mahogany leaf extract initially decreased 5% acetic acid production from the control to 28.01 mM at the 1.5% level of administration of mahogany leaf extract, then increased by 26% to 38.11 mM at the level of 3% administration, but decreased again by 15% to 32.22 mM at the level of 4.5% administration. It shows that the addition of tannin extract to soybean meal affects acetic acid production, with the lowest point at T1 (0.75; 24.70) and the highest point at T2 (3.5; 39.56). A decrease at the level of 4.5% resulted in a higher acetic acid product than the level of 1.5%, and this indicates that the provision of condensed tannin levels at the level of 3-4.5% creates a stable rumen environment so that it does not interfere with the activity of rumen bacteria. Based on the DMRT test, the decrease in acetic acid production at a level of 1.5% was not significantly different from the control. The low level of mahogany leaf tannins used in this treatment caused the fermentative power of carbohydrates in the rumen to have no significant effect on acetic acid production. Nuraliah and Purnomoadi (2015) stated that low levels of tannins in soybean meal in the ration had not affected the fermentation process in the rumen. The same fiber content also caused an

Table 2. Average of Partial VFA (Acetic acid, Propionic, acid and Butyric acids), Methane production, Protozoa number, N-NH3 concentration, and SPM of Protected Soybean Meal Extract Coarse Tannin Condensed Mahogany Leaves in Ration

Parameter	Treatment				Significance
	T0	T1	T2	T3	
VFA					
Acetic acid (mM)	29,67±0,64[a]	28,01±2,1 [a]	38,11±4,11[b]	32,22±6,59 [a]	.002
Propionic acid (mM)	8,40±0,55 [a]	7,69±0,57 [a]	10,59±0,54[b]	8,77±1,61 [a]	.001
Butyric acid (mM)	3,54±0,24 [a]	3,62±0,31 [a]	4,31±0,23[b]	3,75±0,36 [a]	.002
C2:C3 ratio	3,54±0,2	3,46±0,3	3,59±0,3	3,66±0,1	.756
EKH	73,41%	73,70%	73,34%	73,19%	.670
Methane (MJ/H)	14,50±0,31 [a]	14,2±0,88 [a]	19,40±0,58[b]	16,13±2,73 [a]	.000
Protozoa ($x10^5$ sel/ml)	5,85±0,4[c]	4,14±0,5[b]	2,79±0,6 [a]	2,51 ± 0,4 [a]	.000
N-NH_3 (mg/dl)	8,09±0,37 [a]	8,64±0,21[b]	8,64±0,21[b]	9,21 ± 0,42[c]	.000
SPM (mg/100ml)	966,53±13,85 [a]	977,8±13,5 [a]	1006,53±15,5 [a]	1206,63±26,3[b]	.000
RUDP (%)	12,94±0,96 [a]	15,19±0,04[b]	16,50±0,73[C]	17,61±0,97[D]	.008
Dissolved Protein (mg/g)	35,20±1,9 [b]	35,83±2,9 [a]	42,06±2,43[b]	28,13±1,06[c]	.000

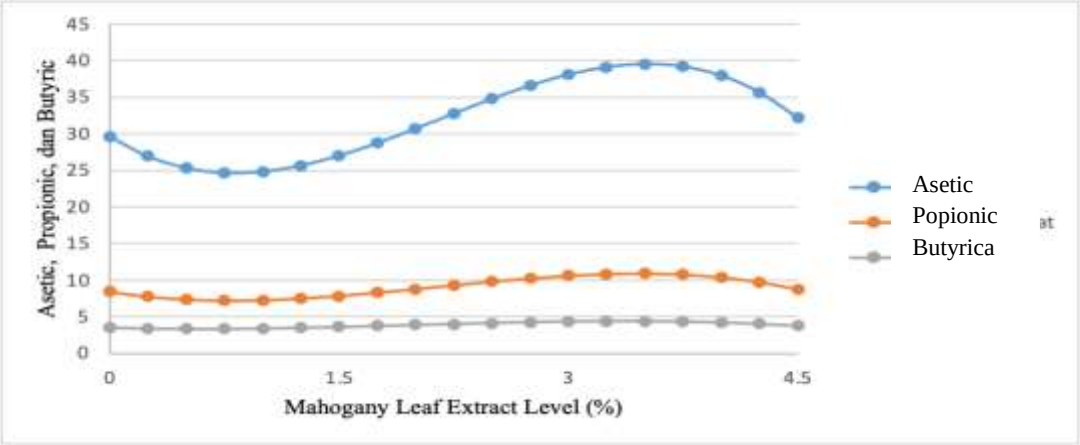

Figure 1. The Relationship between the Addition of Protected Soybean Meal from Mahogany Leaf Extract and Partial VFA (Acetic acid, Propionic acid, and Butyric acid)

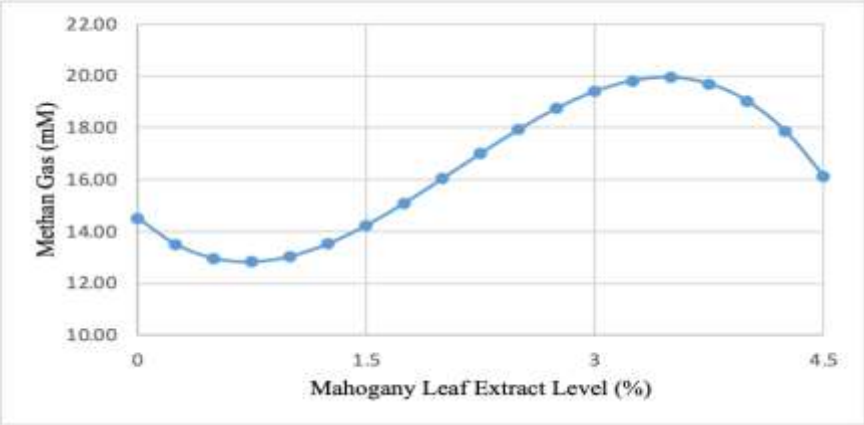

Figure 2. Effect of Addition of Protected Soybean Meal from Mahogany Leaf Extract on Methane Gas

insignificant difference in the percentages. Qori'ah et al., (2016) stated that acetic acid is the primary end product produced from fiber feed ingredients

The increase in acetic acid production at the 3% level illustrates that the mahogany leaf tannins do not interfere with the activity of cellulolytic bacteria, increasing acetic acid production. Tan et al. (2011) reported that the addition of condensed tannins in Quebracho leaves at a level of 1-2% did not interfere with the activity of cellulolytic bacteria. Several factors that affect the concentration of acetic acid include microbial population, rumen pH, and feed fermentability. The high concentration of acetic acid is related to the rumen pH, which is in a balanced state so that the rumen microbes can work well in the fermentation process. Rindawati et al. (2019) stated that mahogany plants have secondary compounds, including flavonoid compounds, alkaloids, phenolics, saponins, and tannins. Alkaloid content in the alkaline mahogany leaf extract is thought to maintain the pH balance of the rumen. The higher acetic acid production compared to the control showed that the addition of mahogany leaf extract was considered to keep the rumen pH stable.

Propionic acid is the primary glucogenic fatty acid produced in the digestion of carbohydrates by ruminants. The lowest mean value of propionic acid was 7.69 mM at the 1.5% mahogany leaf extract level, and the highest was 19.59 mM at the 3% level (Table 2). The results of the analysis of variance (Appendix 3) showed that the provision of protected soybean meal with mahogany extract had a very significant effect ($P<0.01$) on the production of propionic acid, and the orthogonal polynomial test (Appendix 4) showed that the result was cubic with the equation $Y= 8.40 – 3.26\ X + 2.51\ X2 – 0.39\ X3$ coefficient of determination (R^2) = 0.56 and correlation coefficient (R) = 0.75 (Figure 2).

Propionic acid production has the same dynamics as acetic acid production, which is an 8% decrease from control to 28.01 mM at 1.5% level of mahogany leaf extract, then an increase of 27% to 10.59 mM at 3% level but decreased again by 17% to 8.77 mM at 4.5% level (Figure 1) with the highest point of propionic acid at T1 (0.75; 7.21) and the highest point at T2 (3.5; 10.92). The decrease in propionic acid product at the level of 1.5% was lower than the decrease at the level of 4.5%. This situation shows that condensed tannins at a

level of 3-4.5% can increase the production of acetic acid, but at 1.5% it does not increase the production of propionic acid, which indicates the condition does not interfere with rumen activity. Based on the DMRT test, the decrease at the 1.5% level was not significantly different from the control. It showed that the addition of mahogany leaf extract at the 1.5% level had not shown a significant effect because the level of mahogany leaf extract given was low.

The increase in propionic acid production at the level of 3% is due to the mahogany leaves also containing secondary metabolites such as saponins that are reactive to protozoa. At that level, the concentration of saponins was able to inhibit the growth of rumen protozoa. Hidayah (2016) stated that saponins can kill or lyse protozoa by forming complex bonds with sterols to interfere with protozoa development. The decrease in the population of protozoa causes an increase in the population of rumen bacteria, especially amylolytic bacteria, resulting in an increase in the product of propionic acid in the rumen. The content of treatment rations containing high carbohydrates also caused the increased production of propionic acid produced.

The production of acetic and butyric acid at the level of 4.5% mahogany leaf extract in the ratio decreased, this was due to the mahogany leaf extract that was given containing saponins and tannins as protozoan defaunation agents, increasing rumen bacterial population. The increase in microbial activity caused the use of VFA for the microbial protein synthesis process (SPM) to increase, as indicated by the highest production of SPM at the level of 4.5% (Table 2).

The percentage of butyrate is the lowest compared to acetic and propionic acids, but butyrate also has an essential role in providing carbon chains for microbial protein synthesis. The results showed that the average product of butyric acid ranged from 3.54±0.24 (P0) to 3.75±0.36 (P3) (Table 2). The analysis of variance showed that the addition of soybean meal with mahogany leaf extract had a significant effect on butyric acid production. The results of the orthogonal polynomial test for butyrate showed that the provision of protected soybean meal with mahogany extract showed a cubic effect with the equation Y= 3.54 – 0.55 X + 0.54 X2 – 0.09 X3 with a coefficient of determination (r^2) = 0.51 and the correlation coefficient (r) = 0.71 which is shown in Figure 1.

Based on Figure 1, butyric acid production increased to the level of 3% mahogany leaf extract but decreased at 4.5% level. A decrease in the level of 4.5% resulted in higher butyric acid production compared to the control. This situation indicated that the addition of mahogany extract to a level of 4.5% did not interfere with the activity of rumen bacteria. In comparison, a decrease to a level of 4.5% indicated the utilization of butyric acid for microbial protein synthesis as indicated by the highest SPM at a level of 4.5%.

The ratio of acetic and propionic acids (C2/C3) in the protection of soybean meal with mahogany leaf extract ratio tends to increase compared to the control. However, the values are relatively the same ranging from 3.59 to 3.66 (Table 2). The provision of protected soybean meal with mahogany leaf extract had no significant effect (P>0.05) on the C2/C3 ratio because acetic acid production was in line with the production of propionic acid. The results showed that acetic acid production was higher than propionic acid, which means that it is acetogenic. That is, the product of acetic acid rose higher than propionate. Acetogenic describes that the activity of cellulolytic bacteria is more dominant than amylolytic bacteria. Table 2 shows that the addition of protected soybean meal with mahogany leaf extract had no significant effect (P>0.05) on the conversion efficiency of hexose to VFA. The percentage of EFH tends to increase at 1.5% mahogany leaf extract of 73.70%, while in control, level 3% and 4.5% produces EFH of 73.41; 73.43; 73,,19%. It shows that although the 1.5% level did not have the highest partial VFA production, it produced the best energy efficiency compared to other levels and did not interfere with rumen microbial activity as shown by the partial VFA production, which was not significantly different from the control.

Energy efficiency can be seen from methane gas production. The highest methane gas production is 3% mahogany leaf extract, and the lowest is at the 1.5% level (Table 2). Methane is a gas formed from the anaerobic fermentation process of feed ingredients in the rumen by methanogenic bacteria, reflecting the loss of feed energy (Mosoni et al. 2011). The results of the analysis of variance (appendix 3) showed that the addition of the protected soybean meal had a very significant effect (P>0.01) on the production of methane gas. Results of the orthogonal polynomial test show the effect in a cubical way with the equation Y= 14.50 – 5.08 X + 4.29 X2 – 0.68 X3 with the coefficient of determination (r^2) = 0.66 and the correlation coefficient (r) = 0.81 (Figure 2).

Figure 2 shows that the highest methane gas production is at T2(3.5;19.93), and the lowest is at T1(0.75; 18.82). The production of methane gas is directly proportional to the production of acetic acid. The production of methane gas is directly proportional to the production of acetic acid. Methane gas production decreased in addition of 1.5% mahogany leaf extract, but the test results showed that the decrease was not significant. The dynamics of methane gas production same as acetic acid production, which decreases at the level of 1.5%, then increases at the level of 3% and decreases again at the level of 4.5%. The high production of methane gas at the level of 3-4.5% indicates a less efficient use of energy shown by the low EKH compared to the level of 1.5%. Based on Figure 2. the addition of mahogany leaf extract to the

ration has not been able to reduce methane, the data is in line with the results of Jayanegara et al. (2018) which states that condensed tannins have not been able to reduce methane production. The formation of methane gas causes it to be formed by methanogenic bacteria using H2 obtained from acetic acid. Yanuartono et al. (2019) stated that methanogenic bacteria need H_2 gas to create methane gas. The formation of acetate triggers the formation of H_2 so that it will be utilized by methanogenic bacteria to be converted into CH_4. The higher the acetic acid produced, the higher the methane gas produced. Hikmawan et al. (2019) reported in their research that methane gas production was positively correlated with acetic acid production.

Methanogenesis in the digestive system of animal rumen is one of the macromolecular fermentation reaction pathways that produces CH_4 gas through CO_2 reduction with hydrogen gas catalyzed by enzymes produced by methane bacteria. Methanogenic bacteria are often found living attached to the surface of the rumen protozoa to keep getting a constant supply of hydrogen. Still, not all methanogenic bacteria attach to the protozoa. Anggraeny et al. (2021) stated that methanogenic bacteria associated with ciliated protozoa are responsible for the process of methanogenesis in rumen fluid between 9 - 25%.

Results of the analysis of variance (attachment 3) showed that the addition of protected soybean meal with mahogany leaf extract had a very significant effect (P>0.01), and the orthogonal polynomial test showed the impact in a quadratic manner with the equation Y= 5.88 – 1.47 X + 0.16 X2 with a coefficient of determination (r^2) = 0.85 and a correlation coefficient (r) = 0.92 (Figure 3). Figure 3 shows that the protozoa population decreased with the addition of mahogany leaf extract, which was given with the highest point at T0 (0;585) and the lowest point at T3 (4.5;250). The decrease in protozoa in each treatment was significant. This illustrates the presence of protozoa growth disorders due to the addition of protected soybean meal from mahogany leaf extract. Rindawati et al. (2019) stated that mahogany plants have secondary compounds, including flavonoid compounds, alkaloids, phenolics, saponins, and tannins.

Based on the calculation of the total phenol in the tested mahogany leaf extract, 34.71 mg/100 mg DM, the high phenol content disrupted the protozoa population. Since tannins are phenolic compounds that are reactive with cell walls of microorganisms and extracellular enzymes produced by microorganisms. Yulistiani et al. (2011) stated that tannins could suppress the number of protozoa that are predators of bacteria and cause an increase in rumen degradation. The content of saponins in mahogany leaves is also thought to cause a decrease in the population of protozoa. Wahyuni et al. (2014) stated that saponins could lyse protozoa by forming complex bonds with sterols found on the surface of the protozoan membrane.

Protozoa have an essential role in maintaining rumen pH. Protozoa can quickly utilize fermentable carbohydrates for their needs and provide the advantage of slowing down the conversion of fermentable carbohydrates to lactic acid by rumen bacteria to control pH. The decrease in protozoa was marked by an increase in the product of propionic acid when compared to the control, a decrease usually followed by an increase in propionic acid in the production of acetic acid. Still, with the addition of protected soybean meal, the increase in propionic acid was followed by an increase in acetic acid. This shows that the addition of mahogany leaf extract was thought to maintain a stable rumen pH. Alkaloid content in the alkaline mahogany leaf extract is believed to maintain the pH balance of the rumen. The high partial of VFA concentration is related to the rumen pH, which is balanced so that the rumen microbes can work well in the fermentation process.

The fermentation process was also shown the increase in the concentration of N-NH_3 along with the addition of the protection level of soybean cake with mahogany leaf tannins. The concentration of N-NH_3 is one indicator to determine the fermentability of feed protein, microbial activity, and rumen microbial population. The concentration of N-NH_3 describes the amount of feed protein that can be fermented in the rumen, and its value is strongly influenced by the ability of rumen microbes to degrade feed protein and whether or not feed protein is easily degraded (Susilo et al., 2019). Analysis of variance showed that the addition of the protected soybean meal in different levels had a very significant effect (P<0.01) on increasing the concentration of N-NH_3. The orthogonal polynomials test shows the influence linearly with the equation Y = 14.13 + 0.22 X with a coefficient of determination (r^2) = 0.59 and a correlation coefficient (r) = 0.77 (Figure 4).

Figure 4 shows increase concentration of N-NH_3 along with the addition of the concentration of mahogany leaf extract given with the highest production at T3 (4.5; 15.21) and the lowest at T0 (0; 14.09). The content of the treatment ration used had a relatively high crude protein content, namely 13.49%, and the concentrate composition containing coconut meal with a high level of degradability caused a high concentration of N-NH_3 produced. A decrease also followed the increase in the concentration of N-NH_3 in the protozoan population. The decreased protozoa population caused the population of rumen bacteria to increase, including proteolytic bacteria, as indicated by the increased rumen protein fermentation process. Herdian et al. (2014) stated that a decrease in the protozoa population would increase the availability of ammonia in the rumen. Based on the DMRT test, the concentration increase at the 3% level was not

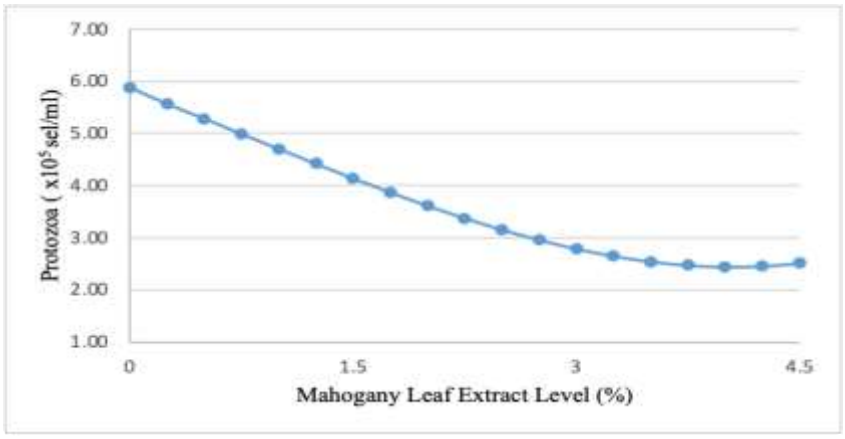

Figure 3. The Relationship between the Addition of Protected Soybean Meal from Mahogany Leaf Extract on the Protozoa Population

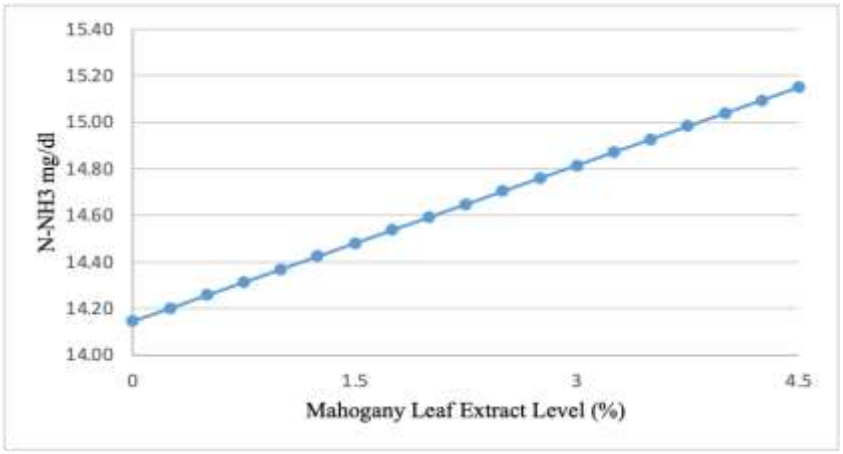

Figure 4. The relationship between the addition of protected soybean meal from mahogany leaf extract to N-NH3

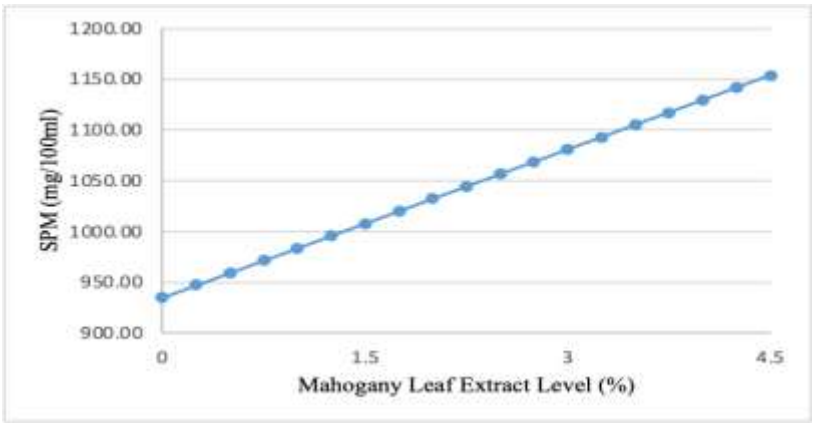

Figure 5. The Relationship between the Addition of Protected Soybean Meal from Mahogany Leaf Extract on SPM

significantly different from the 1.5% level. It showed that the 1.5% level effectively increased the N-NH_3 concentration compared to the control

Concentration of N-NH_3 is related to microbial protein synthesis because microbes in the rumen utilize N-NH_3 as the primary nitrogen source for microbial protein synthesis. Microbial protein synthesis will be optimal if there is a synchronization of the release time between the nitrogen source and the carbon skeleton in the rumen. The high and low concentration of microbial protein synthesis in each treatment was related to the availability of VFA and ammonia, which was influenced by the amount of mahogany leaf extract that protected soybean meal in each treatment. Results of

analysis of variance showed that the addition of mahogany leaf extract had a very significant effect ($P<0.01$) on microbial protein synthesis, the results of the orthogonal polynomial test showed a linear influence with the equation $Y= 935 + 48.6 X$ with a coefficient of determination (r^2) = 0.46 and the correlation coefficient (r) = 0.68 (Figure 5).

Figure 5 shows that microbial protein synthesis (SPM) increased with increasing levels of mahogany leaf extract (Table 2). This increase was due to the addition of Mahogany leaf extract that make the rumen environment stable so that the activity of rumen bacteria increased. The decreased protozoa population causes an increase in bacterial rumen activity so that the feed fermentation process becomes optimal and can provide a nitrogen source and energy source for the microbial protein synthesis process. It is indicated by the availability of carbohydrate fermentation products (partial VFA) and protein ($N-NH_3$). Qori'ah et al. (2016) stated that microbial protein synthesis could run optimally if there is a supply of nitrogen and organic matter in the rumen.

The composition of the treatment ratio had a high protein content of 13.4%, so that it was able to provide ammonia for microbial protein synthesis. Protein in the balance indicates the availability of N for rumen microbes which help microbial growth and production of microbial synthesis in digesting nutrients. The increase in microbial protein synthesis followed by the rise in the concentration of $N-NH_3$ (Table 2) illustrates that the feed ingredients in the treatment have a high level of degradability so that the available concentration of $N-NH_3$ is high even though it has been used for microbial protein synthesis. Suwandyastuti (2013) stated that microbial protein synthesis is influenced by sources of nitrogen intake, whether or not protein is quickly degraded, and the availability of N and energy sources.

The mean Rumen Undegradable Protein (RUDP) was 12.94±0.94 (T0), 15.19±0.04 (T1), 16.50±0.73 (T2), and 17.61±0, 97 (T3) listed in Table 2. RUDP is a feed protein that is not degraded in the rumen, so it can directly undergo enzymatic digestion in the abomasum and intestine. The results of the analysis of variance showed that the addition of protected soybean meal with mahogany leaf extract in the ratio had a very significant effect ($P<0.01$) on RUDP, and the orthogonal polynomial test showed a linear regression effect with the equation $Y= 13.26 + 1.02 X$ with a coefficient of determination (r^2) = 0.83 and correlation coefficient (r) = 0.91 (Figure 6).

Figure 6 shows an increase in RUDP along with the addition of mahogany leaf extract, which was given with the lowest value at the control and the highest at the 4.5% level. This situation shows that increasing level of mahogany leaf extract given will increase the total protein that is not degraded in the rumen. (Yisehak et al. 2016) stated that the size of the protein that escapes degradation in the rumen is influenced by several factors, namely the solubility of the protein, the more excellent the solubility of a protein, the greater the degradation process.

The increase followed the increased content of RUDP in the rumen in the range of ammonia, and this indicates that the degradation of feed protein in the rumen is high. This illustrates that the tannin content in mahogany leaf extract can provide optimal conditions for rumen microbes to degrade feed so that the ammonia produced is high. Another condition that causes high ammonia production is that the treatment ratio contains high protein. The protein is degraded to ammonia and released quickly. The ammonia should be absorbed through the rumen, but there was no absorption by rumen because the study was carried out in vitro. The increasing percentage of RUDP indicates that condensed tannins in mahogany leaves can form complex bonds between condensed tannins and proteins that are resistant to rumen microbial degradation. The condensed tannin complex and protein bonds will be released after reaching the abomasum at a pH of less than 4 to increase the total post-rumen protein. This is because the complex bonds of condensed tannins and proteins will decompose at an acidic pH, and proteins can be digested in the abomasum. Yusiati et al. (2018) stated that condensed tannins could increase protein that escapes rumen degradation due to its ability to bind protein at neutral pH conditions. Still, at acidic pH conditions such as in the abomasum, protein will be released to digested in the abomasum and intestine.

The effectiveness of the protected protein can be seen from the dissolved protein in the post-rumen or abomasum. The study showed that the lowest soluble protein value was in control, and the highest was at the 3% level (Table 2). The analysis of variance showed that the addition of protected soybean meal with mahogany leaf extract showed very significant results ($P<0.01$) for soluble protein. The results of the orthogonal polynomial test show a cubic regression

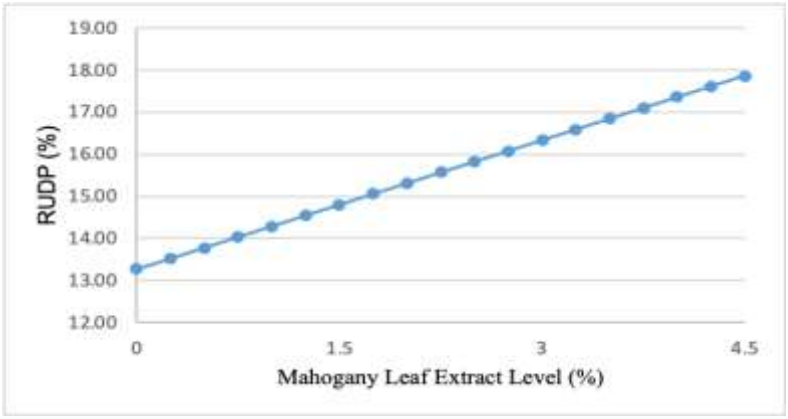

Figure 6. The relationship between the addition of protected soybean meal with mahogany leaf extract to RUDP

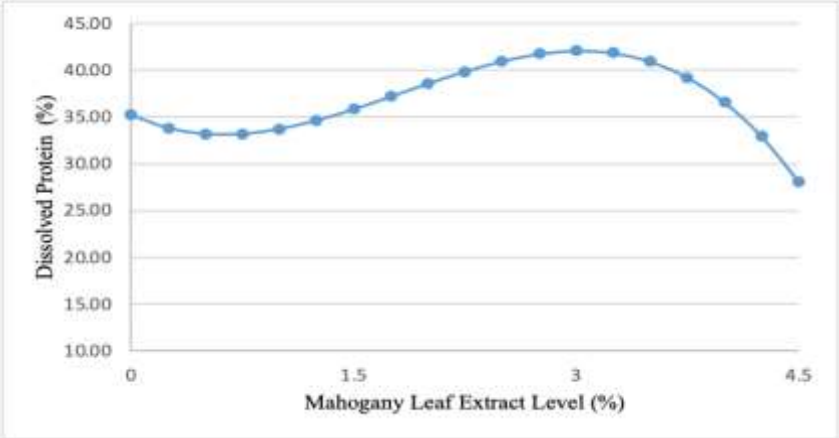

Figure 7. The Relationship between the Addition of Protected Soybean Meal from Mahogany Leaf Extract on Dissolved Protein

response with the equation Y = 35.20 - 7.16 X + 6.96 X2 - 1.27 X3 with a coefficient of determination (r^2) = 0.85 and a correlation coefficient (r) = 0, 92 (Figure 7).

Figure 7 shows that soluble protein experienced an insignificant decrease in the addition of 1.5% soybean meal extract, then increased to a level of 3% and decreased significantly again at a level of 4.5%. The decrease in soluble protein at 4.5% level of mahogany leaf extract resulted in lower soluble protein than the control with the most insufficient production at T3 (4.5; 28.13) and the highest at T2 (3; 42.06). This situation shows that condensed tannins in mahogany leaves can form concentrated tannin bonds with feed protein, resulting in increased post-rumen soluble protein compared to controls. Based on the DMRT test, the total dissolved protein at the level of 1.5% was not significantly different from the control, it showed that the level of 1.5% had not had a significant effect, while at the level of 3%, it showed the best results. The increase in soluble protein showed that the administration of mahogany leaf extract at a level of 3% was able to increase total protein/amino acids, especially in the post-rumen without disturbing rumen microbial activity, as indicated by higher microbial protein synthesis than control and resulted in the best energy efficiency among other treatments. Rimbawanto et al. (2017) stated that the protection of soybean meal using condensed tannins in lamtoro leaves in vitro did not inhibit microbial protein synthesis in the rumen and increased the supply of postrumen microbial protein.

Soybean meal protection aims to increase the total soluble protein without disturbing the activity of rumen bacteria. At the level of 4.5%, soluble protein decreased. This condition indicates that at the level of 4.5%, there is overprotection. Overprotection is when the bond between protein and condensed tannins is too strong so that the solubility of pepsin-HCl becomes low. Giving at the level of 4.5% was not effective, seen from the rumen microbial activity indicated by a decrease in carbohydrate fermentation products (partial VFA). It showed that the condensed tannin level of 4.5% had begun to interfere with the activity of rumen bacteria.

High content of condensed tannins in mahogany leaves can increase the effectiveness of protein protection in soybean meals. In addition, when viewed from the availability, mahogany leaves are underutilized because mahogany is developed for

industrial forest plantations, mahogany wood is mainly used as construction material. The use of mahogany leaves as a protein feed protection agent can increase the usefulness of this plant. The results of this study can be easily carried out by farmers because the addition of mahogany leaves as a source of condensed tannins does not need to go through extraction. In ruminants, the protection process of soybean meal can be done naturally through mechanical digestion in the mouth.
Noted: all the graphics are actually not necessary to present, since all information in the graphics have already been presented in Table 2.

CONCLUSION

The addition of protected soybean meal by mahogany leaf extract at a level of 1.5% produced the best-rumen fermentation products. It was able to optimally increase RUDP and post-rumen soluble protein.

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Characteristics of Libido and Testosterone Concentration of Polled and Horned Bali Bulls after GnRH Injection

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ABSTRAK

Hasbi H, Sonjaya H, Baco S, Amalia R, Gustina S. 2021. Karakteristik libido dan konsentrasi hormon testosteron sapi bali jantan *polled* dan bertanduk setelah penyuntikan GnRH. JITV 26(3): 108-114. DOI: http://dx.doi.org/10.14334/jitv.v23i4.2851.

Sapi Bali merupakan salah satu plasma nutfah asli Indonesia yang dalam pengembangannya ditemukan jenis tidak bertanduk atau *polled*. *Polled* adalah sapi Bali yang tanduknya tidak tumbuh secara alami, namun memiliki karakteristik yang sama dengan yang bertanduk. Akan tetapi ada indikasi bahwa *polled* memiliki libido yang lebih rendah. Penelitian ini bertujuan untuk melihat bagaimana karakteristik libido dan konsentrasi testosteron setelah penyuntikan *gonadotrophin releasing hormone* (GnRH). Pada penelitian ini digunakan masing-masing 7 ekor pejantan sapi Bali *polled* dan bertanduk berumur 3.5-6 tahun. Karakteristik libido diamati dengan mencatat waktu pertama pejantan mencumbu *teaser* sampai terjadinya ejakulasi sedangkan konsentrasi testosteron diukur menggunakan plasma darah yang diambil pada hari ke-0 atau sebelum penyuntikan, hari ke-7, dan hari ke-14 setelah penyuntikan GnRH. Analisa testosteron menggunakan metode *enzyme linked immunosorbent assay* (ELISA). Hasil penelitian menunjukkan bahwa libido sapi *polled* tidak berbeda (P>0.05) dibandingkan bertanduk baik pada hari ke-0, ke-7 dan ke-14 setelah penyuntikan GnRH. Namun, pada sapi *polled* hari ke-7 setelah penyuntikan GnRH nyata lebih rendah dibandingkan hari ke-0 dan ke-14, tetapi pada hari ke-0 tidak berbeda dengan hari ke-14. Konsentrasi testosteron pada hari ke-7 setelah penyuntikan GnRH tidak berbeda (P>0.05) dibandingkan hari ke-0 baik pada pejantan *polled* maupun bertanduk, tetapi pada hari ke-14 setelah penyuntikan nyata lebih tinggi (P<0.05) pada pejantan bertanduk dibandingkan *polled*. Kesimpulan, pejantan sapi Bali *polled* dan bertanduk memiliki libido tinggi dengan skor +1, namun pada hari ke-7 setelah penyuntikan GnRH libido pejantan *polled* lebih rendah dibandingkan bertanduk. Konsentrasi testosteron pejantan *polled* dan bertanduk pada hari ke-7 setelah penyuntikan GnRH tidak berbeda dibandingkan dengan hari ke-0, sedangkan ke-14 lebih tinggi pada pejantan bertanduk.

Kata Kunci: GnRH, Bertanduk, Libido, Sapi Bali *Polled*, Testosteron, GnRH,

ABSTRACT

Hasbi H, Sonjaya H, Baco S, Amalia R, Gustina S. 2021. Characteristics of libido and testosterone concentration of polled and horned Bali Bulls after GnRH injection. JITV 26(3): 108-114. DOI: http://dx.doi.org/10.14334/jitv.v23i4.2851.

Bali cattle are one of the original Indonesian germplasm, which in its development were found to be hornless or polled. Polled are Balinese cattle whose horns do not grow naturally, but have the same characteristics as those with horns. However, there are indications that polled have a lower libido. The purpose of this study was to determine the characteristics of libido and testosterone concentration after gonadotrophin releasing hormone (GnRH) injection. In this study each of 7 male polled and horned Bali cattle aged 3.5-6 years were used. Libido characteristics were observed by recording the first time the male fondled the teaser until ejaculation, while the testosterone concentration was measured using blood plasma taken on day 0 or before injection, day 7th, and day 14th after GnRH injection. Testosterone analysis used enzyme linked immunosorbent assay (ELISA) method. The results showed that the libido of polled was not different (P>0.05) compared to that of the horned on the 0, 7th and 14th days after GnRH injection. However, in polled on the 7th day after GnRH injection, it was significantly lower than on day 0 and 14, but on day 0 it was not different with day 14. Testosterone concentration on day 7 after injection of GnRH was not different (P>0.05) compared to day 0 both in polled and horned bulls, but on day 14 after injection was significantly higher (P<0.05) in horned than polled. In conclusion, polled and horned Bali bulls had high libido with a score of +1, but on the 7th day after GnRH injection, polled had lower libido than horns. The testosterone concentrations of polled and horned on day 7 after GnRH injection were not different compared to day 0, while the 14th day was higher in horned bull.

Key Words: GnRH, Horned, Libido, Polled Bali Bulls, Testosterone

INTRODUCTION

Bali cattle is one of the Indonesia original germplasms which has great potential to produce breeds with high quality, and to supply the needs of animal protein. Bali cattle have several advantages including good adaptability to an environment and low feed quality, compact body shape, high carcass percentage up to 52-58%, low fat meat, so that making it suitable to be developed as beef cattle. In addition, Bali cattle also have a high fertility rate reaching 83%, without being affected by feed quality (Utomo et al. 2017). During development, in province of South Sulawesi, Bali cattle without horns were found that is known as polled.

Polled Bali cattle have horns but do not grow naturally. Although without horns, polled generally have the same characteristics as horned Bali cattle. Comparison of measurements of body dimensions and production characteristics between polled and horned Bali cattle did not show any difference. The polled character of males does not have a negative effect on the growth. It can be seen in the weight of polled Bali cattle which is relatively same as the weight of horned at the same age (Zulkharnaim et al. 2017). However, in reality in the field, there are indications that male polled Bali cattle have a lower libido than horned (Hasbi et al. 2021), which has an impact on the difficulty of semen collection. This is thought to be related to hormonal imbalances, especially testosterone. Therefore, a strategy is needed to increase libido in male polled Bali cattle. One strategy that can be done is to perform gonadotrophin releasing hormone (GnRH) induction. Based on report by Monaco et al. (2015) that injection of GnRH at a dose of 100 µg might enhance temporarily testosterone levels in camels, and reach a peak 2 to 3 hours after administration.

Gonadotrophin releasing hormone (GnRH) is a hormone that is often used to increase reproductive capacity in male and female animals (Franssen et al. 2021). GnRH is secreted to stimulate the release of gonadotrophins and testosterone which are important in the process of spermatogenesis, and also sexual behavior (Kowalczyk et al. 2021). GnRH is a hormone originating from the hypothalamus that stimulates the secretion of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) from the Anterior Pituitary (Khazeni & Varamini 2018). FSH and LH are the main hormones that control germ cell development (Ramaswamy & Weinbauer 2014; Rougier et al. 2019). FSH plays a role in controlling the development of germ cells, and together with LH also plays a role in stimulating the expression of androgen receptors and controlling proliferation and maintaining Sertoli cell function (Aksglaede et al. 2011: Kuiri-Hänninen et al. 2011). Furthermore Kuiri-Hänninen et al. (2011) explained that FSH is a hormone that control the proliferation of Sertoli cells which has an impact on the increase in testicular volume. Meanwhile, LH plays a role in controlling germ cell development and stimulating Leydig cells to produce testosterone (Hasbi & Gustina 2018).

The testosterone plays a role in the initiation, maintenance and restoration of the process of spermatogenesis. Testosterone is synthesized by testicular Leydig cells in response to LH stimulation and plays an important role in maintaining the process of spermatogenesis (Smith & Walker 2014). Level of testosterone is not only responsible for the condition of bull libido, but also for the volume of ejaculation (Monaco et al. 2015). Decreased testosterone concentrations can cause decreased libido, spermatogenesis, and seminiferous tubule diameter. When testosterone levels are high or low (below the normal threshold) it will result in negative feedback to the hypothalamus which results in disrupted spermatogenesis processes. Otherwise, if testosterone levels are normal, it will stimulate the testicles to carry out the process of spermatogenesis (Keefe et al. 2021).

MATERIALS DAN METHODS

Materials

The materials used were 7 bulls polled and 7 horned Bali cattle aged 3.5 to 6 years, 1 Bali anestrus cow as teaser, GnRH (Fertagyl™, Intervet International B.V.,Boxmeer, The Netherlands), and DRG EIA 1559 Kit (DRG Instruments GmbH, Germany).

Methods

GnRH Treatment

Bulls were injected intramuscularly with a single dose of GnRH at a level of 500 µg (Fertagyl™, Intervet International B.V., Boxmeer, The Netherlands).

Characteristics of Libido

Observation of libido characteristics was assessed from a score of -2 to +2. A score of -2 is indicated by the male not responding to climbing, a score of -1 trying to climb but slipping, a score of 0 starting to actively climb, a score of +1 is reaction of attraction to mating and actively climbing by doing one ejaculation, and a score of +2 is reaction of attraction to mating and actively climb by doing more than one ejaculation (Modification of Menegassi et al. 2011; Perumal et al. 2020).

Blood Sampling

These samples were obtained from the jugular vein by damming it in the distal 1/3 of the neck. After the blood is blocked, the area is wiped with a cotton swab moistened with alcohol, and then a sterile needle is inserted at an angle of 30° upward into the vein with the needle hole facing upwards. The blood collected using a vacuum tube containing ±3 ml ethylene diamine tetraacetic acid (EDTA) 5%. Technique of blood plasma separation is done by centrifuging at 2000 rpm for 10 minutes until it separates into 3 layers, plasma, buffy coat, and blood cells. The results of the centrifugation (blood plasma) were collected in a microtube and stored at -20 °C (until hormone analysis was performed). Parameters were measured by the enzyme linked immunosorbent assay (ELISA) method using the DRG EIA 1559 Kit (DRG Instruments GmbH, Germany). Blood samples were taken 3 times, as shown in Figure 1.

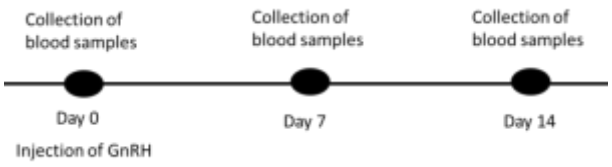

Figure 1. Time of Blood Samples Collection

Data Analysis

The obtained data were analyzed by a comparative test, namely statistical analysis of the t-test (Independent sample t-test), to compare the sample of polled with horned Bali cattle.

RESULTS AND DISCUSSION

Libido Characteristics of Polled and Horned Bali Cattle

Libido is a male's desire to mate which is shown in the form of sexual behavior caused by stimulation (Sam et al. 2017). Libido is one aspect of reproductive function that needs attention. A male's fertility rate and quality of semen will decrease if it is not followed by adequate libido (Kumar et al. 2011). Libido is an expression of engodenous control, especially related to LH or testosterone levels in the blood, as well as stimulation of exogenous through physiological processes and also reproduction experience (Mondal et al. 2019). Libido of a male can be assessed ranging from no response to climbing the teaser to actively climbing by making more than one ejaculation (Menegassi et al. 2011). Meanwhile, according to Waheeb et al. (2018) that the libido index is measured during the semen collection process based on the level of sexual interest for 10 minutes, while the reaction time related time required for bull recognition of the teaser and the completion of copulation in the artificial vagina. The libido characteristics of polled and horned Bali cattle were presented in Figure 2.

Characteristics of libido indicated by the time it took a male to climb the teaser and ejaculate normally. The results of this study (Figure 2) showed that the libido characteristic in polled Bali cattle were not significantly different ($P>0.05$) compared to horned on day 0 or before the GnRH injection, day 7 and 14 after the injection. This finding showed that libido was not affected by the presence or absence of horns in a male. However, on days 0, 7, and 14 showed a tendency to have a higher libido in horned Bali bull. Previous research explained that the libido of polled Bali bull was significantly lower than that of horned (Hasbi et al. 2021). Libido of a male can be influenced by several factors, which are breeds and genetics, age, social relations between males, sexual experience, and climate or environment, stress (Petherick 2005).

Libido is the reaction of a male to climb a female or teaser after being stimulated. Libido can be measured by looking at the time required by a male from being brought near to a female or teaser until false mounting (Salim 2017; Gibson et al. 2020). The results of this study indicated that polled and horned Bali bulls have a high libido with a score of +1, which is the reaction of interest in mating and actively climbing by doing one ejaculation, although it takes a long time 11.18±2.02 to 18.42±5.57 minutes starting from approaching the teaser until the first ejaculation occurred in polled bull and 8.15±3.08 to 12.33±3.39 minutes in horned. Singh et al. (2009) and Kumar et al. (2011)measure level of bulls libido at the semen collection time based on the basis of reaction time, and categorize good libido with reaction time less than 15 min. Meanwhile, Monaco et al. (2015) reported that the average service time required for dromedary camels is 8.2 min.

The results of this study showed that the libido of polled Bali cattle on the 7th day after GnRH injection was significantly lower ($P<0.05$) compared to day 0 or before injection and day 14 after injection, while on day 0 it was not different from day 14 (Figure 2). These results indicated that GnRH injection after day 7 affected libido in male polled Bali cattle, whereas horned Bali cattle had no effect. It indicates that the polled Bali males in this study were more susceptible to stress caused by pain that occurred after GnRH injection treatment. Lieberman et al. (2013) reported that pain sends signals to the hypothalamus. The hypothalamus secretes GnRH which stimulates the anterior pituitary gland to secrete LH, then LH stimulates Leydig cells and produces testosterone.

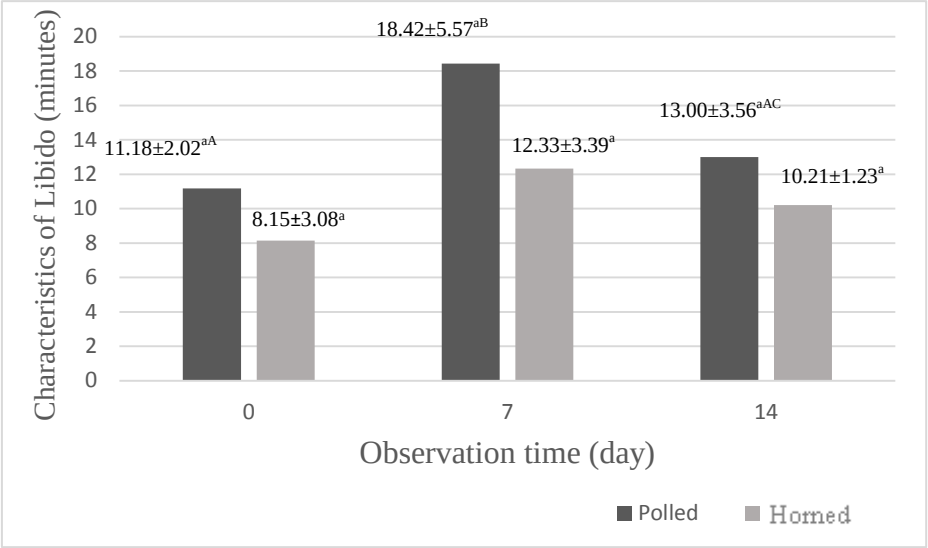

Figure 2. Libido Characteristics of Polled and Horned Bali Cattle after Injection of Gonadotropin Releasing Hormone (GnRH)

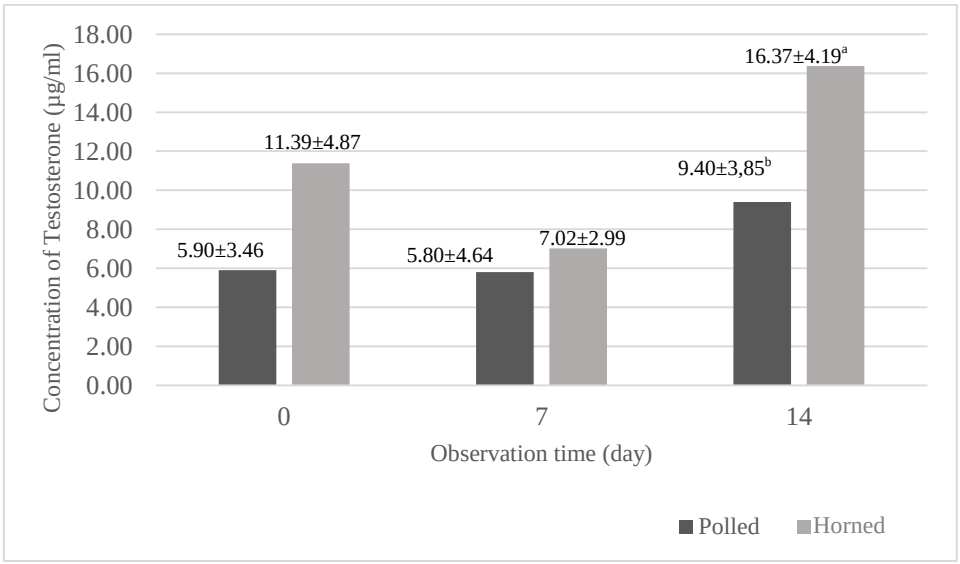

Figure 3. Concentration of the Testosterone in Polled and Horned Bali Cattle after Injection of Gonadotropin Releasing Hormone (GnRH)

Testosterone provides negative feedback to the hypothalamus to inhibit GnRH thereby limiting the rate of testosterone formation. Testosterone is a hormone involved with sexual desire (libido) which provides a sexual stimulus to encourage sexual activity, especially in male animals (Monaco et al. 2015).

Concentration of the Testosterone in Polled and Horned Bali Cattle

Gonadotropin releasing hormone (GnRH) is a hypothalamic neuropeptide that controls the reproductive endocrine system and is thought to be the end product of the central nervous system activator of fertility in all mammals. GnRH initiates the pituitary gland to release the gonadotropins leading to the production of gonadal sex steroids (Salehi et al. 2019).

Injection of GnRH can improve sexual behavior and optimize the quality of spermatozoa (Monaco et al. 2015; Wanlu et al. 2021) through the hormonal cycle. Testosterone is the main androgen in the circulatory system in male animals and is a hormone that affects the level of libido (sexual desire) and provides stimulation for male sexual activity (Rastrelli et al. 2018). The concentration of the hormone testosterone in polled and horned Bali cattle were presented in Figure 3.

Results of the diversity analysis (Figure 3) showed that on the 7th day after GnRH injection, there was no significant difference (P>0.05) in the concentration of the testosterone compared to day 0 or before injection in both polled and horned Bali bulls. Whereas on day e 14th after injection, the testosterone concentration of horned Bali bulls was significantly higher (P<0.05) compared to polled bulls. This result indicated that polled and horned Bali cattle on day 7 after GnRH injection gave the same response, while on day 14 horned Bali cattle gave a high response to GnRH injection. However, the results of this study showed the same pattern between polled and horned Bali bulls, the concentration of testosterone decreased on the 7th day and increased on the 14th day after injection. The concentration of testosterone in polled Bali bulls in this study was 5.90±3.46 - 9.40±3.85 ng/ml and in horned bulls was 7.02±2.99 - 16.37±4.19 ng/ml. The concentration of testosterone in polled is not much different from previous research conducted by Barret et al. (2012) that testosterone concentration in adult cattle ranges from 3-10 ng/ml, while in horned Bali bulls is higher. In dromedary camels, the basal testosterone level is about 3.0 ng/ml (Monaco et al. 2015). In zebu males, the average basal concentration of testosterone is 3.27 ng/ml which are classified as low libido, while normal libido can reach 20 ng/ml (Mondal et al. 2019), in bulls 9.4 ng/ml (Kowalczyk et al. 2021), in holstein bulls 7.7 ng/ml (Devkota et al. 2011).

Hormone concentrations are closely related to external factors that can trigger an increase in testosterone concentrations such as the environment. High or low concentration of hormones are caused by sensory stimuli such as light (eyes), sound (ears), smell (nose), physical stimulation (hot and cold), livestock activity, stress (Duszka et al. 2020; Kırgezen et al. 2021), also testosterone increases in conditions of desire of mating (Rastrelli et al. 2018).

The decrease in testosterone concentration on the 7th day after GnRH injection in this study was thought to be caused by pain and stress due to the injection, which could last a few days after the injection. This condition would improve on the 14th day after the injection so that the testosterone concentration increased again. Lieberman et al. (2013) reported that pain sends signals to the hypothalamus. The hypothalamus secretes GnRH which stimulates the anterior pituitary gland to secrete LH. LH stimulates Leydig cells and produces the hormone testosterone, which provides negative feedback to the hypothalamus to inhibit GnRH thereby limiting the rate of testosterone formation. Furthermore, Rastrelli et al. (2018) reported that fluctuations in testosterone levels indicate that testes functionate normally and has a certain pattern that occurs consistently, and is also an indication of testicular activity and the formation of spermatogenesis.

The testosterone hormone in males is closely related to libido in livestock. The hypothalamus will produce GnRH and parathyroid hormone (PTH) which function to regulate testosterone production and regulate calcium and phosphorus content in the bone including horn. Decreased testosterone will affect parathyroid hormone. Parathyroid hormone, a hormone that has function to regulate calcium and phosphorus content (Levine et al. 2014) while testosterone affects libido levels (Rastrelli et al. 2018).

The results of this study indicated that polled character in Bali cattle do not affect libido, meaning that during the breeding season LH continues to stimulate Leydig cells to produce testosterone even though in small amounts. Apart from internal factors, this condition is also influenced by external factors such as body size and nutrition. Furthermore, Menegassi et al. (2011) reported that the sexual behavior of bulls is influenced by several factors including genetics, environment, nutrition, hormones, sensory acuity, age and experience.

CONCLUSION

Polled and horned Bali cattle bulls have high libido with a score of +1, which is the reaction of interest in mating and actively climbing the teaser by doing one ejaculation. However, on the day 7 after GnRH injection, the libido of polled Bali cattle is lower than horned. The concentration of testosterone in polled and horned Bali cattle on the day 7 after GnRH injection was not different compared to day 0, while it was higher in horned Bali cattle on the day 14 after injection.

ACKNOWLEDGEMENT

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Amantadine resistance of clade 2.3.2 H5N1 Avian Influenza Virus from Waterfowl in Indonesia

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ABSTRAK

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Tujuan penelitian ini adalah mengetahui sensitifitas virus avian influenza subtipe H5N1 clade 2.3.2 asal Indonesia terhadap antiviral amantadin melalui uji molekuler dan *in vitro*. Penelitian dilakukan dengan isolasi dan identifikasi virus, analisis nukleotida, uji sensitifitas amantadin di sel MDCK. Hasil penelitian menunjukkan bahwa titer EID_{50} isolat clade 2.3.2 subtipe H5N1 adalah rata rata $>10^8$ EID_{50}/ml. Analisis pohon filogenetik gen M2 dari keenam virus avian influenza subtipe H5N1 clade 2.3.2 asal Indonesia mempunyai kedekatan dengan virus avian influenza subtipe H5N1 clade 2.3.2 asal Vietnam, Cina, Hongkong. Substitusi protein M2 (V27I) diidentifikasi pada enam isolat subtipe H5N1 clade 2.3.2 asal Indonesia. Avian influenza subtipe H5N1 clade 2.3.2 dapat menyebabkan pembentukan CPE dan uji HA bereaksi positif pada sel MDCK dengan konsentrasi amantadine hydrochloride yang tidak toksik. Hasil analisis genetik gen M2 terhadap resistensi amantadin berkorelasi dengan hasil uji HA dan pembentukan CPE pada sel MDCK sehingga dapat disimpulkan bahwa resistensi amantadin telah diidentifikasi pada virus avian influenza subtipe H5N1 clade 2.3.2 yang diisolasi dari Indonesia

Kata Kunci : Avian influenza subtipe H5N1 clade 2.3.2, Unggas air, Resistensi amantadin, Indonesia

ABSTRACT

Hewajuli DA, Dharmayanti NLP, Wibawan IWT. 2021. Amantadine resistance of clade 2.3.2 H5N1 Avian Influenza virus from waterfowl in Indonesia. JITV 26(3): 115-123. DOI: http://dx.doi.org/10.14334/jitv.v26i3.2764.

The objective of this research was to know the sensitivity of H5N1 clade 2.3.2 AIV from Indonesia to antiviral drug (amantadine) through molecular and in vitro tests. The study was conducted by virus isolation and identification, nucleotide analysis, and susceptibility to the amantadine hydrocloride in MDCK cells. The study result represented that the mean EID_{50} isolates of H5N1 clade 2.3.2 AIV was determined of $>10^8$ EID_{50}/ml. The analysis of phylogenetic tree of M2 gene from six viruses of H5N1 clade 2.3.2 AIV from Indonesia were closed with H5N1 clade 2.3.2 AIV avian influenza viruses from Vietnam, China, Hongkong. The substitution of M2 protein (V27I) was identified in six isolates H5N1 clade 2.3.2 AIV isolated from Indonesia. Avian influenza of clade 2.3.2 H5N1 subtype from Indonesia produced the formation of CPE and the positive HA reaction with non-toxic concentration of amantadine hydrochloride in MDCK cells. The result of genetic analysis of M2 gene for amantadine resistance was related with the results of HA test and the formation of CPE in MDCK cells. These results established that amantadine resistance have been identified in H5N1 clade 2.3.2 AIV viruses isolated from Indonesia

Key Words: Avian Influenza of clade 2.3.2, Waterfowl, Amantadine resistance, Indonesia

INTRODUCTION

Highly pathogenic avian influenza (HPAI) virus has been causing significant outbreaks in poultry in Indonesia since 2003 (Wibawa et al. 2014; Dharmayanti et al. 2014). The avian influenza viruses were grouped into 11 H5 clades actively circulating up to 2012 (WHO/OIE/FAO H5N1 Evolution Working Group 2014). The 2.1 clade of H5N1 viruses were the primary clades of H5N1 viruses circulated in Indonesia throughout among 2003 - 2011 (Dharmayanti et al. 2013). The high mortality of ducks caused influenza A (H5N1) viruses of clade 2.3.2.1 were reported in Java Island, Indonesia, at September 2012 (Dharmayanti et al. 2014). To date, transmission of clade 2.1 H5N1 subtype to humans have been discovered in Indonesia while infection of clade 2.3.2 H5N1 subtype in humans have not been officially reported in Indonesia.

The human case of H5N1 clade 2.3.2 AIV was identified in Bangladesh and Canada (East 2018; Smith & Donish 2015). In Indonesia, the H5N1 clade 2.3.2 AIV have been not detected in human. However, this is

possible that the H5N1 clade 2.3.2 can be transmitted to human in Indonesia. Therefore, the anticipation of clade 2.3.2 transmission to human is necessary. The prophylactic control can use the effective antiviral drugs. The antiviral drugs for the prophylactic control and therapeutics of influenza infection in humans is distinguished into two classes involve the M2 inhibitors (amantadine and rimantadine) and the neuraminidase inhibitors (oseltamivir and zanamivir) (Ison 2013).

The effectiveness of antiviral drugs will depend on the susceptibility H5N1 avian influenza viruses. The substitutions of M2 protein at residues 26, 27, 30, 31, and 34 are the markers of amantadine resistance. The most of H5N1 clade 2.1 AIV were resistant to amantadine in Indonesia (Cheung et al. 2006; Dharmayanti et al. 2010).

The amantadine resistance studies for H5N1 clade 2.1 AIV have been done in Indonesia but the amantadine resistance research for H5N1 clade 2.3.2 AIV have no more in Indonesia. Previous study described substitution at residu 27 (V27I) M2 protein of H5N1 clade 2.3.2 AIV in Indonesia. However, the M2 phenotypic analysis of H5N1 clade 2.3.2 AIV have not yet been performed in Indonesia. The monitoring of H5N1 subtype of 2.3.2 clade isolated from poultry for antiviral resistance is important to control of the disease. The purpose of this research is to study the amantadine susceptibility among avian influenza of 2.3.2 clade isolated from Indonesia based on identification of genetic markers of amantadine resistance and phenotypic analysis in MDCK cells.

MATERIALS AND METHODS

Virus isolation and identification

Samples were sick or inanimate birds that obtained from poultry and live bird markets. In outbreak of avian influenza, samples were collected from inanimate birds. Tissue samples were collected from the cloaca swab, trachea swab, bronchus, brain, intestine, spleen, and lung, and transported to laboratory. The tissue sample was cultivated in three to five embryonated eggs specific pathogen free embryonated chicken eggs of 9-11 days incubation. The eggs are incubated at 37°C (range 35 – 39 ^{o} C) for 2 – 7 days. Viruses were propagated according to the World Organization for Animal Health manual (OIE 2021). The harvested allantoic fluids from specific pathogen free embryonated chicken eggs were stored at – 80^{o}C for futher examination. The hemagglutination agglutination (HA) is used as a sceering test that indicates a high probability of the presence of influenza A viruses. The presence of H5N1 subtype avian influenza were confirmed by Reverse Transcriptation Polymerase Chain Reaction (RT-PCR) methods with the HA primer sequences of RT PCR method referred to the primers design of Dharmayanti et al. 2016 and Lee et al. 2001; the NA primer sequence (Wright et al. 1995), the Matrix primer sequence (Hoffmann et al. 2001). The method of Reed & Muench was used to calculate the virus titer (EID50, log10/ml) in 10 day old embryonated chicken eggs incubated at temperature of 35°C for 40 hour (Reed & Muench 1938).

Six avian influenza virus of 2.3.2 clade strains, designated A/duck/Indonesia/Brs17/2013 (H5N1), A /duck/ Indonesia/Brs 34/2013 (H5N1), A /duck/Indonesia/Brs 62 /2013 (H5N1), A /Muscovy duck /Indonesia/Serang /Srg 17/2016 (H5N1), and A/Duck/Indonesia/Lamongan/Lmn Tr/2016 (H5N1) were isolated from H5N1 outbreaks in Waterfowl in East Java, West Java Provinces and surveillance program in Banten Provinces of Indonesia. Isolates used as representative viruses in this study were selected based on location from outbreaks in the country and species. Furthermore, representative clade 2.3.2 viruses of each year were determined by the amantadine sensitivity of viruses with MDCK cell-based virus reduction assay and genetic analysis of amantadine resistance markers of the M2 protein.

Susceptibility to the adamantanes

The examination of amantadine toxicity had been done before the biological antiviral assay was tested. The examination of amantadine toxicity was intended to determine the non toxic concentration of amantadine hydrochloride (µg/ml) for MDCK cells. The amantadine hydrochloride concentrations tested were 2 µg/ml, 4 µg/ml, 5 µg/ml and 7 µg/ml. Each concentration was replicated 3 times, subsequently observed with a microscope through 3-4 days post infection (Dharmayanti et al. 2010; Cheung et al. 2006).

The amantadine sensitivity of viruses was analyzed in MDCK cell. The phenotypic assay of antiviral activity for each virus were conducted in confluent monolayer of MDCK cells in 96 well plate in triplicate by haemagglutination (HA) reduction assay and described by *Cytophatic Effect* (CPE) (Cheung et al. 2006; Dharmayanti et al. 2010). Monolayers of MDCK cells treated with amantadine hydrochloride at 2µg/ml and 4 µg/ml concentrations (obtained from stock solution of 5 mg/ml in Phosphate Buffered Saline (PBS) were infected with 10^{6-8} EID_{50} virus then cultured for 48 h at 37^{o} C with 5 % CO2. Supernatants of MDCK cell culture were harvested for HA reduction assay. Replication of virus in MDCK cell culture was measured by HA titers of individual well supernatants.

Table 1. Primer sequences of gene (HA, NA, Matrix) that used for RT-PCR assay (Dharmayanti et al. 2016 ; Lee et al. 2001; Wright et al. 1995; Hoffmann et al. 2001)

Primers	Primer sequences
M52C	5'- CTTCTAACCGAGGTCGAAACG-3'
M253	5'- AGGGCATTTTGGACAAAG/TCGTCTA-3'
H5-F	5'-ACACATGCYCARGACATACT-3'
H5-R	5'- CTYTGRTTYAGTGTTGATGT-3'
H5-NLP86F	5'-CAGAGCAGGTTGACACAATC-3'
H5-NLP463R	5'-CCAGGTATGGACATGCTGAG-3'
H5-ID252F	5'-CGAATTCACCAATGTGCCAG-3'
H5-ID889R	5'-GAGTCTGACACCTGGTGTTG-3'
N1-1	5'-TTG CTT GGT CGG CAA GTGC-3'
N1-2	5'-CCA GTC CAC CCA TTT GGA TCC-3'

Nucleotide analysis

The sequencing analysis of M2 gene used the Matrik primer referred to the primers design of Hoffman et al. 2001. The genetic of transmembrane region of the M2 protein was analyzed, and residues (L26, V27, A30, S31, and G34) were the molecular markers of adamantine resistance. The RNeasy kit (QIAGEN) was used for RNA extraction, and RT-PCR was conducted using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (*In vitro*gen).The DNA sequence used the Sanger method with Bioedit versi 7 and the Clustal W program.. The phylogenetic trees were constructed using the MEGA 6 software with neighborjoining algorithm and the Kimura two-parameter model. Tree topology was evaluated by Bootstrap values of 1000 replicates.

RESULTS AND DISCUSSION

Results

Virus isolation

The avian influenza viruses of H5N1 subtype were obtained from the live bird markets and duck farms. Eight isolates of avian influenza H5N1 viruses were isolated from apparently healthy ducks and six isolates from dead ducks at 2016. Detection using RT PCR method indicated that these isolates were avian influenza H5N1 viruses. Their mean EID_{50} was determined as each of $>10^8$ EID50/ml. In another, the mean EID_{50} of influenza A subtype H5N1 viruses isolated at 2013 were $>10^8$ EID50/ml.

Genetic analysis

Phylogenetic tree analysis of the M2 gene of six H5N1 subtype of clade 2.3.2 from Indonesia were closed with H5N1 subtype of clade 2.3.2 from Vietnam, China, Hongkong (Figure 1.). Compared to the A/chicken/East Java/BL-IPA/2003 strain, the M2 gene in 6 representative strains from Indonesia contains amino acid substitutions at positions 27 (V27I). Even though the M2 V27I substitution have been reported in clade 2.3.2 viruses from Indonesia since 2012, the M2 V27I mutation had not been reported previously in clade 2.1.3 viruses from Indonesia.

The results of the M2 gene analysis are shown in Figure 2. The nucleotide sequence of M2 gene of clade 2.3.2 viruses from Indonesia isolated at 2013 and 2016 showed that these viruses are probably still susceptible to amantadine because the established markers of M2 channel inhibitor resistance of amino acid mutations in 26, 27, 30, 31, 34 residues were not found in the M2 protein of these viruses. However, screening of amino acid mutations of the M2 protein in 26, 27, 30, 31, 34 residues are used to determine whether clade 2.3.2 viruses from Indonesia isolated at 2013 and 2016 have maintained its susceptibility to amantadine. Since these amino acid substitutions (V27I) characterized in the M2 protein of clade 2.3.2 viruses from Indonesia isolated at 2013 and 2016 were unusually genetic markers of resistant viruses, these viruses must be verified experimentally to confirm the resistance to amantadine.

Biological antiviral assay

Biological antiviral assay showed that Amantadine hydrochloride at a concentrations of 2 µg / ml and 4 µg /ml were not toxic for MDCK cells, whereas

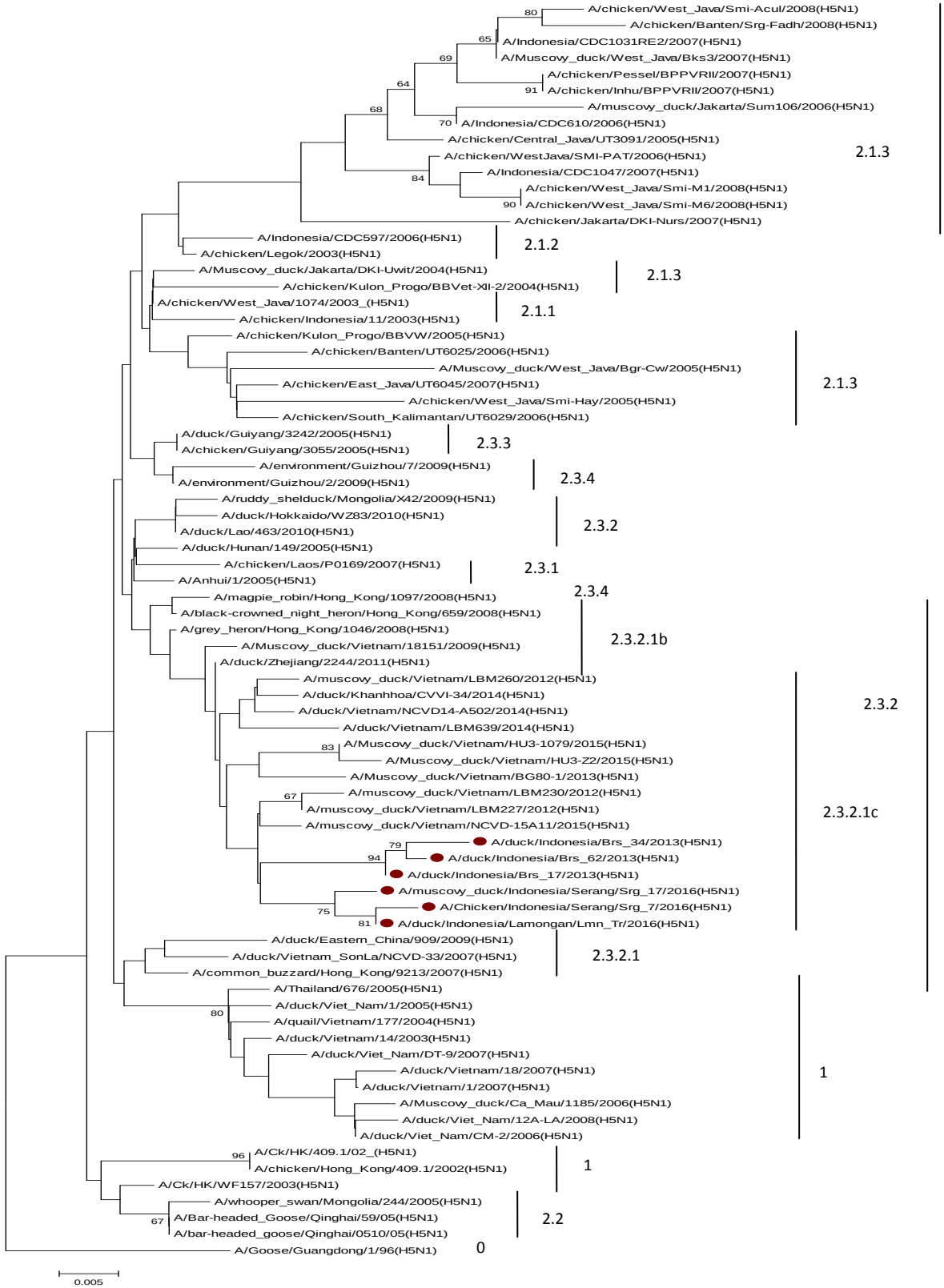

Figure 1. Phylogenetic tree of H5N1 clade 2.3.2 AIV M2 gene sequences. The phylogenetic tree was constructed in MEGA version 6 (www megasoftware.net) using neighbor –joining analysis with 1000 bootstrap replicates and the Kimura 2 parameter model. The H5N1 clade 2.3.2 AIV from waterfowl characterized in this study are indicated with red dot. The M2 tree was rooted are A/Goose/Guangdong/1/96(H5N1).

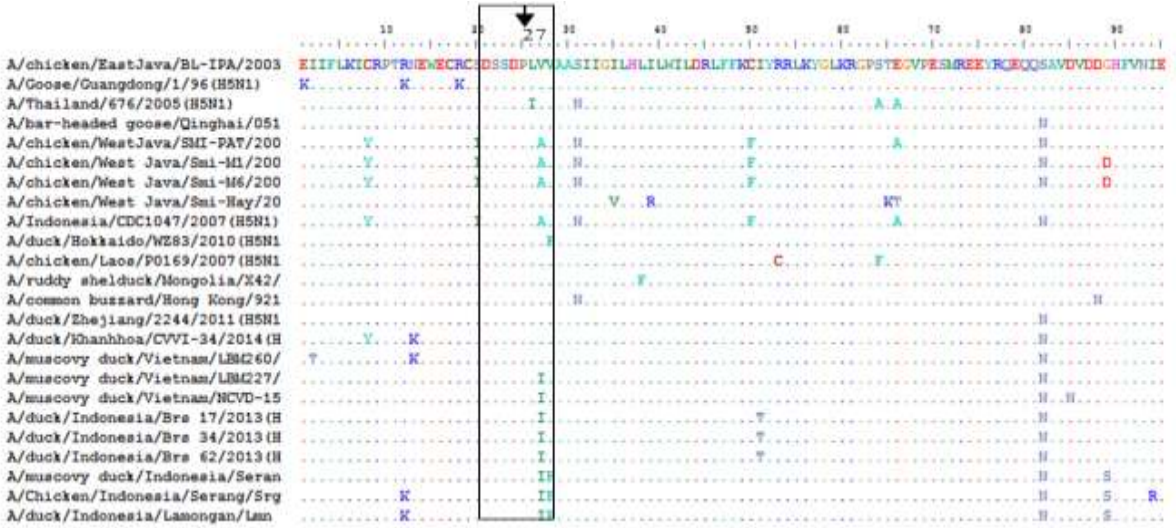

Figure 2. Multiple sequence alignment of M2 protein of avian influenza viruses of clade 2.3.2 H5N1 subtypes from waterfowl

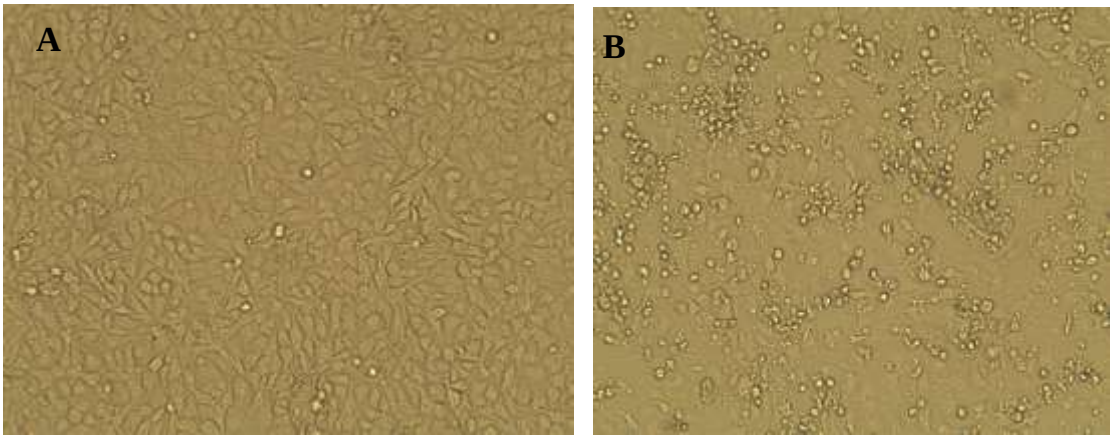

Figure 3. A) MDCK cell that shown non cytopathic effects (CPE). B) Resistence of avian influenza viruses of clade 2.3.2 subtype H5N1 caused CPE in MDCK cell treated with amantadine hydrocloride

amantadine hydrochloride at a concentrations of 5 µg / ml and 7 µg / ml were toxic for MDCK cells. The HA titer of H5N1 subtype of clade 2.3.2 from Indonesia could be discovered on the amantadine-MDCK cells. The mean titers were 3 to 7 log 2. The HA titers and CPE - MDCK cells were influenced by the EID_{50} titers and the period of isolation of clade 2.3.2 H5N1subtype viruses and amantadine hydrocloride concentration. The majority of viruses isolated at 2013 with $\leq 10^7$ EID_{50} gave positive HA reaction ($\leq 2^1$), but the virus with EID_{50} 10^7 gave positive HA reaction with varied titers ($\geq 2^2$) and generated CPE in MDCK cells with diverse concentrations of amantadine hydrochloride. Nevertheless, all of viruses isolated at 2016 with EID_{50} 10^6 - 10^8 were resistance-amantadine hydrochloride in non toxic concentration (2 µg / ml, 4 µg / ml) since these gave positive HA reaction ($\geq 2^2$) and formed CPE in MDCK cells (Figure 3).

Antiviral drugs of amantadine hydrocloride (2 µg / ml and 4 µg / ml concentrations) could not prevented infection of H5N1 subtype of clade 2.3.2 from Indonesia isolated at 2016 with EID_{50} 10^6 - 10^8 in MDCK cells. It is similar for H5N1 subtype of clade 2.3.2 from Indonesia isolated at 2013 with titer $EID_{50}10^8$ for these viruses could infected the amantadine hydrochloride-MDCK cells (2 µg / ml and 4 µg / ml concentrations). However, clade 2.3.2 H5N1 subtype from Indonesia isolated at 2013 ($EID_{50}10^6$ - 10^7) in MDCK cells could be inhibited by the antiviral drug of amantadine hydrocloride (2 µg/ml and 4 µg/ml concentrations). The results revealed that these viruses were unsusceptible to amantadine. The correlation of in vitro assay, HA and molecular analysis results of H5N1 subtype of clade 2.3.2 from Indonesia isolated at 2013 and 2016 are presented in Table 1.

Table 1. The correlation of in vitro assay, HA and genetic analysis results of clade 2.3.2 subtype H5N1 from waterfowl in Indonesia isolated at 2013 and 2016

Avian influenza of clade 2.3.2	EID_{50}	Protein M2	CPE	HA titer	Susceptibility of amantadine with 2 µg/ml and 4 µg/ml amantadine hydrochloride concentrations
A/duck/Indonesia/Brs 17/2013(H5N1)	10^{6}	V27I	No CPE	negative	sensitive
A/duck/Indonesia/Brs 17/2013(H5N1)	10^{7}	V27I	No CPE	negative	sensitive
A/duck/Indonesia/Brs 17/2013(H5N1)	10^{8}	V27I	CPE	positive	resistant (2 µg/ml and 4 µg/ml)
A/duck/Indonesia/Brs 34/2013(H5N1)	10^{6}	V27I	CPE not clear	negative	sensitive
A/duck/Indonesia/Brs 34/2013(H5N1)	10^{7}	V27I	CPE not clear	negative	sensitive
A/duck/Indonesia/Brs 34/2013(H5N1)	10^{8}	V27I	CPE	positive	resistant (2 µg/ml and 4 µg/ml)
A/duck/Indonesia/Brs 62/2013(H5N1)	10^{6}	V27I	CPE	positive	resistant (2 µg/ml and 4 µg/ml)
A/duck/Indonesia/Brs 62/2013(H5N1)	10^{7}	V27I	CPE	positive	resistant (2 µg/ml and 4 µg/ml)
A/duck/Indonesia/Brs 62/2013(H5N1)	10^{8}	V27I	CPE	positive	resistant (2 µg/ml and 4 µg/ml)
A/m.duck/Indonesia/Serang/Srg 17/2016/H5N1	10^{6}	V27I	CPE	positive	resistant (2 µg/ml)
A/m.duck/Indonesia/Serang/ Srg 17/2016(H5N1	10^{7}	V27I	CPE	positive	resistant (2 µg/ml)
A/m.duck/Indonesia/Serang/ Srg 17/2016(H5N1	10^{8}	V27I	CPE	positive	resistant (2 µg/ml)
A/duck/Indonesia/Lamongan/Lmn Tr/2016(H5N1	10^{6}	V27I	CPE	positive	resistant (2 µg/ml and 4 µg/ml)
A/duck/Indonesia/Lamongan/Lmn Tr/2016(H5N1	10^{7}	V27I	CPE	positive	resistant (2 µg/ml and 4 µg/ml)
A/duck/Indonesia/Lamongan/Lmn Tr/2016(H5N1	10^{8}	V27I	CPE	positive	resistant (2 µg/ml and 4 µg/ml)

Discussion

Domestic duck and other wild waterfowl are known as natural reservoir for avian influenza viruses. These waterfowls can take different subtypes of hemagglutinin and neuraminidase subtypes of avian influenza viruses with the silent nature of infections, no sign or mild diseases. Recently, the clade 2.3.2 of avian influenza viruses has been circulated in Southeast Asia including Indonesia and Vietnam (Dharmayanti et al. 2014). The viruses cause severe disease with neurological symptoms, fatality to domestic ducks and predominantly replicates in the brain, heart and lung organs. The excretion of virus through the upper respiratory and conjunctiva can spread virus to environment, poses the high risk of virus spread through cross-contact (Bui et al. 2014). The H5N1 subtype of clade 2.3.2 viruses from Indonesia used in this study were isolated from harvested tissues and tracheal swabs samples of dead or no clinical symptoms ducks through replicated in specific pathogen free (SPF) eggs.

Previous studies had already indicated that avian influenza viruses of H5N1 subtype in this research were clade 2.3.2 viruses based on HA gene. Analysis of the M2 gene phylogenetic tree presented that all avian influenza viruses of H5N1 subtype used in this study were closely correspond to avian influenza viruses of H5N1 subtype in Vietnam, China and Hong Kong (Wan et al. 2011; Naguib et al. 2015; Le & Nguyen 2014; Bi et al. 2015). Futhermore, all of clade 2.3.2 from Indonesia isolated at 2013 and 2016 were closer to cluster of clade 2.3.2 from Vietnam.

Prior to this report, another study (Dharmayanti et al. 2014) had already characterized the M2 Val27Ile mutation of H5N1 subtype viruses of 2.3.2 clade caused duck outbreak in Indonesia at 2012. These studies suggest that the M2 Val27Ile substitution is similar between clade 2.3.2 H5N1 subtype from Indonesia isolated at 2012, 2013 and 2016. The pattern of M2 residues in clade 2.1 subtype H5N1 viruses were different since the majority of viruses circulating in Southeast Asia, especially Indonesia, had single or double substitution on amantadine resistance marker (Val27Ala and Ser31Asn). Amino acid mutation of the M2 protein avian influenza viruses at positions Leu26, Val27, Ala30, Ser31, Gly34, His37 and Trp41 can lead the adamantanes resistance including amantadine and rimantadine. The adamantanes resistance was found in the most influenza A viruses. One or more of M2 amino acids substitution involved amantadine resistance (Cheung et al. 2006; Dharmayanti et al. 2014).

The M2 Val27Ile mutation of six H5N1 subtype avian influenza viruses used in this study was unusually genetic markers of resistant viruses. Analysis of the M2 amino acid confirmed with in vitro test showed that H5N1 subtype of clade 2.3.2 from Indonesia isolated at 2013 and 2016 which had M2 Val27Ile substitution were resistant to amantadine because these viruses induced cytopathic effects in MDCK cells containing the highest non-toxic amantadine concentration.

The M2 Val27Ile, Ser31Asn, Leu26Phe mutations were also found in the other subtype, H4N2, H4N6 and H4N8 subtypes circulated in poultry in China. The single substitution of M2 Val27Ile was characterized in subtype H4N2 and the M2 S31N mutation was identified in subtype H4N6. In addition to single substitution, double substitutions of M2 Val27Ile and Leu26F were also found in subtype H4N8. These mutations are amantadine resistance marker (Liang et al. 2016). The M2 Val27Ile substitution was also identified and characterized in avian influenza viruses received from human clinic samples in Singapore (Lee et al. 2015). This describes that the M2 Val27Ile substitution of human and bird H5N1 subtype of avian influenza viruses may induce amantadine resistance.

The amantadine resistance of subtype H5N1 and seasonal flu viruses has been increasing recently (Zhou et al. 2011). The subtype H5N1 isolated from birds in Northern China has been sustained amantadine resistance up to 83.3%. Amantadine antiviral drugs are intensively used for treatment of H5N1 subtype of avian influenza infections in poultry farms of China. The treatment with amantadine continuously has been intended to prophylaxis so that this condition plays a role important to produce amantadine resistance (He et al. 2008).

The phenotypic analysis (*in vitro* assay) was performed to confirm whether this substitution (V27I) in the M2 gene caused the amantadine resistance. Rapid test of amantadine resistant avian influenza viruses can be demonstrated in the HA assay after viruses replicated in MDCK cell with amantadine drug. The HA test is relatively simple compared to other tests. The avian influenza viruses of subtype H5N1 viruses was resistant to amantadine when HA test showed positive HA reaction ($>2^2$) and the non-toxic concentration of amantadine hydrocloride ($\geq$0.19 µg / ml) (Jacob et al. 2016). The result of HA tests were related with the morphology of MDCK cell and the marker molecular of amantadine resistance of five H5N1 avian influenza viruses clade 2.3.2 from Indonesian. Result in Table 1 showed the amantadine resistant viruses induced the formation of CPE in MDCK cells and the positive HA reaction ($>2^2$) with non-toxic concentration in MDCK cells (2 µg/ml and 4 µg/ml).

The result of this research indicated that resistant viruses may still replicate in MDCK cells containing the highest amantadine concentration. The results of genetic analysis, *in vitro* assay and HA represented that clade 2.3.2 subtype H5N1 from Indonesia isolated at 2013 and 2016 were resistant for amantadine drugs.

Treatment or prophylaxis of avian influenza infection in Indonesian poultry farms with amantadine have not been officially reported so far. The utilization of amantadine to prophylaxis in chickens is one of factors that to play a role for amantadine resistance. The other factor likely to contribute for amantadine resistance is wild birds migration. Migration of wild birds infected with amantadine resistance avian influenza is potential to transmit the amantadine resistance AIV among birds in Indonesia. Previous study (Dharmayanti et al. 2014) revealed that H5N1 subtype of 2.3.2 which has the M2 Val27Ile mutation circulated in Indonesia is probably introduction from overseas viruses.

CONCLUSION

The amantadine resistant avian influenza of clade 2.3.2 H5N1 subtype from Indonesia produced the formation of CPE and the positive HA reaction with non-toxic concentration of amantadine hydrochloride in MDCK cells. The substitution (V27I) was characterized in clade 2.3.2 H5N1 subtype from Indonesia. The result of genetic analysis of M2 gene for amantadine resistance was associated with the results of HA test and the formation of CPE in MDCK cells. These results indicated that amantadine resistance has been identified in avian influenza of clade 2.3.2 subtype H5N1 viruses from Indonesia.

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Interaction Effect of Vitamin E-selenium Supplementation and Metabolic Energy on Reproductive Performance of Joper Breeders

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ABSTRAK

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Efek samping dari oksidasi asam lemak saat sintesis lippoprotein adalah pelepasan oksigen dalam jaringan yang disebut reactive oxygen species (ROS). Stress metabolik pada indukan Joper akibat ketidak seimbangan antara ROS dan antioksidan menyebabkan penuruan produksi dan kualitas telur tetas. Oleh sebab itu perlu penelitian untuk meningkatkan performa reproduksi induk Joper dengan mengkombinasikan suplementasi vitamin E-selenium dan energi metabolik. Penelitian ini menggunakan 200 butir telur tetas hasil persilanngan antara pejantan Sentul umur 60 minggu dengan ayam petelur strain ISA BROWN umur 35 minggu yang diberi pakan perlakuan. Penelitian ini menggunakan Rancangan Acak Lengkap (RAL) Faktorial (2X4). Faktor pertama adalah level energi (2700 dan 2800 kcal/kg) dan faktor kedua dosis suplementasi vitamin E-selenium (0, 25, 50, 75 dan 100ppm). Dosis selenium 1ppm/mg vitamin E. Interaksi antara suplementasi vitamin E-selenium dan energi berpengaruh sangat nyata ($P<0.01$) dalam meningkatkan bobot DOC. Faktor tunggal level energi dan suplementasi vitamin E-selenium berpengaruh nyata ($p<0.05$) dalam menurunkan mortalitas embrio dan meningkatkan daya tetas, sedangkan fertilitas dan kualitas cangkang telur tidak dipengaruhi oleh kedua faktor perlakuan. Kesimpulan dari penelitian ini adalah interaksi antara suplementasi vitamin E-selenium dan level energi dapat meningkatkan bobot DOC Joper, sedangkan faktor tunggal suplementasi vitamin E-selenium dan level energi dapat menurunkan mortalitas *embrio dan meningkatkan daya tetas. Interaksi terbaik pada supelemntasi 100 ppm vitaminE-selenium dan energi 2800 kcal/kg*.

Kata Kunci: Feed suplement, lippoprotein dan indukan Joper

ABSTRACT

Haryuni N, Hartutik, Widodo E, Wahjuningsih S. 2021. Interaction effect of vitamin e-selenium supplementation and metabolic energy on reproductive performance of Joper Breeders. JITV 26(3): 124-131. DOI: http://dx.doi.org/10.14334/jitv.v26i3.2842.

The side effect of fatty acid oxidation during lippoprotein synthesis is the release of oxygen in the tissue called reactive oxygen species (ROS). Metabolic stress in Joper brooders due to an imbalance between ROS and antioxidants causes a decrease in hatching egg production and quality. Therefore, research is needed to improve the reproductive performance of Joper broodstock by combining vitamin E-selenium supplementation and metabolic energy. This study used 200 hatched eggs resulting from a cross between 60 weeks old Sentul males and 35 weeks old ISA BROWN laying hens that had been treated. This study used a Factorial Completely Randomized Design (CRD) (2 x 4). The first factor is the energy level (2700 and 2800 kcal/kg) and the second factor is the dose of vitamin E-selenium supplementation (0, 25, 50, 75 and 100 ppm). Selenium dosage is 1ppm/mg vitamin E. The interaction between vitamin E-selenium supplementation and energy had a very significant effect ($P<0.01$) in increasing DOC weight. The single factor energy level and vitamin E-selenium supplementation significantly ($p<0.05$) in reducing embryo mortality and increasing hatchability, while fertility and eggshell quality were not affected by the two treatment factors. The conclusion of this study is the interaction between vitamin E-selenium supplementation and energy levels can increase the weight of Joper's DOC, while the single factor of vitamin E-selenium supplementation and energy levels can reduce embryo mortality and increase hatchability. The best interaction with 100 ppm vitamin E-selenium supplementation and energy 2800 kcal/kg.

Key Words: Feed supplements, lipoprotein and Joper breeders

INTRODUCTION

The imbalance between demand and supply of native chicken meat in Indonesia is a great opportunity for the development of the native chicken business. In 2019 the demand for native chicken meat was only supplied by 30% (Immawan et al. 2019). Joper (Jowo-cross) is a crossbreed between domesticated males and laying hens (Muharlien et al. 2020). The main priority in the development of the Joper hatchery industry is improving the quality of hatching eggs. Lipoprotein is one of the key factors in assessing the quality of

hatching eggs because it is the main source of nutrition for embryonic development (Ren et al. 2021; Wang et al. 2021).

Reactive oxygen species (ROS) is one of the triggers for metabolic stress which has a major impact on immunity and regulation of body metabolism. ROS is a by-product of fatty acid oxidation in lippoprotein synthesis (Amevor et al. 2021; Zuidhof et al. 2017). To produce good quality hatching eggs, it is necessary to control the triggers of metabolic stress in breeders. Vitamin E-selenium supplementation and increased metabolic energy are solutions to control metabolic stressors in Joper breeders

Vitamin E is a natural antioxidant that can protect tissues from damage caused by ROS. Amevor et al. (2021) reported that selenium plays an important role in various metabolic processes in the body. Vitamin E works synergistically with selenium in lipid peROSidation (Abd El-Hack et al. 2017; Çelebi 2019). Metabolic energy (ME) is used for maintenance (basal metabolism, regulation of body temperature, immune response and activity, growth and egg production (Hadinia et al. 2019). Surplus energy in the body is stored as body fat mass. Body fat mass plays a role in regulating sexual maturity, egg production, egg yolk composition, maximizing the absorption of vitamin E and reproductive hormone precursors (Heijmans et al. 2021; Ren et al. 2021). Research is needed to determine the interaction between ME and the right dose of vitamin E-selenium supplementation in improving hatching egg quality because there is very little information available..

MATERIALS AND METHODS

Place and time

This research was conducted in February 2020. The research location is in the hatchery of Mr. Manto which is located in Rejotangan Village, Tulung Agung Regency, East Java Province, Indonesia.

Artificial insemination

In this study, artificial insemination was performed intravaginally by inserting semen into the cloaca of the Joper breeder as deep as 3-4 cm. This insemination was applied every 4 days at 4 pm. Semen is collected by massaging the area around the abdomen to the cloaca and collected with a graduated tube to measure its volume. Semen dilution using 0.9% NaCl with the ratio between semen and 0.9% NaCl is 1:10. The dose of inseminated semen is 0.2 ml/hens. The concentration of spermatozoa used for artificial insemination refers to the results of the research by Saleh et al. (2019) of 100 million spermatozoa/hens.

Hatchery management

The incubator was cleaned by wiping the inner and outer surfaces of the incubator using a disinfectant and allowed to dry and continued with fumigation. The fumigation of the incubator was carried out by evaporating formalin into a container containing $KMnO_4$. Evaporation is done by pouring formalin 40% in a container containing $KMnO_4$ then the hatching machine is immediately closed and allowed to stand for 24 - 48 hours with the heating condition still on. The fumigation dose per $1m^3$ of hatchery area is 12-15ml of 40% formalin and 6 g of $KMnO_4$. Handling hatching eggs before being put into the semi-authomatic incubator is cleaned by wiping the dirt on the egg shell. Temperature and humidity settings are regulated according to the needs of the embryonic development period. Day 1 to 18 the temperature is set at 37.5 ^{0}C and humidity ranges from 50-60% and on day 19 until hatching the temperature is lowered to 32-33 ^{0}C. On day 20 until hatching humidity is set at 80%. Turning eggs every 1 hour so that in a day there are 24 rounds with a slope of 45^0 (Rahardja et al. 2020). Embryo development was observed every 7 days by candling on the 7th and 14th days after the eggs entered the semi-automatic incubator. Candling is a method used to observe the development of the embryo inside the egg by observing using light.

Breeder Joper and diets

This research is a series of studies from several studies. In previous studies, studies have been carried out on Sentul males to get the best male age which produces good quality semen for artificial insemination where the results of this study are applied to this study. The Joper broodstock used in this study were 400 hens from the ISA BROWN strain and were 35 weeks old with body weight ranging from 1.80-1.85 kg and in good health. These brooders were placed in individual cages with a size of 50 x 40cm and a height of 37cm at the front and 30cm at the back. The hatching eggs of 400 hens were collected and selected, then 200 eggs were taken to be incubated and observed and 200 eggs were used to observe the exterior quality of hatching eggs (egg shell weight and thickness). The total males used were 15 Sentul males aged 60 weeks with body weight ranging from 2.18-2.23 kg and in good health. Sentul roosters are placed separately from the broodstock. These males were placed in individual cages measuring 70 x 50 x 100cm. Sentul rooster was given control feed (E1V0) while Joper broodstock was given treatment feed with the addition of metabolic energy and vitamin E-selenium supplementation according to the treatment. The treatment feed was given 40% at 6 am and 60% at 2 pm. Sentul males and Joper breeders are given drinking water ad libitum.

Table 1. The composition of the experimental feed

Ingredients	Experimental feed									
	E1V0	E1V1	E1V2	E1V3	E1V4	E2V0	E2V1	E2V2	E2V3	E2V4
Corn (%)	48.90	48.90	48.90	48.90	48.90	51.30	51.30	51.30	51.30	51.30
Soy bean meal (%)	21.60	21.60	21.60	21.60	21.60	22.10	22.10	22.10	22.10	22.10
Rice bran (%)	12.20	12.20	12.20	12.20	12.20	12.20	12.20	12.20	12.20	12.20
Meat bone meal (%)	8.00	8.00	8.00	8.00	8.00	8.40	8.40	8.40	8.40	8.40
Grit (%)	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Limestone (%)	3.30	3.30	3.30	3.30	3.30	3.10	3.10	3.10	3.10	3.10
Fat powder (%)	0.00	0.00	0.00	0.00	0.00	1.11	1.11	1.11	1.11	1.11
Complete premix (%)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Monocalcium phospate(%)	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Salt (%)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Sodium bicarbonat (%)	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Vitamin E-selenium(ppm)	0	25	50	75	100	0	25	50	75	100

Calculation of feed composition using Brill Formulation software

Table 2. The nutritional content of the experimental feed

Nutrients	Nutritional content									
	E1V0	E1V1	E1V2	E1V3	E1V4	E2V0	E2V1	E2V2	E2V3	E2V4
ME (kcal/kg)	2,701	2,701	2,701	2,701	2,701	2,800	2,800	2,800	2,800	2,800
Crude protein (%)	19.00	19.00	19.00	19.00	19.00	19.00	19.00	19.00	19.00	19.00
Crude fat (%)	4.40	4.40	4.40	4.40	4.40	5.06	5.06	5.06	5.06	5.06
Crude fiber (%)	3.60	3.60	3.60	3.60	3.60	3.24	3.24	3.24	3.24	3.24
Lysine (%)	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96
Metionin (%)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Methionine + cystine (%)	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87
Threonine (%)	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Triptopane (%)	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22
Calsium (%)	3.99	3.99	3.99	3.99	3.99	3.91	3.91	3.91	3.91	3.91
Total phosphorus (%)	0.83	0.83	0.83	0.83	0.83	0.78	0.78	0.78	0.78	0.78
Phospor avail (%)	0.50	0.50	0.50	0.50	0.50	0.49	0.49	0.49	0.49	0.49
Sodium (%)	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13

Calculation of feed composition using Brill Formulation software
ME = Metabolism energy

Parameters measure

The measurement process in a research is very essential because that is where the numbers are obtained to be processed and analyzed so that the answers to the questions in the research are obtained. The procedure used to obtain data based on the variables observed in this study was as follows:

Egg weight, Egg shell weight (ESW) and Egg shell thickness (EST)

Egg weight (EW) was measured by weighing the hatching eggs. Egg weight measurement was carried out every day and then the average was taken for each week. The weight of the egg shell is one of the determinants of egg quality, especially for hatching eggs. A small eggshell weight indicates a thin eggshell and is not good at hatching. The egg shell weight was measured by breaking the egg and removing the egg white and yolk. Then the weight of the shell was weighed and the results were recorded.The thickness of the egg shell is one of the determinants of the quality of hatching eggs. The thickness of the egg shell is closely related to the ability to hatch. The thickness of the eggshell was measured by breaking the egg, removing the white and yolk and then measuring the thickness of the egg shell using a caliper.

Fertility

Measurement of fertility by calculating the percentage of fertile eggs based on the number of eggs that enter the incubator. Percentage of mortality were calculated based on fertile eggs.

$$\text{Fertility (\%)} = \frac{\text{Number of eggs sett} - \text{Number of clear eggs}}{\text{Number of eggs sett}} \text{ X } 100\%$$

Embryo mortality

Observation of the development of this embryo using light emitted on the surface of the egg. Mortality was measured by splitting unhatched eggs and recording the number of embryos that died.

$$\text{Embryo Mortality (\%)} = \frac{(\text{Number of fertile eggs} - \text{Number of unhatched eggs})}{(\text{Number of fertile eggs})} \text{ X } 100\%$$

Hatchability

Hatchability is the result of fertile eggs until they can hatch and are counted at the end of hatching. The hatchability can be calculated as follows. Percentage of hatchability was calculated based on fertile eggs.

$$\text{Hatchability (\%)} = \frac{\text{Number of Eggs that hatch}}{\text{Number of fertile eggs}} \text{ X } 100\%$$

Body weight of DOC

Hatch weight is the weight of newly hatched chicks. Hatch weight was measured by weighing the newly hatched chicks.

Experimental design

The data obtained were recorded and tabulated and statistically analyzed using ANOVA with a factorial Completely Randomized Design (CRD) with a 2x4 treatment pattern and each treatment using 5 eggs. The first factor was the ME level (2700 and 2800 kcal/kg) (E) and the second factor was the dose of vitamin E-selenium supplementation (0, 25, 50, 75 and 100 ppm) (V). The dose of selenium is 1 ppm/mg vitamin E. Statistical analysis is continued with Duncan's test if the results obtained provide significant or very significant differences in influence.

$$Yijk = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

Where, $Y_{ijk=}$ The results of the observations for the i^{th} level of factor B, j^{th} level of factor B, on the k^{th} test; α_i= General average Effect of factor A at level I; β_j= Effect of factor B on the j level; $(\alpha\beta)_{ij}$= Interaction between A and B at factor A level i, factor B level j; ε_{ijk}= Experimental error for i^{th} level factor A, j^{th} level factor B in the k^{th} replication/group.

RESULTS AND DISCUSSION

Based on the research that has been done, it was found that the effect of metabolic energy level and dose of vitamin E-selenium supplementation in the feed on the reproductive performance of Joper breeders is presented in Table 3 below.

Egg weight

Egg size selection is a selection method that aims to evaluate the feasibility of eggs to be hatched. In this study, interaction between metabolic energy levels with vitamin E-selenium and single factor vitamin E-selenium gave no significant effect ($p>0.05$) on egg weight. The single factor of metabolic energy level had a very significant effect ($p<0.01$) on egg weight. The weight of the eggs before being put into the incubator was 61.13-65.75g. The egg weight obtained in this study is in accordance with the research of Rahman et al. (2016) of 60.95-61.78g and higher than Pagala et al (2020) who got an EW of 56.87g in crosses of bangkok males with laying hens and 47.09g in crosses of broiler breeds with bangkok laying hens. Egg weight based on statistical analysis is known to be influenced by a single factor of ME or vitamin E-selenium supplementation. Statistical analysis showed ME had a more dominant effect on EW than vitamin E-selenium supplementation. The highest EW was obtained from treatment with a ME of 2800kcal/kg and vitamin E-selenium supplementation at a dose of 100 ppm.

Factors that affect EW are the age of laying hens. environmental temperature. strain. feed and body weight. Feed plays an important role in egg production (Pagala et al. 2020). ME factor is the dominant influence on EW. this is because about 99% of the total egg lipid is in the yolk. These lipids are used to supply energy for the developing embryo. The lipid in egg yolk is not free but is in the form of lipoproteins and is derived from the synthesis of ME in feed (Nasr 2015; Liu et al. 2020). Although vitamin E-selenium supplementation did not have a dominant effect on EW. it also had an effect on increasing EW. Slozhenkina et al. (2020) reported that lipids in egg yolk are composed of phospholipids with unsaturated fatty acids that are easily oxidized. Vitamin E-selenium supplementation in this case serves to maintain the integrity of the egg yolk so that it is not damaged by ROS.

Egg shell (weight and thickness)

Interaction between metabolic energy level and single factor energy level and dose of vitamin E-selenium supplementation had no significant effect

Table 3. The average reproductive performance of the Joper broodstock

Treatment	Variable						
	Egg weight (g)	Egg shell weight (g)	Egg shell thickness (mm)	Fertility (%)	Embryo mortality (%)	Hatchability (%)	DOC weight (g)
Metabolic energy level							
E1	62.22±0.05[a]	8.23 ± 0.08	0.08 ± 0.000	90.50±0.58	27.22±0.76[b]	72.78±0.76[a]	42.53±0.19[a]
E2	64.01±0.02[b]	8.33 ± 0.06	0.08 ± 0.001	95.00±0.71	21.28±0.69[a]	78.73±0.69[b]	44.20±0.37[b]
Suplementasion vitamin E-selenium							
V0	62.77 ± 0.52	8.19 ± 0.16	0.08 ± 0.001	92.50 ± 2.50	29.19±2.71[b]	70.81±2.71[a]	41.50±0.42[a]
V1	63.20 ± 0.48	7.88 ± 0.13	0.08 ± 0.001	92.50 ± 0.00	24.81±1.00[a]	75.19±0.00[a]	42.50±0.17[a]
V2	63.10 ± 0.41	8.31 ± 0.09	0.08 ± 0.00	93.75 ± 3.13	25.28±0.14[ab]	74.72±0.14[a]	43.75±0.33[b]
V3	63.23 ± 0.43	8.63 ± 0.13	0.08 ± 0.00	92.50 ± 1.25	21.49 ±1.13[a]	78.51±1.13[ab]	44.00±0.46[bc]
V4	63.25 ± 0.40	8.38 ± 0.19	0.08 ± 0.00	92.50 ± 1.25	20.46±2.45[a]	79.54±2.45[b]	45.08±1.04[c]
Interaction of vitamin E-selenium supplementation and metabolic energy							
V1V0	61.74 ± 0.38	7.88 ± 0.65	0.08 ± 0.00	87.50 ± 8.29	34.61 ± 6.53	65.39 ± 6.53	40.67±0.41[a]
V1V1	62.25 ± 0.37	7.63 ± 0.41	0.08 ± 0.01	92.50 ± 8.29	26.81 ± 3.34	73.19 ± 3.34	42.83±0.50[ab]
E1V2	62.28 ± 0.36	8.50 ± 0.35	0.08 ± 0.01	87.50 ± 8.29	25.56 ± 2.81	74.44 ± 2.81	43.08±0.64[b]
E1V3	62.36 ± 0.34	8.38 ± 0.41	0.08 ± 0.00	95.00 ± 8.66	23.75 ± 4.15	76.25 ± 4.15	43.08±0.83[b]
E1V4	62.45 ± 0.34	8.75 ± 0.25	0.08 ± 0.00	90.00 ± 7.07	25.36 ± 2.86	74.64 ± 2.86	43.00±0.41[b]
E2V0	63.81 ± 0.39	8.50 ± 0.50	0.08 ± 0.01	97.50 ± 4.33	23.78 ± 7.41	76.22 ± 7.41	42.33±0.24[a]
E2V1	64.15 ± 0.81	8.13 ± 0.82	0.08 ± 0.00	92.50 ± 8.29	22.81 ± 1.90	77.19 ± 1.90	42.17±0.17[a]
E2V2	63.93 ± 0.36	8.13 ± 0.22	0.08 ± 0.00	100.0 ± 0.00	25.00 ± 5.00	75.00 ± 5.00	44.42±0.14[bc]
E2V3	64.09 ± 0.20	8.88 ± 0.22	0.08 ± 0.00	90.00 ± 7.07	19.24 ± 3.99	80.76 ± 3.99	44.92±0.28[c]
E2V4	64.05 ± 0.29	8.00 ± 0.61	0.08 ± 0.00	95.00 ± 5.00	15.56 ± 4.44	84.44 ± 4.44	47.17±0.99[d]

a. b a. b. ab. c and d values within a column with a very significant effect ($p<0.01$) in reducing embryo mortality; improve hatchability and increase DOC weight. a very significant effect ($p<0.01$) in increasing DOC weight. a significant effect ($p<0.05$) in reducing embryo mortality and improving hatchability. a very significant effect ($p<0.01$) in increasing DOC weight.

($p>0.05$) on EST and EWS. The ESW obtained in this study was 7.63-8.88 g and the EST was 0.76-0.83 mm. The results obtained are higher than some of the literature. Slozhenkina et al (2020) reported the ESW of Sakini chickens in Nigeria was 4.51 g; Barbe et al. (2020) is 0.31 -0.32 mm and Sapkota et al. (2020) is 0.29-0.43 mm.

The quality of the eggshell has an important role during the incubation period. Eggshell quality is related to mineral metabolism. especially Ca2+. The main component of eggshell is calcium carbonate ($CaCO_3$) which is formed by Ca^{2+} and HCO_3 (Darsi & Zhaghari 2021). One of the declines in the quality of hatching eggs is influenced by the quality of the egg shell. The thin egg shell causes the eggs to be easily damaged and cannot be stored for long. Low egg shell quality is associated with a decrease in the intestinal ability to absorb calcium and an increase in egg size with increasing age of the breeder (Nasri et al. 2020). Egg shell thickness is influenced by heredity and mineral metabolism in the body. Shell thickness is related to calcium metabolism obtained from bone deposits and feed (Sapkota et al. 2020).

Fertility

Fertility is the ability of the breeder to produce day old chick (DOC). Interaction between metabolic energy level and single factor energy level and dose of vitamin E-selenium supplementation had no significant effect ($p>0.05$) on fertility. The fertility obtained in this study ranged from 87.50-100%. The fertility obtained is similar to Saleh et al (2019) of 73.09 - 90.22% and was higher than the study of Pagala et al. (2020) of 66.67-67.67%. Fertility is one of the parameters to measure the success of insemination. The quality of spermatozoa has a major influence on the success of insemination (Pagala et al. 2020; Tesfay et al. 2020). In general. the

fertility rate is influenced by the ratio of males and females. type of insemination. handling of hatching eggs. environment (Adu-Aboagye et al 2020). abnormal spermatozoa and dead spermatozoa. Good quality spermatozoa if spermatozoa abnormality <17% and dead spermatozoa <10% (Feyisa et al. 2018)

Spermatozoa concentration plays an important role in the success of artificial insemination. In this study, the concentration of spermatozoa used for each treatment was the same, namely 100 million spermatozoa/hens. The same thing was also reported by Saleh et al. (2019) in their research which stated that different concentrations of spermatozoa resulted in different fertility where the best fertility was obtained by artificial insemination using semen with a spermatozoa concentration of 100 million spermatozoa/hens.

Therefore. the selection of males is very important for the hatchery industry. Male selection is done by evaluating the quality of semen to determine its reproductive ability. Evaluation of semen quality and its ability to fertilize can be done by analyzing the percentage of live/dead sperm and morphological evaluation (Tesfay et al. 2020). Spermatozoa quality is influenced by several factors including age of the male, maturity, nutritional adequacy and health status of the male. Spermatozoa concentration that is too high or low actually causes a decrease in fertility.

Embryo mortality

The interaction between vitamin E-selenium supplementation and ME had no effect (p>0.05) on embryonic death. Embryo mortality is more influenced by a single factor. ME factors and vitamin E-selenium supplementation factors both have an influence on embryonic death. ME plays more dominant role in reducing embryo mortality than vitamin E-selenium supplementation.

Energy has a dominant role in embryo mortality because ME has an important role in the process of egg yolk formation and egg weight. Egg yolk is a source of nutrition for embryonic development. 75% of the nutrients in egg yolk are lipoproteins (Nasr 2015; Liu et al. 2020). About 99% of the total egg lipid is in the yolk. The lipids in egg yolk are composed of phospholipids with unsaturated fatty acids that are easily oxidized (Slozhenkina et al. 2020). In breeders. surplus energy in the body is stored as body fat mass as a precursor in the synthesis of lipoproteins in egg yolk. The ME also plays a role in the absorption of vitamin E-selenium in the body (Heijmans et al. 2021; Ren et al. 2021). Vitamin E is a natural antioxidant to reduce ROS in egg yolk by reducing lipid peROSidation and oxidation. Oxidation in egg yolks during the hatching process causes the production of toxic malondialdehyde which can cause embryo death. so vitamin E-selenium supplementation is effective for reducing lipid peROSidation and increasing antioxidant capacity in egg yolks which has an impact on increasing hatchability (Barbe et al. 2020).

Embryo mortality is not only influenced by the quality of the hatching eggs but also by the temperature setting in the incubator. The temperature of the incubator is very influential on the mortality of the embryo. Embryo mortality increases when the incubator temperature exceeds the optimal temperature for embryonic development. This is because the embryonic membrane becomes dry so that the embryo will experience nerve. heart. respiratory and kidney disorders (Pagala et al. 2020).

Hatchability

The interaction between metabolic energy level and the dose of vitamin E-selenium supplementation had no significant effect (p>0.05) on hatchability. while the single factor of metabolic energy level had a very significant effect (p<0.01) on hatching weight and the single factor of supplementation dose had a significant effect (p<0.05) on hatchability. The interaction between metabolic energy level and the dose of vitamin E-selenium supplementation had no significant effect (p>0.05) on hatchability. While the single factor of metabolic energy level had a very significant effect (p<0.01) on hatching weight and the single factor of supplementation dose had a significant effect (p<0.05) on hatchability.Hatchability obtained in this study ranged from 65.39-84.44% almost the same as (Pagala et al. 2020) of 85-93%. Hatchability percentage is affected by a single factor of metabolic energy and vitamin E-selenium supplementation. ME more dominant influence on hatchability improvement compared to vitamin E-selenium supplementation. The increase in ME affects the higher hatchability. Nutrient requirements for embryonic development during incubation are stored in albumen. egg yolk and egg shell. The main source of energy for the development of the embryo is obtained from the yolk sac. The energy in egg yolk for embryo development is obtained from the oxidation of fatty acids in egg yolk. Fatty acid oxidation in egg yolk supplies almost 94% of the total energy requirement of the embryo during development (Nasri et al. 2020).

Slozhenkina et al. (2020) reported that the use of antioxidants in broodstock could increase hatchability. The addition of antioxidants can increase hatchability up to 14.60%. The use of antioxidants will help the metabolism to be normal. Agreeing with this. Barbe et al. (2020) stated that vitamin E is a natural antioxidant to reduce ROS in egg yolk by reducing lipid peROSidation and oxidation. Oxidation in egg yolks

during the hatching process causes the production of toxic malondialdehyde which can cause embryo death. so vitamin E-selenium supplementation is effective for reducing lipid peROSidation and increasing antioxidant capacity in egg yolks which has an impact on increasing hatchability (Barbe et al. 2020).

Adu-Aboagye et al. (2020) and Pagala et al. (2020) explain that there are 3 main factors that affect the percentage of hatchability. The first factor is the quality of hatching eggs. This egg quality includes egg size. egg shape. egg weight and nutrient content. Hatching eggs with low nutrient content will produce a low percentage of hatchability. Nutrients present in eggs are the main source of nutrition for embryonic development. The second factor is the handling of hatching eggs before they are put into the incubator. Handling hatching eggs includes the cleanliness of eggs and the length of storage of hatching eggs before incubation. Eggs that are dirty and stored for a long time will result in low hatchability. The third factor is the hatching process. The hatching process is concerned with setting the incubator during incubation. Setting the temperature. humidity and frequency of egg turning has an important role in determining the success of hatching.

DOC weight

Interaction between vitamin E-selenium supplementation and ME had a very significant effect (p<0.01) on DOC weight. Increasing doses of vitamin E-selenium supplementation and ME had an impact on increasing DOC weight. In this study. the weight of DOC ranged from 40.67-47.17g. The quality of hatching eggs has a major role in the weight of the DOC produced. DOC weight is related to metabolic activity during embryonic development. Availability of nutrients for embryonic development is a key factor in DOC weight. Egg yolk is the main source of energy for embryo development. according to Slozhenkina et al (2020) 99% of the constituents of egg yolk are lipids and lipid oxidation in egg yolk is used to supply energy needs for embryonic development. Hadinia et al. (2019) reported that energy intake in the body of the breeder is metabolized for maintenance. growth. and egg production. Surplus energy is stored as body fat mass. Body fat mass plays a role in egg yolk synthesis.

The metabolic activity of the embryo during incubation has an effect on the rate of embryo development. The increased energy requirements during incubation for embryonic development affect metabolism. especially oxidation reactions. Fatty acid oxidation increases with increasing energy requirements. Increased ROS in egg yolk due to high oxidation causes a low metabolic rate. Nasri et al. (2020) explained that the decrease in metabolism during incubation caused organ development and embryonic growth to not be optimal. This can cause the resulting DOC weight to be low. Supplementation of vitamin E-selenium in broodstock feed will increase antioxidant levels in hatching eggs. Barbe et al. (2020) reported that antioxidants in egg yolk will control oxidation by reducing or inactivating ROS before working on embryonic tissue.

CONCLUSION

The conclusion of this study is the interaction between vitamin E-selenium supplementation and energy levels can increase the weight of Joper's DOC, while the single factor of vitamin E-selenium supplementation and energy levels can reduce embryo mortality and increase hatchability. The best interaction with 100 ppm vitamin E-selenium supplementation and energy 2800 kcal/kg.

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Studying the Liver Function in Male Neonates of Rats Born to Sertraline-Treated Mothers

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ABSTRAK

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Sertraline merupakan sebuah antidepresan yang memiliki efek racun pada hati. Penelitian ini dilaksanakan untuk mengevaluasi pengaruh penambahan Sertraline di masa kebuntingan pada fungsi hati tikus neonates jantan. Sebanyak 25 tikus Wistar betina bunting dibagi menjadi empat kelompok dengan jumlah masing masing sebanyak lima ekor. Kelompok control tidak mendapatkan perlakuan penambahan obat apapun, tetapi kelompok eksperimen (Exp) 1, 2 dan 3 menerima 5, 10 dan 20 mg/kg berturut-turut Sertraline sebagai pakan paksa selama masa kebuntingan. Dua puluh dua hari setelah lahir, tikus jantan dibagi menjadi empat kelompok yang terdiri dari 10 ekor berdasarkan pembagian sebelumnya. Setelah ditimbang, sampel darah diambil langsung dari jantung. Kadar serum Alanine transaminase (Alt), Aspartate transaminase (AST), Alkaline phosphatase (Alp), Albumin (Alb), Total protein (TP), dan Bilirubin (Bili) diukur. Kemudian, jaringan hati juga dianalisis secara histopatologis setelah ditimbang. Pada kelompok Exp, terjadi penurunan berat badan, TP dan serum Alb yang signifikan dibandingkan dengan kelompok kontrol ($p<0,05$). Pada kelompok Exp 3, penurunan berat hati yang signifikan diamati dibandingkan dengan kelompok kontrol ($p<0,05$). Pada kelompok Exp 2 dan 3, terjadi peningkatan signifikan kadar serum Alp, Alt, dan Bili dibandingkan dengan kelompok kontrol ($p<0,05$). Sebuah peningkatan yang signifikan dalam tingkat serum AST diamati pada kelompok Exp dibandingkan dengan kelompok kontrol ($p<0,05$). Kerusakan jaringan hati diamati pada ketiga kelompok Exp. Pemberian Sertraline pada tikus betina bunting menyebabkan kerusakan hati dan meningkatkan enzim hati serta parameter biokimia darah pada keturunan jantannya.

Kata Kunci: Albumin, Perkembangan Hati, Enzim Hati, Tikus, Sertraline

ABSTRACT

Safaei V, Shariati M. Studying the liver function in male neonates of rats born to sertraline-treated mothers. JITV 26(4):132-138. DOI: http://dx.doi.org/10.14334/jitv.v26i4.2945

Sertraline is an antidepressant which has toxic effects on the liver. This study was conducted to evaluate the effect of Sertraline administration in pregnancy on liver function of male neonates of rats. Twenty-five pregnant female Wistar rats were divided into 4 groups of 5. The control group did not receive any drug treatments, but experimental (Exp) groups 1, 2 and 3 received 5, 10 and 20 mg/kg Sertraline as gavage throughout the pregnancy, respectively. Twenty-two days after birth, male rats were divided into 4 groups of 10 based on the previous division and after weighing, by taking blood directly from the heart, serum levels of Alanine transaminase (Alt), Aspartate transaminase (AST), Alkaline phosphatase (Alp), Albumin (Alb), Total protein (TP), and Bilirubin (Bili) were measured and the liver tissue was also analyzed histopathologically after weighing. In Exp groups, a significant decrease in body weight, TP and Alb serum levels were observed compared to the control group ($p<0.05$). In Exp group 3, a significant decrease in liver weight was observed compared to the control group ($p<0.05$). In Exp groups 2 and 3, a significant increase in serum levels of Alp, Alt and Bili in was observed compared to the control group ($p<0.05$). A significant increase in AST serum level was observed in Exp groups compared to the control group ($p<0.05$). Liver tissue destruction was observed in all 3 Exp groups. The administration of Sertraline in pregnant female rats causes liver damage and increases liver enzymes and blood biochemical parameters in their male offspring.

Key Words: Albumin, Liver Development, Liver Enzymes, Rat, Sertraline

INTRODUCTION

Depression is a common and recurrent disorder that reduces the functional and cognitional role and even in some cases, it leads to death (Wang et al. 2017). Sertraline is a drug that is widely used in the treatment of depression. It is used in a wide range of psychiatric disorders, including panic disorder, obsessive-

compulsive disorder, and post-traumatic stress disorder. Sertraline also acts as an appetite suppressant in weight loss. The most important drug activity of Sertraline is the inhibition of presynaptic Serotonin reuptake (Singh and Saadabadi, 2021). It also has very weak effects on norepinephrine and neuronal absorption of Dopamine (Suen et al. 2013) and it should not be used in combination with Monoamine oxidase (MAO) inhibitor (Aboukarr and Giudice, 2018). It is believed that Sertraline works by increasing the effects of Serotonin in the brain (Willard et al. 2015). By inhibiting Serotonin reuptake, extracellular Serotonin levels increase, thereby increasing serotonergic neurotransmitters in the brain. This action appears to be responsible for the antidepressant, anti-anxiety, and anti-obsessive effects of Sertraline. Sertraline has little tendency for Norepinephrine transporters or Serotonin, Dopamine, Adrenergic, Histamine, or Acetylcholine receptors. On the other hand, it shows a strong affinity for the Dopamine receptor and the σ_1 receptor (But not for the σ_2) (Albayrak and Hashimoto, 2017). Sertraline is slowly absorbed when taken orally and reaches a maximum plasma concentration 4 to 6 hours after ingestion and 98.5% of it binds to plasma proteins. Based on in vitro studies, Sertraline is metabolized by several cytochrome 450 isoforms (Chen et al. 2020). The active metabolite of Sertraline is Norsetraline (N-Dismethylsertraline) which is significantly less biologically active than Sertraline (McIntyre and Mallett, 2012).

The usual dose of Sertraline in the treatment of major depressive disorders is 50 mg daily, which can be gradually increased to a maximum of 200 mg per day which is guided by the therapeutic response (Honko et al. 2017). The side effects of Sertraline include nausea, diarrhea, insomnia, and sexual dysfunction. Acute secondary hepatitis after Sertraline use is rare, but given the widespread use of Sertraline, awareness of this side effect is important to ensure early diagnosis (Suen et al. 2013). Sertraline is one of the most commonly used antidepressant during pregnancy, and generally recognized safe for this use (Molenaar et al. 2020). Nevertheless, the findings represent that the administration of Sertraline during pregnancy is associated with an increase of this drug in fetal amniotic fluid. Therefore, it seems that the fetus is exposed to this drug through various routes such as placenta and digestion. Also, studies measuring the concentration of Sertraline and its major metabolite, dismethylsertraline, in maternal blood and umbilical cord blood show that the serum concentration of Sertraline in the umbilical cord is almost always lower than the maternal serum concentration (Hostetter et al. 2000, Horackova et al. 2021). The concentrations of Sertraline and dismethylsertraline in breast milk are highly variable and, on average, are equally concentrated as the maternal blood plasma (Pinheiro et al. 2015).

Some studies indicate that hepatotoxicity following Sertraline administration is negligible, but some other studies show that Sertraline is associated with toxicity and liver damage (Tabak et al. 2009; Todorović Vukotić et al. 2021). Because there are not enough findings about the effect of Sertraline on liver tissue and Sertraline is one of the most widely used antidepressants and the effect of this drug in pregnancy on fetal liver function is unknown, therefore, this study was conducted to evaluate the effect of Sertraline on the liver tissue of neonates of male rats born to mothers who have been exposed to different doses of Sertraline during pregnancy. For this purpose, body weight, liver weight, serum levels of liver enzymes and some blood biochemical parameters as well as histopathological changes of the liver were evaluated in this study.

MATERIALS AND METHODS

Animals

In this experimental study, 20 adult female Wistar rats weighing 190±10 g were provided from the animal's house at Islamic Azad University of Kazerun and were kept in the standard conditions at 23±2 ºC, 12 hours of light/darkness, and 70% moisture in 5-individual groups in polycarbonate cages with dimensions of 15× 2.5 × 40 cm with steel mesh roof. In order to adapt to the new environmental conditions, the animals were kept together for 2 weeks. The animals received the same amount of water and food throughout the study without any restrictions. The ethical principles of working with laboratory animals were observed in this study and approved by the Ethics Committee of Islamic Azad University of Kazerun (Ethical code no: IR.IAU.SHIRAZ.51053279901007).

The Experiment Protocol

The pregnant female rats (n=20) were divided into 4 groups of 5 including control, experimental 1 (Exp 1), experimental 2 (Exp2) and experimental 3 (Exp 3). The control group did not receive any drug treatment. Animals in Exp groups 1, 2 and 3 received 5, 10 and 20 mg/kg Sertraline (Zoloft, Pfizer, Germany), respectively, at 9 a.m. every day until the end of pregnancy as a gavage. The doses prescribed in this study were determined based on the previous studies (Mikail et al. 2012; Pereira-Figueiredo et al. 2014). The duration of breastfeeding in male rats from sertraline-treated mothers was 3 weeks.Twenty two days after delivery, male neonates of female rats were divided into 4 groups of 4 as in the previous division. At the end of day 22, male rats were anesthetized with ether (Merck,

Germany), and blood samples were taken directly from the heart, and then liver tissue was removed for histopathological evaluation.

Analysis of Blood Parameters

For blood sampling, the animals were first weighed and then anesthetized using ether and opening the chest; blood was drawn directly from the heart with a 5 ml syringe. In order to perform the agglutination process, blood samples were kept in the laboratory for 30 minutes and then centrifuged at 3000 rpm for 5 minutes. The resulting sera were frozen at -20 ° C until serum levels of Alanine transaminase (Alt), Aspartate transaminase (AST), Alkaline phosphatase (Alp), Albumin (Alb), Total protein (TP) and Bilirubin (Bili) were measured.

Serum levels of AST, ALT, ALP, Alb and TP were measured by RA-1000 auto-analyzer (Technicon, USA) according to the manufacturer's instructions (Pars Azmoon Company, Iran). Serum ALT and AST levels were measured by IFCC (international federation of clinical chemistry) method without the addition of Pyridoxal-50phosphate and serum ALP level was measured by PGKC (Deutsche Gesellschaft Fur Klinische Chemie) method. Also, serum Tp level was measured by photometric method based on Biuret method and serum Alb level was measured by BROMOCRESOL-GREE method. (Letafat et al. 2021)

Liver Tissue Changes Analysis

For histopathological examination of the liver, the abdominal cavity of all animals was opened and the liver was removed. First, the liver weight of all animals was calculated and then the tissue samples were washed with normal saline and fixed in 10% formalin buffer solution. The dehydration process of the samples was performed in 60% to absolute alcohol and the tissue samples were clarified with Xylene. Tissue samples were molded with paraffin and then 10 transverse sections with a thickness of 5 microns were prepared from each sample using a rotating microtome. Under a light microscope (Nikon, Japan), 5 different areas of each cross section were randomly selected and tissue changes were examined. The amount of empty space created due to hepatocyte necrosis was examined in different samples with 40x magnification.

Statistical Analysis

Using Kolmogorov-Smirnov test (SPSS Statistics version 20, SPSS Inc., Chicago, IL), the normal distribution of data was first investigated and then, using one way ANOVA test and Tukey post hoc test, statistical comparison was performed between control and Exp groups at the level of $p<0.05$. Results were presented as mean ±mean standard deviation in the graph (GraphPad Prism 6, Inc., San Diego, CA, USA) and table.

RESULTS AND DISCUSSION

In Exp groups 1, 2 and 3 (Figure 1A) a significant decrease in body weight (Figure 1A) was observed compared to the control group ($p<0.05$). There was no difference in liver weight (Figure 1B) in Exp groups 1 and 2 compared to the control group ($p>0.05$), but in Exp group 3, a significant decrease was observed compared to the control group ($p<0.05$). There was no difference in serum levels of Alt, Alp and Bil (Figure 1C, 1D and 1E, respectively) in Exp groups 1 compared to the control group ($p>0.05$), but in Exp groups 2 and 3, a significant increase was observed compared to the control group ($p<0.05$). A significant increase in serum AST levels (Figure 1F) was observed in Exp groups 1, 2 and 3 compared to the control group ($p<0.05$). Also, a significant decrease in serum levels of TP and Alb (Figure 1G and 1H, respectively) was observed in Exp groups 1, 2 and 3 compared to the control group ($p<0.05$). In the present study, the administration of Sertraline in doses of 5, 10 and 20 mg/kg of body weight caused liver necrosis, weight loss and liver weight loss in Exp groups. Also, serum levels of Alt, AST, Alp and Bili also increased, while serum levels of Alb and TP decreased. Sertraline is one of the most common Serotonin reuptake inhibitors, which is considered as a safe drug during pregnancy (Molenaar et al. 2020). Plasma concentrations of Sertraline may decrease or increase during pregnancy, but no clear association has been found between different doses of Sertraline and its associated clinical effects. Although the rate of Sertraline passage through the placenta is very low, its side effects on fetal tissues and organs are unclear. Sertraline is generally a weak compound that binds to two plasma proteins, Albumin, and more to alpha 1-acid-glycoprotein. Plasma levels of Albumin and alpha-1-acid-glycoprotein decrease during pregnancy so they can affect the plasma concentration of Sertraline (Heinonen et al. 2021). At birth, however, plasma Albumin concentrations in infants are usually higher than in mothers, while the plasma concentration of alpha 1-acid-glycoprotein is one third of the maternal plasma concentration (Ewing et al. 2015).

The enzyme family of cytochrome P450 plays an important role in the metabolism of Sertraline to its major and weak metabolite, dismethylsertraline (Huddart et al. 2020). It seems that any change in the expression of cytochrome P450 family enzymes can affect the plasma concentration of Sertraline. One of these factors is pregnancy, which can induce changes in the activity of metabolic enzymes (Westin et al. 2017).

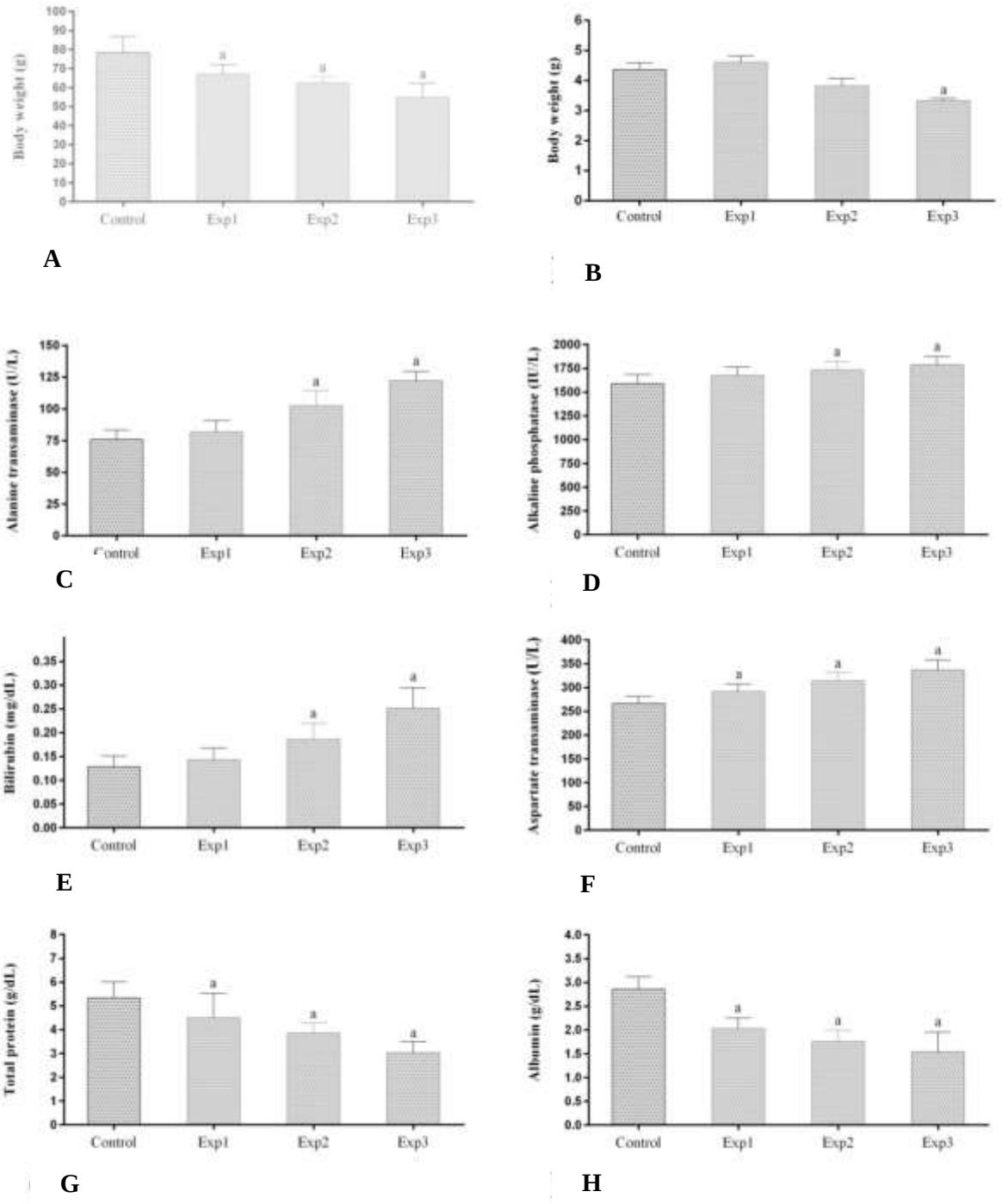

Figure 1. Comparison of mean and standard deviation of mean A) body weight, B) liver weight, C) Alt, D) Alp, E) Bili, F) AST, G) TP and H) Alb levels in control, Exp groups 1, 2 and 3. a ($p<0.05$): compared with the control group.

Serotonin is a neurotransmitter that plays an important role in controlling the appetite and it is directly related to the amount of Tryptophan in the diet and its amount in the brain changes as the amount of Tryptophan decreases or increases. Serotonin receptors inhibit the function of neuropeptide Y, a potent stimulant of hunger and food absorption. Decreased activity of this neuropeptide may be associated with increased activity of Leptin. Leptin is made by adipose tissue in the human body and travels to the brain through the bloodstream, acting on hypothalamic receptors and reduces the appetite (Yabut et al. 2019). Given the abovementioned, it is possible that Sertraline drug increases Leptin by increasing brain Serotonin and

decreasing the function of neuropeptide Y, resulting in decreased appetite and weight loss.

Serotonin reuptake inhibitors are commonly known to reduce serum levels of inflammatory cytokines such as TNF-α and IL-1β in patients with major depressive disorder (Hannestad et al. 2011). However, the effects of these Serotonin reuptake inhibitors, such as Sertraline, appear to be possible at low doses. The studies show that the administration of high doses of Sertraline in rats is associated with increased TNF-α and IL-1β mRNA expression (Sitges et al. 2014). In the present study, with increasing the dose of Sertraline, further destruction of liver tissue was observed. Therefore, due to the dose-dependent effects of the drug, the destruction of the liver parenchyma and the reduction of liver cell density could be a reason for weight loss.

Histopathological examination showed that (Figure 2) in the control group, the liver tissue is healthy and hepatocyte cells are regular and dense (Figure 2A). However, liver tissue degradation was observed in all 3 Exp groups. In Exp group 1, the liver parenchyma was slightly necrotic and some empty spaces were observed between hepatocyte cells (Figure 2B). In Exp groups 2 (Figure 2C) and 3 (Figure 2D), the liver parenchyma had moderate to severe necrosis, respectively, and empty spaces were observed between hepatocytes. In an *in vivo* study, significant morphometric and hepatic histological changes, such as hepatocyte necrosis and hydropic degeneration, were detected in Sertraline-treated rabbits orally (8 mg/kg) for 9 weeks, which was consistent with the histopathological results of this study. It was suggested that, the hepatic necrosis could indicate oxidative stress by glutathione depletion as a consequence of Sertraline toxicity (Almansour et al. 2018). The studies represent that Sertraline consumption during pregnancy is associated with short-term and long-term adverse effects in children (Shen et al. 2017). Other studies also indicate that Sertraline can induce oxidative DNA damage and increase the level of cellular apoptosis (Jajoo et al. 2020). The studies suggest that Sertraline has teratogenic and toxic effects

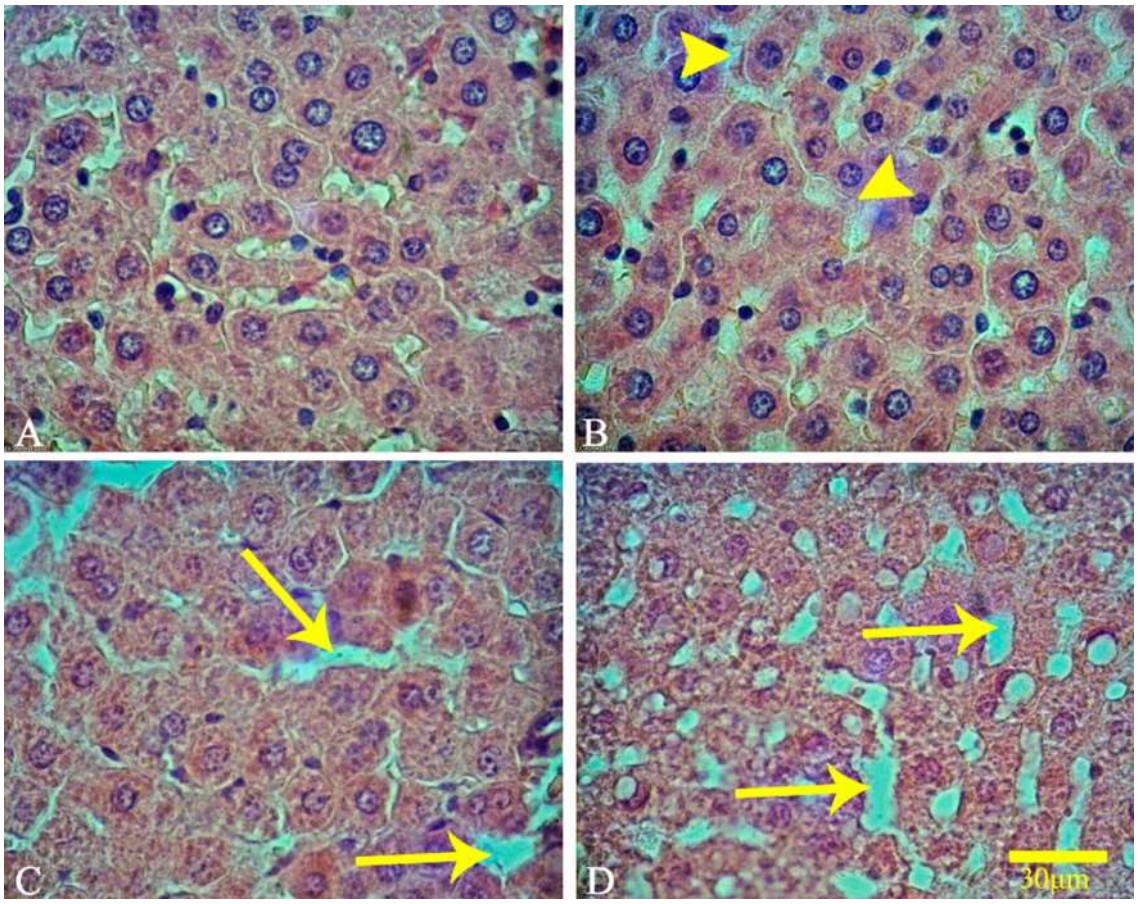

Figure 2. Optical photomicrograph of liver tissue of male rats born to mothers treated with Sertraline. A) In the control group, liver tissue has a normal structure, and healthy hepatocytes are observed. B) In the Exp1 group, very mild necrosis of liver tissue is observed. Damage and structural changes in liver tissue are minor (yellow arrows). C) In the Exp2 group, liver tissue necrosis is observed. Damage and structural changes in liver tissue are moderate (yellow arrows). D) In the Exp3 group, liver tissue necrosis is very severe and large vacuole spaces are observed between hepatocyte cells (yellow arrows). (E&H staining, 40X)

on the fetus. It seems that Sertraline can increase Serotonin (5-HT) receptors by altering Serotonin reuptake. 5-HT, as a messenger molecule, regulates many developmental events such as replication, migration, differentiation, and gene expression. Therefore, Sertraline seems to cause damage to various fetal tissues such as the liver by increasing Serotonin levels and increasing 5-HT (Wise et al. 2020). It has been suggested that some antidepressants, such as Sertraline and Fluoxetine, may inhibit P450 family enzymes, thereby increasing toxic metabolites in the liver. Therefore, it seems that the cause of liver necrosis is the increased serum levels of Alt, AST, Alp and Bili, as well as decreased Alb and TP in this study and the teratogenic and toxic effects of Sertraline on liver's development and function in male rats that is consistent with previous studies (Friedrich et al. 2016).

CONCLUSION

Dose-dependent administration of Sertraline in pregnant rats causes hepatic necrosis, increases serum levels of Alt, AST, Alp, and Bili, and decreases Alb and TP in their male neonates during 28 days. Sertraline at the maximum dose (20 mg/kg of body weight) has the most destructive effects on liver tissue. Therefore, it is recommended to use this drug with more caution during pregnancy.

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Book:

a. Lawrence TLJ, Fowler VR. 2002. Growth of farm animals. 2nd ed. New York (USA): CABI Publishing.
b. Bamualim A, Tiesnamurti B. 2009. Konsepsi sistem integrasi antara tanaman padi, sawit, dan kakao dengan ternak sapi di Indonesia. In: Fagi AM, Subandriyo, Rusastra IW, penyunting. Sistem integrasi ternak tanaman padi, sawit, kakao. Jakarta (Indones): LIPI Press. p. 1-14.
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Blanco EE, Meade JC, Richards WD. 1990. Ophthalmic Ventures, assignee. Surgical stapling system. United States patent US 4,969,591. 1990 Nov 13.

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