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## PREFACE

In this edition, volume 25 No 2, we proudly present articles from animal and veterinary sciences including genetics; reproduction, feed technology, and veterinary. The articles published in this edition are:

"Genetic Diversity of Mitochondrial DNA Cytochrome *b* in Indonesian Native and Local Cattle Populations"; "Non-Genetic and Genetic Effects on Growth Traits from Birth to 120 days of Age of G2 Sapera Goat"; "Efficiency of Dimethyl Sulphoxide and Ethylene Glycol on the Subsequent Development of Vitrified Awassi Sheep Embryos"; "Viability of Peranakan Etawah Liquid Semen Preserved in Tris Substituted with Various Energy Sources"; "Condition of Rumen Fermentation as Impacted by Supplementation of Fermented Rice Brand Using *In Vitro* Gas Production Technique"; "Biological Evaluation of Some Plant Bioactives as Feed Additive to Replace Antibiotic Growth Promoters in Broiler Feeds"; and "Effects of *Silybum marianum* Aqueous Extract and L-carnitine on Stereological Changes in Diazinon-Treated Rat Liver".

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, June 2020

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# Jurnal Ilmu Ternak dan Veteriner

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# Genetic Diversity of Mitochondrial DNA Cytochrome *b* in Indonesian Native and Local Cattle Populations

Prihandini PW, Primasari A, Luthfi M, Efendy J, Pamungkas D

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**ABSTRAK**

Prihandini PW, Primasari A, Luthfi M, Efendy J, Pamungkas D. 2020. Keragaman genetik sitokrom *b* mitokondria DNA pada populasi sapi asli dan lokal Indonesia. JITV 25(2):39-47. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2496

Informasi tentang keragaman genetik ternak asli dan lokal di Indonesia sangat penting dalam pengembangan strategi pemuliaan dan konservasi. Tujuan penelitian ini untuk mengetahui keragaman dan hubungan genetik beberapa populasi sapi asli (Bali) dan lokal Indonesia [(Donggala, Madura, Sragen, Galekan, Rambon, dan Peranakan Ongole Grade x Bali (POBA)]. DNA genom diekstraksi dari sampel darah (n= 75). Parsial sekuen mtDNA cyt *b* (464 bp) diamplifikasi menggunakan teknik *polymerase chain reaction* (primer *forward*: L14735 dan primer *reverse*: H15149). Tiga puluh empat referensi sekuen dari *Bos taurus*, *Bos indicus*, dan *Bos javanicus* digunakan untuk analisis filogenetik. Hasil penelitian, sebanyak 55 situs polimorfik dan 13 haplotipe tersebar di semua populasi, namun, variasi mtDNA cyt *b* tidak ditemukan di populasi Sapi Galekan yang dipelihara di *Beef Cattle Research Station* (BCRS) dan Sapi Rambon. Rataan *haplotype diversity* dan *nucleotide diversity* masing-masing adalah 0,515 ± 0,070 dan 0,0184 ± 0,0045. Jarak genetik tertinggi (0,092) dan terendah (0,000) masing-masing yaitu antara populasi Sapi Bali dan Sapi Donggala dan antara Sapi Galekan (BCRS), Rambon, dan POBA. Berdasarkan analisis mtDNA network dan filogeni, terdapat dua maternal lineage (A dan B) pada populasi sapi Indonesia yang diteliti. Sebagian besar individu (69,33%, tersebar dalam Haplotipe H8-H19) berada dalam lineage B, satu klaster dengan *Bos javanicus*. Disimpulkan bahwa populasi sapi asli dan lokal Indonesia memiliki keragaman genetik yang berbeda-beda; sebagian besar populasi sapi Indonesia memiliki maternal lineage dari *Bos javanicus*.

**Kata Kunci**: Sapi Indonesia, Sitokrom *b*, Keragaman Genetik, Filogeni Analisis

**ABSTRACT**

Prihandini PW, Primasari A, Luthfi M, Efendy J, Pamungkas D. 2020. Genetic diversity of mitochondrial dna cytochrome *b* in indonesian native and local cattle populations. JITV 25(2): 39-47. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2496

Information on the genetic diversity of native and local cattle in Indonesia is vital for the development of breeding and conservation strategies. This study was aimed to assess the genetic diversity and phylogenetic relationship of the Indonesian native (Bali) and local [(Donggala, Madura, Sragen, Galekan, Rambon, dan Peranakan Ongole Grade x Bali (POBA)] cattle populations. Genomic DNA was extracted from blood samples (n= 75). Partial sequences of mtDNA cyt *b*, 464 bp, were amplified using the polymerase chain reaction technique (forward primer: L14735 and reverse primer: H15149). Thirty-four reference sequences of *Bos taurus*, *Bos indicus*, and *Bos javanicus* were included in the phylogenetic analyses. A total of 55 polymorphic sites and 13 haplotypes were observed in the whole breeds. No variable sites of mtDNA cyt *b* were observed in Galekan (kept in BCRS) and Rambon cattle. Overall haplotype diversity and nucleotide diversity were 0.515 ± 0.070 and 0.0184 ± 0.0045, respectively. The highest (0.092) and the lowest (0.000) genetic distances were between Bali and Donggala cattle populations and among Galekan (kept in BCRS), Rambon, and POBA cattle populations, respectively. Both mtDNA network and phylogenetic analyses revealed two major maternal lineages (A and B) of the studied population. Most of the sampled individuals (69.33%, present in haplotype H8-H19) were linked to lineage B, which belonged to the same cluster with *Bos javanicus*. Overall, most of the Indonesian native and local cattle populations had a considerable genetic diversity and shared a common maternal origin with *Bos javanicus*.

**Key Words**: Indonesian Cattle, Cytochrome *b*, Genetic Diversity, Phylogenetic Analysis

## INTRODUCTION

Cattle is one of the most important livestock commodities for Indonesian livestock farmers since they are mostly relying on cattle for their income. To date, several cattle, such as Sumba Ongole, Ongole Grade, Jabres, Sumbawa, Pesisir, Aceh, and Madura have been identified as local cattle in Indonesia, while Bali cattle is the only native cattle breed in the country (Directorate of Livestock Breeding and Production 2020). Although some above-mentioned breeds have been well studied using microsatellite markers (Agung

et al. 2019), there is still a lack of information focused on other cattle breeds, such as Rambon, Galekan, Donggala, and Sragen. As a part of animal genetic resources, it is well known that native cattle possess a considerable number of desirable traits, such as the ability to cope with hot weather environment, low quality of forage, resistance to the internal parasite, and infectious diseases. Therefore, they have a wide variation in morphological and physiological characteristics. Those variations are important in livestock populations to meet current production and future requirements in various environments and changing of objectives.

Recently, a lack of development of native cattle breeds and the introduction of exotic breeds has threatened the genetic diversity of native cattle breeds (Sutarno & Setyawan 2016). Loss of genetic diversity within the breed and genetic erosion are major threats. Besides, genetic resources of locally adapted breeds with their unique characteristics have mostly been neglected. In this respect, it is now understood that it is important to establish conservation strategies to conserve the genetic diversity within and between breeds, especially prevent further losses of biodiversity. However, a lack of sufficient information regarding genetic resources of native cattle, including their current genetic diversity, rate of inbreeding, and genetic blood-mixture leads to the difficulty of making effective conservation strategies. Therefore, providing genetic information of native breeds is necessary for future conservation and breeding strategies.

Up to present, among many molecular markers available, mitochondrial DNA (mtDNA) has been widely employed to predict the genetic diversity and phylogenetic relationship in cattle (Sharma et al. 2015; Hartatik et al. 2019; Xia et al. 2019; Tarekegn et al. 2019; Yan et al. 2019). Unlike genomic DNA, mtDNA is characterized by a lack of recombination, maternal inheritance, and has a simple sequence organization (Harrison 1989). The mutation rate in mtDNA is much more frequent than in the nuclear gene, due to the absence of introns and its efficient repair mechanisms (Andalib et al. 2017). Cytochrome *b* (cyt *b*) is an mtDNA gene, which is widely used for phylogenetic relationship determination in domestic animals, due to its sequence variability and high evolutionary rate (Othman et al. 2017; Tarekegn et al. 2018; Hartatik et al. 2019; Rahmatullaili et al. 2019). Furthermore, mtDNA cyt *b* is a member of the protein-coding genes that has abundant phylogenetic information intraspecies and interspecies and higher variation ratio compared to other functional genes (Browers et al. 1994; Çiftci et al. 2013). Hence, mtDNA cyt *b* is considered to be useful for the determination of genetic diversity and phylogenetic relationships.

Considering the above points, we, therefore, explored the mtDNA cyt *b* to assess the genetic diversity and phylogenetic relationships of the Indonesian native and local cattle populations. This would provide basic data for future conservation and breeding strategies of Indonesian native cattle.

## MATERIALS AND METHODS

### Blood sample collection and DNA extraction

A total of 75 blood samples representing Indonesian native (Bali) and local (Donggala, Madura, Sragen, Galekan, Rambon, and Peranakan Ongole Grade x Bali crossbred) cattle populations were collected. Donggala (as DG; n= 5) cattle samples were collected from Donggala regency of Central Sulawesi province; Madura (as MD; n= 5) cattle samples came from Pamekasan regency of East Java province, Sragen (as SR; n= 9) cattle samples were obtained from Sragen regency of Central Java province; Galekan cattle samples (n= 15) were collected from two different sites including the Beef Cattle Research Station (BCRS) (those kept in BCRS, as TL) and Unit Pelaksana Tugas Daerah (UPTD) of Trenggalek regency of East Java province (those kept in this region, as TU); Bali (as BL; n= 5) and Peranakan Ongole Grade x Bali (as POBA; n= 24) cattle samples were also collected from BCRS; and Rambon (as RM; n= 12) cattle samples were obtained from Banyuwangi regency of East Java province. The genomic DNA was extracted from blood samples using gSYNC™ DNA extraction kit (Geneaid, New Taipei City, Taiwan) and stored at –20°C before further analysis.

### PCR amplification and sequencing

A fragment of 464 bp from the partial mtDNA cyt *b* sequences was amplified using polymerase chain reaction (PCR). The primers used were PR-L14735 (5'-AAA AAC CAC CGT TGT TAT TCA ACT -3') and PF- H15149 (5'- GCC CCT CAG AAT GAT ATT TGT CCT CA -3') (Wolf et al. 1999). The PCR reaction was performed using Sensoquest (Germany) and made up of 2 µl of template DNA (10-100 ng), 0.5 µl of each primer (0.25 µM), 12.5 µl PCR KIT (2x My Taq HS Red Mix gSYNCTMPCR Kit-Bioline-London) and 9.5 µl dd$H_2O$ to make a total volume of 25 µl. The thermal cycling included an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec, with a final extension step at 72°C for 10 min. The PCR products were sequenced using ABI 3730xl genetic analyzer (Applied Biosystems, Foster City, CA, USA).

**Data analysis**

The mtDNA cyt *b* gene sequences from 75 individuals of Indonesian native and local cattle were edited using BioEdit software (Hall 1999) and aligned using the ClustalW (Thompson et al. 1994). The mtDNA cyt *b* diversity measures, such as the number of polymorphic sites (S), nucleotide differences (K), haplotype diversity (Hd), and nucleotide diversity ($\pi$) were calculated using DnaSP version 6.12.01 software (Rozas et al. 2017). Genetic distances based on the Kimura two-parameter model algorithm were estimated using MEGA version 5.0 software (Kumar et al. 2016) and the resulted distance matrices were used to construct a neighbor-joining (NJ) tree with 1000 bootstrapping replicates using the same software of MEGA version 5.0 (Kumar et al. 2016). A median-joining network analysis was performed using NETWORK version 5.0.1.1 software (Bandelt et al. 1999). In this study, thirty-four sequences of *Bos taurus* (GenBank Accession No.: V00654; GQ129207; GQ129208; AY676860; AY676861; AY526085; AY885283; AF492351; EF693798; EU177834; EU177847; EU177852; EU177862; EU177867; EU747736; DQ124389; DQ186203; AY676866; DQ124413), *Bos indicus* (GenBank Accession No.: AF419237; AF492350; AF531473; AY126697; EU096517; EU096518; NC_005971; AY689190; EU096519), and *Bos javanicus* (GenBank Accession No.: D34636; D82889; AY689188; EF197952; DQ459558; DQ459559) from Asian, Europan, and American cattle, and Javan banteng were included in the phylogenetic network analysis.

## RESULTS AND DISCUSSION

**Results**

***mtDNA sequence variation and genetic diversity***

The partial sequences of the mtDNA cyt *b* gene, 464 bp in length, were successfully sequenced for 75 samples representing Indonesian native and local cattle breeds in Indonesia. As shown in Table 1, a total of 55 polymorphic sites and 13 haplotypes were observed in the whole breeds. Bali cattle had the highest number of polymorphic sites (S= 31), while Galekan cattle kept on BCRS and Rambon cattle populations had no polymorphic sites observed (S= 0). The number of haplotypes ranged from 1 (TL and RM) to 5 (TU). The haplotype diversity varied from 0.000 ± 0.000 (RM and TL) to 0.900 ± 0.161 (MD and BL), with an overall Hd value of 0.515 ± 0.070. The nucleotide diversity also varied from 0.000 ± 0.000 (RM and TL) to 0.0579 ± 0.0131 (MD). The overall nucleotide diversity among populations was 0.0184 ± 0.0045 (Table 1).

To elucidate the introgression of the exotic breeds in the studied populations, thirty-four mtDNA cyt *b* gene sequences of *Bos taurus*, *Bos indicus*, and *Bos javanicus* available in GenBank database were included in the haplotype analysis. Of the nineteen haplotypes detected, only six haplotypes (H1, H4, H8, H10, H12, and H13) were shared by more than one population (Table 2). Haplotype H8, present in 52 sequences (69.33%) out of 75 samples of the Indonesian native and local cattle populations and in 3 Javan banteng sequences was found to be the most frequent haplotype. Most of the remaining haplotypes, except H1 and H4, were present in four or fewer samples.

***Genetic distances and phylogenetic analysis***

Pairwise genetic distances among Indonesian native and local cattle populations estimated using the Kimura two-parameter model algorithm are shown in Table 3. The highest genetic distance (0.092) was observed between Bali and Donggala cattle populations, while the lowest genetic distances (0.000) were observed among Galekan cattle kept on BCRS, Rambon, and POBA cattle populations. To determine the phylogenetic network of the Indonesian cattle populations, 19 haplotypes (Table 2) were used to construct the median-joining network (Figure 1). All the haplotypes were grouped into two main lineages (A and B) and most of the Indonesian cattle haplotypes (H8-H19) were distributed in lineage B. Of the 75 individuals sampled, 68 samples (90.67%) were present in lineage B, while only few samples (n= 7, 9.33%) were linked to lineage A.

To confirm the MJ network results, a neighbor-joining (NJ) tree as indicated from the distance matrices was constructed (Figure 2). The results showed that Indonesian native and local cattle were grouped into two major lineages (A and B). Consistent with this result, when 34 reference sequences of the mtDNA gene from the GenBank database were included in the phylogenetic analysis for comparison, it showed that the Indonesian native and local cattle populations were clustered into two major lineages (A and B) (Figure 3). Lineage A was made up of all the cited *Bos taurus* and *Bos indicus* sequences and few sequences of Sragen, Madura, Galekan (at UPTD), and Donggala cattle populations. Interestingly, most sequences of the Indonesian cattle populations (TL, BL, RM, and POBA) were only clustered in lineage B, along with the cited Javan banteng sequences.

**Discussion**

***mtDNA sequence variation and genetic diversity***

Genetic diversity is basic source and a pivotal tool for future genetic improvement and selection programs in livestock populations. Considering this fact, partial sequences of mtDNA cyt *b* (464 bp) from 75 individuals of Indonesian native and local cattle populations were

**Table 1.** Genetic diversity of Indonesian native and local cattle populations based on mtDNA cyt *b* gene partial sequences

| Population | N | S | H | K | Hd | π |
|---|---|---|---|---|---|---|
| Donggala | 5 | 23 | 2 | 9.200 | 0.400 ± 0.237 | 0.0279 ± 0.0165 |
| Madura | 5 | 27 | 4 | 15.800 | 0.900 ± 0.161 | 0.0579 ± 0.0131 |
| Sragen | 9 | 26 | 3 | 6.056 | 0.639 ± 0.126 | 0.0184 ± 0.0125 |
| Galekan (BCRS) | 5 | 0 | 1 | 0.000 | 0.000 ± 0.000 | 0.0000 ± 0.0000 |
| Galekan (UPTD) | 10 | 29 | 5 | 5.956 | 0.667 ± 0.163 | 0.0181 ± 0.0119 |
| Bali | 5 | 31 | 4 | 13.800 | 0.900 ± 0.161 | 0.0437 ± 0.0146 |
| Rambon | 12 | 0 | 1 | 0.000 | 0.000 ± 0.000 | 0.0000 ± 0.0000 |
| POBA | 24 | 2 | 3 | 0.167 | 0.163 ± 0.099 | 0.0005 ± 0.0003 |
| Overall | 75 | 55 | 13 | 5.811 | 0.515 ± 0.070 | 0.0184 ± 0.0045 |

POBA= Peranakan Ongole Grade x Bali crossbreed, BCRS= Beef Cattle Research Station
UPTD=Unit Pelaksana Teknis Daerah of Trenggalek regency
N= number of samples; S= segregating sites; H= number of haplotypes; K= nucleotide differences;
Hd= haplotype diversity; π= nucleotide diversity

**Table 2**. Haplotypes shared among Indoesian native and local cattle populations and reference breeds from *Bos taurus*, *Bos indicus* and *Bos javanicus*

| Haplotype | No. of samples | Population (No. of samples within population) |
|---|---|---|
| H1 | 19 | *Bos taurus* (15), DG (4) |
| H2 | 1 | *Bos taurus* (1) |
| H3 | 1 | *Bos taurus* (1) |
| H4 | 10 | *Bos indicus* (7), MD (2), SR (1) |
| H5 | 1 | *Bos indicus* (1) |
| H6 | 1 | *Bos indicus* (1) |
| H7 | 2 | *Bos taurus* (2) |
| H8 | 55 | *Bos javanicus* (3), DG (1), MD (1), SR (5), TL (5), TU (6), RM (12), POBA (22) |
| H9 | 3 | *Bos javanicus* (3) |
| H10 | 2 | MD (1), TU (1) |
| H11 | 1 | MD (1) |
| H12 | 4 | SR (3), BL (1) |
| H13 | 3 | TU (1), BL (2) |
| H14 | 1 | TU (1) |
| H15 | 1 | TU (1) |
| H16 | 1 | BL (1) |
| H17 | 1 | BL (1) |
| H18 | 1 | POBA (1) |
| H19 | 1 | POBA (1) |

DG=Gonggala, MD=Madura, SR=Sragen, TL= Galekan (kept at the Beef Cattle Research Station), TU= Galekan (kept at the Unit Pelaksana Teknis Daerah of Trenggalek regrecy), RM= Rambon, POBA= Peranakan Ongole Grade x Bali crossbreed, BL= Bali

**Table 3**. Genetic distances among Indonesian native and local cattle populations based on mtDNA cyt *b* gene partial sequences

| Population | Sragen | Galekan (BCRS) | Galekan (UPTD) | Bali | Rambon | POBA | Donggala | Madura |
|---|---|---|---|---|---|---|---|---|
| Sragen | - | | | | | | | |
| Galekan (BCRS) | 0.012 | - | | | | | | |
| Galekan (UPTD) | 0.021 | 0.012 | - | | | | | |
| Bali | 0.029 | 0.019 | 0.029 | - | | | | |
| Rambon | 0.012 | 0.000 | 0.012 | 0.019 | - | | | |
| POBA | 0.012 | 0.000 | 0.012 | 0.019 | 0.000 | - | | |
| Donggala | 0.074 | 0.079 | 0.076 | 0.092 | 0.079 | 0.079 | - | |
| Madura | 0.046 | 0.043 | 0.047 | 0.058 | 0.043 | 0.043 | 0.062 | - |

POBA= Peranakan Ongole Grade x Bali crossbreed, BCRS= Beef Cattle Research Station, UPTD=Unit Pelaksana Teknis Daerah of Trenggalek regency

sequenced to determine their diversity. As a result, a wide range of genetic diversity, from low (Hd ≤0.163 in TU; RM; and POBA) to high (Hd ≥0.900 in MD and BL), was observed. Compared to previous studies, the genetic diversities observed in this study (S= 55; H= 13; Hd= 0.515; $\pi$ = 0.0184) were much higher than those observed in Pasundan (S=1; H= 2; Hd= 0.1818; $\pi$= 0.00045) and Pacitan (S= 2; H= 3; Hd= 0.3778; $\pi$ = 0.00099) cattle of Indonesia (Hartatik et al. 2019), in Chikso cattle of Korea (S= 15; H=13; Hd= 0.4709; $\pi$= 0.00055) (Kim et al. 2013), and in Ethiopian cattle (S= 16; $\pi$ = 0.0010) (Tarekegn et al. 2018) estimated using the same markers, but lower than those observed in Chinese cattle (S= 78; H= 40; Hd= 0.903) analyzed using mtDNA 16S rRNA gene (Yan et al. 2019). A considerable genetic diversity observed in Indonesian native and local cattle populations indicated a lack of artificial selection pressure. Another reason for increased genetic diversity could be the introgression of several exotic breeds leading to genetic admixture, as proposed by Decker et al. (2014). This was reasonable because most of the Indonesian native and local cattle came from multiple maternal origins, such as *Bos taurus*, *Bos indicus*, and *Bos javanicus* (Pamungkas et al. 2012; Sutarno and Setyawan 2016). Although a moderate genetic diversity was observed in the whole breed, however, we did not observe any variable sites within partial sequences of mtDNA cyt *b* gene in Galekan cattle kept at BCRS and Rambon cattle, while very few polymorphic sites (S= 2) were detected in POBA cattle population. These could be caused by the following reasons: a lack of heterozygous individuals present in these populations that might due to selection favoring homozygotes in multiple loci, and sampling bias, of which most of the animals sampled in each population could be collected from similar haplotype origin, and as a consequence, very few numbers of haplotypes were observed in the cyt *b* region (H= 1 in TL, H=1 in RM, and H= 3 in POBA).

Contrarily, Bali and Madura cattle populations represented a high magnitude of mtDNA cyt *b* gene diversity. The remaining cattle populations (DG, SR, and TU), however, still represent a considerable genetic diversity. The high genetic diversity in Bali and Madura cattle, as indicated by the number of segregating sites (S= 31 in BL; S= 29 in MD), might indicate a high mutation rate of the mtDNA cyt *b* occurred in both populations. Similarly, Rahmatullaili et al. (2019) observed high nucleotide substitution in mtDNA cyt *b* in Bali cattle leading to high genetic diversity within the population. Furthermore, a high mutation in mammalian mtDNA is due to replication errors, the poor fidelity of DNA polymerase, and the ROS-saturated environment present within mitochondrion (Li et al. 2019). As indicated by the genetic diversity measures, Bali and Madura cattle populations had not only the highest nucleotide variation but also the highest haplotype and nucleotide diversity. Likewise, a high genetic diversity (S= 118; $\pi$ = 0.0250) was also found in Bali cattle based on mtDNA d-loop sequence analysis (Jakaria et al. 2019). Although a moderate level of genetic diversity was present in Bali cattle based on ETH10 microsatellite marker, the occurrence of inbreeding was observed (Margawati et al. 2018). A wide range of genetic diversities among the native cattle of Indonesia, especially in Bali and Madura cattle populations, however, could be valuable for future genetic improvement and selection of superior animals for economic traits.

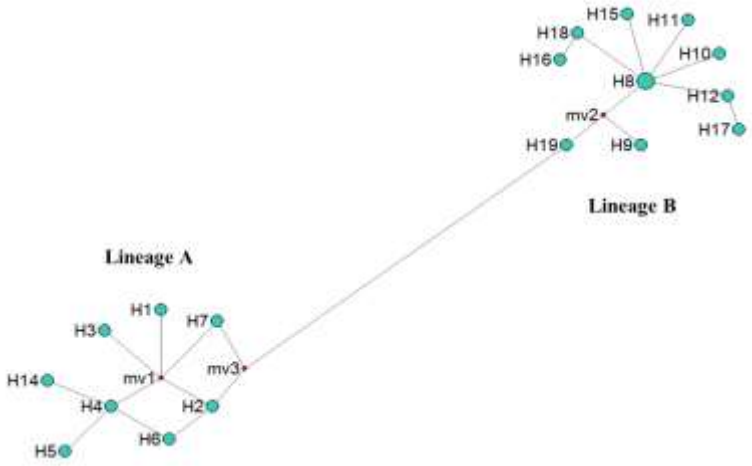


**Figure 1.** Median-joining network of 19 haplotypes based on cyt *b* gene partial sequences (circle areas proportional to sample sizes)

***Phylogenetic tree***

A median-joining network based on the 19 haplotypes observed in this study was constructed to reveal the phylogenetic relationship among the cattle populations (Figure 1). All these haplotypes were grouped into two-major lineages (A and B). Of the 19 haplotypes observed, 11 haplotypes from the Indonesian native and local cattle populations were present in lineage B, along with Javan banteng haplotypes. Besides, Haplotype H8 represented the maternal origin of most of the Indonesian native and local cattle since most of the sampled animals (69.33%) were linked to this haplotype. Only a few haplotypes from the studied populations were linked to lineage A, indicating that *Bos taurus* and *Bos indicus* maternal origins were little interfered with in our studied population. However, 75% of individuals of Donggala cattle were linked to this lineage and grouped in haplotype H1 along with 15 reference breeds of *Bos taurus*, indicating the introgression of *Bos taurus* in this population. Most of *Bos taurus* cattle in Indonesia came from Holstein Friesian (FH) breed (Sutarno and Setyawan 2016), but Limousin and Simmental cattle breeds have also been widely crossed with local breeds (Pamungkas et al. 2012). Therefore, a further comprehensive study should be addressed towards the genetic background of this breed. Since no works of literature are available regarding the genetic background of Donggala cattle so far, this study provides important information that could be valuable for future investigation of this animal genetic resource.

To reveal a more detailed summary regarding the genetic relationships among populations, two NJ trees (Figures 2 and 3) were constructed which supported the results of MJ network analysis. From two lineages observed, lineage A was more specific to *Bos taurus* and *Bos indicus* maternal origins, and very few samples of the studied populations (DG, MD, SR, and TU) were present in this lineage. Lineage B were distributed ubiquitously in the Indonesian cattle population (BL, RM, POBA, TL). This indicated a close genetic relationship among Indonesian cattle populations and higher levels of *Bos javanicus* ancestry in the studied breeds rather than *Bos taurus* and *Bos indicus* ancestries. Similarly, previous studies demonstrated a close genetic relationship between Indonesian cattle (Pasundan and Pacitan) since these breeds have a similar mtDNA maternal origin from *Bos javanicus* (Hartatik et al. 2019), and among Madura, Pasundan, and Pesisir cattle of Indonesia based on microsatellite analysis (Agung et al. 2019). Besides, *Bos javanicus* introgression has also been detected in Indonesian cattle, including in Madura and Galekan cattle tested using mitochondrial and Y-chromosomal analysis (Mohamad et al. 2012), in Aceh cattle detected using mtDNA d-loop sequences (Sari et al. 2016), in Bali, Java, and Limura (Limousin x Madura) cattle (Hartatik et al. 2015), and in Kebumen Ongole Grade cattle using mtDNA cyt *b* (Hartatik et al. 2018). Furthermore, Bali and Madura cattle have a close genetic relationship based on Y-chromosomal microsatellite marker analysis (Winaya et al. 2015). Based on mtDNA and SRY gene analysis, a similar kind of banteng introgression in Bali cattle (124 out of 125 sampled individuals) had been reported, while the remaining one had zebu origin (Mohamad et al. 2012). Using genotypes from 43,043 autosomal single nucleotide polymorphism markers, Decker et al. (2014) also observed banteng introgression in Indonesian cattle breeds (Brebes, Madura, Pesisir, and Aceh) and Chinese Hainan and Luxi cattle. Zebu's introgression into Indonesian native cattle breeds (Aceh, Pesisir, Madura, Brebes) had been reported as well (Gao et al. 2017).

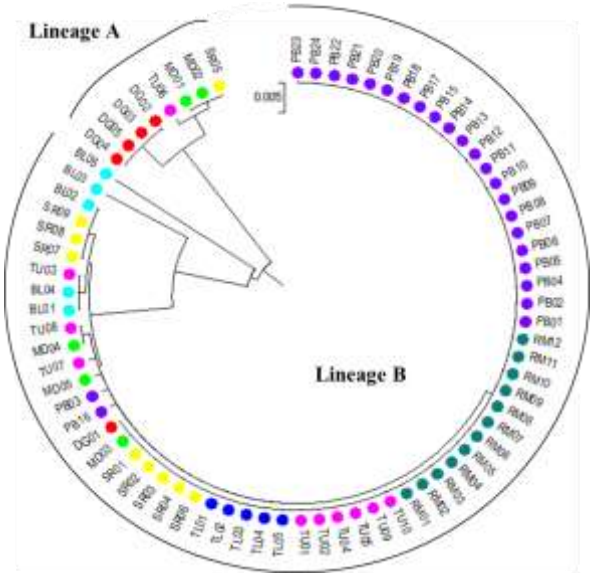


**Figure 2.** A neighbor-joining tree of Indonesian cattle populations tested based on cyt *b* gene partial sequences

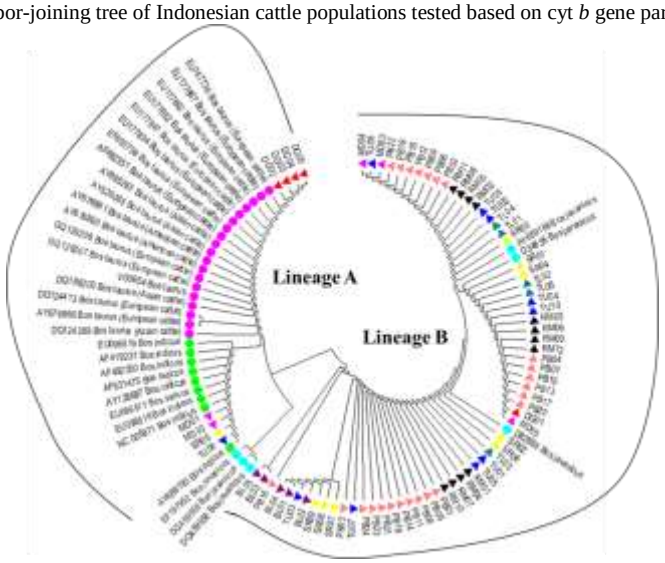


**Figure 3.** A neighbor-joining tree of 75 tested cattle and reference sequences (*Bos taurus*, *Bos indicus* and *Bos javanicus*)

Molecular phylogeny using mtDNA and SRY sequences clearly showed banteng-zebu type in Indonesian cattle, yak-zebu type in Nepal cattle, taurine type in ishima, Mongolian, Korean, and Chinese Yellow cattle, and zebu type in Sri Lanka cattle (Kikkawa et al. 2003). Asian domestic cattle like in Indonesia, however, may be hybrids and came from hybridization between multiple species from *Bos taurus*, *Bos indicus*, *Bos javanicus*, and *Bos grunniens* (Kikkawa et al. 2003; Jia et al. 2010).

Results of the present study and previous studies highlighted a considerable proportion of Javan banteng ancestry in most of the Indonesian cattle. However, since the introduction of exotic breeds from *Bos taurus* (Holstein Friesian, Simental, and Limousin) as well Indian zebu cattle (Ongole breed) are continuously increasing, the blood proportion of the Indonesian cattle might change in future. Citing data from some previous studies, some Indonesian cattle breeds came from *Bos indicus* and *Bos javanicus* as well as their crosses. For instance, Rambon cattle are derived from *Bos indicus* x *Bos javanicus*; Madura cattle are a crossbreed between Balinese cattle (*Bos javanicus*) and zebu cattle (*Bos indicus*); Bali cattle are directly domesticated from wild banteng (*Bos javanicus*); and Aceh cattle are derived from the crossing between Ongole (*Bos javanicus*) and Bali (*Bos javanicus*) cattle (Mohamad et al. 2012; Sari et al. 2016; Sutarno et al. 2015; Sutarno & Setyawan

2016; Hartatik et al. 2019). In general, the results of the phylogenetic analysis obtained from this study were consistent with those of previous studies.

## CONCLUSION

In this work, we demonstrated different diversities of mtDNA cyt *b* gene within each population of Indonesian native and local cattle breeds, ranging from very low (TU; RM; and POBA) to high (MD and BL). The phylogenetic analysis revealed a quite close genetic relationship among the selected Indonesian cattle populations, which mostly belonged to *Bos javanicus* maternal origin. Finally, this study provided important data for future utilization of Indonesian cattle breeds and could be valuable to define breeding strategies.

## ACKNOWLEDGEMENT

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# Non-Genetic and Genetic Effects on Growth Traits from Birth to 120 days of Age of $G_2$ Sapera Goat

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**ABSTRAK**

Anggraeni A, Saputra F, Hafid A, Ishak ABL. 2020. Pengaruh non-genetik dan genetik sifat pertumbuhan pada saat lahir hingga umur 120 hari dari kambing $G_2$ Sapera. JITV 25(2):48-59. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2498

Informasi ragam genetik dan lingkungan diperlukan dalam program seleksi. Balai Penelitain Ternak (Balitnak) saat ini sedang melakukan seleksi awal pada sifat pertumbuhan dari kambing Sapera (50% Saanen, 50% PE). Studi ini bertujuan untuk mengetahui pengaruh non-genetik dan genetik terhadap sifat pertumbuhan sejak umur lahir – 120 hari dari anak-anak kambing $G_2$ Sapera. Data bobot badan dan sejumlah ukuran tubuh diperoleh dari anak-anak umur lahir (105 ekor) sampai 120 hari (51 ekor). Data pertumbuhan setiap interval umur 30 hari dihitung secara interpolasi linier. Kontribusi dari pengaruh non-genetik dianalisa menerapkan Model Linier Umum untuk data tidak berimbang dengan pengaruh tetap mempertimbangkan jenis kelamin, tipe kelahiran, bulan- dan tahun lahir. Pengaruh genetik menerapkan model linier campuran mempertimbangkan pejantan sebagai variabel acak. Nilai heritabilitas dihitung dengan metode *paternal halfsib*. Faktor non-genetik sebagian besar tidak berpengaruh secara nyata (P>0,05) terhadap bobot badan dan ukuran tubuh pada kisaran umur pengamatan. Bobot umur 90 hari dan 120 hari dari anak jantan secara nyata lebih tinggi daripada anak betina (P<0,05). Tipe dan tahun lahir berpengaruh nyata (P<0.05) terhadap bobot badan dan beberapa ukuran tubuh pada umur tertentu. Namun bulan lahir tidak ada yang berpengaruh nyata (P>0,05) pada sifat pertumbuhan. Ragam genetik ($h^2$) dari bobot badan dan ukuran tubuh relatif rendah, yaitu $h^2$ bobot badan = 0,11-0,19 dan $h^2$ ukuran tubuh = 0,03-0,24. Pengecualian untuk bobot badan umur lahir dan 30 hari ($h^2$ = 0,59 dan 0.29), serta pada umur 30 hari dan 60 hari untuk lingkar panggul ($h^2$ = 0,13-0,54). Sifat pertumbuhan anak kambing $G_2$ Sapera dipengaruhi jenis kelamin dan tahun lahir tetapi tidak banyak dipengaruhi genetik pejantan.

**Kata Kunci**: Kambing Perah, Pertumbuhan, Genetik, Non Genetik

**ABSTRACT**

Anggraeni A, Saputra F, Hafid A, Ishak ABL. 2020. Non-genetic and genetic effects on the growth traits from birth to 120 days of age of $G_2$ Sapera goat. JITV 25(2):48-59. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2498

Information on non-genetic and genetic factors is required in the selection program. Indonesian Research Institute for Animal Production (IRIAP) has been conducting a selection of the growth traits of Sapera goat (50% Saanen, 50% PE). This research was aimed to study non-genetic and genetic effects on growth traits from birth to the age of 120 days old of the 2nd generation ($G_2$) of Sapera goat. Data on body weight and measurement were collected from kids at birth (105 head.) to the age of 120 days old (51 head). The 30 days interval growth data were calculated by linear interpolation. Non-genetic effects were analyzed by General Linear Model for unbalanced data by considering sex, type of birth, the month of kidding, and year of kidding as fixed variables. The genetic component was analyzed by a mixed linear model by considering sire as a random variable. Heritability was estimated by the paternal half-sib method. Non-genetic factors mostly had no significant effect (P> 0.05) on body weight and measurement. The 90 days old and 120 days old males had higher weights than females (P<0.05). Birth type and year of kidding had significant effects (P<0.05) on body weight and some measurements at certain ages. No significant months of kidding effect on the growth traits (P>0.05). Heritability values of body weight ($h^2$ = 0.11-0.19) and body sizes ($h^2$ = 0.03-0.24) were relatively low. Except high heritability values for birth weight and for body weight at 30 days old ($h^2$ = 0.59 and 0.29), and for hip girth at 30 days old and at 60 days old ($h^2$ = 0.13-0.54). The growth traits of $G_2$ Sapera kids were affected by sex and year of kidding and slightly influenced by genetic (sires) factors.

**Key Words**: Dairy Goat, Growth, Genetic, Non-Genetic

## INTRODUCTION

Dairy goat agribusinesses show a positive trend, so intensive dairy goat development has been growing especially in some locations in Java Island. To support this intensive dairy goat development, the availability of breeding stocks for possessing high genetic potency of milk production, and an adaptive tropical climate is required. This is, one of the other ways, attempted by crossing local female goats to male dairy goats from

exotic breeds (Devendra 2012); Anggraeni & Praharani 2017; Josiane et al. 2020). Crossbreeding of the local PE or Peranakan Etawah female to Saanen male was done by Indonesian Research Institute for Animal Production (IRIAP) for the expectation of resulting complementary effect of high milk production from the Saanen breed and a good tropical adaptation from PE breed (Anggraeni & Praharani 2017; Anggraeni et al. 2020). Genetic improvement by crossing should be followed by selection activities to gather the superiority of both traits passed through to their offspring. Estimation of non-genetic and genetic factors related to growth trait is needed to develop a proper selection program and to achieve a good response of selection in dairy breeding program (Gholizadeh et al. 2010; Caro-Petrovic et al. 2012; Kuthu et al. 2017; Josiane et al. 2020).

The potency of milk production of a dairy goat can be seen earlier from the growth traits of the kid such as body weight and body measurements (Waheed & Khan 2011; Kuthu et al. 2017; Anggraeni et al. 2020). Phenotypes of growth are the expression of genetic, environment, and interaction of both (Kuthu et al. 2017; Selvam 2018; Josiane et al. 2020). Animals in high growth potency will be more tolerant in less suitable environmental conditions compared to the low growth ones (Přibyl et al. 2008). Some non-genetic factor had a significant effect on body weight at earlier ages such as sex, type of birth, seasons of birth, and year of birth (Bharathidhasan et al. 2009; Mabrouk et al. 2010; Caro-Petrovic et al. 2012; Supakorn & Pralomkarn 2012; Kaunang et al. 2013; Kugonza et al. 2014; Dudhe et al. 2015; Josiane et al. 2020; Mohammed et al. 2018). Male usually expressed higher body weight and body size than the female kid. Some studies reported the differences in birth weight and weaning weight of both sexes around 5.0-12.2% and 6.0-20.0% (Bharathidhasan et al. 2009; Mabrouk et al. 2010; Caro-Petrovic et al. 2012; Supakorn & Pralomkarn 2012; Kugonza et al. 2014; Josiane et al. 2020). Whereas single birth kid usually expressed higher body weight and growth rate against twin and triplet kids (Bharathidhasan et al. 2009; Mabrouk et al. 2010, Supakorn & Pralomkarn 2012; Dudhe et al. 2015; Josiane et al. 2020; Mohammed et al. 2018). Interaction between kidding season and year of kidding can also affect the body weights and growth rate of a young goat (Bharathidhasan et al. 2009; Supakorn & Pralomkarn 2012; Caro-Petrovic et al. 2012; Dudhe et al. 2015; Selvam 2018; Mohammed et al. 2018).

Genetic variances are important to know the strengthening of a trait to be inherited to offspring. Estimation of genetic parameter becomes useful information to predict the effectiveness of the selection method and to obtain selection responses in achieving genetic improvement (Kuthu et al. 2017; Rout et al. 2018; Josiane et al. 2020). Heritability values as an indicator of additive genetic variability of birth weight and weaning weight at the earlier age of goat were reported quite varied from low to high. Estimated heritability resulted from some models of analyses from some goat breeds were reported for birth weight by $h^2$ = 0.11-0.41 and weaning weight by $h^2$ = 0.110.43 (Caro-Petrovic et al. 2012; Supakorn & Pralomkarn 2012; Rout et al. 2018; Josiane et al. 2020). Body dimension that reflects the growth of body skeleton can be another indicator to do an initial selection on milk production in dairy goat (Waheed & Khan 2011; Anggraeni et al. 2020). Heritability values of the morphometrics from various goat breeds were reported from low to high (Waheed & Khan 2011; Josiane et al. 2020). Heritability values at 3 mo. interval age from birth to one year old of some goat breeds were reported from low to medium, successively chest girth by $h^2$ = 0.16 (0.09-0.24), body length by $h^2$ = 0.05 (0.0003-0.11), and wither height by $h^2$ = 0.13 (0.07-0.21) (Josiane et al. 2020).

Information on non-genetic and genetic factors that affect the growth trait as an early indicator in selecting milk production of $G_2$ Sapera goat was necessary. The purpose of this study was to examine the effect of non-genetic and genetic factors on the growth trait providing body weight and body measurement from birth to 120 days of age of $G_2$ Sapera goat at IRIAP dairy goat station in Ciawi, Bogor, West Java.

## MATERIALS AND METHODS

### Location

This research was conducted at the dairy goat station of the Indonesian Research Institute for Animal Production (IRIAP), Ciawi Subdistrict, Bogor District, West Java. The IRIAP was located in an area of about 23 Ha in Banjar Waru Village, Ciawi Subdistrict, Bogor Regency, at the altitudes of 450 to 500 m asl with rainfall between 3,500 to 4,000 mm per year.

### Materials

In this study, the 2$^{nd}$ generation ($G_2$) Sapera goat (50% Saanen, 50% PE) was used for a total number of 105 kids consisting of 47 males and 58 females during the year of birth of 2018 and 2019. The number of observed kids: at birth, 30 days, 60 days, 90 days (weaning age), and 120 days were successively 105 head, 105 head, 104 head, 104 head., and 51 head. These kids were the offsprings of the $G_1$ Sapera parents

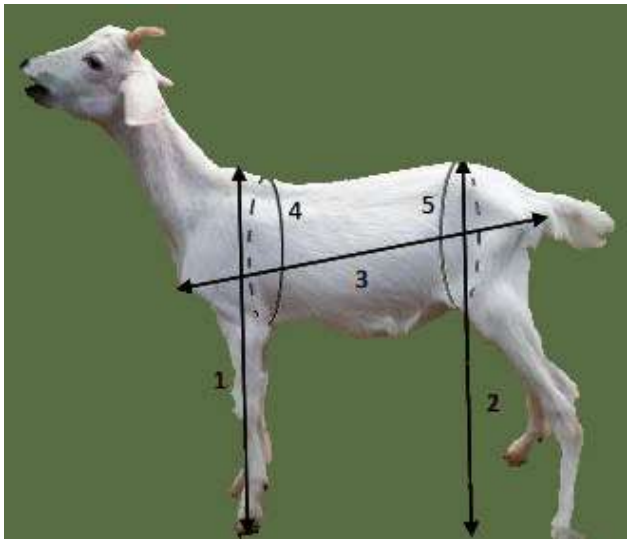


Figure 1. Body measurement of 2nd generation Sapera Goat: 1= shoulder height (SH), 2= hip height (HH), 3= body length (BL), 4= chest girth (CG), 5= hip girth (HG)

(9 bucks, 46 does). The $G_1$ Sapera females were previously synchronized by PGF2α hormone. Mating was done naturally in the flocks with a ratio of a buck to does by 1:5-10. The birth occurred during February, March, and April in 2018 and 2019. However, birth in March was not too many.

**Management**

Kids after one day of birth were kept separately from the mothers in kid colony cages. Colostrum was given during the first four days after birth. Then milk was given by bottle twice a day. Kids at 2 weeks old were feed by legume of *Calliandra*, as well as a small number of concentrates. Weaning kids were feed by grasses ad-libitum and concentrate around 0.3 kg per day. Post-weaning goats were fed by grasses around 1.4 kg and concentrate for 0.4 kg per day. Male and female kids were kept in the same pen from birth until the weaning age, then female kids were kept separately to males about 6-10 heads per cage.

**Variables**

Data of body weight (kg) were weighed using a sitting scale at an accuracy level of 0.1 kg. Body measurements (cm) were measured for shoulder height (SH), hip height (HH), body length (BL), chest girth (CG), and hip girth (HG). A measuring tape with a length of 150 cm at a sensitivity of 0.1 cm was used to measure chest girth and hip girth. A measuring stick with a length of 100 cm at a sensitivity of 0.1 cm was used to measure shoulder height, body length, and hip height.

The body weight and body sizes were recorded at the interval within 7-30 days. Excepting birth weight, individual records of both body weight and measurement were standardized to a 30 days interval by interpolation method to obtain body weight and body size at the ages of 30 days, 60 days, 90 days; and 120 days.

Non-genetic factors were observed for the following components and subcomponents: sex for male and female; type of birth for single, twin and triplets; months of kidding of February and March (1st) and for April (2nd); and year of kidding for 2018 and 2019.

**Statistical analysis**

***Non genetic component***

Effect of the non-genetic factor on each body weight and body measurement at each observed age of the $G_2$ Sapera kid were analyzed by ANOVA using Least Square Means for unbalanced data by PROC GLM of the SAS packets (SAS, 1999). The GLM statistical equation was as follow:

$$Y_{ijklmn} = \mu + S_j + T_k + M_l + Y_m + \varepsilon_{ijklmn}$$

where,

| | | |
|---|---|---|
| $Y_{ijklmn}$ | : | body weight or body measurement of $n^{th}$ kid, $j^{th}$ sex, $k^{th}$ birth type, $l^{th}$ month of kidding, and $m^{th}$ of year of kidding. |
| M | : | overall population mean |
| $S_j$ | : | effect of $j^{th}$ sex of the kid |
| $T_k$ | : | effect of $k^{th}$ type of birth |
| $M_l$ | : | effect of $l^{th}$ month of kidding |
| $Y_m$ | : | effect of $m^{th}$ year of kidding |
| $\varepsilon_{ijklmn}$ | : | residual error |

In the case of interaction between two factors resulted in no significant effect (P>0.05), then interaction was removed from the model to simplify the calculation process. Different LSMs between subclasses were tested by the Tukey test.

***Heritability***

Mixed Model was used to calculate sire variance as a random component, while fixed variables included sex, birth type, and year of birth. The statistical equation of the Linear Mixed Model was as follows:

$$Y_{ijklm} = \mu + S_j + T_k + Y_l + s_m + \varepsilon_{ijklmn}$$

where,

| | | |
|---|---|---|
| $Y_{ijklm}$ | : | body weight or body measurement of $n^{th}$ kid, $j^{th}$ sex, $k^{th}$ birth type, $l^{th}$ year of kidding, and $m^{th}$ sire |
| $\mu$ | : | overall population mean |
| $S_j$ | : | fixed effect of $j^{th}$ sex of kid |
| $T_k$ | : | fixed effect of $k^{th}$ type of birth |
| $Y_l$ | : | fixed effect of $l^{th}$ year of kidding |
| $s_m$ | : | random effect of $m^{th}$ sire |
| $\varepsilon_{jklmn}$ | : | residual error |

The assumed matrix model to estimate heritability value was as follows:

$$Y = XT \pm Zu \pm e$$

where,

| | | |
|---|---|---|
| Y | : | vector of observation (n x 1) |
| T | : | vector of fixed variables (t x 1) |
| u | : | vector of random variables (b x 1) |
| X | : | matrix related to fixed variables (n x t) |
| Z | : | vektor of error (n x 1) |
| e | : | vektor of error (n x 1) |

Heritability values were computed by paternal half-sib analysis using VARCOMP procedures. Estimated heritability was obtained by the following equation (Mioč et al. 2011):

$$h^2 = \frac{4\sigma^2 s}{\sigma^2 e + \sigma^2 s}$$

where:
$h^2$ : heritability value
$\sigma^2 s$ : component between sires
$\sigma^2 e$ : variance component of the kids within sire

## RESULTS AND DISCUSSION

### Non-genetic effect

Least square means (LSM) and standard error (SE) of the growth trait include body weight and body size from birth to 120 days of age of $G_2$ Sapera kid classified by sex is in Table 1, for the type of birth in Table 2, for months of kidding in Table 3, and year of kidding in Table 4. The growth of animal can be reflected by the development of body weight in line with the age. Morphometrics is commonly measured based on body measurements of animals. Morphometrics reflects the development of body conformation and body skeleton. Several body measurements become important variables in the selection activity as an initial indicator of milk production due to the existence of a high positive genetic correlation between the two in dairy goat (Waheed & Khan 2011).

### Sex

The effect of sex on the body weight of $G_2$ Sapera goat in Table 1 shows that the least-square means (LSMs) of the body weight of the male kid was not statistically different from the female kid across the ages (P>0.05). The exception was at weaning age where males were significantly heavier than females (P<0.05). LSMs of body weight of male and female at birth were 3.04±0.08 kg and 2.92±0.07 kg respectively, while at weaning age (90 days) were 9.88±0.37 kg and 8.98 ±0.25 kg respectively. The different weaning weight by the sex was 0.90 kg or 10.02%. However, by observing body weight at another age, it seemed different in body weight from birth to 120 days of the age between a male kid and a female kid by 2.40-7.72%.

These results were still consistent with some previous studies. Male kid to female kid of Croatian goat had almost the same birth weight (2.34 kg vs. 2.27 kg), gaining a faster growth rate (125.15 gr/d. vs 106.96 gr/d.), thereby achieving a higher weaning weight (23.46 kg vs. 22.58 kg) (Mioč et al. 2011). Bharathidhasan et al. (2009) reported body weight at the age from birth, 10 weeks, 20 weeks and 30 weeks of male to female were heavier successively by 10.0% (2.2 kg vs. 2.0 kg), 6.0% (7.1 kg vs. 6.7 kg), 12.1% (11.1 kg vs. 9.9 kg), and 20.3% (15.0 kg vs. 13.3 kg). Heavier birth weight and weaning weight of male kid against female kid were reported from many goat breeds around 5.0-12.2% and 6.0-20.0 % respectively (Bharathidhasan et al. 2009; Mabrouk et al. 2010; Caro-Petrovic et al. 2012; Supakorn & Pralomkarn 2012; Kugonza et al. 2014; Dudhe et al. 2015; Josiane et al. 2020). The effect of sex on growth could be attributed to the different hormonal status between males and females.

Effect of sex on morphometrics of the $G_2$ Sapera goat (Table 1) shows that both linear body size including shoulder height, hip height, and body length and non-linear body sizes providing chest girth and hip girth of the two sexes were not significantly different (P>0.05) for all ages. The exception was for 120-d female kid had larger body length than the male kid (P>0.05). LSMs of body length of males and females at this age were 51.87±0.67 cm and 49.11±0.66 cm, so the difference of both was 2.76 cm (5.62%). Observation of

**Table 1.** Least square means (LSM) and standard error (SE) of body weight and body measurements from birth to 120 days old by sex

| Traits | Sex | At Birth | | 30 days old | | 60 days old | | 90 days old | | 120 days old | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N | LSM±SE | N | LSM±SE | N | LSM±SE | N | LSM±SE | N | LSM±SE |
| BW (kg) | Female | 58 | 2.92±0.07[a] | 58 | 4.78±0.12[a] | 58 | 6.94±0.17[a] | 58 | 8.98±0.25[a] | 30 | 10.04±0.35[a] |
| | Male | 47 | 3.04±0.08[a] | 47 | 5.13±0.14[a] | 46 | 7.21±0.23[a] | 46 | 9.88±0.37[b] | 21 | 10.25±0.32[a] |
| SH (cm) | Female | 58 | 35.50±0.48[a] | 58 | 40.82±0.35[a] | 58 | 47.50±0.52[a] | 58 | 52.84±0.58[a] | 30 | 52.23±0.44[a] |
| | Male | 47 | 34.98±0.63[a] | 47 | 41.22±0.36[a] | 46 | 47.86±0.51[a] | 46 | 54.33±0.71[a] | 21 | 53.12±0.51[a] |
| HH (cm) | Female | 58 | 35.54±0.55[a] | 58 | 42.73±0.35[a] | 46 | 50.40±0.53[a] | 58 | 55.05±0.66[a] | 29 | 54.55±0.56[a] |
| | Male | 47 | 36.01±0.64[a] | 47 | 42.98±0.34[a] | 58 | 50.49±0.57[a] | 46 | 56.00±0.80[a] | 21 | 55.59±0.60[a] |
| BL (cm) | Female | 58 | 29.54±0.67[a] | 58 | 37.90±0.33[a] | 58 | 44.55±0.59[a] | 58 | 52.36±0.90[a] | 30 | 49.11±0.66[a] |
| | Male | 47 | 30.21±0.66[a] | 47 | 38.28±0.38[a] | 46 | 44.87±0.72[a] | 46 | 52.67±1.00[a] | 21 | 51.87±0.67[b] |
| CG (cm) | Female | 58 | 31.60±0.58a | 58 | 39.00±0.33[a] | 58 | 46.41±0.48[a] | 58 | 49.89±1.12[a] | 30 | 50.17±0.49[a] |
| | Male | 47 | 31.52±0.64[a] | 47 | 39.14±0.34[a] | 46 | 46.68±0.45[a] | 46 | 51.30±0.75[a] | 21 | 51.90±0.44[a] |
| HG (cm) | Female | 58 | 24.67±0.99[a] | 58 | 35.77±0.30[a] | 58 | 44.66±0.78[a] | 58 | 48.46±0.78[a] | 30 | 54.87±0.85[a] |
| | Male | 47 | 24.67±1.37[a] | 47 | 35.99±0.40[a] | 46 | 44.71±0.93[a] | 46 | 50.03±1.05[a] | 21 | 56.27±1.00[a] |

BW: body weight, SH: shoulder height, HH: hip height, BL: body length, CG: chest girth, HG: hip girth
Different letters between row for a and b were statistically significant difference ($P<0.05$) and for a and c were very significant difference ($P<0.01$)

the body measurement by the respective age presented by male body sizes was slightly larger than females. Even at the birth male kid to female kid had shorter shoulder height and chest girth, while the hip circumference of both was similar. The high difference in body size of male kid to a female kid from birth to 120 days of age were found for hip height by 0.09-1.04 cm (0.6-1.9%) and body length by 0.31-2.76 cm (0.7-5.6%). While the difference between the two at the age of 30 d. to 120 d. kid was: for shoulder height by 0.40-1.49 cm (0.01-2.97%), chest girth by 0.14-1.54 cm (0.36-3.32%, and hip girth by 0.05-1.57 cm (0.11-0.61%). The difference in body size, when compared to those in body weight, at the respective age, due to sex differences was relatively low. This result indicated that the growth of the body skeleton was slower than those of the body weight of the kid.

Body measurement at the birth of the $G_2$ Sapera goats in this study was quite larger than those of the local Sirohi goat in India as reported from the study by Dudhe et al. (2015). Body length, wither height, and body girth of these kids at birth was 28.3 cm, 31.0 cm, 31.2 cm respectively. For the development of a selection index method based on body weight and body dimension to improve milk production in Dhofari goat breed in Turkey, varying phenotypes were found for body length, body height, heart girth, and rear girth at birth, successively 31.4 cm (27.0-37.0 cm), 34.7 cm (30.0-41.0 cm), 33.6 cm (25.0-42.0 cm), and 37.0 cm (27.0-47.0 cm). The sex reported by Jafari and Hashemi (2014) affects the number of body measurements from local Makuie sheep in Iran. Male had greater body dimension to female as indicated by differences in wither height, rump height, and body length successively by 4 cm (12.9%), 3.8 cm (4.8%), 1.1 cm (2.2%), 2.6 cm (4.1%) and 1.0 cm (3.3%). Dudhe et al. (2015) in Sirohi goat in India also found the influence of sex on body size as shown by the larger body dimension of male kid over a female at the age of birth, 3 months (weaning), 6 months 9 months and one yearold successively for body weight by 2.51%, 3.80%, 3.05%, 3.02%, and 2.87%, body length by 2.36%, 3.41%, 2.86%, 6.81% and 3.24%; and chest girth by 2.53%, 3.67%, 3.03%, 2.98% and 2.85%. The significant influence of sex factor on the growth trait may be due to physiological characteristics and endocrinal system, type, and measure of hormone secretion, especially sexual hormones.

**Type of birth**

The effect of birth type on growth trait of both body weight and body size from birth to 120 d. age of the $G_2$ Sapera goat is presented in Table 2. A significant difference by birth type on body weight only evidenced at the birth ($P < 0.01$) instead of the other age. Single, twin and triplets kid had birth weight by 3.37±0.12 kg, 3.01±0.06 kg, and 2.56±0.12 kg respectively, so that the weight benefit of the single kid to twin is 0.36 kg (11.96%) and to triplets is 0.81 kg (31.64%). Factors that cause smaller birth weight in multiple births might be due to the capacity of the mother uterus when pregnant to accommodate more fetuses than a single fetus. The capacity of the uterus in twin pregnancy will cause fetal competition in getting nutrients from the mother, thus causing low birth weight.

Results show that birth type affected body weight inconsistently after birth, even twin body weights could be greater against single birth weight, although the differences were not significant. These results differed from some previous studies that reported a significant effect of birth type on the body weight from various goat breeds (Bharathidhasan et al. 2009; Mabrouk et al. 2010; Supakorn & Pralomkarn 2012; Josiane et al. 2020). Birth weight, weaning weight and daily growth rate of both ages in Barbari goat from the single kid was successively 1.94±0.08 kg, 7.16±0.44 kg, and 55.56±4.80 gr/d. being higher than twins that were successively 1.83±0.06 kg, 6.71±0.40 kg, and 55.45±4.41 gr/d respectively (Bharathidhasan et al. 2009). Mabrouk et al. (2010) found significant differences in body weight of the single kid over the twin at each one-month interval of the age, from birth to five months old, sequentially 7.66%, 13.48%, 21.38%, 19.74%, 27.44%, and 17.67%. Several studies also reported that single kid had benefits against the twin for birth weight by 6.01-37.62% and weaning weight by 3.58-25.37 %; while those from single kid against triplets for birth weight by 25.37-37.62% and weaning weight by 25.37-31.35% (Bharathidhasan et al. 2009; Supakorn & Pralomkarn 2012; Mioč et al. 2011; Dudhe et al. 2015; Mohammed et al. 2018).

The individual body measurement observed in the Sapera $G_2$ kid increased by age (Table 2). However, a significant effect ($P < 0.05$) of birth type was only found on shoulder height at birth and 30 days of age, as well as chest circumference at 120 days of age. Whilst there was no significant effect of birth type on body size ($P > 0.05$). At birth and weaning age, a shoulder height of the single kid (36.74±1.18 cm, 43.64±0.83 cm) and twin (36.10±0.46 cm, 43.07±0.29) was significantly higher than triplets (34.49±1.02 cm, 41.85±0.49 cm). The difference between single kid to twin was by 0.64 cm (1.77%) and to triplet by 2.25 cm (6.52%), while at the 30 days old the difference was respectively by 0.57 cm (1.32%) and 1.79 cm (4.28%). At the 120 days old, chest girth of the single kid (53.06±0.8 cm) differed from the twin (50.48±0.36 cm) and the triplets (49.56±0.89 cm). The superiority of the chest girth of the single kid to twin was 2.58 cm (5.11%) and to triplets by 3.5 cm (7.06%).

**Table 2.** Least square means (LSM) and standard error (SE) of body weight and body measurements from birth to 120 days old by type of birth

| Traits | Type of Birth | At Birth | | 30 days old | | 60 days old | | 90 days old | | 120 days old | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | N | LSM±SE | N | LSM±SE | N | LSM±SE | N | LSM±SE | N | LSM±SE |
| BW (kg) | 1 | 13 | 3.37±0.12[a] | 13 | 4.88±0.25[a] | 13 | 7.28±0.17[a] | 13 | 9.27±1.69[a] | 9 | 10.75±0.74[a] |
| | 2 | 66 | 3.01±0.06[b] | 66 | 4.96±0.11[a] | 65 | 6.96±0.46[a] | 65 | 9.54±0.49[a] | 31 | 10.11±0.31[a] |
| | 3 | 26 | 2.56±0.12[c] | 26 | 4.90±0.20[a] | 26 | 6.98±0.27[a] | 26 | 9.49±0.46[a] | 11 | 9.58±0.40[a] |
| SH (cm) | 1 | 13 | 36.74±1.18[a] | 13 | 43.64±0.83[b] | 13 | 50.73±1.12[a] | 13 | 53.90±4.08[a] | 9 | 55.50±1.11[a] |
| | 2 | 66 | 36.10±0.46[a] | 66 | 43.07±0.29[b] | 65 | 50.37±0.48[a] | 65 | 53.50±4.08[a] | 31 | 55.24±0.54[a] |
| | 3 | 26 | 34.49±1.02[b] | 26 | 41.85±0.49[a] | 26 | 49.64±0.84[a] | 26 | 52.18±1.73[a] | 10 | 54.49±0.77[a] |
| HH (cm) | 1 | 13 | 36.10±1.18[a] | 13 | 43.07±0.83[b] | 13 | 50.37±1.12[a] | 13 | 56.90±4.08[a] | 9 | 55.50±1.11[a] |
| | 2 | 66 | 36.74±0.46[a] | 66 | 43.64±0.29[b] | 65 | 50.73±0.48[a] | 65 | 55.50±4.08[a] | 31 | 55.24±0.54[a] |
| | 3 | 26 | 34.49±1.02[b] | 26 | 41.85±0.49[a] | 26 | 49.64±0.84[a] | 26 | 54.18±1.73[a] | 10 | 54.49±0.77[a] |
| BL (cm) | 1 | 13 | 29.00±1.45[a] | 13 | 38.23±0.98[a] | 13 | 44.35±1.26[a] | 13 | 50.37±3.81[a] | 9 | 51.75±1.42[a] |
| | 2 | 66 | 30.47±0.52[a] | 66 | 38.24±0.27[a] | 65 | 45.41±0.61[a] | 65 | 53.08±1.26[a] | 31 | 50.56±0.65[a] |
| | 3 | 26 | 30.16±0.90[a] | 26 | 37.80±0.59[a] | 26 | 44.37±0.81[a] | 26 | 52.09±1.66[a] | 11 | 49.16±0.91[a] |
| CG (cm) | 1 | 13 | 31.29±1.45[a] | 13 | 39.22±0.64[a] | 13 | 47.13±1.10[a] | 13 | 50.45±2.43[a] | 9 | 53.06±0.84[b] |
| | 2 | 66 | 31.76±0.52[a] | 66 | 39.13±0.28[a] | 65 | 46.50±0.39[a] | 65 | 50.53±0.96[a] | 31 | 50.48±0.36[a] |
| | 3 | 26 | 31.63±0.90[a] | 26 | 38.82±0.56[a] | 26 | 46.00±0.73[a] | 26 | 50.80±1.27[a] | 11 | 49.56±0.89[a] |
| HG (cm) | 1 | 13 | 23.92±2.58[a] | 13 | 35.54±0.64[a] | 13 | 42.97±1.92[a] | 13 | 47.60±2.05[a] | 9 | 52.26±1.29[a] |
| | 2 | 66 | 24.17±1.04[a] | 66 | 36.14±0.30[a] | 65 | 46.50±0.78[a] | 65 | 50.14±1.29[a] | 31 | 5258±0.85[a] |
| | 3 | 26 | 25.93±1.47[a] | 26 | 35.96±0.55[a] | 26 | 44.58±0.97[a] | 26 | 49.99±2.32[a] | 11 | 51.88±1.52[a] |

BW: body weight, SH: shoulder height, HH: hip height, BL: body length, CG: chest girth, HG: hip girth
Different letters between row for a and b were statistically significant difference (P<0.05) and for a and c were very significant difference (P<0.01)

Body measurement of single kid were higher than multiple kid were obtained by a number of previous studies (Jafari & Hashemi 2014; Dudhe et al. 2015; Sarma et al. 2019). Dudhe et al. (2015) studied in native Sirohi goat in India at birth, 3 months, 6 months, 9 months and 12 months old, found larger body size of single kid to twin for body height successively by 2.19 cm (7.32%), 2.11 cm (4.18%), 2.10 cm (3.71%), 1.40 cm (2.29%), and 1.34 cm (2.02%); body lengths by 2.21 cm (8.12%), 1.87 cm (3.98%), 2.20 cm (4.16%), 1.42 cm(2.51%), 1.11 cm (1.79%); and body girth by 2.16 cm (7.17%), 2.17 cm (4.29%), 2.06 cm (3.58%), 1.25 cm (2.03%) and 1.48 cm (2.22%).

**Month and year of kidding**

Months of kidding of the $G_2$ Sapera kid in this study were classified based on two months of kidding, namely the first for February and March in 2018 and the second for April in 2019. Table 3 shows that month of kidding gave no significant effect on body weight and body size at all ages ($P>0.05$). This shows that the growth trait did not vary within the two months of kidding observed. This was reasonable as the two months of kidding were still in one kidding season (end of the rainy season). This result differed from those obtained by Mabrouk et al. (2010) for goat in the arid region of Tunisia where the month of birth (January-March and December) affected body weight and body size at birth to age 5 months old. These results were still in line with the study of Sarma et al. (2019) that found a significant effect of the seasons on early growth trait of mountain goat in India. Differences in body weight and body measurement associated with differences in the birth season of goat and also sheep were reported from some studies (Bharathidhasan et al. 2009; Supakorn & Pralomkarn 2012; Caro-Petrovic et al. 2012; Dudhe et al. 2015; Selvam 2018; Mohammed et al. 2018).

Table 4 shows that years of kidding (2018 and 2019) did not have a significant effect on body weight and all body measurements from birth until the weaning age. Significant year of kidding effects on body weight and body size were found at 120 days old kid ($P<0.05$) rather than shoulder height and chest girth ($P>0.05$). It seemed body weight and body measurements slightly decreased in the latter year of kidding might be due to the older age does. Most of these does get older (5-8 yr. old) at year of kidding 2019 than 2018. This is in line with the findings from some studies stating that body weight and body size of goat decreased after the does reaching the peak phase of production (Mabrouk et al. 2010; Anggraeni 2014).

However different results were reported by Dudhe et al. (2015) in Sirohi goat in India that found a significant influence year of kidding on body weight, body height, body length, and girth size at the successive three-month interval from birth to 12 months old. The significant influence of birth year either on body weight or body size was also obtained by some previous studies (Supakorn & Pralomkarn 2012; Caro-Petrovic et al. 2012; Jafari & Hashemi 2014; Dudhe et al. 2015; Selvam 2018). Differences due to year of kidding might be caused by differences in management, food availability (quantity and quality), disease, and climate condition (rainfall, relative humidity, and temperature) (Jafari & Hashemi 2014; Dudhe et al. 2015).

**Genetic effect**

Heritability is part of phenotype variances that resulted from differences in heredity among the genes and gene combination of individual genotype. Prediction of response to selection and efficiency of a breeding program to result in genetic progress rely on the information on the heritability of the trait under consideration. Estimated $G_2$ Sapera goat heritability from birth to 120 days of age are presented in Table 5. Heritability value of body weight of the kid at birth was high ($h^2 = 0.59$), and at 30 d. age ($h^2 = 0.29$) was moderate. Whereas heritability value at the age of 60 days old ($h^2 = 0.11$), 90 days old ($h^2 = 0.16$), and 120 days old ($h^2 = 0.15$) were low. Likewise, heritability value of body measurement in all ages were low providing shoulder height ($h^2 = 0.10$-$0.18$), hip height ($h^2 = 0.04$-$0.08$), body length ($h^2 = 0.03$-$0.09$), and chest girth ($h^2 = 0.09$-$0.15$). Heritability by moderate value however was obtained for chest girth at 30 d. age ($h^2 = 0.24$), and moderate to high value for hip girth ($h^2 = 0.23$-$0.34$). Classification of the heritability was by referring to Mioč et al. (2011) stated highly heritable trait for $h^2$ value exceeded 0.40, whilst lowly heritable traits for $h^2$ value below 0.15.

Heritability value of $G_2$ Sapera kid growth trait in this study was calculated from the sire variance component. So that the variance of body weight and body measurement likely due to the variations between sires. Dudhe et al. (2015) reported a high heritability value of body measurement that was estimated by the sire component. Results of some studies explained that sire significantly influenced morphometric trait showing the presence of additive genetic variability among these traits and the significant effect of sire might be related to the appearance of the family of the buck used.

A previous study by Dudhe et al. (2015) obtained a relatively high value of the heritability of growth trait in Sirohi goat. Heritability estimated in Dhorfari goat in Egypt for body weight, body length, body height, hip girth, and rear girth were successively 0.41, 0.33, 0.82, 0.90. While Waheed & Khan (2011) reported genetic additive variation within a wide range for goat growth

**Table 3.** Least square means (LSM) and standard error (SE) of body weight and body measurements from birth to 90 days old by month of kidding.

| Traits | Month of Kidding | At Birth | | 30 days old | | 60 days old | | 90 days old | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | LSM±SE | N | LSM±SE | N | LSM±SE | N | LSM±SE |
| BW (kg) | Feb-March | 53 | 2.84±0.08[a] | 53 | 4.38±0.09[a] | 53 | 7.62±0.16[a] | 53 | 10.52±0.24[a] |
| | April | 52 | 3.12±0.06[a] | 52 | 5.50±0.11[a] | 51 | 6.53±0.20[a] | 51 | 8.35±0.37[a] |
| SH (cm) | Feb-March | 53 | 35.08±0.52[a] | 53 | 41.57±0.33[a] | 53 | 48.13±0.50[a] | 53 | 54.53±0.39[a] |
| | April | 52 | 35.40±0.55[a] | 52 | 40.46±0.38[a] | 51 | 47.24±0.54[a] | 51 | 52.64±0.82[a] |
| HH (cm) | Feb-March | 52 | 33.03±0.50[a] | 53 | 44.85±0.34[b] | 53 | 51.37±0.54[a] | 53 | 56.37±0.38[a] |
| | April | 53 | 38.52±0.67[a] | 52 | 40.85±0.36[a] | 51 | 49.12±0.56[a] | 51 | 54.69±0.92[a] |
| BL (cm) | Feb-March | 53 | 30.18±0.55[a] | 53 | 38.86±0.30[a] | 53 | 45.63±0.51[a] | 53 | 53.70±0.54[a] |
| | April | 52 | 29.57±0.73[a] | 52 | 37.33±0.34[a] | 51 | 43.78±0.62[a] | 51 | 51.32±0.73[a] |
| CG (cm) | Feb-March | 53 | 31.35±0.57[a] | 53 | 39.43±0.27[a] | 53 | 47.47±0.48[a] | 53 | 52.32±0.44[a] |
| | April | 52 | 31.77±0.51[a] | 52 | 38.68±0.33[a] | 51 | 45.62±0.47[a] | 51 | 48.87±1.33[a] |
| HG (cm) | Feb-March | 53 | 21.92±0.91[a] | 53 | 36.30±0.34[a] | 53 | 48.02±0.81[a] | 53 | 54.73±0.75[a] |
| | April | 52 | 27.42±0.58[a] | 52 | 35.46±0.33[a] | 51 | 46.35±0.59[a] | 51 | 53.76±0.99[a] |

BW: body weight, SH: shoulder height, HH: hip height, BL: body length, CG: chest girth, HG: hip girth
Different letters between row for a and b were statistically significant difference ($P<0.05$) and for a and c were very significant difference ($P<0.01$)

**Table 4.** Least square means (LSM) and standard error (SE) of body weight and body measurements from birth to 90 days old by year of kidding.

| Traits | Year of kidding | At Birth | | 30 days old | | 60 days old | | 90 days old | |
|---|---|---|---|---|---|---|---|---|---|
| | | N | LSM±SE | N | LSM±SE | N | LSM±SE | N | LSM±SE |
| BW (kg) | 2018 | 54 | 3.02±0.08[a] | 54 | 5.51±0.11[a] | 53 | 8.26±0.19[a] | 53 | 10.92±0.36[b] |
| | 2019 | 51 | 2.94±0.07A[a] | 51 | 4.34±0.09[a] | 51 | 5.88±0.15[b] | 51 | 7.95±0.23[a] |
| SH (cm) | 2018 | 51 | 36.35±0.50[a] | 54 | 41.17±0.36[a] | 53 | 47.98±0.52[a] | 53 | 55.05±0.69[a] |
| | 2019 | 54 | 34.14±0.57[a] | 51 | 40.87±0.34[a] | 51 | 47.38±0.52[a] | 51 | 52.12±0.40[a] |
| HH (cm) | 2018 | 54 | 38.61±0.51[a] | 54 | 44.75±0.37[a] | 53 | 51.04±0.55[a] | 53 | 58.18±0.84[b] |
| | 2019 | 51 | 32.94±0.67[a] | 51 | 40.96±0.33[a] | 51 | 49.45±0.55[a] | 51 | 52.88±0.38[a] |
| BL (cm) | 2018 | 54 | 28.96±0.71[a] | 54 | 40.03±0.33[a] | 53 | 48.03±0.60[a] | 53 | 58.94±0.66[b] |
| | 2019 | 51 | 30.80±0.56[a] | 51 | 36.16±0.29[a] | 51 | 41.38±0.50[a] | 51 | 49.08±0.44[a] |
| CG (cm) | 2018 | 54 | 33.43±0.49[a] | 54 | 40.56±0.32[a] | 53 | 47.65±0.46[a] | 53 | 53.97±0.69[b] |
| | 2019 | 51 | 29.68±0.59[a] | 51 | 37.55±0.27[a] | 51 | 45.43±0.49a | 51 | 47.22±0.41[a] |
| HG (cm) | 2018 | 54 | 28.32±0.58[a] | 54 | 36.95±0.33[a] | 53 | 44.62±0.59[a] | 53 | 52.89±0.99[a] |
| | 2019 | 51 | 21.02±0.91[a] | 51 | 34.81±0.34[a] | 51 | 44.74±0.84[a] | 51 | 50.60±0.75[a] |

BW: body weight, SH: shoulder height, HH: hip height, BL: body length, CG: chest girth, HG: hip girth
Different letters between row for a and b were statistically significant difference (P<0.05) and for a and c were very significant difference (P<0.01)

**Table 5.** Heritability value ($h^2$) and standard error (SE) of body weight and body measurement from birth to 120 days old.

| Traits | At Birth | 30 days old | 60 days old | 90 days old | 120 days old |
|---|---|---|---|---|---|
| Body weight | 0.59±0.12 | 0.29±0.09 | 0.11±0.04 | 0.16±0.05 | 0.15±0.10 |
| Shoulder height | 0.10±0.08 | 0.11±0.07 | 0.13±0.05 | 0.18±0.05 | 0.16±0.10 |
| Hip height | 0.04±0.07 | 0.08±0.06 | 0.03±0.07 | 0,04±0.07 | 0.05±0.08 |
| Body length | 0.03±0.11 | 0.05±0.07 | 0.08±0.06 | 0.15±0.06 | 0.09±0.08 |
| Chest girth | 0.15±0.09 | 0.24±0.07 | 0.16±0.06 | 0.14±0.06 | 0.09±0.12 |
| Hip girth | 0.13±0.08 | 0.34±0.10 | 0.35±0.08 | 0.28±0.09 | 0.23±0.09 |

trait. There was also a correlation between total milk production and weaning weight (rg = 0.37) and body sizes (body length, body height, and body girth) (rg = 0.41-0.49) in Beethal goat in India. For lactation does, body height, hip height, and body length were with heritability values from low to moderate ($h^2$ = 0.10-0.24). Contrarily, Kumar et al. (2016) found that heritability value of body measurements was high for body length, body height, and chest circumferences successively by 0.62±0.18, 0.63±0.15, 0.61±0.16, and 0.63±0.18.

Rout et al. (2018) reported heritability value of body weight of Jamnapari goat, analyzed by animal model and sire model, tended decreased by the age of which higher heritability value of body weight was at birth (0.14 and 0.11) and weaning (0.16 0.43), whilst lower heritability values were after weaning ages, i.e. at the ages: of 6 months (0.19, 0.37), 9 months. (0.12, 0.11), and 12 months (0.11, 0.13). This showed a permanent environment factor during the pregnancy period of does had an important effect on the initial body weight. Similarly, Supakorn & Pralomkarn (2012) stated that the initial body weight from birth to weaning age was not only determined by the genetic potency and its interaction with the environment but also significantly affected by the maternal effect. Based on these results the high heritability value of body weight at birth and weaning of the $G_2$ Sapera kid in this study might be quite large contributed by maternal permanent environment. Beside of that, low heritability value for certain age either in the specific body weight or most of the body size of $G_2$ Sapera kid probably because both the $G_1$ Sapera and G2 Sapera goat were in a very closed population and in the use of few male (2 Saanen bucks) to initially produce the $G_1$ Sapera goat at IRIAP.

## CONCLUSION

The growth traits of $G_2$ Sapera kids were affected by sex and year of kidding and slightly influenced by genetic (sires) factors. While heritability value ($h^2$) of both body weight and body measurement was low indicating a relatively narrow genetic difference of growth at the early age.

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# Efficiency of Dimethyl Sulphoxide and Ethylene Glycol on Subsequent Development of Vitrified Awassi Sheep Embryos

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**ABSTRAK**

Mardenli O, Mohammad MS, Hassooni HA. 2020. Efisiensi dimetil sulfoksida dan etilena glikol pada perkembangan subsequen embrio domba Awassi yang divitrifikasi. JITV 25(2):60-67. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2459

Penggunaan krioprotektan dalam vitrifikasi dapat menurunkan kerusakan embrio dan meningkatkan daya hidupnya. Penelitian ini dilakukan di laboratorium bioteknologi reproduksi Fakultas Pertanian Universitas Aleppo. Penelitian ini bertujuan untuk melihat efesiensi dimetil sulfoksida (DMSO) dan etilena glikol (EG) terhadap viabilitas dan daya hidup embrio muda domba Awassi Siria. Embrio divitrifisasi dalam tiga larutan krioprotektan: A. DMSO 3 ml, B. EG 3 ml dan C yang tersusun dari kombinasi DMSO (1,5 ml) dan EG (1,5 ml). Setelah proses *thawing*, embrio yang telah divitrifikasi dalam larutan C memiliki tingkat pembelahan paling tinggi (P<0,01) dibandingkan pada larutan A dan B secara berturut-turut untuk tahap 2-16 pembelahan sel (50,00% Vs 30,77% dan 36,36%); morula (9,00% Vs 44,44% dan 40,00%); dan blastosis (92,86% Vs 58,33% dan 50,00%). Pada tahap blastosis, tahap 2-16 pembelahan sel embrio yang telah divitrifikasi dalam larutan C memiliki tingkat kekuatan dibandingkan dalam larutan A dan B secara berturut-turut sebesar 39,20% Vs 23,08% dan 22,73%. Jumlah embrio tertahan menurun secara signifikan (P<0,05) setelah proses pencairan dalam 3 larutan tertama tahap morula dan blastosis secara berturut-turut sebesar 0,00 dan 3,70% respectively (larutan C). Tidak terdapat perbedaan yang signifikan yang terlihat pada ketiga tipe embrio dalam semua tahap dan larutan meskipun terlihat rentang jarak yang luas. Hal ini menunjukkan maksimum penggunaan larutan campuran DMSO dan EG yang dianjurkan untuk hasil yang baik adalah 1:1 dalam proses vitrifikasi embrio domba.

**Kata Kunci:** Domba Awassi, Dimetil Sulfoksida, Etilena Glikol, Produksi Embrio *In Vitro*, Vitrifikasi

**ABSTRACT**

Mardenli O, Mohammad MS, Hassooni HA. 2020. Efficiency of dimethyl sulphoxide and ethylene glycol on subsequent development of vitrified Awassi sheep embryos. JITV 25(2):60-67. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2459

The use of cryoprotectants in vitrification would reduce the critical damages to the embryos, thus increase the survival rates. This research was conducted in the laboratory of reproductive biotechnology at the faculty of Agriculture of Aleppo University. The study aimed to evaluate the viability and survivability of early Syrian Awassi embryos under the influence of dimethyl sulphoxide (DMSO) and ethylene glycol (EG) following vitrification. Embryos were vitrified in three solutions of cryoprotectants (A: DMSO (3 ml), B: EG (3 ml), and C which was composed of a combination of DMSO (1.5 ml) and EG (1.5 ml)). After thawing, embryos that had been vitrified in C solution achieved the highest rates of cleavage (P< 0.01) comparing with A and B solutions for 2-16 cell stage (50.00% Vs 30.77% and 36.36%), morula (9.00% Vs 44.44% and 40.00%) and blastocyst stage embryos (92.86% Vs 58.33% and 50.00%) respectively. Down to the hatching blastocyst stage, 2-16 cell stage vitrified embryos in C solution achieved an encouraging rate comparing with A and B solutions (39.20% Vs23.08% and 22.73% respectively). The rates of arrested embryos decreased significantly (P< 0.05) after thawing across the three solutions especially the morula and blastocyst stage (0.00 and 3.70% respectively) (C solution). No significant differences were observed in the three types of embryos across all stages and solutions despite the large range among these rates. Given the apparent benefit of the participatory effect of cytoprotectants, it is advised to use a mixture of DMSO and EG (1:1) in vitrification of ovine embryos.

**Key Words:** Awassi Sheeep, Dimethyl Sulphoxide, Ethylene Glycol, *In Vitro* Embryo Production, Vitrification

## INTRODUCTION

Recently, cryobiology is considered the most important science in embryo technology, especially the *In Vitro* Embryo Production applications (IVEP).

Despite the great progress achieved by this technology in the field of farm animal industry, there are still some outstanding issues that need solutions. In order to preserve the embryos produced *In vitro*, it was necessary to face many prominent obstacles, the most

important of which is the decrease in the survivability rates of frozen embryos during the blastomere stage, also, the high accumulated content of lipids (especially triacylglycerides) in embryonic cells, which have harmful effects (Palasz et al. 2008). There are two methods in the cryopreservation of embryo: vitrification and programmed slow freezing (Arav 2014). In literature, some considerations were identified in the programmed slow freezing, Thompson et al. (2011) indicated that subjecting embryos to a rate of 1 °C $min^{-1}$ is considered a typical cooling rate for mammalian embryos. Despite the huge costs, equipment, and multiple steps of slow freezing, it has been observed a decrease in both survival and implantation rates (Bromfield et al. 2009).

Vitrification method depends basically on the use of high concentrations of cryoprotectants and the very fast freezing rates (Moussa et al. 2014). Due to the chemical and molecular properties of DMSO and EG, the use of these two compounds as cryoprotectants agents (CPAs) has prevailed in vitrification strategy, The prominent role of DMSO is in reducing the electrolytic concentration in the residual chilled contents within and around of a biological cell, on the other hand, EG alters the hydrogen bonding while mixing it with water during cryopreservation (Bhattacharya 2018). Within the vitrification scenario, the toxic effects of the cryoprotectants were not neglected, as both DMSO and EG are characterized by the minimal toxic effect (Best 2015). However, as for all cell lines, the cytotoxicity of DMSO could increase at a high concentration of this agent (Fahy 2010).

Therefore, the present study was designed to evaluate the efficiency of the cryoprotectants levels (DMSO, EG, and combination of DMSO and EG 1:1) that used in vitrification methods on morphological survival and subsequent development of Awassi sheep early embryos produced *in vitro* following vitrification.

## MATERIALS AND METHODS

### Animal, and oocyte recovery

Ovaries of Awassi ewes were collected from a local slaughterhouse in Aleppo city and transported to the reproductive biotechnology laboratory at Aleppo University (about 1 h) in Dulbecco's PBS (DPBS). Cumulus oocyte complexes (COCs) were collected by the slicing method from follicles 3-8 mm. COCs with evenly granulated cytoplasm and with 3 or more layers of cumulus cells attached were selected for further work.

### *In vitro* maturation (IVM)

COCs were matured as described previously by Salvador et al. (2011) with some modifications. COCs were washed three times in phosphate buffer saline solution (PBS) supplemented with 50 µg /ml gentamicin, and cultured in 50-µl microdrop of maturation medium (TCM-199) supplemented with 0.255 mM sodium pyruvate, 10% heat-treated estrus cow serum, 5 µg/ml FSH, 25 mM Hepes and 100 µM cysteamine and incubated under paraffin oil for 27 h at 39 $^{o}$C in a humidified atmosphere of 5% $CO_2$ in the air for 27 hours.

### Sperm preparation and i*n vitro* fertilization (IVF)

Following maturation, presumptive COCs were denuded of surrounding cumulus cells by vortexing for 1 min in 2 ml HEPES-TALP and washed three times in HEPES-TALP supplemented with 2% bovine serum albumin (BSA) and twice in IVF-TALP. Oocytes were transferred into four-well plates containing 250 µl of Fertil-TALP. The fertilization medium (TALP) was supplemented with a final concentration of 10 µg/ml heparin-sodium salt, 500 µM epinephrine, and 250 µM penicillamine. Frozen-thawed Awassi ram semen was prepared for IVF using previously described methods by Salvador et al. (2011) with some modifications. Briefly, two frozen semen straws were thawed in a water bath at 38 °C for 30 seconds and emptied in a centrifuge tube with 4ml of Hepes-TALP medium. The tube was centrifuged at 200 x g for 10 minutes. The resulting aliquot of sperm pellet was resuspended (1:1) with the Hepes-TALP medium. Then 2 ml of Hepes-TALP medium was added to 50 µl of aliquots of spermatozoa and placed at the bottom of a conical tube for Swim-up. After 1 h, 0.5 ml of the sperm suspension was collected from the upper part of the tube and centrifuged at 200 x g for 10 min. The resulting sperm pellet was resuspended with heparin containing (100 µg/ml) Hepes-TALP medium and incubated for 45 min at 38.5 $^{o}$C. The sperm concentration was assessed in a hemocytometer and the sperm pellet was resuspended in TALP to give a final concentration of 3 x $10^9$ sperms /ml. The sperm suspension was added to each fertilization well to obtain a final concentration of $1.5\times10^6$ spermatozoa/ml. Plates were incubated for 17 h under 5% $CO_2$ in the air with maximum humidity (>95%) at 38.5 $^{o}$C. Resulting zygotes were rinsed with PBS and examined under an inverted microscope to detect second polar body formation.

### *In vitro* culture

Following IVF, presumptive zygotes were gently vortexed in PBS to remove spermatozoa or cumulus cells remaining attached to these zygotes. All zygotes were washed twice in PBS and the same in TCM-199 before being transferred into the culture wells. Zygotes were Cultured in TCM-199 under mineral oil in a humidified atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ at 38.5 $^{o}$C. Fetal calf serum (FCS, 10%) was added 24 h for six days (Silva et al. 2010).

**Table 1**. Volumes of DMSO and EG used in vitrification of Awassi sheep embryos (ml)

| Cryoprotectant | Solution | | | | | |
|---|---|---|---|---|---|---|
| | A | | B | | C | |
| | VS* | ES** | VS | ES | VS | ES |
| DMSO | 3 | 1.5 | - | - | 1.5 | 0.75 |
| EG | - | - | 3 | 1.5 | 1.5 | 0.75 |

VS*: Vitrification solution, ES**: Equilibrium solution

**Embryos cryopreservation**

The resulting embryos were vitrified as described previously by Ghorbani et al. (2012), with some modifications. Briefly, both of vitrification solution (VS) and equilibrium solution (ES) comprised of TCM-199 culture media supplemented with 0.4% calf serum (CS) and different volumes (ml) of the cryoprotectants dimethyl sulphoxide (DMSO), ethylene glycol (EG) and combination of DMSO and EG 1:1 divided into three solutions A, B, and C, each solution contained two parts: VS and ES as it is shown in Table 1. TCM-199 culture media was added to both VS and ES solutions, while only 0.5 M sucrose was added to ES solution, to give a final volume of 10 ml for each solution. Embryos were treated to vitrification by putting them in ES solution for 8 minutes at moderate room temperature (stage 1) and transferred to VS solutions for 1 minute exactly (stage 2), during that time (1 minute) every 6 embryos were put in straw (0.25 mm) and closed well by special devices and plunged immediately in liquid nitrogen. Embryos were left in liquid nitrogen for three days (72 hours).

**Embryos thawing, culture and survivability determination**

Frozen embryos were thawed across two steps as described previously by Ghorbani et al. (2012) with some modifications as follows: Cryoprotectant was removed by transferring the embryos in two successive baths containing decreasing concentrations of sucrose and a fixed concentration of calf serum (CS): 20% calf serum +1 M sucrose; 20% CS+0.5 M sucrose supplemented with TCM-199 culture media to give a final volume of 10 ml. Embryos were placed into the first solution at room temperature (22–25 $^{o}$C) for 1 min, then placed into the second solution for 3 min, before they were placed in TCM-199 culture media for an additional 5 min. Embryos were then cultured in 500 ml of TCM-199 at 38.8 $^{o}$C, in presence of 5% $CO_2$, 5%$O_2$, and 90% $N_2$ at 100% humidity. Embryos survivability were identified after freezing depending on the increase in the number of cells in early embryos (2-16 cell), morulae and blastocyst were identified depending on the subsequent development depending on the re-expansion of the embryos sizes and by the increase in the outer diameter and the arrival of to the hatching blastocyst stage.

**Embryo grading**

Embryos were graded according to their quality (exterior shape) into three main groups based on the classification of Wintner et al. (2017) with some modification as follows:

- Type 1: Cells are of equal size; no fragmentation is seen.
- Type 2: Cells are of equal size; minor fragmentation only.
- Type 3: Cells are of equal or unequal size; fragmentation is moderate to heavy.

**Reagents**

The chemicals used were from Sigma Chemical Co (St. Louis, USA) unless mentioned otherwise.

**Statistical analysis**

The experiment was designed according to the single-factor experimental design for several traits. Pearson Chi-square of contingency table and exact Fisher test were used to analyze the data among groups of vitrification cryoprotectants solutions for different stages of survived embryos using SAS, 14.3 Software package (SAS Institute 2017).

## RESULTS AND DISCUSSION

**Result**

***Survivability and development of embryos following vitrification***

Our results show that the total rates of survived Awassi sheep embryos in different stages of embryonic development vitrified in C solution which composed of a combination of DMSO and EG cryoprotectants was

greater (P < 0.01) compared to those vitrified in the tow solutions A and B (vitrified by using single type DMSO or EG): 76.3 % versus 44.1 % and 42.4% respectively. The survival rates of blastocyst and hatching blastocyst for vitrified embryos in C solution was high (P< 0.05) compared to those vitrified in the two solutions A and B: 92.8% versus 58.3% and 50% respectively. Moreover, the survival rate of vitrified embryos reaching to morulae stage in C solution was superior (P< 0.05) compared to those vitrified in both solutions A and B: 90% versus 44.4% and 40% respectively. Although the survival rate of 2-16 cell stage embryos vitrified in C solution was slightly greater compared to those vitrified in the two solutions A and B: 50% versus 30.8 % and 36.4 % respectively, but these differences were not significant (Table 2).

In detail, as shown in Table 3, total rates of cleavages differed significantly (P < 0.01) through the various stages of embryonic development where the embryos vitrified in C solution attained higher values: 50.0%, 90.0%, and 92.9%, respectively, the rates of embryos reached to blastocyst and hatching blastocyst stages increased significantly (P < 0.01) from 2-16 cell to blastocyst stage across the three solutions A, B and C, it should be noted that these rates were high and doubled for embryos that were subjected to C solution (39.20%,85.00% and 89.29 % for 2-16 cell, morula and blastocyst stage respectively).

### *Lyses and arrest of embryos*

The rates of arrested embryos generally converged (P < 0.05) in 2-16 cell stage embryos across the three solutions A, B and C while these rates were virtually zero in the morula and blastocyst stages for embryos that were subjected to C solution (0.00 and 3.70% respectively) (Table 4). Rates of lyses were completely absent at morula and blastocyst stage embryos that were vitrified in C solution. It was also observed a low rate of lyses of embryos that were vitrified in a single solution (A and B solutions) (Table 4). The general difference between these rates was 20%, noting that there was no significant difference among these rates.

### *Embryo quality*

There were no significant differences in the quality of developed embryos after thawing despite the high rates of Type3 embryos in 2-16 cell stage embryos across the three solutions A, B, and C: 75.0%,75.0%, and 80.0%, respectively (Table 5).

## Discussion

Vitrification is considered a modern, potential, important, and essential method that has replaced the traditional freezing methods, especially the slow freezing (Moussa et al. 2014). Its advantages include reducing the cost of freezing, speed, and simplicity of the application as well as its effective use on oocytes, sperm, ovaries, and cellular tissues (Chen & Yang 2009).

In general, our results (Table 2) came close with many of the studies that have confirmed the possibility of using different types of cryoprotectants in vitrification method in many species such as in mice embryos (Momozawa et al. 2017), and cows (Caamaño et al. 2015). Moreover, the survival rates of the embryos in the current study are in agreement with the result of Riha & Vejnar (2004) who observed a very high survival rate (80%) of embryos vitrified in solution composed of 25% v/v EG + 25% v/v DMSO in culture medium supplemented with 20% v/v of FCS. While current results are slightly greater than the results of Donnay et al. (1998) who observed a high rate (67%) of *In vitro* development in embryos vitrified in solution composed of 25% v/v EG + 25% v/v G.

The survival rates of both morulae and blastocyst stages vitrified in C solution are higher than those

**Table 2.** Total rates of survival and development of vitrified Awassi sheep embryos following thawing-culturing *in vitro*

| Cryoprotectants solutions* | Stages of embryos | | | | | | Total rates of survived embryos | |
|---|---|---|---|---|---|---|---|---|
| | 2- 16 cell | | Morulae | | Blastocyst and hatching blastocyst | | | |
| | No. | % | No. | % | No. | % | No. | % |
| A | 8 | 30.76 | 8 | 44.44[a] | 14 | 58.33[a] | 30 | 44.11[a] |
| B | 8 | 36.36 | 8 | 40.00[a] | 12 | 50.00[a] | 28 | 42.42[a] |
| C | 14 | 50.00 | 18 | 90.00[b] | 26 | 92.85[b] | 58 | 76.31[b] |
| Sig | NS[1] | | P < 0.05 | | P < 0.05 | | P < 0.01 | |

Values with different subscripts (a and b) differ within column at assigned probability
NS1: not significant; A: DMSO, B: EG, C: DMSO+EG

**Table 3.** *In vitro* development of various stages of Awassi sheep embryos following verification

| Developmental stage | Vitrification solution* | Vitrified (No.) | Recovered (%) | Morphologically normal (%) | Cleavages (%) | Blastocyst and hatching blastocyst (%) |
|---|---|---|---|---|---|---|
| | A | 26 | 57.69 (15/26) [a] | 50.00 (13/26) [a] | 30.77 (8/26) [a] | 23.08 (6/26) [a] |
| 2-16 cell | B | 22 | 63.64 (14/22) [a, b] | 54.55 (12/22) [a] | 36.36(8/22) [a, b] | 22.73 (5/22) [a, b] |
| | C | 28 | 78.57 (22/28) [a, b, c] | 64.29 (18/28) [a, b] | 50.00 (14/28) [a, b] | 39.20 (11/28) [a, b] |
| | A | 18 | 88.89 (16/18) [b, c, d] | 77.78 (14/18) [a, b, c] | 44.44 (8/18) [a, b] | 44.44 (8/18) [a, b] |
| Morula | B | 20 | 90.00 (18/20) [c, d] | 75.00 (15/20) [a, b, c] | 40.00 (8/20) [a, b] | 35.00 (7/20) [a, b] |
| | C | 20 | 90.00 (18/20) [c, d] | 90.00 (18/20) [c] | 90.00 (18/20) [c] | 85.00 (17/20) [c] |
| | A | 24 | 95.83 (23/24) [c, d] | 91.67 (22/24) [c] | 58.33 (14/24) [b] | 50.00 (12/24) [b] |
| Blastocyst | B | 24 | 91.67 (22/24) [c, d] | 83.33 (20/24) [b, c] | 50.00 (12/24) [a, b] | 45.83 (11/24) [a, b] |
| | C | 28 | 96.43 (27/28) [d] | 92.86 (26/28) [c] | 92.86 (26/28) [c] | 89.29 (25/28) [c] |
| Sig | | | P < 0.01 | P < 0.05 | P < 0.01 | P < 0.01 |

Values with different subscripts (a, b, c and d) differ within column at assigned probability
A: DMSO, B: EG, C: DMSO+EG

**Table 4.** Rates of lyses and arrest of embryos following thawing of various developmental stages of Awassi sheep embryos *in vitro*.

| Developmental stage | Vitrification solution* | Vitrified (No.) | Lyses (%) | Arrested (%) |
|---|---|---|---|---|
| 2-16 cell | A | 26 | 20.00 (3/15) | 26.67 (4/15) [a] |
| | B | 22 | 14.29 (2/14) | 28.57 (4/14) [a] |
| | C | 28 | 13.64 (3/22) | 22.73 (5/22) [a, b] |
| Morula | A | 18 | 12.50 (2/16) | 37.50 (6/16) [a, b, c] |
| | B | 20 | 11.11 (2/18) | 44.44 (8/18) [a, b, c] |
| | C | 20 | 0.00 (0/18) | 0.00 (0/18) [c] |
| Blastocyst | A | 24 | 8.70 (2/23) | 30.43 (7/23) [c] |
| | B | 24 | 9.09 (2/22) | 36.36 (8/22) [b, c] |
| | C | 28 | 0.00 (0/27) | 3.70 (1/27) [c] |
| Sig | | | NS | P < 0.05 |

Values with different subscripts (a, b and c) differ within column at assigned probability
NS: not significant. A: DMSO, B: EG, C: DMSO+EG

**Table 5.** Rates of embryo quality types following of various developmental stages of Awassi sheep embryos *in vitro*

| Developmental stage | Vitrification solution* | Embryo quality | | |
|---|---|---|---|---|
| | | Type 1 (%) | Type 2 (%) | Type 3 (%) |
| 2-16 cell | A | 0.00 (0/4) | 25.00 (1/4) | 75.00 (3/4) |
| | B | 0.00 (0/4) | 25.00 (1/4) | 75.00 (3/4) |
| | C | 20.00 (1/5) | 0.00 (0/5) | 80.00 (4/5) |
| Morula | A | 16.67 (1/7) | 33.33 (2/6) | 50.00 (3/6) |
| | B | 25.00 (2/8) | 37.50 (3/8) | 37.50 (3/8) |
| | C | 0.00 (0/0) | 0.00 (0/0) | 0.00 (0/0) |
| Blastocyst | A | 14.29 (1/7) | 42.86 (3/7) | 42.86 (3/7) |
| | B | 25.00 (2/8) | 37.50 (3/8) | 37.50 (3/8) |
| | C | 0.00 (0/1) | 100 (1/1) | 0.00 (0/1) |
| Sig | | NS[1] | NS | NS |

NS[1]: not significant
A: DMSO, B: EG, C: DMSO+EG

obtained by Gibbons et al. (2011) where survival rates of same two stages in goats were 41% and 50% respectively, and in sheep 64% and 64% respectively.

As evidenced in Tables 2, 3, differences were observed (P<0.01) among the rates of morulae and blastocyst stages vitrified in C solution compared with those vitrified in the tow solutions A and B indicating that using a combination of two types of cryoprotectants in vitrification helps improving embryos survivability in many stages compared to single type usage. As a result, the solidarity effect of the cryoprotectants reduces the toxicity levels in frozen embryos. Also, current results were less than the rates reached by Bagis et al. (2005) for the same solution (C solution) but with different concentrations of DMSO and EG, the values were 79%, and 43%, respectively, as well as the rate of the resulting morulae in B solution, came less than the same obtained by Bagis et al. (2005) by using EG in vitrification (69% and 52% respectively). Shirazi et al. (2010) found differences (P<0.01) in survival rates of morulae and blastocyst stages compared to 2-16 cell stage embryos in tow vitrification solutions (3.4M G + 4.8M EG and 2.7M G, + 3.4 M DMSO)**.** As for the influence of cryoprotectants on embryos survivability of 2-16 cell stage embryos, our results showed that survival rates of embryos vitrified in the three solutions A, B, and C was higher than those obtained by Martínez et al. (2006) where survival rates were less than 10%, as well as the current results, were higher than the results obtained by Han et al. (2000) which not exceeded 20%. However, the current results of the 2-16 cell stage are considered encouraging because of the importance of this stage in recent embryo technology applications, and because of the rarity in studies interested in this stage of embryonic development. These differences in the former results due to the influence of the types of cryoprotectants, the way of adding them (single or contributor), and to the solidarity effect of cryoprotectants (Leibo & Pool 2011). The differences, also, can be attributed to developmental stage and the content of lipid of frozen embryos, Gajda et al. (2011) found a slight difference among the survival rates at different stages of embryonic development, where the rates rose at morulae and blastocyst stages which they usually characterized by a decrease in the level of lipids in their cells. Also, the length of exposing embryos period to ES and VS solutions affects the survival rates of embryos before freezing (Păcală et al. 2012). In our study, the survival rates of 2-16 cell stage embryos came low compared with those in morulae and blastocyst on the one hand and low survival rates of morulae compared to blastocyst on the other hand, this, can be explained to the difference in size of the embryonic cells in different stages of development. Tachikawa et al. (1993) noted that the large size of the cells in 2-16 cell stage makes them more sensitive to the stresses of osmotic pressure and toxicity of cryoprotectants during penetrating cell embryos unlike morulae and blastocyst which characterized by the small size of their cells compared to 2-16 cell stage embryos, and thus, survival rates in blastocyst were high compared to those in the earlier stages of embryonic development.

In the current study, despite the insignificance, rates of lysed embryos came high in blastomere embryos comparing to morula and blastocyst stages (Table 4), in

literature, many studies referred that the cryodamage in morulae and blastocyst stage is higher than in the blastomeres (Gupta & Lee 2010). Balaban et al. (2008) noted that the cryodamage may affect negatively at various cooling rates by causing a perturbation in metabolism.

Absence of significance in the quality of the embryos was clarified despite the high rates of embryos of Type 3 of 2-16 cell stage embryos in the current study (Table 5), it seems that the factors that control the quality of embryos produced *in vitro* are many and a bit complicated, but the most important factor that can justify the absence of differences in the quality of embryos in most studies is the physiological and reproductive status of the animal in the period immediately preceding slaughter. Santos et al. (2008) attributed the low quality of embryos and oocytes to the effects of negative energy balance.

## CONCLUSION

It concluded from this study that using a combination of the cryoprotectants DMSO and EG in vitrification led to high survival rates of embryos compared to those vitrified in single type (DMSO or EG). Also, the vitrification of 2-16 cell stage embryos in the same solution led to encouraging rates of survivability despite the slightness in rates values.

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# Viability of Peranakan Etawah Liquid Semen Preserved in Tris Substituted with Various Energy Sources

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## ABSTRAK

Zakiya NAH, Yanti AH, Setyawati TR. 2020. Daya simpan semen cair kambing Peranakan Etawah dalam tris yang disubstitusi dengan sumber energi berbeda. JITV 25(2): 68-73. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2026

Pengunaan semen cair untuk keperluan inseminasi buatan pada kambing Peranakan Etawah (PE) merupakan alternatif untuk menggantikan semen beku yang produksinya terkendala sarana yang terbatas dan mahal. Produksi semen cair lebih cepat dibandingkan semen beku, tetapi masa simpan semen cair yang dipreservasi dengan media pengencer standar seperti tris kuning telur sangat singkat. Tujuan penelitian ini adalah untuk mengetahui viabilitas semen kambing PE dalam pengencer tris kuning telur yang disubstitusi sumber energi berupa glukosa, galaktosa, dan manosa serta sumber energi yang paling efisien untuk preservasi semen. Penelitian ini dilaksanakan pada bulan Agustus hingga September 2018 di Balai Inseminasi Buatan Lembang, Jawa Barat. Penelitian ini didesain dengan rancangan acak kelompok (RAK) yang terdiri atas tiga perlakuan yang dibagi menjadi lima kelompok. Semen segar kambing PE dipreservasi menggunakan media pengencer yang telah dimodifikasi sumber energinya. Hasil penelitian menunjukkan bahwa penggunaan glukosa pada media pengencer semen kambing PE menghasilkan motilitas terbaik di antara perlakuan lainnya (64,29 ± 9,2%). Viabilitas tertinggi ditemukan pada media pengencer dengan substitusi fruktosa (86,76 ± 2,3%). Viabilitas semen cair yang paling lama ditemukan pada media pengencer dengan substitusi glukosa yaitu dengan masa simpan enam hari.

**Kata Kunci**: Glukosa, Semen Cair, Motilitas, Kambing PE, Viabilitas

## ABSTRACT

Zakiya NAH, Yanti AH, Setyawati TR. 2020. Viability of Peranakan Etawah liquid semen preserved in tris substituted with various energy sources. JITV 25(2): 68-73. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2026

The use of liquid semen for artificial insemination program of Etawah crossbreed goat (PE) is an alternative to replace frozen semen which is constrained by limited and expensive facilities. Production of liquid semen is faster than frozen semen, but the viability of liquid semen which preserved with a standard extender such as tris egg yolk is very short. The purpose of this study was to determine the viability of PE goat semen in egg yolk tris substituted with energy sources such as glucose, galactose, and mannose and to determine the most efficient energy source for semen preservation. This research was conducted from August to September 2018 at the Artificial Insemination Center in Lembang, West Java. This study was designed in a randomized block design (RBD) consist of three experimental groups divided into five groups. Fresh semen of PE goats were preserved using extender which energy source has been modified. Results showed that using glucose in PE goat semen extender produced the best motility among other groups (64.29 ± 9.2%). The highest viability was found in extender with fructose substitution (86.76 ± 2.3%). The longest viability of liquid semen was found in the extender with glucose substitution. It lasted for six days.

**Key Words**: Glucose, Liquid Semen, Motility, PE Goat, Viability

## INTRODUCTION

Peranakan Etawah (PE) goat is a type of dairy goat that has high productivity. The demand for PE goat milk has increased since it is considered to contain richer nutrition compared to cow's milk, such as higher phosphor and has even been recommended as a milk substitution for infants, children, and adults who are allergic to cow's milk. This PE goat demand could be fulfilled through artificial insemination.

Artificial Insemination (AI) is a reproductive technology that is able to improve genetic quality of livestock and produce good quality offspring by utilizing superior males. AI involves males' semen to be taken and preserved to become frozen semen or liquid semen. Frozen semen treatment is sometimes constrained by the availability of facilities such as containers and liquid nitrogen for cryopreservation. In addition, Herdiawan (2004) stated that the quality of spermatozoa in frozen cow semen decreased by around

30-60%. The decline in quality can be seen from a decrease in viability up to 70% and low fertility of surviving spermatozoa.

The quality of liquid semen for AI is very dependent on the extender. One of the main components in the extender is nutrition, such as an energy source for spermatozoa endurance.

Rehman et al. (2013) stated that various kinds of sugar can be used as semen nutrition, such as glucose, trehalose, ribose, raffinose, saccharose, galactose. Carbohydrates commonly used in semen extender to maintain motility for longer spermatozoa are monosaccharides. The effectiveness of various types of monosaccharides in semen extender can be different because each monosaccharide crosses a different reaction path. This study aims to determine the viability of EC goat semen in tris egg yolk extender substituted with energy sources such as glucose, galactose, and mannose; and to find out the best type of energy source in EC goat semen extender.

## MATERIALS AND METHODS

### Semen collection and preservation

Semen was obtained from two 6-year-old male Etawah goats weighing 70 kg. The goat's semen is collected using an artificial vagina to obtain two ejaculates each. The semen used for preservation must meet the following requirements, namely thick to moderate consistency, spermatozoa motility ≥70%, spermatozoa concentration more than 2.5 x $10^9$, and total spermatozoa abnormalities ≤20% (Arifiantini 2012). Fresh semen from one ejaculate is divided into four parts and diluted each with different diluents (Table 1). The diluted semen is then packaged in a tube and stored in a refrigerator at 4$^o$C.

### Semen assessment

Assessment is done on fresh semen and post preservation semen. Fresh semen evaluation covers all macroscopic and microscopic aspects. The macroscopic evaluation includes volume, color, consistency (with thick, moderate, watery criteria), and degree of acidity (pH). The microscopic evaluation includes motility, concentration, viability, and spermatozoa abnormalities. Evaluation of post preservation semen includes motility, viability, and abnormal spermatozoa every 24 hours until spermatozoa motility reaches 50%.

Motility assessment is done by homogenizing one drop of fresh semen with three drops of physiological NaCl on the object-glass and covered with a glass cover and assessed subjectively using a microscope with 200 times magnification. The proportion of progressively active spermatozoa and those that are passive are compared. Spermatozoa concentrations are assessed by the Neubauer counting chamber.

Observation of viability and abnormality is done by making semen smear using eosin 2%. One drop of semen was dropped on the object-glass, then 3 drops of semen were dropped on it and homogenized, then a smear was made. The object-glass is dried on a heating table at ±40$^o$C for 5 minutes. The preparations are observed at magnification 400 times under a microscope. Observation of viability and abnormality was carried out by observing 200 spermatozoa minimum.

### Data analysis

Data in the form of motility, viability, and spermatozoa abnormalities in each treatment were analyzed with SPSS Statistics 23. A comparison of each treatment group was tested with two-way ANOVA and followed by Duncan's test with 95% confidence level.

## RESULTS AND DISCUSSION

### Quality of goat fresh semen

The assessment results showed that all macroscopic and microscopic factors of fresh semen were normal and qualified for the preservation stage (Table 2). Fresh semen looks creamy. This is in line with the statement of Ariantie et al. (2014) which states that PE goat semen is generally creamy. In fact, according to Arifiantini (2012), healthy goat semen has a broader spectrum of colors, ranging from milky white, creamy, to clear. The volume of semen ranges from 1–1.5 ml/ejaculate with an average volume of 1.4 ml/ejaculate. This volume is within the normal range for goat semen. According to Arifiantini (2012), the normal volume of goat semen ranges from 0.5–2 ml/ejaculate. The average pH of semen is 6.84. The value is still within the normal range for goat semen, which is 6.4-7.2 (Ramukhithi et al. 2011). The consistency of semen is thick and quite normal.

The percentage of fresh semen motility of PE goats ranged from 70−80% with an average value of 74%. This value is classified as normal and meets the minimum motility standard allowed for the preservation stage, which is 70−90% Arifiantini (2012). The average concentration of spermatozoa in fresh semen is normal, which is 2.48x$10^9$/ml and ranges from 2.5-2.545x$10^9$/ml. According to Ariantie et al. (2014), PE goat spermatozoa concentrations were in the range of 2.4-3.5x$10^9$/ml. This value allows the semen to proceed to the preservation stage which requires a minimum concentration of 2.5x$10^9$/ml.

**Table 1**. Composition of extender

| Composition | Variation on extender | | | |
|---|---|---|---|---|
| | K | $P_1$ | $P_2$ | $P_3$ |
| Tris aminomethan (g) | 0.6 | 0.6 | 0.6 | 0.6 |
| Citric acid monohydrate (g) | 0.33 | 0.33 | 0.33 | 0.33 |
| Energy source: | | | | |
| Fructose (g) | 0.4 | 0 | 0 | 0 |
| Glucose (g) | 0 | 0.4 | 0 | 0 |
| Galactose (g) | 0 | 0 | 0.4 | 0 |
| Manose (g) | 0 | 0 | 0 | 0.4 |
| Aquabidest (ml) | 20 | 20 | 20 | 20 |
| Yolk (ml) | 4 | 4 | 4 | 4 |
| Penicillin (g) | 3 | 3 | 3 | 3 |
| Streptomycin (g) | 3 | 3 | 3 | 3 |

**Table 2**. Average value of goat fresh semen quality tested macroscopic and microscopic

| Assessment | Value | Criteria |
|---|---|---|
| Macroscopy | | |
| Colour | Creamy | Normal |
| Volume (ml) | 1.4 | Normal |
| pH | 6.84 | Normal |
| Consistency | Thicky | Normal |
| Microscopy | | |
| Motility (%) | 74 | Normal |
| Concentration ($x10^9$/ml) | 2.4802 | Normal |
| Viability (%) | 85.6 | Normal |
| Abnormality (%) | 1.3 | Normal |

**Table 3**. Motility, viability, and abnormality of post preservation semen

| Extender | Motility (%) | Viability (%) | Abnormality (%) |
|---|---|---|---|
| K (fructose) | $58.43 \pm 9.9^a$ | $86.76 \pm 2.3^a$ | $0.57 \pm 0.6^a$ |
| $P_1$ (glucose) | $64.29 \pm 9.2^b$ | $82.83 \pm 3.6^b$ | $0.51 \pm 0.6^a$ |
| $P_2$ (galactose) | $56.57 \pm 8.7^a$ | $83.56 \pm 3.8^b$ | $0.56 \pm 0.6^a$ |
| $P_3$ (mannose) | $57.71 \pm 9.6^a$ | $81.69 \pm 3.7^b$ | $0.53 \pm 0.7^a$ |

The viability of fresh semen was in the range of 70-92% with an average of 85.6%. The percentage of total spermatozoa abnormalities ranged from 0.5−3% with an average of 1.3%. In addition, all abnormalities found in the form of folded tail and classified as secondary abnormalities. Based on Purwantara et al. (2010), reference to the INS-Indonesian National Standard which allows a maximum of 20% sperm abnormality rate including both primary and secondary abnormalities, it is expected that the primary sperm

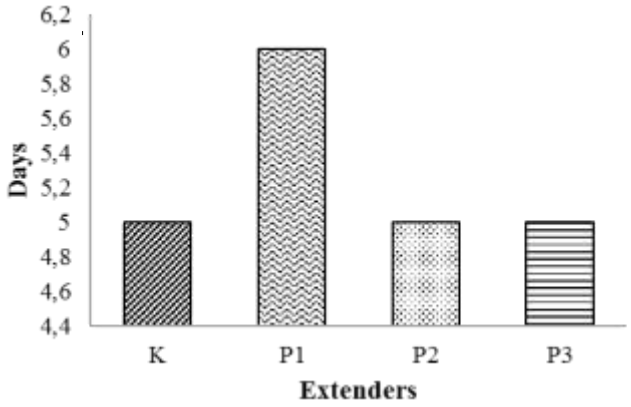


**Figure 1.** Storage age of goat semen in various extenders

abnormality rate does not exceed 10%. Thus, the abnormality value is still classified as normal and qualifies for preservation because of the total abnormal spermatozoa ≤20% (Arifiantini 2012).

**Quality of goat on post preservation semen**

The spermatozoa motility of the P1 extenders was significantly different when compared to the K, P2, and P3 extenders. The highest motility (64.29±9.2%) occurred in P1 extenders, followed by K extenders (58.43±9.9%), P2 (56.57±8.7%), and P3 (57.71±9.6%) (Table 3). This is in line with the statement of Maisse (1994) in Nynca et al. (2016) who concluded that fructose and glucose are comparable in terms of its value as an extender.

The superiority of glucose as an energy source was also evidenced by the longer storage age of the P1 extender, which lasted for six days. This calculation was based on how long the spermatozoa able to maintain the motility percentage above 50%. Motility parameters represent the activeness of spermatozoa in movement (motile) and the nature of the life of the cell so that it is appropriate to be benchmark viability of semen. Extenders K and P3 were recorded to last only for five days before their motility dropped below 50%. Nevertheless, the initial motility of extenders K, P1, P2, and P3 is almost the same, which ranged 70−74%.

The use of glucose as an energy source for spermatozoa cells involves the conversion of glucose into fructose first. Glucose is reduced to sorbitol through the enzyme aldose reductase. Sorbitol then dehydrogenates and forms fructose by sorbitol dehydrogenase. The result of fructose will be metabolized via the glycolysis pathway. Fructose gets a phosphate donor with a phosphofructokinase to fructose bisphosphate. The aldolase enzyme breaks down fructose bisphosphate into glyceraldehyde-3-phosphate (G3P). G3P forms 1,3-bisphosphoglycerate by dehydrogenase. The kinase enzyme converts 1,3-BPG to phosphoglycerate. The enzyme phosphoglycerate converts phosphoglycerate to phosphoenolpyruvate. The conversion of pyruvate phosphoenol to pyruvate by the pyruvate kinase enzyme is an exergonic reaction that produces 2 ATP.

Galactose and mannose substitutions had no significant effect on maintaining spermatozoa motility (Table 3). The utilization of galactose and mannose by cells is preceded by an overhaul involving various enzymes (McKee & McKee 2011). The two monosaccharides should be broken down to become fructose, then undergo a glycolysis reaction to form energy. Galactose reshuffle begins with the conversion of galactose to galactose-1-phosphate (G1P) through the galactokinase enzyme. The galactose-1-phosphate uridylyltransferase enzyme converts G1P into uridine diphosphate galactose (UDPGal). This compound is the intermediary that converts galactose to glucose. UDPGal through the enzyme UDP-galactose-4-epimerase produces UDP-glucose (UDPGlu) which is then converted to glucose-1-phosphate (G1P). The compound undergoes phosphate donors via phosphoglucomutase to glucose-6-phosphate (G6P) enzyme. G6P is converted to fructose and then fructose passes through the glycolysis pathway to produce energy. Likewise, mannose must be converted to fructose-6-phosphate (F6P) by hexokinase to start the glycolysis reaction.

The amount of energy produced by galactose and mannose is the same as the amount of energy produced by glucose and fructose, which is 2 ATP. However, the breakdown of galactose and mannose must be preceded by the conversion of these compounds into fructose so that the glycolysis reaction can begin. This process will delay energy consumption by cells which can result in the death of spermatozoa. Dead spermatozoa can become toxic substances and increase free radicals

(Setiadi et al. 2000). This condition can poison other spermatozoa and will gradually reduce the value of motility as well as the viability of spermatozoa.

The success of fertilization and insemination is determined not only by motility but also by the viability or viability of spermatozoa cells. The ability to survive these cells is much influenced by environmental conditions that exist, both from the natural conditions of the female reproductive organs and seminal plasma and artificial conditions such as the addition of semen extenders (if intended for artificial insemination activities). The addition of extenders and their modification into semen aims to extend the life span of cells so that cells can last longer and the probability of conception increases.

The percentage of spermatozoa viability of K extender substituted by fructose was significantly different from those of P1, P2, and P3 extender, with an average of 86,76 ± 2.3%. Apart from being a source of energy, fructose is a monosaccharide that can be used as cryoprotectant agents to avoid cold shock to cells. Bucak et al. (2012) state that cold shock is one of sublethal damage which emerge with other condition, they are ice crystal formation, oxidative stress, osmotic changes and lipid-protein reorganizations within the cell membranes, resulting in the loss of motility and viability. Cold shock can be prevented through the composition of air in cells. Water molecules in the cell can be substituted by fructose so that the cell is more stable during the temperature transition to the preservation period. This also happens as the sugar prevents sperm cells against cold shock during cold storage at extreme temperatures (Rehman et al. 2013). Cell membranes help in maintaining internal conditions and physiological activities to keep them running normally.

Water substitution by sugar also occurs in monosaccharides such as glucose, galactose, and mannose. However, spermatozoa cells more quickly substitute monosaccharides of the same type as it has low molecular weight molecules that lead to the ability to pass through the plasma membrane of spermatozoa and provide energy to function in metabolism and normal physiological manner (Naing et al. 2010). Monosaccharides from extender are used by cells just before the seminal plasma fructose runs out to use energy and save cells from cold shock. Therefore, the viability of extender P1, P2, and P3 is still relatively high (Table 3). This can be caused by monosaccharides in these three types of extender can still be used by cells as energy sources and cryoprotectant agents.

The reduced water composition after monosaccharides substitute water in the cell plasma will prevent crystallization and tear in the plasma membrane. This is caused by the ability of energy sources (sugar) as a cryoprotectant agent. This agent prevents the spermatozoal membrane from integrity damage which could consequently interfere with the fertilizing capacity of spermatozoa (Bohlooli et al. 2012).

Spermatozoa abnormalities were found in all extenders, although the percentage was very small and not significantly different between groups (Table 3). Different types of extenders do not have an impact on the tendency for abnormal spermatozoa. Besides, there was no significant increase in the percentage of abnormalities. This indicates that morphological abnormalities in spermatozoa are disorders that have occurred before semen is ejaculated.

All recorded abnormalities are folded tail types and are classified as secondary abnormalities. According to Susilawati (2011), secondary abnormalities can occur after spermatogenesis up to ejaculation and in the processing stage either. Disorders experienced by spermatozoa can be in the form of chemical factors such as contamination with urine or blood or physical factors such as heating that can take place during post-ejaculatory treatment. A very small percentage of abnormalities in this study were caused by the use of sterile semen collection tools and proper post-ejaculation treatment. Prevention of contamination of the semen is done by sterilizing the artificial vagina before use. Besides, the goat is always bathed before the collection of semen. Such treatment can minimize the possibility of exposure to spermatozoa from contaminants so that secondary abnormalities can be avoided.

## CONCLUSION

In conclusion, this study demonstrated that substitution of glucose in tris extender for Peranakan Etawah liquid semen can maintain the storage age longer among other extenders up to six days and show up the highest motility and the lowest abnormality. However, further studies still needed to observe the effect of glucose substitution in various concentrations.

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# Condition of Rumen Fermentation as Impacted by Supplementation of Fermented Rice Brand Using *In Vitro* Gas Production Technique

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**ABSTRAK**

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Gas metana merupakan salah satu gas yang dihasilkan oleh ternak ruminansia dari fermentasi di dalam rumen. Adapun tujuan dari penelitian ini adalah untuk mengetahui produksi monakolin K pada fermentasi bekatul menggunakan kapang *Monascus purpureus* dan pengaruh suplementasi bekatul yang difermentasi dengan kapang *Monascus purpureus* pada pakan rumput gajah terhadap produk fermentasi dan produksi gas metana cairan rumen secara *in vitro*. Penelitian dilakukan dalam 2 tahap, yang pertama analisis kandungan monakolin K yang terkandung dalam bekatul yang difermentasi dengan kapang *Monascus purpureus* sebanyak 0, 4, 8, dan 12% (v/w) dari substrat (bekatul). Analisis kandungan monakolin K pada substrat dilakukan dengan menggunakan HPLC. Penelitian tahap II yaitu analisis *in vitro* produksi gas dari 3 perlakuan pakan yaitu rumput gajah (kontrol), rumput gajah:bekatul (1:1), dan rumput gajah: bekatul fermentasi (kandungan monakolin K tertinggi (12% dari substrat)) (1:1). Hasil penelitian tahap I menunjukkan bahwa fermentasi bekatul dengan dosis inokulum sebanyak 12% dari substrat memiliki kandungan monakolin K tertinggi (1,39 µg/ml). Hasil fermentasi *in vitro* pakan menunjukkan bahwa perlakuan pakan tidak berpengaruh (P>0,05) terhadap kandungan ammonia, VFA, pH, aktivitas CMC-ase, protein mikroba, KCBK dan KCBO. Namun penambahan bekatul yang difermentasi pada pakan rumput gajah dapat menurunkan (P <0,05) produksi gas metana ($CH_4$) sebesar 50% dan populasi protozoa sebesar 80%. Berdasarkan hasil tersebut dapat disimpulkan bahwa penambahan bekatul fermentasi yang mengandung monakolin K ke dalam pakan dapat menurunkan produksi gas metana secara *in vitro* tanpa mempengaruhi karakteristik fermentasi rumen.

**Kata Kunci**: Fermentasi, *In Vitro*, Metana, Monakolin K, *Monascus purpureus*

**ABSTRACT**

Zuratih, Budhi SPS, Bachruddin Z. 2020. Condition of rumen fermentation as ımpacted by supplementation of fermented rice brand using ın vitro gas production technique. JITV 25(2):74-80. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2066

Methane is one of the gases produced by ruminants during feed fermentation in the rumen. This experiment was aimed to investigate the production of monacolin K in rice bran fermented by *Monascus purpureus* mold and the influence of the supplementation of fermented rice bran using *Monascus purpureus* mold on elephant grass basal diet on fermentation products and methane production in an in vitro gas production method. The study consisted of two experiments. The first experiment analysis of monacolin K production in fermented rice bran using *Monascus purpureus*. Fermentation is done by the addition of *Monascus purpureus* at levels 0, 4, 8, and 12% (v/w) of substrate (rice bran) with 3 replications. Monacolin K in the substrate was analyzed using HPLC. The second experiment was the evaluation of supplementation of fermented rice bran to elephant grass basal diet using in vitro gas production. The treatment diet evaluated were *Pennisetum purpureum* (control), *Pennisetum purpureum*:rice bran (1:1 ratio), and *Pennisetum purpureum*:rice bran fermented. Each treatment was replicated 3 times. Results from the first experiment shows that rice bran with the highest monacolin K content was in rice bran fermented at 12% by *Monascus purpureus*. Result from the second experiment showed that supplementation of fermented rice bran to *Pennisetum purpureum* basal diet did not affect rumen ammonia concentration, VFA, protein microbial production, and dry matter and organic matter digestibility. However, methane production ($CH_4$) was reduced (P<0.05) by 50%, and the protozoal population was decreased (P<0.05) by 80%. It is concluded that supplementation of fermented rice brands containing monacolin K was able to reduce methane production and the protozoa population without affecting feed fermentation.

**Key Words**: Fermentation, *In Vitro*, Methane, Monacolin K, *Monascus purpureus*

## INTRODUCTION

Global warming has been widely discussed in Indonesia and even in the world. Livestock is one of the contributors to greenhouse gas emissions (GHG). One source of GHG emissions from the livestock subsector is methane gas (CH4) from the enteric fermentation and manure removed by ruminants. Beef cattle are the

largest contributor to CH4 emissions (69.41%) compared to other ruminants (Widiawati et al. 2016).

The efforts to reduce CH4 emissions from ruminants have been carried out in Indonesia, one of them is the use of secondary metabolites of plants such as essential oils, saponins, and tannins (Benchaar & Greathead 2011; Bodas et al. 2012). However, the use of the compounds produced by bacteria and molds is still rarely done, such as the use of secondary metabolites from *Monascus purpureus* which has the potential to reduce about 30% methane production (Morgavi et al. 2013).

*Monascus purpureus* is one of the molds used in the production of food and medicine. Besides, this mold was known to produce the compounds of biologically active such as inhibitors 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) (Shi & Pan 2011). These compounds inhibit methanogenic growth in the rumen by *in vitro* (Beltowski et al. 2009).

Based on this fact, this research was aimed to determine the effect of monacolin K as a secondary metabolite of *Monascus purpureus* on methane gas production in rumen.

## MATERIALS AND METHODS

Materials used were mold of Monascus from the Central Microbiology Inter-University Laboratory (PAU), rice bran, elephant grass, and rumen fluid of Filial Ongole Cattle (PO) owned by Faculty of Animal Husbandry, Gadjah Mada University.

This research was conduct in 2 phases. The first phase about the fermentation of rice bran with the addition of 0, 4, 8, and 12% (v/w) inoculum of Monascus from the total substrate (rice bran), and the second phase was the evaluation of the effect of using fermented rice bran as an additional feed on the base feed of elephant grass against methane production by *In vitro* gas test according to the Menke & Steingass 1988 method.

### Fermentation of rice bran by *Monascus purpureus*

A total of 50 g substrates was transferred to 250 ml Erlenmeyer flasks, added distilled water (40% DM) then autoclaved at $121^0$C for 30 min, left it until the temperature becomes 25-$30^0$C. After that, the *Monascus purpureus* was inoculated into the substrate as much as 0, 4, 8, and 12% (v/w) from the dry weight of the substrate, then incubated for 9 days. Each level of inoculation was repeated 3 times. After the fermentation finished, weighed the product and dried in an oven at $100^0$C for 30 minutes to inactivate the mold, then continue baking at $50^0$C for 24 hours. After that, stir evenly, ground and samples were taken to measure the pH and levels of monacolin K.

### PH measurement and analysis of monacolin K levels of fermented rice bran using HPLC

pH measurement of the substrate was carried out by a digital pH meter. A total of 1-gram substrate was dissolved with 10 ml of distilled water and then the pH was measured using a digital pH meter that had previously calibrated using a pH buffer of 4 and 7. Measurement of monacolin K content was performed by using HPLC at the Toxicology and Pharmacology Laboratory of the Faculty of Pharmacy UGM. A total of 1 mg the fermented product was milled and dissolved with 9 ml of ethanol 67% (v/v) then stirred at $50^0$C for 2 hours. After that, precipitated and then the supernatant was taken to be analyzed for monacolin K levels using HPLC according Zhang et al. 2013.

### *In vitro* gas production (Menke & Steingass 1988)

The feeds were tested using *in vitro* gas production from 3 treatments, namely elephant grass (control), elephant grass: rice bran (1:1), and elephant grass: fermented rice bran (1:1); each treatment consisted of 3 replications. The fermented rice brand used was the best result of the first phase, based on AOAC method (2006).

The fermentation medium was prepared by mixing 474 ml of McDougall's buffer, 0.12 ml of mineral B solution, 237 ml of buffered solution, 237 ml of mineral A solution, 1.22 ml of resazurin solution and 49.5 ml of reduced solution ($Na_2S$) put into the Erlenmeyer 2 L. then mixed with 2,000 ml of rumen fluid while continuously flushed with $CO_2$ in anaerobic conditions before being put into a syringe glass. The ratio of rumen fluid and the medium is 1:2 (v/v) (Karlsson et al. 2009).

Approximately 300 mg of each test feed was put into the glass syringe which contains 30 ml of fermentation medium. All glasses were then incubated in a modified water bath at 39oC for 72 hours then its gas production was observed. At 0, 1, 2, 4, 6, 8, 12, 24, 36, 48, and 72 h measurement volumes were recorded; samples of gases produced were taken in Vacutainer® tubes for CH4 concentration analysis using Gas Chromatography (GC) and then released. At the end of this incubation (72 h), the liquid phase was centrifuged at a rate of 3,000 g. Its filtrate was used for testing rumen fermentation parameters (ammonia levels, VFAs, pH, and methane gas production) and microbial activity (CMCase, microbial proteins, and protozoal). The remaining material was filtered through sintered crucibles to determine *in vitro* apparent dry matter and

organic matter degradability. The residual dry matter and organic matter contents were determined to refer to the AOAC (2006). Dry matter (DM) and ash contents were determined by drying at 105 $^0$C for 8 h and at 550 $^0$C for 6 h, respectively.

Ammonia was determined according to Chaney & Marbach (1962). A total of 0.5 mL filtrate was centrifuged at 10,000 g for 10 minutes, then 20 µL of the sample supernatant was added with 2.5 mL LC (a mixture of phenol 50 mg Na nitroprusside and 10 gr crustal phenol which dissolved with aquadest to 1 L volume) and 2.5 mL LD (a mixture of hypochlorite and 5 gr NaOH, 21.31 gr $Na_2HPO_4$ anhydrous or 269.7125 gr $Na_2HPO_4$ 1 $2H_2O$ which dissolved with 100 mL aquadest and 25 mL sodium hypochlorite 5%) then homogenized.

Measurement of VFA produced during *in vitro* fermentation was carried out according to Filípek & Dvořák (2009). A total of 0.2 mL of filtrate was added with 1 mL of metaphosphoric acid, then centrifuged at 10,000 g for 10 minutes. A total of 1µL of the supernatant sample was taken and injected into gas chromatography.

CMase activity was carried out using 0.1 acetate buffered solution; 1% CMC solution; cyanide carbonate solution; $Na_2CO_3$ solution; and 0.05% potassium ferricyanide solution (Halliwell & Lovelady 1981).

Protozoal populations were taken from the incubation medium at the end of fermented (72 h). The population was counted in the counting chamber thick as 0.2 mm using a microscope with a magnification of 40 times (Diaz et al. 1993).

The determination of microbial protein levels was measured according to the Lowry method (Plummer 1987). A total of 0.5 mL sample was put into a test tube then added with 2.5 mL of Lowry B solution then homogenized and allowed to stand for 30 minutes. After that, it was added 0.25 mL of Lowry A solution and allowed to stand for 10 minutes at room temperature then read using a spectrophotometer at a λ of 750 nm.

The data obtained were statistically analyzed using a completely randomized directional pattern design using the SPSS Program version 16.0. If there are differences, the analysis continued with the Duncan Test.

## RESULTS AND DISCUSSION

### Content of monacolin K of fermented rice bran

The effect of *Monascus purpureus* level on pH and monacolin K production of rice bran is presented in Table 1. Table 1 shows that the inoculum dose of *M. purpureus* had a significant effect (P <0.05) on the pH and production of monacolin K substrate. The pH of the substrate with the addition of 12% inoculum was still within the normal range for the growth of *M. purpureus* is 7-8. This result was also supported by an increase of monacolin K levels. The highest production of monacolin K in this study was in the addition of 12% (v/v) inoculum that is 1.39 µg/ml or equivalent to 154 mg/kg. This result was lower than that reported by Morgavi et al. 2013 which produced 570 mg/kg monacolin K with fermented rice used as substrate and ammonium sulfate addition to increasing monacolin K production. This is caused by the carbohydrate content of rice bran is lower than rice, while the effectiveness of the fermentation of Monascus in producing monacolin is influenced by carbohydrate content (Liu et al. 2020). Besides, the addition of ammonium sulfate can also increase the content of monacolin K (Su et al. 2003).

### Characteristics of rumen fermentation

The effect of fermented rice bran addition to elephant grass basal diet on rumen fermentation characteristics of 72 h in vitro incubations is presented in Table 2.

Table 2 shows that the addition of fermented rice bran using *M. purpureus* did not affect rumen ammonia levels. Ammonia levels in this study were 25 to 28 mg/100 mL, which was still in the normal range to support the growth of rumen microbes that is 10.21 to 35.76 mg/100 mL (Olijhoek et al. 2016). Ammonia levels in the rumen are indicative of protein degradation. Protein will be degraded to oligopeptides, then to peptides and amino acids, then the process of amino acid deamination will produce ammonia (Goldberg 2013). Besides, ammonia levels in the rumen also describe degradation and protein synthesis process by rumen microbes. If the feed is protein-deficient, the ammonia concentration in the rumen will be decreased, and the growth of rumen microbes will be slow, that causes decreased digestibility of feed (Suharti et al. 2019). As in this study, with the same ammonia levels in each treatment caused no difference in the digestibility of dry matter and organic matter (Table 4).

The addition of the fermented rice bran did not affect average VFA levels (acetate, propionate, and butyrate) and the acetate: propionate ratio. In this study, the proportion of acetate was higher than propionate. This caused the feed in the fermented liquid to contain a lot of fiber. Glucose-rich food increased propionate production while fiber-rich feed increased acetate production (Suryani et al. 2014). The addition of rice bran without or with fermentation using *M. purpureus* did not affect (P <0.05) on pH (Table 2). The addition of fermented rice bran in this study resulted in a range of pH values that were still within the normal pH range for the rumen fermentation process, which is 6-7. These results were in line with that reported by Candyrine et al. (2018), that the addition of 2 mg/kg body weight/day lovastatin to goat feed resulted in rumen pH of 6.59.

**Table 1.** Average pH and monacolin K production of rice bran after fermentation with *Monascus pupureus* at various levels

| Parameters | *Monascus purpureus* % DM | | | |
|---|---|---|---|---|
| | 0 | 4 | 8 | 12 |
| pH | 6.53[a] ± 0.06 | 8.50[b] ± 0.30 | 9.00[c] ± 0.21 | 8.20[b] ± 0.06 |
| Monacolin K (µg/ml) | 0.00[a] ± 0.00 | 0.01[a] ± 0.01 | 1.07[b] ± 0.14 | 1.39[c] ± 0.21 |

[a,b,c] Means within rows and subtitles followed by distinct superscripts differ (Duncan test at 5%)

**Table 2.** Ammonia levels, VFA, and pH of fermented rice bran with *M. purpureus* used as inoculum of 72 hours *in vitro* incubations

| Parameters | Feed | | |
|---|---|---|---|
| | Elephant grass | Elephant grass:rice bran (1:1) | Elephant grass:fermented rice bran (1:1) |
| Ammonia (mg/100 mL) | 25.62 ± 0.34 | 28.05 ± 1.60 | 25.80 ± 1.95 |
| VFA (%) | | | |
| Acetate | 78.00 ± 1.83 | 77.06 ± 0.51 | 76.39 ± 0.84 |
| Propionate | 15.77 ± 0.53 | 14.28 ± 0.40 | 15.99 ± 1.48 |
| Butyrate | 6.23 ± 2.08 | 8.66 ± 0.44 | 7.63 ± 0.67 |
| Acetate:propionate | 4.95 ± 0.17 | 5.40 ± 0.18 | 4.81 ± 0.50 |
| pH | 6.25 ± 0.04 | 6.26 ± 0.08 | 6.30 ± 0.07 |
| Methane (mL/100 mg DM) | 5.52 ± 0.71[b] | 5.53 ± 1.29[b] | 2.75 ± 1.14[a] |

[a,b] Means within rows and subtitles followed by distinct superscripts differ (Duncan Test at 5%)
VFA: Volatile Fatty Acid

The addition of fermented rice bran reduced methane production (P <0.01) to 50.2% (Table 2). These results were in line with that reported by Morgavi et al. (2013) that the use of rice bran fermented with *Monascus sp*. and hay with a ratio of 1: 1 in sheep reduced 30% methane production *in vitro*. This result caused by the *Monascus sp*. that produces secondary metabolites such as monacolin K. Monacolin K is an HMG-CoA reductase inhibitor, which is an enzyme that plays a role in cholesterol formation (Sharpe & Brown 2013a). With monacolin K, the formation of cholesterol will be disrupted so that the development of protozoal inside will also be disrupted because cholesterol is one of the constituent components of the protozoal cell membrane. Protozoal live in symbiosis with methanogenic bacteria (methane-producing bacteria) in the rumen. Methanogenic bacteria get a constant supply of hydrogen from protozoal, so a decrease in the protozoal population in the rumen will indirectly reduce methane production (Martin et al. 2010).

**Microbial activity**

Effect of fermented rice bran addition to elephant grass basal diet on rumen microbial activity of 72 h *in vitro* incubations is presented in Table 3.

**CMCase: Carboxymethyl cellulase**

Carboxymethyl cellulose is a cellulose degradation enzyme which is a polysaccharide contained in the feed (Sitoresmi et al. 2009). Results showed that the addition of rice bran without or with fermentation using *M. purpureus* did not affect the activity of CMCase fermentation fluid. Results were in line with that reported by Candyrine et al. (2018), the use of HMG-CoA reductase inhibitors (mevastatin and lovastatin) did not affect the growth of a fiber-degrading bacteria in the rumen.

**Table 3.** CMCase activity and the number of protozoal of fermented rice bran with *M. purpureus* used as inoculum of 72 h *in vitro* incubations

| Parameters | Feed | | |
|---|---|---|---|
| | Elephant grass | Elephant grass:rice bran (1:1) | Elephant grass:fermented rice bran (1:1) |
| CMCase (U/g) | 1.98 ± 0.67 | 2.21 ± 0.54 | 2.23 ± 0.15 |
| Protozoal ($x10^3$)/mL | $24.39^{ab}$ ± 13.81 | $30.67^{b}$ ± 6.29 | $6.31^{a}$ ± 4.83 |
| Microbial protein (mg/mL) | $0.20^{a}$ ± 0.06 | $0.36^{b}$ ± 0.04 | $0.18^{a}$ ± 0.63 |

[ab] Means within rows and subtitles followed by distinct superscripts differ (Duncan test at 5%)

**Table 4**. The digestibility of dry matter and organic matter of fermented rice bran with *M. purpureus* used as inoculum of 72 h *in vitro* incubations

| Parameters | Feed | | |
|---|---|---|---|
| | Elephant grass | Elephant grass:rice bran (1:1) | Elephant grass:fermented rice bran (1:1) |
| Dry matter digestibility | 41.63 ± 8.53 | 43.68 ± 0.47 | 44.46 ± 8.79 |
| Organic matter digestibility | 41.06 ± 6.12 | 41.63 ± 1.96 | 43.74 ± 12.20 |

The addition of fermented rice bran using *M. purpureus* reduced the number of rumen fluid protozoal. As stated earlier, secondary metabolites (monacolin K) produced by *M. purpureus* are HMG-CoA reductase inhibitors (Sharpe & Brown 2013b). Monacolin K compounds competed with HMG-CoA reductase enzymes in binding HMG-CoA so that will be inhibited the formation of mevalonic acid, which is the stage of cholesterol formation. Cholesterol is one of the constituent components of the protozoal cell membrane so that with monacolin K the growth of protozoa in the rumen will also be disrupted, causing the protozoal population in the rumen to be reduced. In this study, the use of fermented rice bran reduced 74.13% the protozoa population. These results are in line with that reported by Dinesh et al. (2014), the addition of statin compounds (atorvastatin and simvastatin) can inhibit the growth of *Leishmania donovani* which is one type of protozoal.

The addition of fermented rice bran using *M. purpureus* did not affect the microbial protein. Microbial protein derived from bacteria, fungi, and protozoa in the rumen. The use of rice bran in the diet increased the population of protozoa and rumen microbes, which caused the addition of carbohydrates and fiber in the rice bran. As reported by Martínez et al. (2010), an increase in the ratio of carbohydrates in the feed will increase the protozoal population in the rumen. Whereas, the addition of fermented rice bran decreased the protozoal population due to the presence of Monacolin K. Therefore, the protozoal population decline causes a decrease in microbial protein production.

**Effect of fermented rice bran on the digestibility of dry matter and organic matter**

Effect of fermented rice bran addition to elephant grass basal diet on the digestibility of dry matter and organic matter of 72 h *in vitro* incubations are presented in Table 4.

The results show that the addition of rice bran without or without fermentation using *M. purpureus* did not affect (P> 0.05) digestibility of dry matter and organic matter diet *in vitro*. These results are in line with that reported by Candyrine et al. (2018), the addition of fermented oil palm cake using *Aspergillus terreus* (lovastatin 850 mg/kg DM) on goat diet, did not affect the total rumen microbial population and feed digestibility. This result also indicated that there is not significantly different on the VFA, NH3 (Table 2), and rumen microbial protein (Table 3)..

## CONCLUSION

Fermentation of *Monascus purpureus* in rice bran produced Monacolin K with the best results at the level of 12% DM. The fermented rice bran reduced methane production by 50%, protozoal population, and microbial proteins without affecting ammonia production, pH, CMCase enzyme content, and nutrient rumen fluid in *vitro*. Monacolin K derived from *M. purpureus* has the potential to be used as an additive to animal feed for reducing methane production in the rumen. *In vivo* research needs to be done to see the benefits of using *M. purpureus* as a food additive in reducing emissions of enteric methane.

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# Biological Evaluation of Some Plant Bioactives as Feed Additives to Replace Antibiotic Growth Promoters in Broiler Feeds

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**ABSTRAK**

Sinurat AP, Pasaribu T, Purwadaria T, Haryati T, Wina E, Wardhani T. 2020. Evaluasi biologis zat bioktif beberapa tanaman sebagai imbuhan pakan pengganti antibiotik pada ayam broiler. JITV 25(2):81-90. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2501

Antibiotik (AGP) sudah lama digunakan sebagai imbuhan pakan untuk meningkatkan pertumbuhan dan efisiensi penggunaan pakan dalam industri perunggasan. Namun, banyak negara, termasuk Indonesia sudah melarang penggunaannya dan banyak upaya dilakukan untuk mengganti antibiotik agar produktifitas broiler tetap tinggi. Bioaktif tanaman adalah salah satu alternatif yang mungkin dapat menggantikan AGP. Suatu penelitian dilakukan untuk mengganti AGP dengan campuran beberapa sumber zat aktif tanaman yaitu asap cair cangkang mete, tanaman meniran dan daun cengkeh. Delapan (8) ransum perlakuan disusun dengan kandungan gizi yang sama, terdiri dari kontrol negatif (NC), kontrol positif (NC+AGP), ransum NC yang ditambahkan bioaktif tanaman bentuk cair dalam 3 dosis dan ransum NC yang ditambahkan bioaktif tanaman bentuk tepung dalam 3 dosis. Setiap ransum diberikan pada ayam broiler mulai umur sehari hingga 35 hari dengan 6 ulangan tiap perlakuan dan 10 ekor/ulangan. Performan dan indikator respon immunitas ayam diamati selama penelitian. Hasil menunjukkan bahwa penambahan bioaktif tanaman bentuk tepung tidak dapat meningkatkan performan ayam. Penambahan bioaktif tanaman maupun AGP tidak mempengaruhi imunitas ayam broiler. Namun, bioatif tanaman dalam bentuk cair dengan dosis rendah dapat meningkatkan performan ayam broiler, bahkan lebih baik dari peningkatan dengan penambahan antibiotik. Oleh karena itu, kombinasi bioaktif tanaman dalam bentuk cair dengan dosis rendah dapat digunakan sebagai pengganti antibiotik imbuhan pakan (AGP).

**Kata Kunci**: *Antibiotic Growth Promoters*, Bioaktif Tanaman, Broiler

**ABSTRACT**

Sinurat AP, Pasaribu T, Purwadaria T, Haryati T, Wina E, Wardhani T. 2020. Biological evaluation of some plant bioactives as feed additives to replace antibiotic growth promoters in broiler feeds JITV 25(2):81-90. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2501

Antibiotics (AGP) have been used as feed additives to promote growth and feed efficiency in poultry production. However, many countries include Indonesia now ban the use of AGP and attempts are made to replace the antibiotic to maintain good performances of broilers. Plant bioactives is one of the alternatives that could replace the AGP. An experiment was conducted in an attempt to replace the AGP in broiler feed with a mixture of some plant bioactives (liquid smoke of cashew nutshell, *Phyllanthus niruri*, and clove leaves). Eight (8) dietary treatments were formulated to have similar nutrients consist of negative control (NC), positive control (NC+AGP), diets supplemented with liquid plant bioactives in 3 levels and diets supplemented with powder plant bioactives in 3 levels. Each diet was fed to 6 replications of 10 birds each from 1 to 35 days old. The performances and the immune response of the broilers due to the treatments were observed. Results showed that the powder plant bioactives could not improve the performance of broilers. None of the feed additives (AGP or plant bioactives) affect the immune systems of the broilers. However, liquid plant bioactives in low dose improved the performance of broilers better than the AGP and therefore is suitable to replace the antibiotic as feed additives in broiler diet.

**Key Words**: Antibiotic Growth Promoters, Broilers, Plant Bioactives

## INTRODUCTION

Since the 1940's some antibiotics, known as antibiotic growth promoters (AGP) have been commonly supplemented into poultry feed to improve performances of chickens. The improvement is shown in the mortality reduction, and enhancement in productivity and feed utilization (Dibner & Richards 2005). Questions about the advantages and disadvantages of using AGP began after Swan Report was submitted by the UK scientific commitee to the parliament in 1969. The report highlighted that "the

administration of antibiotics to farm livestock possess certain hazards to human and animal health since it has led to the emergence of strains of bacteria which are resistant to antibiotics". Since then, many countries have banned or restricted the use of AGP. Indonesia has started to ban the use of AGP as stated in the Act since 2009 (RI 2009), although the regulation was effectively applied since January 2018.

Some ingredients or materials have been investigated and used in the livestock industry to replace the AGP commercially, such as enzymes, organic acids, plant bioactives, probiotic, prebiotic and synbiotic (Sinurat et al. 2017). Most of these products are imported and more expensive when compared to commercial AGP. Supplementation of exogenous enzymes improves the condition of gastrointestinal (GI) tracts and reduces the microbial population in intestinal such as *C. perfringens* (Sun et al. 2015) and *Campylobacter* (Wealleans et al. 2017). Supplementation of organic acids into feed lower the pH in the GI of animals, penetrate the cell wall of bacteria and disrupt their growth and therefore can be used as an alternative to AGP (Hassan et al. 2010; Khodambashi et al. 2013; Khan & Iqbal 2016; Cengiz et al. 2012). Other substances such as probiotics and prebiotics have been shown to decrease the population of *E. coli,* but increase the population of bifidobacteria and lactobacilli and improved broiler performances similar to the AGP (Afrouziyeh et al. 2014; Mazhari et al. 2016).

Some plants contain active substances or Phyto bioactives that can be used to replace AGP in animal feed. The bioactives are defined as secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals (Blomhoff 2010). Sarica et al. (2007) reported that herbal powder (thyme and garlic) was as effective as AGP (flavomycin) in improving the growth performance of broilers and reducing the population of total aerobic bacteria and *E. coli* in the small intestine. Some studies also showed that the combination of plant bioactives with enzymes (Sarica et al. 2007) or with probiotics, and organic acids (Manafi et al. 2016) showed as good or even better than the AGP in improving performance and immunity of broilers against some diseases. Plant bioactives obtained from *Aloe vera* leaves have been reported effectively to inhibit the growth of some microbes such as *Salmonella hadar* and *E. coli* (Sinurat 2013) and improved the feed conversion efficiency when supplemented in the broiler or laying hens diet (Bintang et al. 2005; Sinurat et al. 2004; Sinurat 2013). Sharifi et al. (2013) also tested four (4) medicinal plants: cumin (*Cuminum cyminum*), peppermint (*Mentha piperita*), yarrow (*Achillea millefolium*) and poley (*Teucrium polium*) and found the only peppermint could be used as a feed additive to replace the AGP (flavomycin).

As a tropical country, Indonesia is rich in plants that contain bioactives. Some plants have been used traditionally for human healthy food, drinks, and medicines in Indonesia (Affandi et al. 2004). Many studies conducted on plant bioactives aimed to replace the AGP in poultry feed. Most of the studies were based on the active substances found in the extract of one or single plant to inhibit the growth of pathogenic microorganisms or bacteria (Sarica et al. 2007; Oleforuh-Okoleh et al. 2014; Ameri et al. 2016; Sarker et al. 2016; Pasaribu & Wina 2017; Yesuf et al. 2018; Mashayekhi et al. 2018) although some studies used a combination of some plant bioactives (Ertas et al., 2005, Zaki et al., 2016). A new approach was initiated to find feed additives by combining 3 plant bioactives with high activity as anti-bacteria, anti-fungi, and antioxidant. Sinurat et al. (2018) evaluated 12 plants and found 3 plant bioactives that are potentially used to replace the AGP, i.e., liquid smoke of cashew nuts (*Anacardium occidetale*) shells (CNSL) effective to inhibit the growth of pathogenic bacteria, clove (*Syzygium aromaticum*) leaves extract effective to inhibit the growth of fungi and leaf flower (*Phyllanthus niruri*) extract effective as an antioxidant. Combinations of the three bioactives have been tested *in vitro* with similar results to AGP in inhibiting the growth of *E. coli* and *Salmonella* spp. (Pasaribu et al. 2018). Based on those studies, an experiment was designed to study the effectiveness of these bioactives mixture on the performances of broilers.

## MATERIALS AND METHODS

Three plant bioactives, i.e., liquid smoke of cashew nut (*Anacardium occidentale*) shell (CNSLL), an extract of leaf fruits (*Phyllanthus niruri*) plants (LF) and extract of clove (*Syzygium aromaticum*) leaves (CL) were used in this study. Those bioactive were reported to have the highest activity as anti-bacteria, antioxidant, and anti-fungi as reported previously in an *in vitro* study (Sinurat et al. 2018). A further *in vitro* study showed that the effective combination of the three bioactives to inhibit the growth of *E. coli* and *Salmonella* spp. was at the concentration of 0.0313 % *CNSL,* 0.0313% LF extracts and 0.0157% LF extract. Therefore, this combination was used as a basis in this feeding trial. The combined feed additives were formulated in 2 forms, *i.e.* in liquid or extract form (LPB) and powder form (PPB). The LPB composed of 8.9% LF extract, 88.8% CNSL, and 2.3% CL extract and the PPB were formulated to contain similar bioactives contents, i.e., 26.7% LF powder, 66.6% CNSL and 6.7% CL powder (Pasaribu et al. 2018). Since the CNSL was already in liquid form, therefore the volume or weight of LF and CL were adjusted to meet the concentration of the combined feed additives.

**Tabel 1.** Composition of negative control-starter and grower diet

| Ingredients (%) | Starter | Grower |
|---|---|---|
| Maize | 60.73 | 62.00 |
| Meat and bone meal | 6.00 | 4.00 |
| Soybean meal | 30.27 | 29.50 |
| Vegetable oil | 1.14 | 2.19 |
| Limestone | 0.18 | 0.77 |
| DL-Methionine | 0.41 | 0.24 |
| Threonine | 0.08 | 0.01 |
| L-Lysine | 0.35 | 0.12 |
| Sodium bicarbonate | 0.10 | 0.10 |
| Salt | 0.20 | 0.20 |
| Vitamin Premixes | 0.03 | 0.03 |
| Mineral Premixes | 0.05 | 0.05 |
| Dicalcium phosphate | 0.36 | 0.69 |
| Choline Chloride | 0.10 | 0.10 |
| Total, % | 100.00 | 100.00 |
| Nutrient Content (calculated): | | |
| Dry matter, % | 87.60 | 88.90 |
| Crude fibre, % | 3.30 | 3.27 |
| Metabolisable energy, kcal/g | 2900.00 | 3000.00 |
| Crude Protein, % | 22.50 | 21.00 |
| Crude fat, % | 4.69 | 5.56 |
| Calcium, % | 0.90 | 1.00 |
| Available Phosphorous, % | 0.50 | 0.45 |
| Digestible Lysine, % | 1.270 | 1.010 |
| Digestible Methionine, % | 0.679 | 0.492 |
| Digestible Methionine + Cystine, % | 0.940 | 0.76 |
| Digestible Tryptophan, % | 0.219 | 0.213 |
| Digestible Threonine, % | 0.800 | 0.680 |

Two control diets, *i.e.*, a negative control- without AGP (NC) and positive control (PC), i.e., the NC+40 ppb zinc bacitracin were formulated. Experimental diets were formulated as a starter diet fed from day old to 21 d and grower diet fed from 22 to 35 days old broiler chickens. The composition of the control diet is shown in Table 1. The effect of plant bioactives was studied by supplementing the LPB or PPB into the NC diet in three doses, respectively. The levels of supplementation tested were: low LPB (1810 ml/ton diet), medium LPB (3620 ml/ton diet), high LPB (5430 ml/ton diet), low PPB (2350 g/ton diet), medium PPB (4700 g/ton diet) and high PPB (7050 g/ton diet). Similar levels were added into the starter (0 to 21 days) and grower (22 to 35 days) diets. Therefore, 8 dietary treatments were tested in this trial, i.e.:

a. Negative Control diet without AGP (NC)
b. Positive Control diet, i.e, NC+40 g zinc bacitracin 500 g/ ton diet (PC).
c. Low level liquid plant bioactives: NC+1810 ml/ton diet (Low LPB)
d. Medium dose liquid plant bioactives: NC+3620 ml/ton diet (Medium LPB).

e. High level liquid plant bioactives, i.e., NC+5430 ml/ton diet (High LPB).
f. Low-level powder plant bioactives, i.e., NC+2350 g/ton diet (Low PPB).
g. Medium level powder plant bioactives, i.e., NC+4700 g/ton diet (Medium PPB).
h. High-level powder plant bioactives, i.e., NC+7050 g/ton diet (High PPB).

Each diet was fed to 60 birds (10 birds per pen with 6 replications) reared in a conventional litter broiler house. Rearing management of the birds was conducted to normal standard procedures where the feed and water were given *ad libitum*. Feed intake, body weight at 1 day, 21 days, 28 days, and 35 days old, were measured and the survival rates were recorded. At 34 days old, blood samples were taken from the wing vein of 1 bird from each pen. The blood samples were sent to the laboratory to measure the lymphocyte, monocyte, and heterophil as an indication of the immunity levels. At the end of the trial, 1 bird from each cage was slaughtered to measure the carcass percentage, weights of abdomen fat, liver, Fabricius bursa, and spleen.

All data obtained were subject to analyses of variance in a completely randomized design. The difference between treatments was determined by Duncan test if the analyses of variances were significant at $P<0.05$.

## RESULTS AND DISCUSION

The performance of the birds during the starter period (1 to 21 days old) and grower period (1 to 28 days old and 1 to 35 days old) are presented in Tables 2, 3, and 4, respectively. Body weight of the birds at the beginning of the trial or 1 day old was very similar which indicates their homogeneity. The body weight at 21 days old was not significantly ($P>0.05$) affected by supplementation of AGP nor by the plant bioactives. However, numerically the heaviest birds were found when the feed was supplemented at low concentration of liquid plant bioactives (778 g) and the lighter birds were found when the birds were fed without feed additives or the negative control (679 g). The body weight of birds feed with the AGP was in between (720 g). The growth improvement was 6.0% and 14.6% due to AGP and liquid bioactives supplementation, respectively as compared to the negative control.

Body weight of broilers at 28 and 35 days old were also not significantly ($P>005$) affected by supplementation of the AGP nor by the plant bioactives. The treatments effect on body weight at 28 and 35 days have a similar trend as in the starter period. The heaviest birds (1199 g at 28 days and 1751 g at 35 days) were achieved when fed with a diet supplemented with low dose liquid plant bioactives. However, the degree of improvement due to AGP or liquid plant bioactives was decreasing as the birds were older. The body weight improvement due to AGP supplementation were 6.0%, 2.0% and 1.3% at 21, 28 and 35 days old and body weight improvement due to low dose liquid plant bioactives supplementation were 14.6%, 7.9% and 4.2% at 21, 28 and 35 days, respectively. Therefore, it was consistent that the highest improvement in body weight was achieved in birds fed a diet with low dose liquid plant bioactives, followed by those fed diets with AGP during the starter and grower period.

Body weight improvement in broilers due to AGP supplementation varied according to some reports such as from 2% to 9% (Miles et al. 2006), 5.2 % (Costa et al. 2017), 10.1 % (Mashayekhi et al. 2018) and 14.4 % ( Emami et al. 2012). Reports on the use of plant bioactives as feed additives have been reported with different degrees of improvement on the body weight gain of broilers. Ertas et al. (2005) showed 16.3% body weight gain improvement by the inclusion of 200 ppm mixed essential oils. Mashayekhi et al. (2018) showed 7.3% BWG improvement by the inclusion of 0.5% eucalyptus leaf powder. However, supplementation of mixed medicinal plant leaves in the powder form reduced the body weight gain of broilers by 2.9% (Aroche et al. 2018), which is similar to our findings in this research. The results found in this experiment indicated that the liquid plant bioactives produce a better body weight improvement as compared to the AGP and therefore, could be used as growth promoters to replace the AGP. The non-significant results found in this trial may be due to large variations among the replications.

The feed intake during the starter and grower periods were not significantly ($P>0.05$) affected by the supplementation of AGP nor by the plant bioactives. Supplementation of plant bioactives in powder form tends to increase the feed intake of the broilers and the highest feed intake was found in broilers fed on a low dose of powder plant bioactives, i.e., 1206, 1976 and 2954 g/bird and the lowest feed intake was found in broilers fed medium dose of liquid plant bioactives, i.e., 1086, 1820 and 2732 g/bird during 1 to 21, 1 to 28 and 1 to 35 days period, respectively.

Plant bioactives were added without adjustment to the nutrient contents of the diet. The AGP (40 ppb) and the liquid plant bioactives (0.180 to 0.543%) were added in small quantities, while the powder plant bioactives were added in larger quantities (0.235 to 0.705%) due to make equal bioactives concentration in feed. Therefore, broilers fed with powder plant bioactives tried to meet their nutrient requirements by increasing the feed intake. This trend was only observed during the starter period (1 to 21 days), although the differences were not statistically significant. The effect on the feed intake during the grower period however was not consistent and could not be explained. Based on the literature, there was no consistent effect of AGP and

**Table 2.** Performance of broilers fed diet supplemented with antibiotic growth promoters (AGP) or plant bioactives during stater period (1 to 21 daysay)

| Treatments | Body Weight DOC (g) | Body Weight 21 d (g) | Feed intake (g/bird) | FCR* | Liveability (%) |
|---|---|---|---|---|---|
| Negative Control (NC) | 53.9±2.5 | 679.2±90.1 | 1,116 ±103 | 1.630ab±0.102 | 100.0±0.0 |
| Positive Control (NC + AGP) | 53.2±4.0 | 720.1±84.4 | 1,122±88 | 1.593b±089 | 100.0±0.0 |
| NC + Low LPB | 52.9±3.7 | 777.7±75.7 | 1,1401±81 | 1.498b±0.87 | 98.3±4.1 |
| NC + Medium LPB | 53.0±2.8 | 689.0±37.1 | 1,086±53 | 1.578b±0.80 | 100.0±0.0 |
| NC + High LPB | 54.2±2.8 | 714.0±103.3 | 1,103±127 | 1.579b±0.091 | 98.3±4.1 |
| NC + Low PPB | 53.5±3.7 | 693.1±114.4 | 1,206 ±132 | 1.785a±0.146 | 100.0±0.0 |
| NC + Medium PPB | 52.2±3.5 | 681.4±84.0 | 1,169±111 | 1.790a±0.154 | 96.7±5.2 |
| NC + High PPB | 53.5±3.4 | 713.9±84.6 | 1,137±104 | 1.632ab±0.132 | 98.3±4.1 |
| Significance (P) | 0.99 | 0.58 | 0.49 | 0.003 | 0.47 |

LPB = Liquid plant bioactives; PPB = Powder plant bioactives
*Different superscripts within the same column showed significant differeneces (P<0.05)

**Table 3.** Performance of broilers fed diet supplemented with plant bioactives during 1 to 28 days

| Treatments | BW 28 days (g) | Feed intake (g/bird) | FCR 1-28 days | Liveability (%) |
|---|---|---|---|---|
| Negative Control (NC) | 1,111 ± 146 | 1,897 ± 146 | $1.759^{abc}$ ± 0.141 | 95.0 ± 8.4 |
| Positive Control (NC + AGP) | 1,133 ± 175 | 1,920 ± 140 | $1.714^{bc}$ ± 0.112 | 96.7 ± 8.2 |
| NC + Low LPB | 1,199 ± 71 | 1,921 ± 122 | $1.647^{c}$ ± 0.063 | 96.7 ± 8.2 |
| NC + Medium LPB | 1,069 ± 81 | 1,820 ± 67 | $1.726^{bc}$ ± 0.152 | 93.3 ± 5.2 |
| NC + High LPB | 1,134 ± 120 | 1,897 ± 150 | $1.694^{bc}$ ± 0.088 | 98.3 ± 4.1 |
| NC + Low PPB | 1,100 ± 133 | 1,976 ± 168 | $1.830^{ab}$ ± 0.113 | 93.3 ± 5.2 |
| NC + Medium PPB | 1,033 ± 108 | 1,877 ± 183 | $1.920^{a}$ ± 0.176 | 91.7 ± 9.8 |
| NC + High PPB | 1,090 ± 130 | 1,902 ± 144 | $1.808^{abc}$ ± 0.137 | 91.7 ± 7.5 |
| Significance (P) | 0.48 | 0.78 | 0.02 | 0.68 |

LPB = Liquid plant bioactives; PPB = Powder plant bioactives
*Different superscript within the same column showed significant different (P<0.05)

**Table 4.** Performance of broilers fed diet supplemented with plant bioactives during 1 to 35 days

| Treatments | BW 35 days (g) | Feed intake (g/bird ) | FCR | Liveability (%) |
|---|---|---|---|---|
| Negative Control (NC) | 1,681 ± 151 | 2,881 ± 250 | $1.790^{bc}$ ± 0.095 | 95.0 ± 8.4 |
| Positive Control (NC + AGP) | 1,700 ± 176 | 2,849 ± 277 | $1.774^{bc}$ ± 0.067 | 95.0 ± 8.4 |
| NC + Low LPB | 1,751 ± 94 | 2,943 ± 182 | $1.734^{c}$ ± 0.062 | 96.7 ± 8.2 |
| NC + Medium LPB | 1,649 ± 120 | 2,732 ± 127 | $1.817^{bc}$ ± 0.093 | 88.3 ± 11.7 |
| NC + High LPB | 1,702 ± 126 | 2,917 ± 219 | $1.745^{c}$ ± 0.052 | 98.3 ± 4.1 |
| NC + Low PPB | 1,671 ± 140 | 2,954 ± 276 | $1.840^{abc}$ ± 0.061 | 93.3 ± 5.2 |
| NC + Medium PPB | 1,569 ± 129 | 2,801 ± 308 | $1.945^{a}$ ± 0.145 | 90.0 ± 11.0 |
| NC + High PPB | 1,650 ± 118 | 2,800 ± 298 | $1.893^{ab}$ ±0.126 | 86.7 ± 8.2 |
| Significance (P) | 0.48 | 0.71 | 0.04 | 0.21 |

LPB = Liquid plant bioactives; PPB = Powder plant bioactives
*Different superscript within the same column showed significant different (P<0.05)

other feed additives on feed intake of broilers. Ertas et al. (2005), Fascina et al. (2017), and Mashayekhi et al. (2018) showed that no significant effect of AGP supplementation on the feed intake of broilers. However, Emami et al. (2012) showed an increase in feed intake of broilers due to AGP supplementation. Some reports showed that supplementation of plant bioactive or phytogenic did not alter the feed intake in broilers (Fascina et al. 2017), although Aroche et al. (2018) showed a depression in feed intake due to supplementation of mixed medicinal plant leaves powder.

The feed conversion ratio (FCR) of broilers during starter ($P<0.01$) and grower ($P<0.05$) periods were significantly affected by the supplementation of the feed additives. The liquid plant bioactives improved the FCR by 3.2 to 8.8% during the starter period (1 to 21 days) as compared to the negative control and the most efficient feed conversion was found when the birds were fed at a low dose of liquid plant bioactives. However, supplementation of plant bioactives in powder form did not show any improvement on the FCR. During the starter period, the most efficient birds to convert the feed were those supplemented with low dose liquid plant bioactives (FCR= 1.498) and the less efficient birds were those fed diet supplemented with medium-dose powder plant bioactives (1.790), while the FCR of birds fed the AGP was slightly better than the negative control (FCR= 1.593). Supplementation of low dose liquid plant bioactives improved the FCR by 8.8% while supplementation of the AGP improved the FCR 2.32% as compared to the negative control. A similar trend on the FCR during the grower period (1 to 28 days and 1 to 35 days) also occurred but the degree of improvement was decreasing as the birds older. The FCR improvement due to low dose liquid plant bioactives was 8.8%, 6.8%, and 3.2% and the improvement due to AGP supplementation was 2.32%, 2.6%, and 0.9% from 1 to 21 daysays, 1 to 28 and 1 to 35 days period, respectively.

The liquid plant bioactive was more effective than the powder plant bioactive, although were added at the same antibacterial activity measured by *in vitro* assay (Pasaribu et al. 2018). This might be due to the different specificity of the bioactive compounds in the liquid and powder plant bioactive. In the liquid form, the bioactive compounds were extracted with methanol which might contain more phenolic or antioxidant compounds, and not only the antibacterial compounds. While the bioactive compounds in the powdered form were extracted by fluids that exist in the gastrointestinal tract which is dominated by water and less phenolic compound could be extracted (Altemimi et al. 2017). The liquid form might give a more bioactive effect to broiler performance than that the powder form. The study of other compounds other than antibiotics that influence broiler performance is interesting. It is well known that most medicines are also prepared by extraction which increasing the purity and specificity of bioactive.

The FCR improvement on broilers due to AGP supplementation varied from 3.2% (Fascina et al. 2017), 4.7% (Ahmed et al. 2016), 6.7% (Mashayekhi et al. 2018), and 7.3% (Ertas et al. 2005). Many efforts on the use of plant bioactives as feed additives to replace the antibiotic have been reported. Different plant bioactives have been investigated with positive or negative results. Ertas et al. (2005) reported a 14.2% improvement on the FCR of broilers fed with mixed essential oil. Mashayekhi et al. (2018) reported a 4.1% FCR improvement due to eucalyptus leaf powder. Ahmed et al. (2016) reported that peppermint oil supplementation at 250 mg/kg diet improved the feed efficiency to a similar improvement by the AGP (4.7%) but the supplementation in powder forms did not affect the body weight gain nor the feed efficiency in broilers. Asadi et al. (2017) also reported that supplementation of peppermint powder in broilers diet improved the body weight gain (14.2 %) and the feed efficiency (6.4%). On the other hand, other reports showed that the peppermint essential oils (Emami et al. 2012) or powder (Gurbuz & Ismael 2016) could not improve body weight and FCR of broilers. Fascina et al. (2017) also showed that a phytogenic additive (mixed of turmeric extract, citrus extract, grape seed extract, cinnamon oil, boldo leaves, and fenugreek seeds) did not improve the FCR in broilers.

This experiment showed that the low dose liquid plant bioactives were the most effective to improve the performance of the broilers. Zhu et al. (2019) also reported an improvement in broiler performance by supplementing low dose commercial plant extract in the diet while increasing the dose to double did not make further improvement. A higher dose of plant bioactive did not improve the performance of the chickens. This might be due to excessive concentration of the active components such as total phenol, tannin, and saponin (Sinurat et al. 2018). Similar results also reported by Attia et al. (2017) which indicated that a high content of bioactive such as tannins in the diet may decrease nutrient digestion and absorption.

The survival rates of the chickens during the starter period were very high, i.e. 96.7–100.0%, and were not significantly affected by the treatments ($P>0.05$). The cumulative survival rates during 1 to 28 d old and 1 to 35 d old periods were quite low due to the occurrence of chronic respiratory disease (CRD) at 26 d old. However, the survival rates were not significantly affected by treatments ($P>0.05$).

One of the compounds in plant bioactives tested in this trial was extract or powder of leaf fruit which has a high antioxidant level (Sinurat et al. 2018). Therefore,

the inclusion of this substance in the feed was expected to increase the immune system in the blood circulation of the chickens. The effect of the treatments on the leucocyte differential counts in the blood is presented in Table 5 and the effect on the immune organs is presented in Table 6. The heterophils and monocyte in the blood of the broilers were not significantly (P>0.05) affected by supplementation of the AGP, but the lymphocyte (L) was significantly (P<0.05) affected. The highest percentage of lymphocyte was found in birds fed a diet supplemented with low dose liquid plant bioactives (75.2%) and the lowest percentage was found in birds fed a diet supplemented with high dose powder plant bioactives (67.0%). These results showed that neither the supplementation of AGP nor the plant bioactives (except the high dose of powder plant bioactives) significantly (P>0.05) affect the phagocyte levels as compared to the negative control. The ratio between heterophils and lymphocyte (H:L ratio) was also not significantly (P>0.05) affected by the treatments.

The H:L ratio in the blood has been shown as a good indication of stress in chickens. Chickens with the high-stress condition will have a higher H:L ratio in the blood (Scanes 2016). This was also shown in broilers challenged with coccidiosis (Moraes et al. 2019). Supplementation of AGP or plant bioactives is expected to improve the immunity of the birds, hence decrease the H:L ratio in the blood. Moraes et al. (2019) also showed that the supplementation of feed additives did not alter the H:L ratio in unchallenged birds, but alleviate the H:L ratio in broilers challenged with coccidiosis. Helal et al. (2015) showed that feeding the AGP did not affect the lymphocyte, heterophils, monocyte, and the H:L ratio in the blood of broilers. Wahjuni (2017) also showed that the extracts of *Phyllanthus niruri* L. decreased the number of lymphocytes on infected broilers with enterotoxin *Escherichia coli'*s antibiotics resistant. Some reports showed that supplementation of blend plant extract (Attia et al. 2017) or mixed powder of medicinal plants (Aroche et al. 2018) into feed increased the immunity of broilers significantly as measured by the titer antibody levels or by immunoglobulin concentrations in the serum.

The relative weight of some immune organs of broilers due to AGP or plant bioactives supplementation in the diet is presented in Table 6. Results showed that none of the immune organ's weight (liver, spleen, and bursa of facbricius) was significantly (P>0.05) affected by the feed additives. The relative weight of immune organs such as bursa fabricius, spleen, and thymus were increased by feeding AGP or organic acids in (Abdel-Fattah et al. 2008; (Mohamed et al. 2014). However, Fascina et al. (2017) showed that supplementation of feed additives such as AGP, phytogenic or organic acids did not affect the weight of the liver, bursa of fabricius, spleen, and thymus of broilers.

**Table 5.** The Leucocyte differential count in blood of broilers at 34 days old as affected by feeding different additives

| Treatments | Leucocyte differential count | | | |
|---|---|---|---|---|
| | Heterophils (H) % | Monocyte % | Lymphocyte (L) % | H:L Ratio |
| Negative Control (NC) | 16.3 | 9.5 | 74.7[a] | 0.22 |
| Positive Control (NC + AGP) | 18.7 | 9.2 | 74.2 [a] | 0.25 |
| NC + Low LPB | 17.5 | 9.5 | 75.2[a] | 0.24 |
| NC + Medium LPB | 17.7 | 7.5 | 74.5[a] | 0.24 |
| NC + High LPB | 18.0 | 10.2 | 71.8[ab] | 0.25 |
| NC + Low PPB | 19.7 | 9.8 | 72.2[a] | 0.27 |
| NC + Medium PPB | 16.7 | 8.2 | 75.2[a] | 0.23 |
| NC + High PPB | 19.0 | 12.3 | 67.0[b] | 0.29 |
| Significance (P) | 0.93 | 0.34 | 0.04 | 0.79 |

LPB = Liquid plant bioactives; PPB = Powder plant bioactives
*Different superscript within the same column showed significant different (P<0.05)

**Table 6.** The relative weight of some immune organs of broilers at 35 d old fed with antibiotic or plant bioactves as additives

| Treatments | Liver (% live weight) | Spleen (% live weight) | Bursa of fabricious (% live weight) |
|---|---|---|---|
| Negative Control (NC) | 2.32 | 0.112 | 0.072 |
| Positive Control (NC + AGP) | 1.99 | 0.113 | 0.062 |
| Negative Control (NC) | 2.13 | 0.123 | 0.050 |
| Positive Control (NC + AGP) | 1.97 | 0.125 | 0.073 |
| NC + Low LPB | 2.00 | 0.128 | 0.048 |
| NC + Medium LPB | 2.14 | 0.118 | 0.063 |
| NC + High LPB | 2.03 | 0.140 | 0.075 |
| NC + Low PPB | 2.08 | 0.132 | 0.063 |
| NC + Medium PPB | 0.56 | 0.78 | 0.45 |

LPB = Liquid plant bioactives; PPB = Powder plant bioactives

Wallace et al. (2010) have listed the use of some plant material and plant extracts as feed additives in poultry nutrition. Some have beneficial on the immunity status and general performance (body weight, feed efficiency), increase muscle proportion, and reduce lipid contents in meat. Some of the plant bioactives did not have any effect on the performance, even some have a detrimental effect on the performances and immune responses of the chickens. Therefore, the effect of plant bioactives used as feed additives in poultry could not be generalized.

Definitive mechanisms on how the plant bioactive improves the performance of chickens could not be concluded from this experiment. To replace the AGP, plant bioactives used in this experiment were formulated to have three functions, i.e., antibacterial (represented by liquid smoke of cashew nutshell), anti fungi (represented by clove leaves) and immunomodulators (represented by leaf fruits) as reported by Sinurat et al. (2018). The mixture of these three materials, especially the liquid or extract form may have worked synergistically as they improved the performance i.e., the feed conversion ratio of the broilers better than the AGP.

The results of this experiment showed that plant bioactives showed different effects on the performance of the broilers when fed in different forms (powder or liquid) and concentrations. This experiment showed that the best performance improvement was achieved when the plant bioactives fed in a low dose which gave higher improvement than the AGP. It is interesting to explore if a lower dose than used in this experiment could perform a similar improvement to minimize the cost of the feed additives.

## CONCLUSION

It is concluded that supplementation of low dose liquid plant bioactive into the diet, improved the performance of broilers, especially the feed conversion efficiency. Supplementation of the plant bioactive in powder form however did not show any effect on the performance of the broilers.

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# Effects of *Silybum marianum* Aqueous Extract and L-carnitine on Stereological Changes in Diazinon-Treated Rat Liver

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## ABSTRAK

Masoumi F, Shariati M, Mokhtari M. 2020. Pengaruh sari ekstrak *Silybum marianum* dan *l-carnitine* pada perubahan stereologi hati tikus yang diberikan perlakuan diazinon. JITV 25(2):91-98. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2467

Sebagai senyawa organiphosphorus, diazinon (DZN) dapat menggangu fungsi jaringan hati dengan menghambat asetinkolinesterase dan menyebabkan tekanan oksidatif. Dalam penelitian ini, pengaruh ektrak sari Silybum marianum (SMAE) dan L-carnitine (LC) pada perubahan stereologi dan histopatologi hati tikus jantan yang diberi perlakuan DZN diamati. Tikus-tikus ditempatkan dalam 9 kelompok dengan masing-masing sebanyak 8 ekor terdiri dari kontrol, plasebo dan kombinasi DZN, SMAE dan LC. SMAE dan bahan kimia lainnya diberikan kepada tikus coba secara oral selama 30 hari. Setelah itu, jaringan hati semua tikus coba dikeluarkan. Bagian jaringan hati disiapkan untuk melihat penanda stereologis termasuk volume dan berat hati, volume hepatosit, volume vena sentral, volume sinusoidal, volume jaringan ikat, laju peradangan, dan jumlah inti hepatosit. Juga, jaringan sampel dievaluasi secara histopatologis. Pengobatan dengan DZN secara signifikan mengurangi volume dan berat hati, volume hepatosit, volume vena sentral, volume sinusoidal, dan jumlah nukleus hepatosit dibandingkan dengan kelompok plasebo dan kontrol tetapi secara signifikan meningkatkan peradangan dan volume jaringan ikat hati. Namun, pemberian SMAE dan LC bersamaan dengan DZN meningkatkan volume dan berat hati, volume hepatosit, volume vena sentral, volume sinusoidal, volume jaringan ikat, dan jumlah inti hepatosit sendiri dibandingkan dengan pengobatan DZN. Peradangan hati juga menurun secara signifikan dibandingkan dengan pengobatan DZN tetapi jika dibandingkan dengan kelompok plasebo dan kontrol, hal itu meningkat secara signifikan. Pemberian SMAE dan LC secara simultan memiliki efek perlindungan pada jaringan hati dan dapat mengurangi cedera hati yang disebabkan oleh DZN pada tikus.

**Kata Kunci**: Diazinon, L-carnitine, Hati, Tikus, *Silybum marianum*

## ABSTRACT

Masoumi F, Shariati M, Mokhtari M. 2020. Effects of *Silybum marianum* aqueous extract and l-carnitine on stereological changes in diazinon-treated rat liver. JITV 25(2):91-98. DOI: http://dx.doi.org/10.14334/jitv.v25i2.2467

As an organophosphorus, Diazinon (DZN) impairs liver tissue function by inhibiting acetylcholinesterase and causing oxidative stress. In this study, the effects of *Silybum marianum* aqueous extract (SMAE) and L-carnitine (LC) on the stereological and histopathological changes of the liver in DZN-treated male rats were investigated. The rats in this study were placed into 9 groups of 8 each containing control, placebo, and a combination of DZN, SMAE, and LC. The animals received SMAE and chemicals orally for 30 days. At last, the liver tissue of all animals was removed. Then, tissue sections from the liver were provided to study the stereological markers including liver volume and weight, hepatocytes' volume, central venous volume, sinusoidal volume, connective tissue volume, inflammation rate, and a number of the hepatocytes' nuclei. Also, the sample tissues were evaluated histopathologically. Treatment with DZN significantly reduced the liver volume and weight, hepatocyte volume, central venous volume, sinusoidal volume, and hepatocyte nucleus number compared to placebo and control but it significantly increased the inflammation and volume of liver's connective tissue. However, co-administration of SMAE and LC with DZN improved liver volume and weight, hepatocyte volume, central venous volume, sinusoidal volume, connective tissue volume, and hepatocyte nucleus number alone compared to the DZN treatment. Liver inflammation was also significantly decreased compared to the DZN treatment but comparing to the placebo and control groups, it increased significantly. Simultaneous administration of SMAE and LC has protective effects on liver tissue and can reduce DZN-induced liver injury in rats.

**Key Words:** Diazinon, L-carnitine, Liver, Rat, *Silybum marianum*

## INTRODUCTION

Medicinal herbs are currently considered as a viable alternative to chemical drugs due to their ease of access, reduced side effects and reasonable prices (Karimi et al. 2015). Most medicinal plants can prevent the damage caused by free radicals due to their antioxidant properties (Nasri 2013). Some crude herbal extracts used in traditional medicine are rich in compounds with preventive and protective properties, especially in the liver (Xiong & Guan 2017).

*Silybum marianum* (SM) is one or two year old plant from Cichorium family that grows in warm climates. This plant is native to western and central Europe, northern India, and today grows in southern Europe, Africa, China and Australia, South America, and Asia and is widely distributed in Iran. The fruits of this plant contain a group of flavonoid compounds called silymarin. Silymarin is composed of silybin, silydianine, silychristine, and isosilybin (Bijak 2017; Abenavoli et al. 2018). The reports have suggested that silymarin may exert its effects on liver cells in three ways: 1) It binds to the membrane receptor of the liver cells that uptakes the toxins and modifies their phospholipid composition to prevent their uptake, 2) Because it is a potent antioxidant, it inhibits lipid peroxidation by preventing metabolism abnormalities, especially in liver cells, 3) By stimulating protein synthesis, it induces liver cell regeneration (Surai 2015).

Nowadays, various pesticides are used in the agricultural industry to increase the quality and quantity of crops to combat pests. Diazinon toxin is an organophosphate compound used to control insects in the agricultural industry. Diazinon is a colorless, oily and liquid toxicant that inhibit the acetylcholinesterase enzyme which is essential for the functioning of the nervous system (Duysen et al. 2012).

Diazinon, as an environmental pollutant, has a lengthy half-life in the environment and therefore can be hazardous to human health (Jones et al. 2015). Diazinon increases the risk of genetic syndromes, including Turner syndrome, by altering sperm chromosomes (Slotkin & Seidler 2012). The expanding use of organophosphates, especially DZN, and numerous reports in recent years on the effects of anomalies of these pesticides on various developmental processes has raised many concerns about the harmful effects of such toxins on human health. Organophosphorus appears to alter cell function, gene mutation, stop mitotic division, fetal malformation, stop DNA synthesis, and induce cell death. Therefore, based on the reported effects of these toxins, they are classified in the group of cytotoxic and genotoxic compounds (Slotkin & Seidler 2012; Newcomb et al. 2005). Recent studies have shown that acute and chronic toxicity with organophosphorus compounds, including DZN, induces free radicals through the induction of oxidative stress and thereby changes the balance of the body's antioxidant system and provides the conditions for pathological changes in the body (Anbarkeh et al. 2014). Indeed, exposure to DZN causes severe histopathologic damages in the liver including sinusoidal dilatation, disrupt of hepatocytes, vacuolization of hepatocyte cytoplasm, and centrilobular necrosis (Beydilli et al. 2015).

L-carnitine (LC) is produced in the body through diet, biosynthesis and utilizing essential amino acids like lysine and methionine (Ghoreyshi et al. 2019). Transferring long-chain fatty acids from the mitochondrial inner membrane for beta-oxidation and producing ATP in diverse tissues are among the important physiological roles of LC. L-carnitine prevents oxidative stress and regulates nitric oxide, cellular respiration and activity of enzymes involved in oxidative stress. Its role as a free radical scavenger in aging has been also described (Murali et al. 2015).

The liver, as the most important organ of the body's metabolism, plays a key role in many essential physiological processes such as glucose homeostasis, production of essential plasma proteins, lipoproteins and lipids, production and secretion of bile acids and storage of vitamins (Trefts et al. 2017). In addition, due to its major role in detoxification of toxins with internal and external origin, liver is constantly exposed to various types of high concentrations of toxins. There is extensive evidence that free radicals and reactive oxygen species play a key role in initiating and regulating the different stages of liver disease. Even small amounts of antioxidants found in food and body are used to protect body against different types of oxidative damage which are caused by oxygen free radicals (Hodges & Minich 2015). This study was conducted to investigate the effects of LC and SMAE alone and in compound form on the stereological and histopathological changes of liver tissue in DZN-treated male rats.

## MATERIALS AND METHODS

### Animals

In this experimental study, 72 adult male Wistar rats were obtained from the animal house of Islamic Azad University, Kazerun, Iran, which was approximately 10 weeks old and weighing 220±20 g, 22±2$^{o}$C, 12 hours on the light and 12-hour on the darkness and 70% humidity were the standard conditions in which the animals were kept. To adapt to the new environmental conditions, the animals were kept for 2 weeks with the mentioned standard conditions. During the study, the animals had adequate and free access to pelleted food and water. All

experiments were undertaken based on Iran Veterinary Organization rules and regulations for working with laboratory animals and the ethical committee of the Islamic Azad University of Kazerun, Iran approved all the ethical considerations on animal care. (Ethical Code No: IR.Kiau.15230509971001).

**Preparing the plant extract**

The stems and seeds of the SM were first dried and then powdered. One hundred g of the powder was added to 500 ml of distilled water and mixed well and then kept at room temperature for 24 hours. The resulting mixture was stirred using a magnetic heating stirrer at 60° C for 1 hour. The extract was centrifuged at 10,000 rpm for 20 minutes and then filtered. The extract was kept in the refrigerator until use (Sajadi et al. 2016).

**Preparing chemicals**

L-carnitine was purchased from Merck INC. (Germany) and DZN with a purity of 95% was purchased from Sam Gol Company (Iran).

**Experimental protocol**

Animals were randomly divided into 9 equal groups (*n*= 8) of control, placebo, DZN15, SMAE100, LC300, SAME100+LC300, DZN15+LC300, DZN15+SMAE300 and DZN15+SMAE100+LC300 as follows:

a. Control group: received nothing except water and food.
b. Placebo group: only received 1 mL distilled water orally.
c. DZN15 group: received 15 mg/kg DZNtoxin orally at 6 p.m.
d. SMAE100 group: received 100 mg/kg of SMAE orally at 8 a.m.
e. LC300 group: received 300 mg/kg LC orally at 8 a.m.
f. SAME100+LC300 group: received 100 mg/kg of SMAE and 300 mg/kg LC orally at 8 a.m.
g. DZN15+LC300 group: received 15 mg/kg DZN toxin at 6 p.m. and 300 mg/kg LC orally at 8 a.m.
h. DZN15+SMAE300 group: received 15 mg/kg DZN toxin at 6 p.m. and 100 mg/kg SMAE orally at 8 a.m.
i. DZN15+SMAE100+LC300 group: received 15 mg/kg DZN toxin at 6 p.m. and 100 mg/kg SMAE along with 300 mg/kg LC orally at 8 a.m. SAME, DZN and LC doses were selected based on previous studies (Sajadi et al. 2016; Hussei et al. 2014).

On the day 31$^{st}$, all animals were anesthetized with ether (Merck, Germany) and then were euthanized by cervical dislocation. Then, the abdominal area of each animal was opened and the liver tissue was removed. The removed specimens were fixed in 10% formalin buffer solution for fixation and blocked in paraffin after tissue passage and serial sections were prepared using a microtome machine for stereological and histopathological studies.

**Stereological study**

At the last stage, the rats' weight was measured and then forfeited. The liver was weighed, and the initial volume (V primary) was attained via the Scherle method (Scherle 1970; Zare et al. 2019). Isotropic identical random sections were acquired by the orientation method". Then, on average, 9-12 slabs were picked from each liver randomly. A circle was pressed out from a liver slab by a trocar. All the collected slabs and the circular pieces were implanted in the same paraffin block 5 µm and 25 µm sections were gained. After staining of 25 µm tissue sections with Hematoxylin-Eosin, they were mounted with a coverslip. The diameters of the circular piece of the liver and the area of the circle were getting quantified once more to approximately obtain the global grade of liver tissue shrinkage. The shrinkage degree was calculated using the formula below:

Degree of shrinkage: $1- (\frac{AA}{AB})^{1.5}$

where, AA is the area of the circular piece after and AB is the area of the circular piece before handling and staining, respectively.

**Estimation of liver's hepatocytes, sinusoids, central veins, connective tissue volume or fibrosis in the experimental group**

The following formula was used to assess the total volume of the hepatocytes, sinusoids, central veins, connective tissue or fibrosis, and inflammatory area.

$$Vv(\text{structure}) = \sum_{i=1}^{n} p\,(\text{structure}) / \sum_{i=1}^{n} (\text{reference})$$

where, "$\Sigma P_{structure}$" was considered as the number of points hitting the profiles of the hepatocytes, sinusoids, central veins, connective tissue and inflammatory area tissue and "$\Sigma P_{reference}$" was considered as the number of points hitting the liver:

$V(_{structure})$=Vv (structure/liver) ×V final

**The hepatocytes' nuclei total number**

Using the Stereolite software and the optical dissector method with the following formula, the total number of hepatocytes 'nuclei was evaluated as mentioned before:

$$NV = \frac{\sum_{i=1}^{n} Q}{(\sum P \times h \times a/frame)} \times \frac{t}{BA}$$

where ΣQ was considered as the number of the whole hepatocytes 'nuclei which were counted in all the dissectors, h was considered as the height of the optical dissector, a/frame was considered as the area of the counting frame, Σp was considered as the total number of the counted frames, BA was considered as the microtome block advance to cut the block, and finally, it was considered as the mean of the final section thickness.

**Statistical analysis**

Using SPSS software version 20, the normality of data was confirmed by the Kolmogorov-Smirnov test and the data analyzed using one-way ANOVA and LSD test at $P<0.05$. The results were expressed as mean ± standard deviation in the diagrams using GraphPad Prism software version 6.

## RESULTS AND DISCUSSION

**Results**

***Stereological findings***

Liver weight and volume, hepatocyte volume, central venous volume, and sinusoid volume and hepatocyte nucleus number (Figure 1A-1F) were significantly decreased in the DZN15 group compared to the control and placebo groups ($P<0.05$). There was no significant difference between the SMAE100, LC300, and SAME100+LC300 groups with the control and placebo groups ($P>0.05$). No significant difference was observed in DZN15+LC300 and DZN15+SMAE100 groups with the control and placebo ($P>0.05$).

***The inflammation rate***

The level of inflammation (Figure 1G) was significantly increased in the DZN15 group compared to the control and placebo groups ($P<0.05$). No significant difference was observed between SMAE100, LC300 and SAME100+LC300 groups with control and placebo groups ($P>0.05$). A significant decrease in the inflammation of liver tissue was observed in DZN15+LC300, DZN15+SMAE100 and DZN15+SMAE100+LC300 groups compared to DZN15 group ($P<0.05$), however, there was a significant increase compared to control and placebo groups ($P<0.05$).

***Connective tissue volume***

The volume of connective tissue (Figure 1H) was significantly increased in the DZN15 group compared to the control and placebo groups ($P<0.05$). There was no significant difference between the SMAE100, LC300, SAME100+LC300, DZN15+LC300, DZN15+SMAE100, and DZN15+SMAE100+LC300 groups compared to control and placebo groups ($P>0.05$).

***Histopathologic findings***

Histopathological findings show no evidence of hepatocyte injury and central vein in control (Figure 2A) and placebo (Figure 2B) groups. Hepatocytes are placed regularly without any damage and the liver tissue is perfectly normal.

In the DZN15 group (Figure 2C), hyperemia, destruction of sinusoids, lobular center vein coagulation necrosis, hepatocyte atrophy, and moderate to severe vacuolar cell resorption of interstitial cells were observed. Lymphocytic infiltration, apoptosis, and congestion were also observed in this group. Coagulation necrosis or fragmentation and lubrication of the cell nuclei and eosinophilization of their cytoplasm were detected and the inflammatory response around necrosis was severe. Liver hepatocytes were damaged and disrupted in such a way that the disorder and the pancreatic nucleus were largely observed in them. In this group, an increase in inflammatory cells and fibrosis was visible. No sign of tissue changes or tissue damage was observed in the SMAE100 (Figure 2D), LC300 (Figure 2E), and SAME100+LC300 (Figure 2F) groups.

Histopathological changes were reduced in the DZN15+LC300 group (Figure 2G) but in some parts of the hepatic tissue, hyperemia, sinusoids destruction, lobular center vein coagulation necrosis and mild to moderate vacuolar cell degradation in interstitial cells were observed. Lymphocytic inflammation and the presence of vacuolar spaces in the cytoplasm were observed in groups with a relative improvement of liver tissue compared to the DZN15 group. In the DZN15+SMAE100 group (Figure 2H), some local inflammation and lymphocytic infiltration were observed at some points. Apoptosis, congestion, hyperemia, and ballooning of hepatocytes were also observed. Coagulation necrosis or pycnosis, cell division, and lysis of the cells and eosinophilization of their cytoplasm were detected and the inflammatory response around necrosis was mild.

In DZN15+SMAE100+LC300 group (Figure 2I), histopathologic lesions were significantly reduced. There was also a significant decrease in congestion, hyperemia and tissue inflammation so that the histological structure of liver tissue was almost normal and comparable to control and placebo groups.

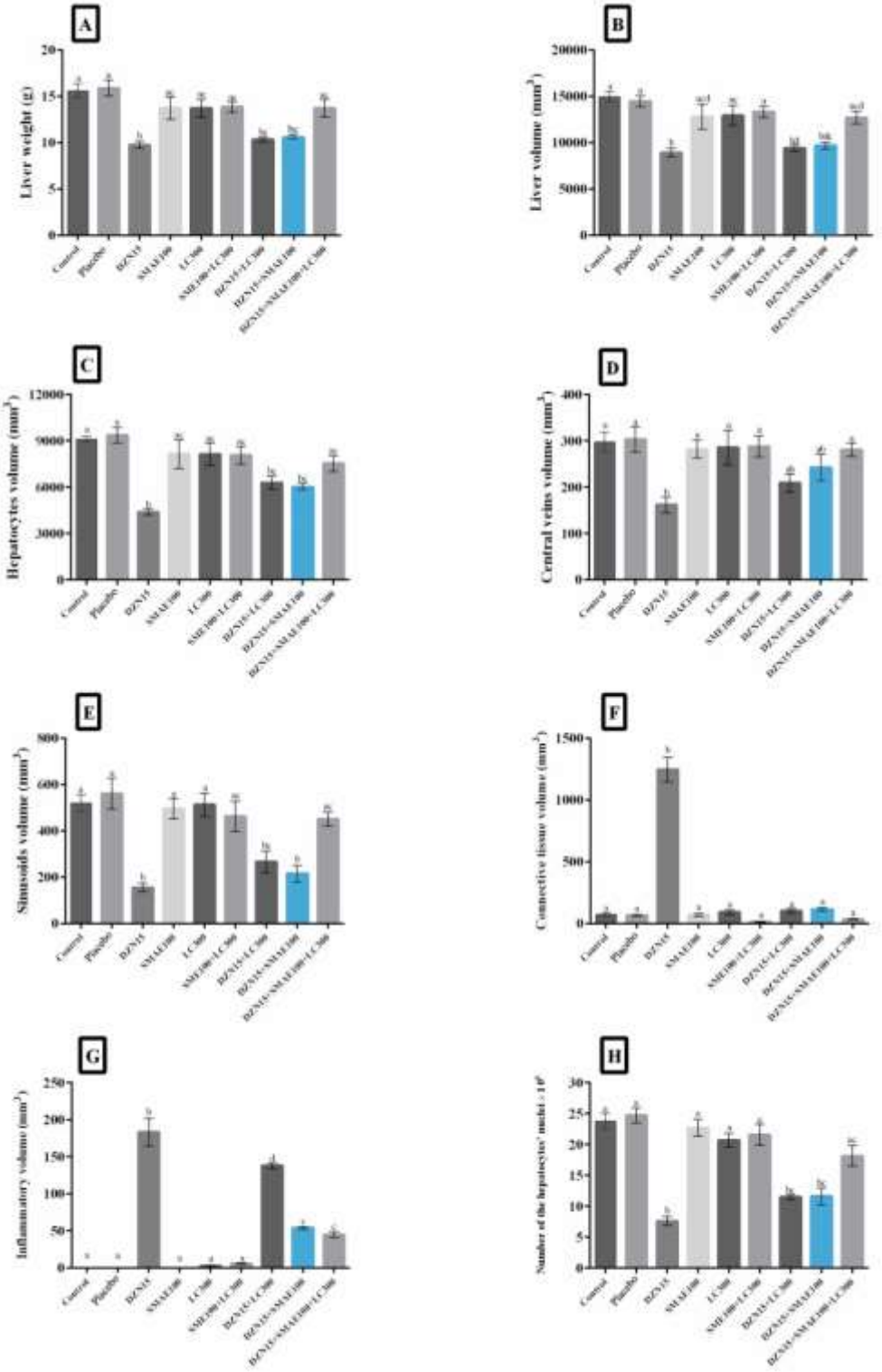


**Figure 1.** Evaluation of the liver weight (A), total volume of the liver (B), hepatocytes (C), central veins (D), sinusoids (E), connective tissue or fibrosis (F) inflammatory area (G) and number of the hepatocytes' nuclei (H) in the control, placebo, Diazinon (DZN15), Silybum *marianum* (SMAE100), L-carnitine (Lc300), SMAE100+LC300, DZN15+ LC300, DZN15+SMAE100, and DZN15+SMAE100+LC300 groups. a, b, c and d: According to *post-hoc* Tukey test which was used to make intergroup comparisons groups with same superscripts were not significantly different at ($P>0.05$). However, dissimilar letters indicate a significant difference ($P<0.05$).

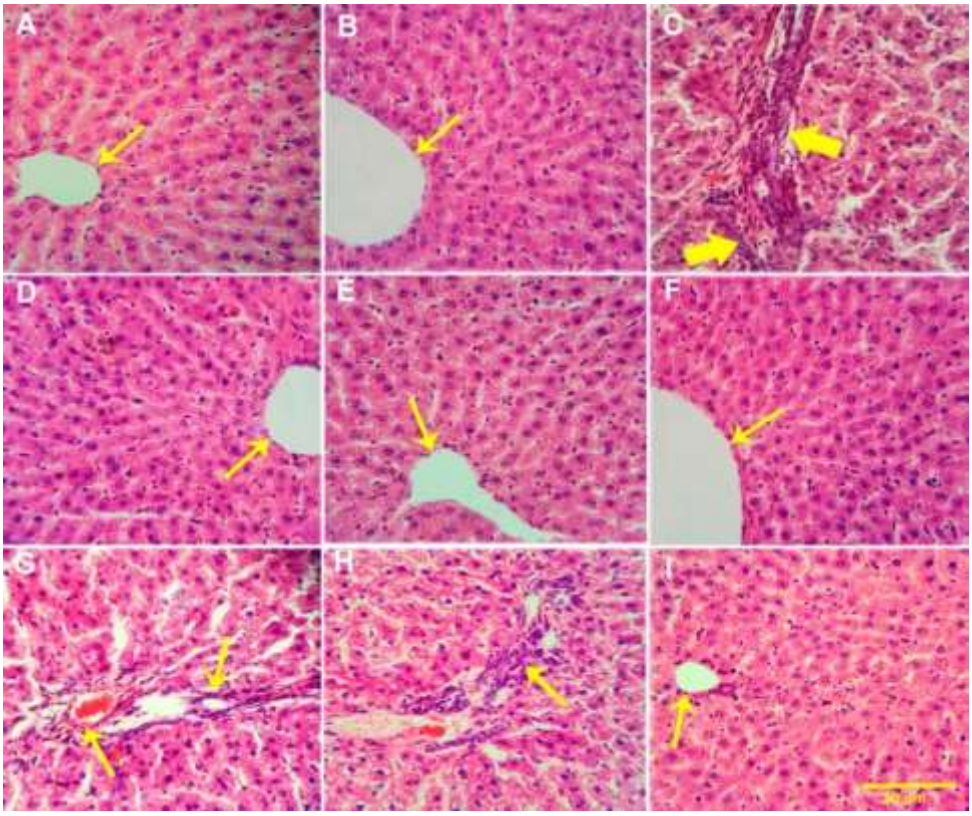


**Figure 2.** Photomicrograph of the rat's livers in the control (A), placebo (B), DZN15 (C), SMAE100 (D), LC300 (E), SMAE100+LC300 (F), DZN15+LC300 (G), DZN15+SMAE100 (H), DZN15+SMAE100+LC300 (I) groups. Stained with hematoxylin and eosin (H&E) stain and magnification at ×100. Control (A), and placebo (B), rats with normal hepatocytes and normal central vein (yellow thin arrow). In the DZN15-treated rat (C), a little number of hepatocytes' nuclei, lesser sinusoidal space volume and central vein volume, and increase bridge of the connective tissue or fibrous tissue (yellow pick arrows) can be seen. No structural changes were detected in the rat treated with SMAE100 (D), LC300 (E) and SMAE100+LC300 (F). DZN15 rats treated with LC300 (G), and SMAE100 (H), showed lymphocytic inflammation (yellow thin arrow) and vacuolization of cytoplasm. In the rat treated with DZN15+SMAE100+LC300 (I), a smaller number of hepatocytes' nuclei, normal central vein (yellow thin arrow), lesser accumulation of the fibrous tissue, and larger sinusoidal space can be observed

## Discussion

In the present study, treatment with DZN toxin increased liver weight and volume, hepatocyte volume, central venous volume, sinusoidal volume, and hepatocyte nucleus volume and in contrast, it increased inflammation and liver fibrosis. The studies have shown that organophosphate toxins react with macromolecules and cell macromolecules and caused cellular and genetic damage (Li et al. 2015).

Organophosphorus insecticides can produce free radicals and disrupt the body's antioxidant systems. There is a balance between the production and removal of free radicals in natural conditions and oxidative stress is caused by the imbalance in these processes. Due to their tendency to absorb electrons, free radicals can damage important macromolecules such as proteins, lipids, and DNA (Li et al. 2015; Pearson & Patel 2016).

Organophosphates degrade various cells and tissues of the body by increasing lipid peroxidation, cell apoptosis, and the production of free radicals, and they also inhibit the antioxidant activity of some enzymes like superoxide dismutase, glutathione peroxidase and catalase (Prokić et al. 2017; Eroglu et al. 2013).

Histopathologic damage in DZN toxin-treatment groups appears to be associated with increased oxidative stress and the induction of cell death. Also, the results of this study show that in groups treated with SMAE and LC, improvement of stereological and histopathologic indices is seen in comparison with the DZN toxin treatment group.

Considering the stereological and histopathological results of this study, it is concluded that the mechanism of SMAE and LC is to prevent oxidative stress induced by DZN in different parts of the liver. In living beings, there are two antioxidant systems to counteract the damaging effects of free radicals and oxidative stress which includes enzymatic antioxidant defense (Superoxide dismutase, glutathione peroxidase, catalase) and non-enzymatic include ascorbic acid, alpha-tocopherol, bilirubin, uric acid, polyphenols and

carotene (Eroglu et al. 2013; Soto-Méndez et al. 2016). These compounds minimize the damage caused by free radical activity by preventing the production of free radicals and repairing the damaged tissues (Valko et al. 2016). Therefore, it seems that the SMAE and LC in rats treated with DZN, due to the function of phenolic compounds (SMAE) and their antioxidant properties, inhibits the toxic and oxidative effect of DZN and plays a protective role for body cells.

Abdel-Daim et al. (2016) studied, using DZN toxin at a dose of 20 mg/kg for 4 weeks the level of biochemical parameters associated with liver injury was increased significantly like hepatic enzymes of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and gamma-glutamyltransferase. However, sesame oil and lipoic acid supplementation were able to reduce the toxic effect of DZN in rats by inhibiting free radicals and enhancing antioxidant activity.

In the study of Messarah et al. (2013), the protective effect of *Curcuma longa* and vitamin E on DZN-induced oxidative damage in rat liver was investigated. Diazinon increased lipid peroxidation and thiobarbituric acid reactive substrate levels. Diazinon increased alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase. In this study, it was shown that *Curcuma longa* and vitamin E can inhibit the toxic effects of DZN.

The antioxidant and protective properties of SM against a variety of free radical species have been proven in some studies (Negahdary et al. 2015; Zahkouk et al. 2015). *In vitro* studies have shown that the antioxidant properties of SM against oxidative stress injury are similar to the biological antioxidant glutathione (GSH) and even significantly greater than vitamin E (Surai 2015). However, the antioxidant effects of SM have not been fully understood but some of its mediating features have been shown through the cleansing of free radicals, reducing the activity of the enzymes responsible for producing free radicals, maintaining the integrity of the electron transport chain in the mitochondria, maintaining optimal redox state of the cell by activating a wide range of antioxidant and non-antioxidant enzymes and it has been shown mainly through transcription factors including nuclear factor erythroid-2-related factor 2 (Nrf2) and nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-kβ) (Negahdary et al. 2015; Surai 2015).

## CONCLUSION

Generally, the results of this study indicate that diazinon (DZN) toxin causes severe damage to rat liver tissue. Diazinon appears to exert its toxic effects on liver tissue by increasing inflammatory cells and fibrosis. On the other hand, concomitant use of SMAE and LC has protective and beneficial effects in DZN-treated rat's liver. The protective effects of the SMAE and LC appear to be probably due to the reduction of tissue inflammation.

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10. **Citation in text:**

The citation consists of the author's last name and publication year.

**Example:**

a. One author: ..... grow slower than lamb fed cattle's milk (Supriyati 2012). Supriyati (2012) formulates.....
b. Two authors: ..... expect, end maintenance weight (Khasrad & Rusdimansyah 2012). Khasrad & Rusdimansyah (2012) argued.....
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d. The same author cited from 2 different papers: (Purwadaria et al. 2003a, 2003b).
e. The author with the same family name is written consecutive: (Dawson J 1986; Dawson M 1986).
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