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PREFACE

In this edition, volume 24 No 3, we proudly present articles from animal and veterinary sciences including genetics; reproduction and food technology. The articles published in this edition are:

"Plasma B-Endorphin and Cortisol Profiles around Periparturient Period at Stressful Conditions in Egyptian Buffalo"; "Effect of the Addition of Insulin-Transferrin-Selenium on In Vitro Maturation and Fertilization of Bali Cattle Oocytes"; "Retained Placenta in Relation with Blood Components in Egyptian Crossbred Cattle"; "Antimicrobial and Anti-inflammation Activities of Fraction and Single Peptides Derived from Mare Milk Protein"; " Characterisation of M2e Antigenicity using anti-M2 Monoclonal Antibody and anti-M2e Polyclonal Antibodies"; and "Identification of Resources in the System of Broiler Farming Business";.

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

Chief Editor

Bogor, September 2019

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LIST OF CONTENT

Plasma B-Endorphin and Cortisol Profiles around Periparturient Period at Stressful Conditions in Egyptian Buffalo

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ABSTRAK

Abed El-Hady HA, El-Malky OM, Mourad RS, Al-Gezery IS. 2019. Profil plasma B-endorphin dan kortisol sekitar periode *peripartum nutrient* pada kondisi stres pada kerbau Mesir. JITV 24(3): 87-94. DOI: http://dx.doi.org/10.14334/jitv.v24i3.2003

Penelitian ini bertujuan untuk menentukan hubungan antara gangguan reproduksi berupa stres dengan plasma β-endorphin dan kortisol pada kerbau saat proses kelahiran. Perubahan parameter ini dapat digunakan sebagai parameter objektif untuk melihat hubungan kondisi stres dengan persalinan. Periode periparturien, sesaat sebelum dan setelah melahirkan, adalah waktu yang sangat krusial bagi sapi perah karena harus menyesuaikan perubahan fisiologis, metabolisme dan endokrin. β-endorphin dan kortisol dengan cepat meningkat sebagai respon terhadap stres pada sapi. Studi ini menentukan kadar plasma darah β-endorphin dan kortisol kerbau dengan gangguan reproduksi (distosia dan retensi plasenta) dan skor kondisi tubuh rendah selama periode periparturien. Dua puluh kerbau Mesir multipara pada periode akhir kehamilan digunakan selama dua bulan sebelum kelahiran. Konsentrasi β-endorphin lebih tinggi pada kelompok gangguan reproduksi. Sedangkan, konsentrasi β-endorphin adalah 134,9±4,8 untuk RP, 121,3±4,9 untuk distosia, 114,2±8,4 untuk BCS Rendah dan 113,5±6,5 pg/ml untuk kontrol. Pada periode yang lebih dekat proses kelahiran baik plasma β-endorphin dan kortisol menunjukkan tren peningkatan nilai secara bertahap selama -2, -1 hari dan nol waktu di semua kelompok. Hal yang sama juga terlihat pada konsentrasi β-endorphin dan kortisol pada periode postpartum dengan penurunan nilai yang diamati pada semua kelompok setelah proses kelahiran hingga sebulan atau lebih. Kerbau dengan gangguan reproduksi menunjukkan nilai β-endorphin dan kortisol yang relatif tinggi. Perbedaan yang signifikan (P≤0,01) diamati antara kelompok. Secara umum, kerbau dengan gangguan reproduksi memiliki dampak yang nyata pada plasma darah β-endorphin di sekitar proses kelahiran.

Kata kunci: Kerbau, β-endorphin, Kortisol, Distokia, RetensiPlasenta

ABSTRACT

Abed El-Hady HA, El-Malky OM, Mourad RS, Al-Gezery IS. 2019. Plasma B-endorphin and cortisol profiles around periparturient period at stressful conditions in Egyptian buffalo. JITV 24(3): 87-94. DOI: http://dx.doi.org/10.14334/jitv.v24i3.2003

The study purpose was to determine the relationship between reproductive disorders as a stress factor with plasma β–endorphin and cortisol in buffalo around parturition and changes in these parameters could be used as an objective measure of the stress associated labour. The periparturient period, the period immediately before and after calving, is a challenging time for dairy cattle that must cope with physiological, metabolic and endocrine changes. β-endorphin and cortisol rapidly increased in response to stress in cattle. The study determined the level of blood plasma β-endorphin and cortisol of buffalo with reproductive disorders (dystocia and retained placenta) and Low body condition score during periparturient period. Twenty multiparous Egyptian buffalo at late pregnancy period were used for two months before parturition. β-endorphin concentrations were higher in reproductive disorders groups. Whereas, β-endorphin concentrations were 134.9±4.8 for RP, 121.3±4.9 for dystocia, 114.2±8.4 for Low BCS and 113.5±6.5 pg/ml for control. At the closer period around parturition both of plasma β-endorphin and cortisol followed the same trend toward a gradually increased value during -2,-1days and zero time in all groups. A concomitant trend was noticed in β-endorphin and cortisol concentrations in postpartum period with values decreased were observed in all groups after parturition continued for month or more. Buffalo with reproductive disorders were showed a high relative values in β-endorphin and cortisol. Significant differences (P≤0.01) were observed between the groups. Generally, buffaloes with reproductive disorders had a clear impact on blood plasma β-endorphin around parturition process.

Key words: Buffalo, β-endorphin, Cortisol, Dystocia, Retained Placenta.

INTRODUCTION

The periparturient period, defined as the period immediately before and after calving, is a challenging time for dairy cattle that must cope with physiological, metabolic and endocrine changes, as well as a variety of environmental and management-related stressors. Also, these challenges likely contribute to the high incidence of disease observed during the weeks following parturition. Moreover, changes in behavior during the

period around parturition can be used to identify animals that are ill or at risk of disease (Sepúlveda-Varas et al. 2013). Also termed the transition period, is defined as the period from 3 weeks prepartum to 3 weeks postpartum, and is marked by several changes in the endocrine and immune systems in preparation for colostrogenesis, parturition and lactogenesis (Sordillo et al. 2009). Studies have verified that the incidence of metabolic and production-related diseases including milk fever, mastitis, fatty liver disease, ketosis, metritis, hypomagnesemia and abomasal displacements are highest during the periparturient period and complications from dystocia and retained placenta commonly occur (Ribeiro et al. 2013).

β-endorphin and cortisol rapidly elevate in response to stress in cattle. β-endorphins are neuropeptides derived from Proopiomelanocortin which is found in the anterior pituitary gland and placenta mRNA (Scott et al. 1993; Ettema & Santos 2004). Dystocia has been a long-standing problem in animals industry and causes trauma for animals and lead to increased rates of uterine infections, reproductive disorders such as retained placenta (Akar & Gazioglu 2006). The synthesis of β-endorphin can be occurred by immune system cells which possess mRNA transcripts for Proopiomelanocortin and T-lymphocytes, and in ovarian follicles, but the relatively low levels found in reproductive tissues indicate that it exerts autocrine or paracrine effects in the ovary (Hamada et al. 1995)**.** Ovarian function can be affected by β-endorphin by modulating LH and FSH secretions then exerting an inhibiting effect on GnRH secretion (Kaminski et al. 2000)**.** Dairy cows respond with elevated circulating levels of β-endorphin when placed in an unfamiliar room for milking (Bruckmaier RM et al. 1993). The relationship among changes in β-endorphin and cortisol levels during stress and whether or not any of these hormonal changes can be used as an objective indicator of stress in buffaloes. Then, it would be beneficial to investigate whether dystocia, retained placenta and Low body condition score stimulate β–endorphin and cortisol releasing or not.

MATERIALS AND METHODS

Twenty multiparous Egyptian buffalo cows which belonged to the experimental station of Animal Production Research Institute, Agriculture Research Center, Egypt. Those cows ranged from 400 to 620 kg live body weight, 5 to 11 years old and 3 to 8 parities at late pregnancy period were used for 2 months before the expected parturition date. From 10 days before the expected birth date the birth canal of each animal was monitored by rectal palpations and the body temperature was checked daily to predict the probable time of parturition. All animals were housed in semi-open pans then they were transferred to the maternity unit before 1 or 2 d of the expected birth date. After delivery dams were subjected to the regular managerial practices of the breeding stock. Follow-up using ultrasound sonar for ovarian activity after birth to study link functions, the growth of ovarian follicles and first ovulation occurs after birth and its association with the occurrence of heat or not.

Parturitions followed during the period extend 4 months extended from October to January. Animals were divided into 4 groups, 5 animals in each one: control group (A): delivered spontaneously at term with no obstetrical assistance. Dystocia group (B): delivery was at term but was accompanied by dystocia. RP group (C): delivery was at term but was accompanied by retained placenta. Low BCS group (D): delivery was at term but animals showed Low body condition score. Blood samples of 10 ml were collected from the jugular vein into clean, dried and heparinized evacuated 10 ml tubes, kept on ice, centrifuged at 2500 rotation/m for 20 minutes to obtain the plasma and stored at -20°C until extraction of β-endorphin that measured by RIA (Osawa T et al. 1998). Cortisol concentrations quantified by enzyme immunoassay (Nakao T et al. 1981).

Samples were collected by a permanent catheter was inserted under local anesthesia provided with an extension tube that was fixed to the neck with adhesive tape. It was, therefore, possible to withdraw blood without having to restrain the animal, as follow: a weekly sample started from 8 months till parturition, a sample at 1-2 days before the expected date of parturition, a sample at 0 times of parturition and 3 h after delivery and finally; samples at days 1, 3, 5 of parturition and every 10 days until corpus luteum formation that following first ovulation. The day of first ovulation was estimated by determination of plasma progesterone concentration show ≥1 ng/ml and rectal palpation (Mourad 2017)**.**

Data were statistically analyzed using descriptive statistics, ANOVA and Duncan test was used to determine the significant differences among means at α=0.05 via SAS computer program (SAS 2003)**.**

RESULTS AND DISCUSION

β-endorphin and Cortisol at late pregnancy period

Alterations in plasma β-endorphin and cortisol levels during the late pregnancy period at 2 months before parturition in the different experimental groups were shown in Table 1a & 1b. Results revealed to a gradually increased in plasma β-endorphin value reaching a maximum level during the end of the third trimester in association with the approach of parturition and/or labor pains. Reproductive disorders groups RP

Table 1a. Plasma β-endorphin during pregnancy period (3rd trimester) in buffalo

Groups	β endorphin concentration (pg/ml)							
	Number of Weeks before parturition (Mean ±SE)							
	Two months before parturition				One month before parturition			
	8th w	7th w	6th w	5th w	4th w	3th w	2nd w	1st w
Control	105.7±5.	109.7±6.	109.1±5.	109.9^{b}±5.5	111.5^{b}±6.2	114.1^{b}±6.9	121.8^{b}±7.9	126.1^{b}±8.
Dystocia	106.8±5.	107.6±6.	109.5±5.	117.5^{ab}±4.7	120.4^{ab}±6.	125.2^{ab}±6.	136.9^{ab}±3.3	146.4^{ab}±2
RP	116.5±5.	121.6±5.	125.9±5.	133.9^{a}±5.7	137.6^{a}±4.7	140.5^{a}±5.9	144.0^{a}±3.1	159.0^{a}±2.
Low BCS	102.8±7.	109.8±7.	110.3±7.	106.0^{b}±8.0	113.3^{b}±6.9	118.0^{b}±8.4	125.2^{ab}±9.8	127.9^{b}±1
P -value	0.45	0.40	0.18	0.03	0.03	0.04	0.04	0.01

a, b Means bearing different superscripts in the same column are significantly different (P<0.05).

Table 1b. Plasma Cortisol during pregnancy period (3rd trimester) in buffalo

Groups	Cortisol concentration (ng/ml)							
	Number of Weeks before parturition(Mean ±SE)							
	Two months before parturition				one month before parturition			
	8th w	7th w	6th w	5th w	4th w	3th w	2nd w	1st w
Control	19.8^{b}±1.4	19.8^{b}±1.6	21.2^{b}±1.4	22.6±1.4	23.8^{c}±1.2	24.8^{c}±1.9	19.2^{b}±1.4	18.8^{c}±0.7
Dystocia	25.6^{a}±0.9	23.4^{ab}±0.9	25.4^{ab}±0.5	23.2±1.6	35.0^{b}±2.7	36.6^{a}±2.2	32.0^{a}±2.3	32.8^{b}±2.1
RP	25.0^{a}±1.4	25.2^{a}±1.3	26.4^{a}±2.1	25.8±.6	41.0^{a}±1.8	31.6^{b}±1.2	34.2^{a}±3.3	41.2^{a}±2.0
Low	21.4^{b}±1.1	20.6^{b}±1.2	21.4^{ab}±1.9	23.4±1.3	28.4^{c}±1.3	25.2^{c}±0.9	24.0^{b}±1.1	23.0^{c}±1.3
P –value	0.01	0.03	0.07	0.62	0.00	0.00	0.00	0.00

a, b Means bearing different superscripts in the same column are significantly different (P<0.05)

Table 2a. Plasma β endorphin around parturition period (*periparturient*)in buffalo

Groups	β endorphin concentration (pg/ml)						
	Times around parturition (Mean ±SE)						
	Before parturition		during	After parturition			
	-2 days	-1 day	Zero time	3 h.	1 day	3 days	5 days
Control	117.4^{c}±5.2	123.3^{b}±5.6	127.6^{bc}±6.4	124.7^{c}±6.3	94.4^{d}±4.5	81.7^{d}±1.6	77.5^{d}±1.9
Dystocia	130.0^{b}±1.4	133.3^{b}±3.1	146.9^{b}±1.7	146.5^{b}±0.5	145.9^{b}±1.2	137.5^{b}±1.6	127.8^{b}±1.7
RP	140.4^{a}±2.5	149.8^{a}±8.4	169.0^{a}±2.2	168.2^{a}±2.4	164.5^{a}±0.9	160.6^{a}±2.7	152.4^{a}±1.8
Low	123.6^{bc}±3.1	126.1^{b}±2.2	137.6^{c}±2.1	134.0^{d}±1.6	123.5^{c}±1.7	120.2^{c}±2.1	119.1^{c}±2.2
P –value	0.00	0.01	0.00	0.00	0.00	0.00	0.00

a, b Means bearing different superscripts in the same column are significantly different (P<0.05)

Table 2b. Plasma cortisol around parturition period (*periparturient*) in buffalo

Groups	Cortisol concentration (ng/ml)						
	Times around parturition (Mean ±SE)						
	Before parturition		during	After parturition			
	-2 days	-1 day	Zero time	3 h.	1 day	3 days	5 days
Control	20.0^{d}±0.7	21.8^{d}±0.7	30.6^{b}±0.2	24.0^{d}±1.5	20.4^{d}±0.5	18.4^{d}±0.4	19.6^{b}±0.4
Dystocia	35.0^{b}±1.1	41.4^{b}±1.3	52.6^{a}±2.5	47.6^{b}±1.1	42.8^{b}±1.9	38.8^{b}±2.1	35.4^{a}±1.6
RP	42.2^{a}±1.5	44.4^{a}±1.0	57.2^{a}±1.6	53.0^{a}±0.7	48.0^{a}±1.1	43.2^{a}±0.9	38.8^{a}±1.9
Low	26.8^{c}±1.1	27.8^{c}±0.6	30.6^{b}±1.7	28.2^{c}±0.4	27.4^{c}±0.8	23.6^{c}±0.8	20.2^{b}±0.6
P –value	0.00	0.00	0.00	0.00	0.00	0.00	0.00

a, b Means bearing different superscripts in the same column are significantly different (P<0.05).

and dystocia showed higher β-endorphin concentration, started from the 5th week before parturition till the last week before parturition followed by groups Low BCS and control with significant differences at (P≤0.05). Table 1a show the highest values for studied groups RP, Dystocia, Low BCS and control at the last week before parturition were 159.0±2.5, 146.4±2.8, 127.9±11.0, 126.1±8.1 pg/ml, respectively.

Meanwhile, cortisol values showed significant differences at (P≤0.05) between groups in the late pregnancy period. The same trends of β-endorphin values were notated in cortisol values between studied groups. Table 1b show that RP and dystocia groups showed an increased cortisol level as 41.2±2.0 and 32.8±2.1 ng/ml, respectively; at the end of this period close to parturition may be due to stress at the last week of parturition as compared to other groups Low BCS and control as 23.0±1.3 and 18.8±0.7 ng/ml, respectively.

The intensity and duration of the β-endorphin and cortisol responses were dependent on stress. In farm animals, many factors such as pregnancy, parturition processes and periparturient disorders can be considered a natural event that causes various physiol-ogyical stressors (Sathya et al. 2005). Moreover, cows at the time of parturition exposed to severe stress which resulted in hyper-adrenocortical activity with higher levels of circulating steroids like cortisol and biochemical blood parameters (Hoyer et al. 1990).

In buffaloes, the gradual increase in plasma cortisol value during the prepartum period peaked on the day of calving and could be explained by prepartum anxiety and myometrial contractions associated with the stress of parturition (Sathya et al. 2005). In late pregnancy period, the increased activity of β-endorphin producing cells either in the pituitary gland, from placenta or due to direct fetal input into whole blood β-endorphin level may be related to the observed increase in β-endorphin value (Evans et al. 1986).

In cows at parturition, the stress of labor and delivery doesn't seem to influence plasma β-endorphin levels in cows and no relation was found between β-endorphin concentration and type of calving. Whereas, β-endorphin values in dystocia group tended to be higher than the normal group. Cows with abnormal calving may have more stress than the normal one, leading to an increase of peripheral β-endorphin level. Besides, there was a concomitantly trend between both of plasma β-endorphin and cortisol profile (Aurich et al. 1990).

β-endorphin and Cortisol around, before, at and after parturition

Table 2a and 2b illustrated the mean values of plasma β-endorphin and cortisol in blood peripheral of buffalo cows around the labor period. Data showed that both of plasma β-endorphin and cortisol followed the same trend tendency toward a gradually increased value during the period, started from 2 days and 1 day before parturition till the 0 time of parturition (rupture of the amnion and delivery) in all studied groups as control, dystocia, RP and Low BCS, respectively. Table 2a and 2b show the highest values recorded in abnormal delivery groups RP and dystocia followed by groups Low BCS and control. The values of plasma β-endorphin were 169.0±2.2, 146.9±1.7, 137.6±2.1, and 127.6±6.4 pg/ml, respectively; and cortisol were 57.2±1.6, 52.6±2.5, 30.6±1.7, and 30.6±0.2 ng/ml, respectively. Immediately after parturition in 3 h till 5 days (1, 3 and 5 days), a decreases values were noticed in both of β-endorphin and cortisol concentrations. Lowest values in both of β-endorphin and cortisol concentrations in the periparturient period were significant (P≤0.05) between the studied groups on day 5 after parturition, which groups RP, dystocia, Low BCS and control were 152.4±1.8, 127.8±1.7, 119.1±2.2, and 77.5±1.9 pg/ml for β-endorphin concentrations and 38.8±1.9, 35.4±1.6, 20.2±0.6, and19.6±0.4 ng/ml for cortisol concentrations, respectively. These results showed a close association between β- endorphin and cortisol levels in buffalo cows through a few days after parturition.

In accordance to the present results, plasma cortisol level increased in both cows showed or not higher levels of β-endorphin nearest and/ or at calving. Furthermore, in normal parturition cases, a distinguish of β-endorphin excretion was noticed when the uterine constriction and labor pain increased as a result of amniorrhexis (Osawa Takeshi et al. 2000).

In the periparturient period, a significant alteration in both of β-endorphin and cortisol levels. Cows with normal parturition reached the highest levels of both 2 hormones after rupture of the amniotic sac directly, while, dystocia group was seen just after parturition. They also suggest that dystocia cows may have suffered intensive stress at the time of parturition rather than any other time resulting in excretion of β-endorphin into plasma at parturition time. Adding to that, during periparturient period β-endorphin and cortisol concentrations were higher in dystocia group comparison to normal parturition one and there are no significant differences between groups (Osawa et al. 1998).

In cattle, levels of plasma cortisol are elevated during labor, resulting from the increasing demands for glucocorticoids to initiate the lactation and the fact that estrogen reduces the metabolic clearance rate for cortisol (Aurich et al. 1993). It has been reported that plasma cortisol concentrations showed higher values in cows suffering from dystocia than that with normal parturition (Hydbring et al. 1999). On the other hand, dystocia disorder seems to be more stressful for

Table 3a. Plasma β-endorphin at the postpartum period in buffaloes

Groups	β endorphin concentration (pg/ml)					
	Times at the postpartum period per day (Mean ±SE)					
	10 d	20 d	30 d	40 d	50 d	60 d
Control	77.2^{c}±5.2	74.5^{c}±5.8	74.5^{c}±5.9	72.2^{c}±6.5	71.0^{c}±4.0	70.9^{c}±5.6
Dystocia	120.3^{b}±1.5	115.1^{b}±1.6	115.5^{b}±1.4	117.5^{b}±2.5	107.6^{a}±5.8	103.5^{a}±3.7
RP placenta	145.1^{a}±7.6	136.3^{a}±5.3	125.6^{a}±5.6	122.0^{a}±1.2	122.1^{a}±2.2	122.1^{a}±2.1
Low BCS	112.3^{b}±3.9	116.1^{b}±1.4	114.9^{ab}±1.4	106.1^{b}±2.0	106.3^{b}±1.3	101.8^{b}±1.5
P –value	0.00	0.00	0.00	0.00	0.00	0.00

[a, b] Means bearing different superscripts in the same column are significantly different (P<0.05).

Table 3b. Plasma Cortisol at the postpartum period in buffaloes

Groups	Cortisol concentration (ng/ml)					
	Times at the postpartum period per day (Mean ±SE)					
	10 d	20 d	30 d	40 d	50 d	60 d
Control	16.4^{c}±1.1	16.8^{c}±1.1	20.8^{b}±0.8	21.2^{b}±0.7	19.8^{d}±0.8	21.8^{b}±1
Dystocia	29.2^{b}±1.2	29.0^{b}±1.4	35.4^{a}±2.2	35.0^{a}±0.8	33.2^{b}±1.4	36.8^{a}±1.5
RP	37.6^{a}±1.7	37.6^{a}±1.9	37.4^{a}±2.1	36.6^{a}±1.7	38.0^{a}±1.1	38.4^{a}±0.9
Low BCS	19.6^{c}±0.7	20.4^{c}±1.6	22.0^{b}±0.7	24.8^{b}±1.3	25.2^{c}±2	24.0^{b}±1.6
P –value	0.00	0.00	0.00	0.00	0.00	0.00

[a, b] Means bearing different superscripts in the same column are significantly different (P<0.05).

Table 4a. Plasma β-endorphin during lactation periods in buffaloes

Groups	β endorphin concentration (pg/ml)							
	Times during lactation period (Mean ±SE)							
	1 month		2 month		3 month		4 month	
	4w	2w	4w	2w	4w	2w	4w	2w
Control	73.1^{c}±1.9	75.3^{c}±4.1	96.1^{c}±1.7	96.2^{c}±1.7	107.2^{b}±4.3	112.2^{a}±4.2	111.3^{a}±4.8	112.7^{a}±
Dystocia	104.1^{b}±1.6	107.9^{b}±1.7	95.9^{c}±1.9	95.5^{c}±2.3	98.1^{c}±2.7	97.5^{b}±2.2	98.7^{b}±1.9	98.6^{b}±1
RP	120.5^{a}±2.4	127.9^{a}±1.4	119.6^{a}±2.2	117.3^{a}±2.1	119.9^{a}±2.4	119.2^{a}±2.7	119.2^{a}±2.7	123.1^{a}±
Low	107.2^{b}±2.5	111.4^{b}±1.6	101.5^{b}±1.0	104.1^{b}±1.3	101.9^{bc}±0.7	111.4^{a}±3.5	111.1^{a}±4.0	117.6^{a}±
P –value	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00

[a, b] Means bearing different superscripts in the same column are significantly different (P<0.05).

Table 4b. Plasma Cortisol during lactation periods in buffaloes

Groups	Cortisol concentration (ng/ml)							
	Times during lactation period (Mean ±SE)							
	1 month		2 month		3 month		4 month	
	4w	2w	4w	2w	4w	2w	4w	2w
Control	20.8^{c} ±0.7	20.8^{c}±0.7	21.6^{c}±0.9	22.6^{c}±0.6	22.8^{d}±1.2	24.0^{d}±0.6	24.8^{d}±0.6	24.6^{d}±0.9
Dystocia	31.2^{b} ±1.2	31.2^{b}±1.0	31.4^{b}±0.7	34.4^{b}±0.7	34.8^{b}±1.1	37.8^{b}±1.4	42.8^{b}±1.0	43.8^{b}±1.7
RP	36.8^{a}±1.3	37.6^{a}±1.5	38.2^{a}±1.7	38.2^{a}±1.2	40.8^{a}±0.6	42.2^{a}±0.5	50.4^{a}±1.5	51.6^{a}±1.2
Low	19.4^{c}±0.8	20.6^{c}±0.4	21.2^{c}±0.4	24.2^{c}±0.2	28.2^{c}±0.9	30.2c±0.7	32.0^{c}±1.3	32.0^{c}±1.3
P –value	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[a, b] Means bearing different superscripts in the same column are significantly different (P<0.05).

animals than that the normal one (Heuwieser et al. 1987). Besides, dystocia increases the stress of the cow's health and reproduction (Ettema and Santos 2004).

By the present results, significantly (P≤0.01) higher plasma levels of β-endorphin in both buffaloes and cows with retained placenta than that with normal animals. They suggested that β-endorphin would be considered as a factor which probably influences the retention of placenta in farm animals(El-Azab et al. 1988). Moreover, at parturition values of β-endorphin were higher in cows suffering from dystocia and retained placenta reached up to 79% in compare with normal one that reached 42% (Osawa et al. 2000).

Cortisol is a gluconeogenic hormone thereby favoring hyperglycemia at the expense of body proteins, used as an objective indicator for the quantification of stress in cows (Osawa et al. 2000). Also, a markedly increased in plasma glucocorticoid was shown in mammals during parturition (Nakao Toshihiko and Grunert 1990). Similarly, in cattle, levels of plasma cortisol are elevated during labor, resulting from the increasing demands for glucocorticoids to initiate the lactation and the fact that estrogen reduces the metabolic clearance rate for cortisol (Aurich et al. 1993).

β-endorphin and cortisol during the postpartum period

Table 3a and 3b showed the concentrations of plasma β-endorphin and cortisol in postpartum period of buffalo cows. Data illustrated reduces values were observed in all groups after parturition continued for two months in different groups for plasma β-endorphin with ranged from 145.1±7.6 to 122.1±2.1, 120.3±1.5 to103.5±3.7, 112.3±3.9 to 101.8±1.5, and 77.2±5.2 to 70.9±5.6 but plasma cortisol have slowly increased for two months postpartum and ranged from 37.6±1.7 to 38.4±0.9, 29.2±1.2 to 36.8±1.5, 19.6±0.7 to 24.0±1.6, and 16.4±1.1 to 21.8±1 for groups RP, dystocia, Low BCS and control, respectively. Groups RP and dystocia which suffering from reproductive disorders showed relatively high value in β-endorphin and cortisol concentrations than that in the other groups Low BCS and control. Highly significant differences (P≤0.01) were observed between the studied groups. All groups were shown the same trend from increases values in plasma β-endorphin and cortisol levels at the end of postpartum days.

Dystocia significantly elevated cortisol level as compared to normal parturition. Adding that, the rises in the cortisol concentration in dystocia heifers appear to be induced during calving stress (Nakao & Grunert 1990). The cortisol level during the first stage of parturition was significantly higher in the serum of parturient animals suffering from dystocia (Mohammad & Abdel-Rahman 2013). In this respect, there is an incidence of powerful acute stress due dystocia followed by an outpouring of ACTH hormone, which in turn caused the adrenal cortex to increase its secretion of glucocorticoids, including cortisol (Kindahl et al. 2002).

β-endorphin and cortisol concentration during the lactation period:

Table 4a and 4b represented that there was a very highly significant difference at (P≤0.001) between concentrations of plasma β-endorphin and plasma Cortisol during lactation periods in buffalo cows. β-endorphin concentrations and cortisol values increased during lactation periods in buffaloes till the highest values in the fourth month of lactation stage in groups RP, dystocia, Low BCS and control as following: 51.6±1.2, 43.8±1.7, 32.0±1.3, and 24.6±0.9, respectively. A comparable study showed that β-endorphin release was not affected by milking frequency and not correlated with the magnitude of prolactin release (Lacasse & Ollier 2014). Furthermore, milking frequency did not affect cortisol production (O'Driscoll et al. 2012). The increase of cortisol concentrations during milking was not likely induced by ACTH because there were no changes in ACTH concentrations (Tančin et al. 2000), although the control of the adrenal glucocorticoid secretion has generally been supposed to exclusively depend on the release of ACTH (Bruckmaier R & Wellnitz 2008). A comparison of the results obtained in dairy cows after machine milking with published data showed a similar pattern of cortisol concentrations, with a cortisol increases after milking compared to baseline values (Sutherland & Huddart 2012). β-endorphin and cortisol concentration at stages of estrus cycle of buffaloes.

Table 5a and 5b represented that there was a very highly significant difference at (P≤0.001) between concentrations of plasma β-endorphin and Cortisol in plasma during the estrus cycle stages (Metestrus, Early diestrus, Latediestrus, and Follicular) in buffalo cows. The highest values of plasma β-endorphin and Cortisol in plasma were found during the Follicular estrus cycle stage in groups RP, dystocia, Low BCS and control of plasma β-endorphin were 130.7±2.4, 130.6±2.3, 117.0±3.0, and 107.2±3.6, (pg/ml) and Cortisol values were 51.4±1.6, 43.4±1.3, 32.0±2.4, and 24.0±2.0 (ng/ml), respectively for the same groups. This result is comparable to the findings in sheep in which there were different responses of plasma β-endorphin and cortisol to a change of environment (Fordham et al. 1991). It was reported that in response to acute stress, plasma β-endorphin, ACTH and cortisol levels in sheep increased and then β-endorphin declined while ACTH and cortisol levels remained elevated (Shutt et al. 1988). The different profiles of β-endorphin and cortisol may be related to the half-life of plasma degradation of these two hormones.

Table 5a. Plasma β-endorphin at stages of estrus cycle in buffaloes

Groups	β-endorphin concentration (pg/ml)			
	Stages of estrus cycle (Mean ±SE)			
	Metestrus	Early diestrus	Late diestrus	Follicular
Control	98.0^{c}±2.3	103.5^{b}±3.6	103.4^{c}±2.7	107.2^{c}±3.6
Dystocia	114.6^{ab}±4.3	122.0^{a}±3.6	123.9^{a}±3.0	130.6^{a}±2.3
RP	118.6^{a}±2.4	122.6^{a}±1.8	128.3^{a}±1.4	130.7^{a}±2.4
Low BCS	107.9^{b}±1.9	109.6^{b}±2.1	112.9^{b}±3.0	117.0^{b}±3.0
P –value	0.00	0.00	0.00	0.00

[a, b] Means bearing different superscripts in the same column are significantly different (P<0.05)

Table 5b. Plasma Cortisol at stages of estrus cycle in buffaloes

Groups	Cortisol concentration (ng/ml)			
	Stages of estrus cycle (Mean ±SE)			
	Metestrus	Early diestrus	Late diestrus	Follicular
Control	20.8^{c}±1.7	22.2^{c}±2.5	22.4^{c}±1.5	24.0^{d}±2.0
Dystocia	36.0^{b}±1.2	39.8^{a}±1.9	42.2^{a}±1.9	43.4^{b}±1.3
RP	42.0^{a}±2.4	43.2^{a}±2.6	44.4^{a}±1.3	51.4^{a}±1.6
Low BCS	26.4^{c}±2.4	29.4^{b}±2.4	31.2^{b}±2.8	32.0^{c}±2.4
P –value	0.00	0.00	0.00	0.00

[a, b] Means bearing different superscripts in the same column are significantly different (P<0.05).

CONCLUSION

In conclusion, β-endorphin and cortisol secretion tend to increase at the time of calving. The Opioid system is a subject of attention not only due to its complexity but also its impact on key functions of the organism. Plasma β-endorphin level in buffalo during pregnancy state increased gradually through the gestation period. It reaches its highest level during the third trimester due to stressful state. Also, it reaches its peak at the time of parturition by appearing its analgesic action. Meanwhile, it decreases gradually towards the re-establishment of the ovarian cycle. It is concluded that ß-endorphin, like oxytocin, is released in an episodic manner during parturition in cows and that both hormones are released concomitantly in conjunction with uterine and abdominal contractions and distension of the uterine cervix.

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Effect of the Addition of Insulin-Transferrin-Selenium on In Vitro Maturation and Fertilization of Bali Cattle Oocytes

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ABSTRAK

Iskandar H, Sonjaya H, Yusuf M, Hasbi. 2019. Pengaruh penambahan Insulin-Transferrin-Selenium pada pematangan dan fertilisasi *in vitro* oosit sapi Bali. JITV 24(3): 95-102. DOI: http://dx.doi.org/10.14334/jitv.v24i3.2020

Penelitian ini dilakukan untuk mengetahui pengaruh penambahan Insulin Transferrin Selenium (ITS) pada medium terhadap tingkat maturasi dan fertilisasi oosit sapi Bali secara *in vitro*. Ovarium sapi Bali disayat untuk menghasilkan oosit, lalu oosit dikoleksi dan diseleksi berdasarkan kualitasnya. Oosit tersebut lalu dimaturasi 24 jam dan difertilisasi 18 jam di dalam inkubator 5% CO2 dan 38,5°C. Oosit diwarnai dengan aceto orcein 2%, lalu diamati di bawah mikroskop. Penelitian ini menggunakan Rancangan AcakLengkap (RAL) dengan empat perlakuan penambahan ITS (P0 kontrol; P1 (5 ng/ml); P2 (10 ng/ml); dan P3 (15 ng/ml)) dan 4 ulangan. Parameter yang diamati yaitu tahap tingkat maturasi oosit yang terdiri dari germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase-I (M-I) dan metaphase-II (M-II), tingkat fertilisasi (0 pronukleus (0 PN), 1 pronukleus (1 PN), 2 pronukleus (2 PN) dan lebih dari 2 pronukleus (>2 PN)). Hasil penelitian menunjukkan bahwa persentase tingkat maturasi oosit tertinggi cenderung pada tahap M-II yaitu dicapai oleh oosit P1 dengan pemberian ITS sebanyak 5 ng/ml sedangkan persentase tingkat fertilisasi tertinggi pada tahap PN-2, yaitu dihasilkan oleh oosit P3 dengan pemberian ITS sebanyak 15 ng/ml. Kesimpulan dari penelitian ini adalah bahwa pemberian ITS sebanyak 5 ng/ml cenderung menghasilkan tingkat maturasi yang terbaik dan untuk tingkat fertilisasi yang terbaik cenderung pada pemberian ITS sebanyak 15 ng/ml.

Kata Kunci: Ovarium sapi Bali, insulin-transferrin-selenium, maturasi, fertilisasi

ABSTRACT

Iskandar H, Sonjaya H, Yusuf M, Hasbi. 2019. Effect of the addition of Insulin-Transferrin-Selenium on ın vitro maturation and fertilization of bali cattle oocytes. JITV 24(3): 95-102.DOI: http://dx.doi.org/10.14334/jitv.v24i3.2020

This study was conducted to determine effect of the addition of Insulin Transferrin Selenium (ITS) on in vitro maturation and fertilization of Bali cattle oocytes. Bali cattle ovary is sliced to obtain oocytes, then oocytes were collected and selected based on their quality. Oocyte then maturated for 24 hours and fertilized for 18 hours in an incubator of 5% CO2 and 38.5°C. Oocyte was stained with 2% acetoorcein, then observed under a microscope. This study was done based on a Completely Randomized Design (CRD) with four treatments of ITS addition (P0 control; P1 (5 ng/ml); P2 (10 ng/ml); and P3 (15 ng/ml)) in 4 replications. Parameters observed were the stage of oocyte maturation level consisting of germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase-I (MI) and metaphase-II (M-II), fertilization rate (0 pronucleus (0 PN) , 1 pronucleus (1 PN), 2 pronucleus (2 PN) and more than 2 pronucleus (>2 PN)). Results showed that the highest percentage of oocyte maturation rate tends to be in the M-II stage, which is achieved by P1 oocytes with ITS addition as much as 5 ng/ml while the highest percentage of fertilization rate at PN-2 stage, which was produced by P3 oocytes with ITS addition as much as 15 ng/ml. It is concluded that the addition of ITS at 5 ng / ml tends to produce the best maturation rate and for the best level of fertilization tends to be as much as 15 ng / ml of ITS addition.

Key words: Bali cattle ovary, Insulin Transferrin Selenium, maturation, fertlization

INTRODUCTİON

At the time of in vitro maturation, oocytes develop metabolism with the condition of O_2 at higher concentrations. This condition triggers an increase in free radical production (Reactive Oxygen Species/ROS) resulting in oxidative stress conditions. This high level of ROS can damage cell membranes due to membrane lipid perioxidation. An enzymatic antioxidant system found in mammalian cells, namely superoxside dismutase, glutathione peroxidase, and catalase can function as ROS scavenger (Cetica et al. 2001). Therefore, the addition of antioxidants is needed in the process of oocytes maturation to inhibit cell damage due to ROS and increase the rate of cell growth in oocytes.

Antioxidant supplements such as α-tocopherol, glutathione (GSH) and Insulin Transferrin Selenium

(ITS) were added to the oocytes maturation medium with the aim of scavenging free radicals. Research (Kim et al. 2005; Lee et al. 2005) showed administration of insulin and insulin like growth factor (IGF) into the medium, could increase oocytes growth potential, the level of embryo cleavage during in vitro maturation (IVM), and in vitro Cultured (IVC) in pigs.

Insulin Transferrin Selenium (ITS) act as antioxidants in biological systems in rats (Wu et al. 2007; Gutteridge 1986). The use of ITS as a supplement has been carried out for rat oocytes (De La Fuente et al. 1999), ITS and epidermal growth factor (EGF) succeeded in managing prepubertal rat oocytes and preantral follicles by 92.2% (Gao et al. 2007), goats (Herrick et al. 2004), pig oocytes (Jeong et al. 2008) significantly increase the concentration of glutathione. The combination of ITS can be used both in media maturation and complex and non-complex fertilization, ITS is a supplement that can stimulate oocytes growth. However, studies and scientific information regarding the effect of Insulin Transferrin Selenium (ITS) in Bali cattle are still very limited.

Problems arising from the low quality of the results of in vitro maturation IVM and in vitro fertilization (IVF) oocytes by cell damage due to oxidative stress caused by increased production of free radicals or Reactive Oxygen Species (ROS) in vitro. Free radicals are atoms or molecules that have electrons that are not paired with orbitals (Gutteridge 1986). In order to get stability chemically, free radicals cannot maintain their original form for long periods and soon bind to the surrounding material. Free radicals will attack stable molecules the closest one and takes electrons, the substances that electrons take up will become radical also free so that it will start a chain reaction, which eventually couse cell damage (De La Fuente et al. 1999). Free radical activity can be reduced by administering antioxidants (Gao et al. 2007). Antioxidants are compounds that can inhibit, delay, prevent or slow down oxidation reactions even in small concentrations. Bali cattle is a germ plasm asset of Indonesian native cattle with some specific advantages, among them: very good reproductive properties and high fertility so that the addition of ITS in the media of maturation and fertilization is expected to provide a better response. Also the addition of antioxidant supplementation materials ITS into the maturation and fertilization medium aims to prevent the effects of ROS, so that the oocytes metabolic process can take place normally.

ITS is a media supplement in in vitro maturation that suppress the influence of free radical compounds that trigger the oxidative environment. So this study was conducted to evaluate the effect of ITS on the maturation and fertilization of Bali cattle oocytes in vitro.

MATERIALS AND METHODS

Sample collection

Bali cattle ovary obtained from Tamangapa Animal Slaughter house, Makassar City, South Sulawesi province and taken to the Fertilization and Embryo In Vitro Laboratory of Hasanuddin University with a distance of 10 km in a solution of 0.9% NaCl plus 100 IU/mL of penicillin and 100 µg/mL of streptomycin sulfate. The number of ovaries is ten out of five Bali cattle. Incubator temperature used is 37° C, 5% CO_2.

Oocytes collection

Ovaries obtained from Makassar Tamangapa RPH use physiological solutions of 0.9% NaCl first rinsed twice on 0.9% NaCl. Washed and dried, glass dishes were prepared, then filled with media of phosphate buffered saline (PBS). The ovaries were placed on the glass dish, then chopped. Oocytes are collected in a dish that has been filled with collection media (Phosphate Buffered Saline/PBS + 10% Fetal Bovine Serum/FBS). Only oocytes of A and B quality were used. Quality A oocyte (having a uniform and compact cumulus with surrounded by five or more layers of cumulus cells), quality B oocytes (characterized by uniform oocytes and having cytoplasm the dark with a complete complement of corona radiata but surrounded by no more than five layers of cumulus cells).

In Vitro Oocytes Maturation

Collected oocytes were washed in each maturation medium twice, then ripening in TCM-199 (Sigma, USA) supplemented with 0.3% bovine serum albumin (BSA), 10 IU/ml pregnant mare serum gonodrptropin (PMSG) (Intergonan, Intervet Deutschland GmbH), 10 IU/ml human chorionic gonadrotropin (hCG) (Chorulon, Intervet international BV Boxer-Holland), 50 µg/ml gentamycin (Sigma, USA), and the addition of Transferrin Selenium Insulin (ITS) with different concentrations (controls (0 ng/ml), 5 ng/ml, 10 ng/ml and 15 ng/ml) (Romar & Funahashi 2006; Shirazi & Sadeghi 2007).

In Vitro Fertilization,

Stage of fertilization based on (Suzuki et al. 2000) is as follow:

Frozen semen straw was thawed at 37°C for 20 seconds, then put it into the prepared spermatozoa washing medium to be centrifuged for 5 minutes at 1800 rpm of speed. The supernatant was discarded and then the deposited sperm was added with the second washing medium. It was centrifuged again with the

same time and speed. The centrifuged supernatant was removed and the sperm was diluted with fertilization media and made in the form of a drop in the fertilization dish. The coating used ±3 ml mineral oil to cover the entire surface of the fertilization media. Mature oocytes were removed from the incubator, then taken and placed on the media wash and washed twice. Washed oocytes were placed in a fertilization dish containing of sperm drop which then incubated for ≥16 hours.

Fixation

Fixation process was started by removing fertilization dish from incubator after ≥16 hours of fertilization. The fertilized oocytes were then removed and washed three times (all sperms were not involved and only a few cumulus cells surround the oocytes). The fertilized oocytes are washed again 2-3 times and then moved to become preparations and glue the objects glass and glass cover using vaselin (adhesive). The fixation was used ethanol: acetic acid for three days. Then the preparation was rinsed using absolute ethanol for one hour.

Cell Coloring

Cell staining preparations used 2% acetone orcein then rinse again with 25% acetic acid. Oocyteswere examined under an inverted microscope to observe the maturation and fertilization rates.

Observed parameters

The parameters observed in this study were the level of oocyte maturation and the rate of fertilization. Oocyte maturation rate according to (Sonjaya et al. 2016) was germinal vesicle (GV) phase which was characterized by the presence of a nuclear membrane and a nucleoli clearly visible on the edge; Germinal Vesicle Breaking Down (GVBD) phase which was characterized by the tearing of the core membrane so that the nucleoli not clearly visible. Metaphase-I (M-I) phase was characterized by the presence of homologous chromosomes that were paired and lined up in the equatorial plane. Metaphase-II (M-II) phase was characterized by the presence of a polar body I and the same chromosome arrangement as the M-I stage, anaphase and telophase phases.

Oocyte maturation rates may be calculated according to the formula below:

$$\frac{The\ number\ of\ oocytes\ under\ going\ as\ tage\ of\ maturation}{The\ number\ of\ oocytes\ being\ saturated} X100\%$$

Meanwhile, fertilization rates according to Syaiful et al. (2011) was fragmented oocytes or oocytes that do not achieve metaphase II development (0 PN); oocytes that have one pronucleus (1 PN) consisting only of female pronucleus; oocytes that have two pronucleus (2 PN) consisting of male and female pronucleus; fertilized oocytes that have two or more pronucleus (> 2 PN).In vitro fertilization rates may be calculated according to the formula below:

$$\frac{The\ number\ of\ oocytes\ under\ going\ as\ tage\ of\ fertilization}{Number\ of\ fertilized\ oocytes} X\ 100\%$$

Data analysis

The level of maturity and oocyte fertilization were analyzed by Completely Randomized Design (CRD) Contingency Table with the following formula (Steel & Torrie 1993):

$$X^2 = \frac{[(BxC) - (AxD]^2\ E}{(A+C)(B+D)(A+B)(C+D)}$$

TREATMENT	X	Y	
ITS 1	A	B	A+B
ITS 2	C	D	C+D
	A+C	B+D	A+B+C+D=E

Table 1. Bali cow oocytes maturation level with Insulin Transferrin Selenium (ng/ml) addition in different media

Treatments (ITS addition)	Oocytes count	Maturation level (%)			
		GV	GVBD	M-I	M-II
Control (0 ng/ml)	36	0.00 ± 0.00[a]	9.00 ± 10.39[ab]	25.75 ± 6.13	26.00 ± 0.00[b]
5 ng/ml	26	0.00 ± 0.00[a]	0.00 ± 0.00[a]	17.50 ± 12.26	25.25 ± 9.91[b]
10 ng/ml	34	18.00 ± 0.00[b]	11.00 ± 13.11[ab]	19.25 ± 14.22	18.00 ± 0.00[ab]
15 ng/ml	32	9.00 ± 10.39[c]	20.00 ± 4.00[b]	22.00 ± 4.61	13.50 ± 9.00[a]

GV= germinal vesicle; GVBD= germinal vesicle break down; M-1=metaphase-I (MI); M-II= metaphase-II; [a,b]different superscript in the same column show real differences (P≤0.05)

RESULTS AND DISCUSSION

Level of Bali cow oocytes maturation with addition of Insulin Transferrin Selenium (ITS) in maturation media

The observation of the maturation rate with the addition of different level of Insulin Transferrin Selenium (ITS) in maturation media is presented in Table 1. The analysis results showed that the addition of ITS to oocytes had no significant effect (P> 0.05) on oocytes maturation rates. However, the addition of ITS 0 ng/ml-5 ng/ml into maturation media in this study tends to have a higher maturation rate compared to the addition of ITS 10 ng/ml and 15 ng/ml. This means that the addition of ITS of 0 ng/ml-5 ng/ml into the medium of maturation is sufficient to increase the oocytes maturation rate and support the development of oocytes to reach the stage of MII.

The number of oocytes used for each treatment was 40 oocytes, but at the time of observation there were several oocytes which were lost where in P0 the remaining oocytes were 36, P1 (26 oocytes), P2 (34 oocytes) and P3 (32 oocytes). By the glass cover at the time of fixation it is not well pressed so that when the cell staining occurs, some oocytes lost. Also the treatment of an increased ITS dose causes an irregular decrease in oocytes. This is possible, ITS content can increase GSH concentration.

The tendency of different oocytes maturation percentage shows that ITS supplementation in maturation media has an influence on oocytes maturation and can be used as an indicator of increasing cell life force, with increasing oocytes to stage M-II. This means that supplementation of ITS 5 ng/ml into the medium of maturation can reduce the rate of apoptosis. Whereas with ITS supplementation of 10 ng/ml-15 ng/ml, oocytes up to M-II stage tend to decrease, this is because the dose given exceeds the good dose so that free radicals at maturation that should be suppressed actually have an impact on oocyte reduction until stage M-II.

ITS supplementation on the maturation medium can reduce the oxidation reaction of ROS, because the content of selenium in the ITS medium is the main element of the antioxidant gluthathione peroxidase (GSx) and reduced gluthathione (GSH) which serves to prevent damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides (Djuwita et al. 2012). According to Hwang et al. (1992) Glutathione (GSH) is considered the most abundant molecule among endogenous antioxidants. GSH is a reduced peptide consisting of three-residues (γL-glutamyl-L-cysteinyl glycine) which can donate an electron with the consequence that two electron donating GSH molecules form oxidized GSSG. In humans, GSH is almost uniquely present in a quite high concentration (1–10 mM) which allows to scavenge.

ROS either directly or indirectly Werdhany (1999), GSH is an antioxidant that play a role in preventing the formation of new free radicals and reducing existing free radicals. In addition, the transferrin and selenium contents are important for the GSH peroxidase catalyst activity (Cerri et al. 2009).

Cytoplasm of oocytes strongly supports the spread of organelles and interactions between other organelles, as well as the presence of compact cumulus cells that can support the maturation of oocytes through metabolites produced and secreted through a gap junction mechanism to oocytes cells (De Loos et al. 1989).

In this study only oocytes that had complex cumulus (categories A and B) were used in the in vitro maturation process (Figure 2). According to (De Loos et al. 1989) the oocytes that well included were compact, multilayered and compact cumulus cells, homogeneous ooplasm, total bright and transparent COC (Cumulus Oocytes Complex). The presence of cumulus cells supports the occurrence of oocytes maturation in vitro to stage M-II and related to cytoplasmic maturation (Lapathihis et al. 2002). In providing nutrients for oocytes and helping synthesize proteins to form a pellucid zone at prophase stage. Egg cells without cumulus after being matured, might lost many proteins while in egg cells with intact cumulus, protein will survive.

During in vitro maturation of bovine oocytes the presence of cumulus cells surrounding the oocytes is very helpful to the development of blastocysts (Boediono & Suzuki 1996).

Observations on Bali cattle oocytes ranging from un-denaturated oocytes to oocytes after staining with various levels of core maturation can be seen in Figure 1 and Figure 2.

Factors that support the success of oocytes core maturation (Zheng & Sirard 1992) are the expansion of the cumulus maturation cells of the nucleus that reaches M-II and cytoplasmic maturation. According to Motlík & Fulka (1976) the cumulus cells that surround the oocytes will be wide and brightly colored. Oocytes have reached maximum maturation and are ready to fertilize if they have reached stage M-II in the process of meiotic division (Figure 2).

The meiosis process begins with the GV stage, which is characterized by a clear core membrane and a clear colored necloulus ring followed by the rupture of the core stage or GVBD, necloulus disappears and the one pole body has been formed (Tsafriri 1985), while stoplasma maturation includes the addition of yellow grains eggs in the cytoplasm, formation of the pellucid zone sheath, and the formation of cortical granules (Djuwita et al. 2000).

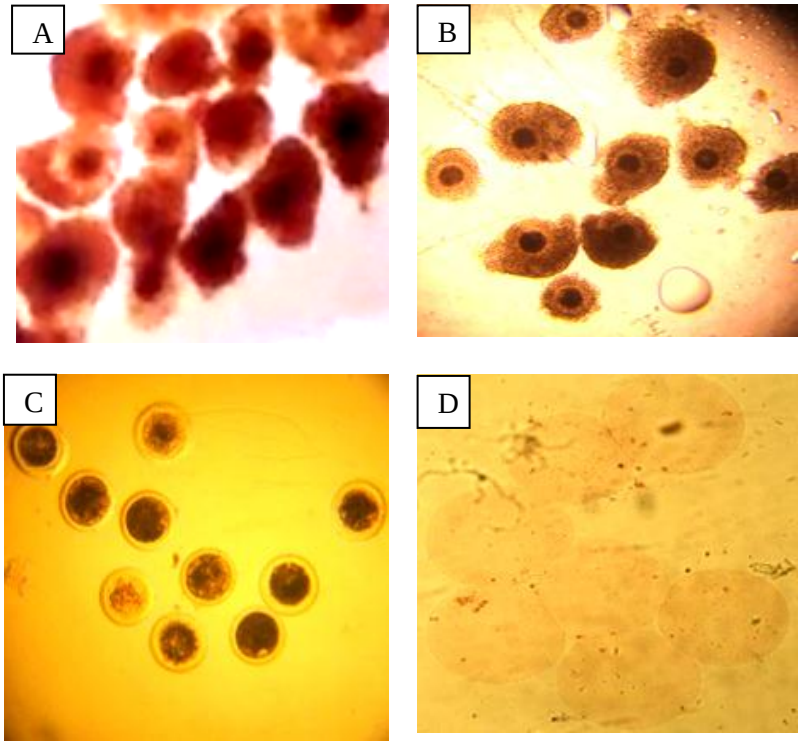

Figure 1. Bali cow oocytes changes from un-denaturated to after staining with various levels of core maturation. (A) oocytes before maturation, (B) oocytes after maturation,(C)oocytes after denudation, (D) oocytes after staining

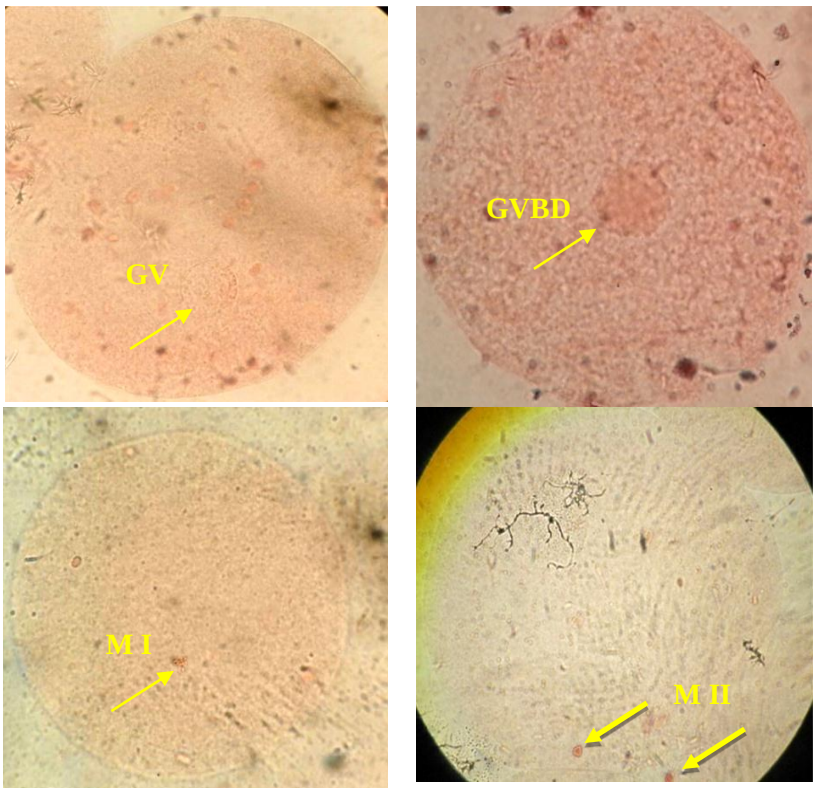

Figure 2. Bali cow oocytes maturation core status after in vitro maturation. Arrow marks indicate core status at stage (A) oocytes vesicle germinal stage, (B) oocytes vesicle germinal stage breaks down, (C) oocytes stage metaphase-I, (D) mature oocytes metaphase-II stage

Table 2. Bali cow oocytes pronucleous formation (%) and fertilization rates with different level of Insulin Transferrin Selenium addition

Treatment (ITS addition)	Oocytes count	Pronucleusformation(%)				Fertiluzation rate
		0 PN	1 PN	2 PN	>2 PN	
Control	36	22.75±16.35^{b}	9.00±10.39	11.00±13.11	17.50±12.26	26.00±0.00^{a}
5 ng/ml	26	0.00±0.00^{a}	9.00±10.39	22.00±4.61	17.50±12.26	31.00±6.27a^{b}
10 ng/ml	34	9.00±10.39$_{ab}$	9.00±10.39	23.75±7.22	22.00±4.61	34.25±7.88^{ab}
15 ng/ml	32	0.00±0.00^{a}	9.00±10.39	20.00±4.00	27.50±7.14	36.00±3.46^{b}

PN= pronucleous; ^{a,b}different superscripts in the same column show real differences (P≤0.05)

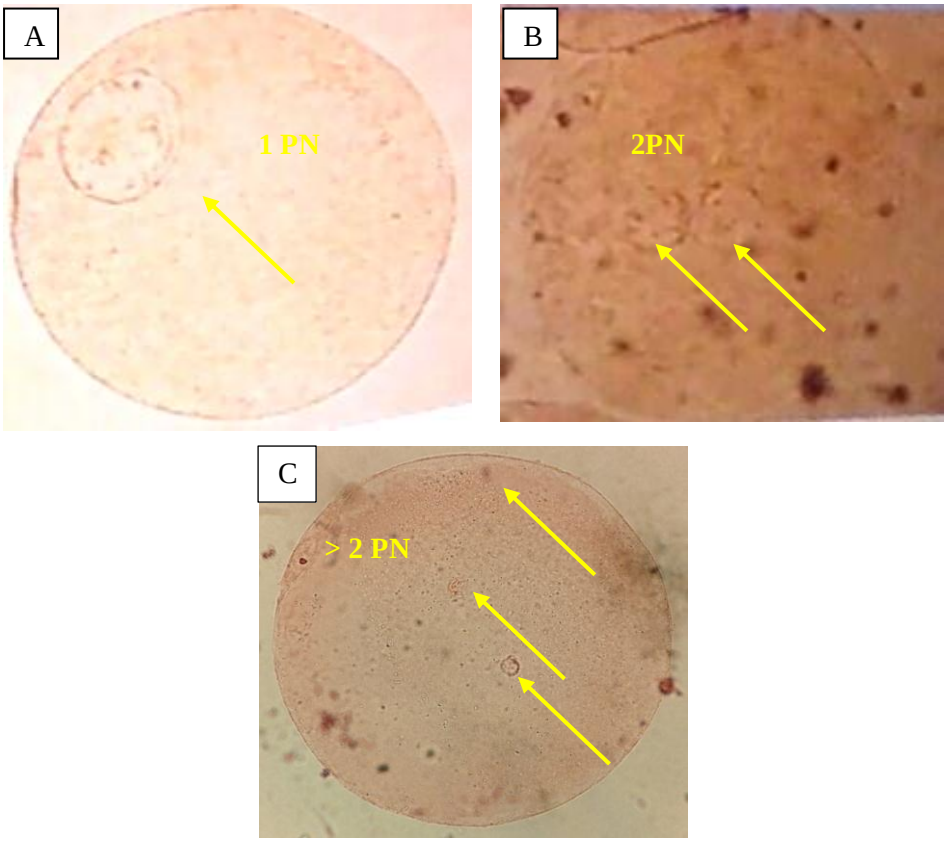

Figure 3. Bali cow pronucleous formation. PN= pronucleous; (A) oocytes stage 1 pronucleus; (B) fertile oocytes stage 2 pronucleus; (C) fertile oocytes stage >2 pronucleus

Bali cow oocytes fertilization rate with different addition of Insulin Transferrin Selenium (ITS)

The results of observing the degree of fertilization with the addition of different Insulin Transferrin Selenium (ITS) Insulin are presented in Table 2.Results of the analysis showed that the effect of the addition of ITS on the oocytes had no significant effect (P> 0.05) on the level of oocytes fertilization. However, with the addition of ITS 15 ng/ml the fertility rate tends to be higher than the addition of 5 ng/ml and 10 ng/ml. This means that the addition of ITS of 15 ng/ml increased the level of oocytes fertilization and was better than other treatments. Rusiyantono et al. (2000) obtained a value of 65.4% in tissue culture medium (TCM 199)+Essensial media, while Djuwita et al. (1995) obtained a value of 31.7% in TCM 199+ media for fetal serum (EFS) and 25.6% in TCM 199+FCS media.

Meanwhile, the role of ITS for P2 and P0 and those who were not given ITS (control) was higher but the fertility rate was low. This is thought to be related to the role of ITS which can optimize cell growth better when fertilized. Furthermore, it is also suspected that the low level of fertilization in the P2 medium supplemented with ITS or without the addition of ITS results in differences in protein metabolism needed to improve oocytes competency (Orsi & Leese 2004).

Fertilization failure is characterized by the presence of one pronucleus and in this study varied. Fertilization failure is influenced by several factors, among others: the level of oocytes maturation in both the nucleus and cytoplasm is incomplete (Boediono et al. 2000); adequate and failure of spermatozoa to experience condensation in the oocytes cytoplasm causing a failure of male pronucleus formation (Crozet et al. 1995). The incidence of polyspermi in this study varied. The incidence of polyspermi may be caused by various factors including the concentration of spermatozoa (Nadir et al. 1993; Long et al. 1994), the length of incubation of spermatozoa and oocytes (Long et al. 1994) and not perfect blockade of vitelin (Dandekar & Talbot 1992).

Another factor that also affects the ability of in vitro fertilization is the production of ROS. Dead spermatozoa produce ROS causing membrane lipid peroxidation, reducing membrane fluidity and sperm function. High ROS destroys the metabolism of spermatozoa in the media of in vitro fertilization. Kim et al. (2002) said ROS increased under in vitro conditions using 5% CO_2.

Fertilized oocytes are characterized by the formation of PN (Figure 3). Oocytes that have undergone a maturation process for 24 hours are then fertilized for 18 hours in an incubator with CO_2 and the same temperature when matured. Before the fertilization process, the sperm is frozen first in thawing and diluted on the fertilization medium.

CONCLUCIONS

Addition of Insulin Transferrin Selenium (ITS) to mediums of 10-15 ng/ml did not significantly influence the maturation and fertilization of Bali cattle oocytes in vitro.

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Retained Placenta in Relation with Blood Components in Egyptian Crossbred Cattle

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ABSTRAK

Mourad RS. 2019. Retensiplasentadanhubungannyadengankomponendarahpadasapimesirpersilangan. JITV 24(3): 103-111 DOI: http://dx.doi/10.14334/jitv.v24i3.2002

Retained placenta (RP) adalah salah satu gangguan reproduksi utama pada sapi perah dan terjadi jika plasenta tidak dilepaskan dalam kurun waktu kurang lebih 12 jam setelah melahirkan dan 3 jam setelah pembukaan. Penelitian ini dilakukan pada 14 sapi normal dan 32 sapi dengan RP di unit dokter hewan. Sapi yang dipilih adalah sapi betina dalam masa nifas selama kurang lebih 6 hingga 12 jam di Menoufia, Mesir. Hasil penelitian menunjukkan bahwa terdapat peningkatan yang signifikan pada konsentrasi komponen darah pada sapi normal dibandingkan dengan sapi yang mengalami RP, kecuali pada globulin, sodium dan mangan. Konsentrasi plasma komponen biokimia menunjukkan hasil yang signifikan antara musim panas dan musim dingin pada kolesterol, total protein dan albumin tetapi tidak ada perbedaan pada glukosa, globulin dan A/Grasio. Konsentrasi plasma lebih tinggi di musim dingin dari pada di musim panas di semua elemen makro kecuali kalium dan rasio Ca/P. Juga, konsentrasi plasma lebih tinggi di musim dingin dari pada di musim panas di semua elemen mikro kecuali mangan. Konsentrasi plasma komponen biokimia tidak signifikan antara paritas kecuali dalam rasio A/G. Tidak ada konsentrasi plasma yang signifikan antara musim panas dan musim dingin di semua elemen makro. Tetapi, ada perbedaan yang signifikan ($P \leq 0,01$) dalam konsentrasi plasma Cu dan Cd antara musim panas dan musim dingin, dan ada perbedaan yang signifikan ($P \leq 0,05$) pada Fe dan Se tetapi, tidak ada yang signifikan dalam Co, Zn, Mn, dan Mo.

ABSTRACT

Mourad RS. 2019. Retained placenta in Relation with Blood Components in Egyptian crossbred cattle. JITV 24(3): 103-111. DOI: http://dx.doi/10.14334/jitv.v24i3.2002

Retained placenta (RP) is one of the main reproductive disorders in dairy cattle and happened if the placenta is not released out within certain duration around 12 h post calving and 3 h post foaling. The present study was carried out in the veterinary units on 14 normal cows and 32 cows with RP. Cows were chosen after about 6 to 12 h from parturition in Menoufia governorate, Egypt. Results show that there was significant increase of concentrations of blood components in normal cows than those in RP cows except in globulin, sodium and manganese. Plasma concentrations of biochemical components were significant between summer and winter in cholesterol, total protein and albumin but it was no difference in glucose, globulin and A/G ratio. Plasma concentration is higher in winter than in summer in all macro elements except potassium and Ca/P ratio. Also, Plasma concentration is higher in winter than in summer in all micro elements except Manganese. Plasma concentrations of biochemical components were not significant between parities except in A/G ratio. No significant in plasma concentration between summer and winter in all macro elements. But, there was significant deference ($P \leq 0.01$) in plasma concentration of Cu and Cd between summer and winter, and it was significant deference ($P \leq 0.05$) in Fe and Se but, there was no significant in Co, Zn, Mn, and Mo.

Keywords: Cattle, plasma constituents, macro- micro-minerals, retained placenta.

INTRODUCTION

Retained placenta (RP) is one of the main reproductive disorders in dairy cattle. That causes economic losses in the herd due to decreased milk production, illness and treatment cost, beside a decreased market value of the animal (Semacan & Sevinç 2005). Fetal membranes "placenta" is a vital organ for prenatal transfer of nutrients, oxygen and immunity from the mother to the fetus. It is normally released in the short time post parturition. The RP as a reproductive disorder happened if the placenta is not released within certain duration around 12 hours post calving and 3 hours post foaling (Mohamed & Amer 2009; Taylor et al. 2010).

The risk factors associated with RP include twins, dystocia, stillborn calf, induced parturition, abortion, milk

fever, and increasing age, as well as conflicting seasonal effects (Gröhn & Rajala-Schultz 2000). Metabolic disorders play an important role in pathology of puerperal period. Deficiency of energy often causes the atony or hypotony of the uterus and this is the main cause of its late involution and cleaning. Energy disturbances can start a few weeks before calving (Kuzma et al. 1996). Cows with RP have to increased uterine activity in the days after calving (Frazer 2005). The incidence of retained placenta in dairy cattle averages around 4% and appears to be worsening (Esslemont & Kossaibati 2002). Cows with RP have an increased risk of metritis within one month postpartum and longer days to first service and services per conception (Correa et al. 1993). Other causes of RP as: genetic and breed predisposition, time of calving, length of pregnancy and the age of cows (Laven & Peters 1996). There is a fluctuation in the occurrence of RP in the same herd in different years and among herds in the same year. The interesting is that high milk efficiency in the previous lactation is not a risk factor of placental retention (Gröhn et al. 1995). The aim of this study was to determine blood plasma components in case of RP in Egyptian crossbred cattle.

MATERIALS AND METHODS

This study was carried out in veterinary units in Shebin El-kom, Menoufia, Egypt. 46 postpartum crossbred cows aged from 4 to 11 years, 345 to 620 kg live body weight and within the 2 to 6 parity were subjected in this study. There is influence between various parities. The experimental cows included fourteen normal cows as a control group and thirty two cows with RP. Cows were chosen after about 6 to 12 hours from parturition. Cows rations consisted of a commercial concentrate feed mixture as 60% yellow corn, 15% wheat bran, 23% soybean meal, 1% NaCL and 1% mineral mixtures, green clover (*Trifolium alexandrinum*) and rice straw, Animals were milked twice daily morning and evening milking.

Blood samples collected from jugular vein into clean heparinized test tubes were centrifuged at 3000 rpm for 20 minutes, then blood plasma was carefully separated and stored frozen at –20˚C. Plasma digested by adding 10 ml concentrate H2SO4 and two drops of H_2O_2 to 1 ml of blood plasma and heated. The digested sample diluted with distillated water at a ratio of 1:50. Concentration of macro- (Na, K, Mg, and Ca) and micro- (Mn, Cd, Se, Co, Cu, Mo, Fe, and Zn) elements were determined from blood plasma using an atomic absorption spectrophotometer "Unicam 929 AA". However, concentration of cholesterol, glucose, total protein, albumin, and inorganic phosphorus was determined calorimetrically according to (Allain et al.). Concentration of globulin was calculated by subtracting albumin and total protein concentration.

Data obtained were statistically analyzed using descriptive statistics, ANOVA and Duncan test was used to determined the significant differences among means at α=0.05 via SAS computer program (SAS 2003).

RESULTS AND DISCUSSION

Blood Biochemistry of cattle with normal and retained placenta

Biochemical components

Concentration of blood biochemical components including cholesterol (CH), glucose (G), total protein (TP), albumin (AL), globulin (GL), AL/GL ratio and are depicted in Table 1.

Results showed that there were very highly significant increases in all concentrations of blood biochemical components in normal cows than those in retained fetal membranes cows except in globulin (GL). Time around calving is a critical period in high-yielding cows with regard to hormonal changes, metabolism, immunity, health, further fertility, and milk productivity (LeBlanc 2013). During the time of retained fetal membranes, the immune system is depressed in periparturient cows (Goff 2008).

Normal blood serum metabolic in cows were as following: glucose ranged from 3 to 4 mmol/l, albumin ranged from 30 to 49 g/l and, cholesterol ranged from 1.8 to 5.2 mmol/l (Winnicka 1997).

Glucose level decreasing cause decreased the activity of aspartate aminotransferase during the last month of pregnancy so it is the main factor in happening of RP (Markiewicz et al. 2001).

Cows with retained placenta had glucose (RP= 1.99 ± 0.80, Normal= 2.35 ± 0.67 mmol/l) and cholesterol (RP= 2.08 ± 0.83, Normal= 2.17 ± 0.84 mmol/l) levels similar to the lower limit of physiological normal (2.22 mmol/l of glucose and 2.33 mmol/l of cholesterol) (Kaczmarowski et al. 2006). There was no significant difference in the serum total protein between buffalo cows with RP and normal parturient buffalo cows (Dutta and Dugwekar 1983).

Table 1. Blood biochemical components in normal and RP crossbred cattle

Biochemical components	Case	Mean	±SE	Min	Max	P Value
Cholesterol (mg/dl)	Normal	214.45	±0.59	210.30	218.10	0.00
	RP	170.77	±6.35	128.71	231.71	
Glucose (mg/dl)	Normal	74.75	±0.71	72.59	78.65	0.00
	RP	42.90	±0.79	38.99	51.79	
Total protein (mg/dl)	Normal	7.25	±0.14	6.38	7.68	0.00
	RP	5.96	±0.08	5.00	6.67	
Albumin (mg/dl)	Normal	6.08	±0.1	5.48	6.35	0.00
	RP	4.65	±0.07	4.02	5.54	
Globulin (mg/dl)	Normal	1.17	±0.05	0.90	1.33	0.30
	RP	1.31	±0.09	0.40	2.05	
A/G Ratio	Normal	5.29	±0.2	4.71	6.61	0.00
	RP	1.29	±0.02	1.09	1.47	

Normal =14 healthy cows, RP=32cows with retained placenta. Significant different at (P≤0.05)

Table 2a.Blood plasma macro elements in normal and RP crossbred cattle.

Case	Macro	Mean ± SE (mg/dl)		Min	Max	P-Value	Macro	Mean ± SE (mg/dl)		Min	Max	P-Value
Normal	Na	147.09	±0.58	143.89	149.56	0.11	Ca	10.46	±0.24	9.14	12.02	0.00
RP		159.07	±4.81	140.28	215.30			8.08	±0.19	6.45	10.30	
Normal	K	6.23	±0.15	5.23	7.20	0.00	P	5.46	±0.16	4.60	6.40	0.00
RP		32.47	±0.29	29.00	36.55			4.69	±0.08	4.18	5.57	
Normal	Mg	5.4600	±0.09	4.91	5.72	0.00	Ca/P Ratio	1.92	±0.04	1.61	2.00	0.00
RP		2.4195	±0.14	2.01	4.10			1.72	±0.02	1.30	1.94	

Normal =14 healthy cows, RP=32 cows with retained placenta. Significant different at (P≤0.05)

Table 2b. Blood plasma micro elements in normal and RP crossbred cattle.

Case	Micro	Mean ±SE (µg /dl)		Min	Max	P-Value	Micro	Mean ±SE (µg /dl)		Min	Max	P-Value
Normal	CO	4.46	±0.07	3.89	4.65	0.00	Se	0.59	±0.01	0.58	0.66	0.00
RP		2.72	±0.04	2.15	2.89			0.39	±0.00	0.36	0.45	
Normal	CU	0.82	±0.05	0.65	1.13	0.00	Fe	4.21	±0.10	3.68	4.72	0.00
RP		0.41	±0.01	0.35	0.45			6.06	±0.31	2.95	8.00	
Normal	Zn	1.26	±0.14	0.66	1.84	0.00	Mo	0.14	±0.00	0.13	0.15	0.00
RP		0.88	±0.03	0.43	1.00			0.03	±0.00	0.02	0.03	
Normal	Mn	0.35	±0.02	0.20	0.45	0.12	Cd	0.89	±0.11	0.49	1.54	0.00
RP		0.42	±0.03	0.08	0.56			0.42	±0.00	0.40	0.46	

Normal =14 healthy cows, RP=32 cows with retained placenta. Significant different at (P≤0.05)

Macro and Micro-Elements

Concentration of blood macro- and micro- elements in plasma of normal cows and those with retained fetal membranes are shown in Table 2a and 2b. Results showed that concentrations of all blood macro elements were statistically higher in normal cows than those with retained fetal membranes except Na and K elements. Also there was a very highly significant difference ($P \leq 0.01$) between all macro elements in normal cows and those with retained fetal membranes except in Na.

In high yielding cows, the most important pathogenic factor causing RP during parturition is immune suppression caused by metabolic disorders. These metabolic conditions and health effects are adequately connected with high milk production in cows (Goff 2008).

Serum Ca and blood glucose levels were significantly lower ($P<0.01$) in buffaloes with retention of fetal membrane than normal parturient buffaloes. They suggested that the lower Ca concentration and lower energy level might be responsible for the occurrence of retention of fetal membranes in buffaloes (Mandali et al. 2002).

The disturbance in the Ca metabolism and its utilization by the tissue results in atony of the internal organs. Moreover, during advanced stages of pregnancy, there is excessive mobilization of Ca, then less availability of glucose and Ca to the uterine tissues results in atony of the uterus, with decreased contraction and RP (Mohanty et al. 1994). In addition, immune-mediated etiology of mineral disturbances observed especially during hypocalcaemia - milk fever in dairy cows (Kimura et al. 2006; Gray et al. 2007).

Table 2b showed that plasma concentrations of Co, Cu, Zn, Se, Mo, and Cd in normal cows were higher than those with retained fetal membranes. On the other hand, the blood plasma concentrations of Mn, and Fe were statistically higher in cows with retained fetal membranes than in those normal cows. Also there was a very highly significant difference ($P \leq 0.01$) between all micro elements in normal cows and those with retained fetal membranes except in Mn.

The oxidative stress and its immune-suppressive consequences also in relation to RP are caused not only by deficiency of vitamins E and A, but also by a lack of micro- or macro-elements such as Se, Zn, Cu, and Cr in diet (Kendall and Bone 2006). Copper is essential for normal phagocytosis (Spears and Weiss 2008).

Blood serum metabolic and mineral limits in cows as follows: glucose (3–4 mmol/l), albumins (30–49 g/l), Mg (> 0.7 mmol/l), P (> 1.3 mmol/l), for Normal cows and Ca (2.4–3.0 mmol/l), cholesterol (1.8–5.2 mmol/l), may be helpful during potential biochemical blood metabolic monitoring in dairy cows in terms of RP (Winnicka 1997). In addition; the normal limits for the most important trace elements in serum as Cu (9.2 μmol/l), I (3–6 μg/l), Mo (>1.1 μmol/l), Fe (25–35 μmol/l), Co (0.38–1.85 μmol/l) or in plasma as Se (> 110 μg/l), Zn (80–120 μg/dl), Mn (5–6 μg/l) (Mordak et al. 2017).

Se deficiency is associated with an increased risk of retained placenta and perhaps mastitis. It is thought that Se deficiency reduces the immune response in the cow. The mechanisms are largely unknown (Erickson et al. 2015). Sedependant glutathione peroxidase (GSHPx) has been found to be associated with the incidence of RP with low levels in plasma being reported with higher incidence of RP (Brzezinska-Slebodzinska et al. 1994). Vitamin E and Se supplementation of dairy cows, concluding that supplementing diets with both or either alone could reduce the risk of RP (Mee 2004). The use of inorganic Se may indeed prevent severe Se deficiencies but supplementation with Se yeast proved beneficial in providing enhanced Se status and antioxidant function at times of greatest stress and disease challenge, such as in the periparturient period (Givens et al. 2004).

Effect of season on the occasion of retained placenta in cattle

Table 3 showed that plasma concentrations of biochemical components were highly significant ($P \leq 0.01$) between summer and winter in cholesterol, total protein and albumin but was no difference in between the two seasons in the concentrations of glucose, globulin and A/G ratio. Effect of season is also significant according to temperature and nutrition (Cady 2008).

The season is correlated with birth weight of calves. Extremely low and high temperatures affect birth weight (Deutscher et al. 1999). The distribution of retained placenta in cattle as following: 27.5% cases occurred in spring, 32.5% in summer, 7.5% in autumn and 32.5% in winter (Sharma et al. 2017).

Table 4a shows that there is very highly significant different ($P \leq 0.01$) in plasma concentration between summer and winter in all macro elements except K and Ca/P ratio. The incidence of RP varies from 4 – 16.1% in cow and it can be much higher in problem herds. It increases during summer with increased parity and following birth of male fetus (El-Malky et al. 2010).

There were no significant differences in RP percentage between winter and summer feeding as well as between male or female birthing (Gaafar et al. 2010). Other causes of RP as time of calving “more frequent

Table 3. Seasonal variations of blood biochemical components day in crossbred cattle with RP

Items	Case	Mean	±SE	Min	Max	P -Value
Cholesterol (mg/dl)	Summer	168.33	±7.38	128.71	231.71	0.00
	Winter	206.42	±3.49	181.71	217.25	
Glucose (mg/dl)	Summer	49.09	±2.81	38.99	77.90	0.06
	Winter	57.57	±3.52	39.00	78.65	
Total protein (mg/dl)	Summer	6.07	±0.11	5.00	7.23	0.00
	Winter	6.76	±0.18	5.99	7.68	
Albumin (mg/dl)	Summer	4.76	±0.11	4.02	6.28	0.00
	Winter	5.55	±0.16	4.53	6.35	
Globulin (mg/dl)	Summer	1.31	±0.09	0.40	2.05	0.41
	Winter	1.21	±0.07	0.75	1.70	
A/G Ratio	Summer	2.36	±0.38	1.09	6.61	0.54
	Winter	2.72	±0.41	1.14	4.77	

Summer (n=27), Winter (n=19). Significant different at (P≤0.05)

Table 4a. Seasonal variations of plasma macro elements in crossbred cattle with RP

Case	Macro	Mean ± SE (mg/dl)	Min	Max	P-Value	Macro	Mean ± SE (mg/dl)	Min	Max	P-Value
Summer	Na	147.19 ± 0.71	140.28	155.01	0.00	Ca	8.39 ±0.30	6.45	12.02	0.02
Winter		167.13 ± 7.57	140.28	215.30			9.39 ±0.28	7.45	10.30	
Summer	K	26.93 ±2.19	5.23	36.55	0.12	P	4.76 ±0.12	4.18	6.40	0.03
Winter		21.01 ±2.98	5.87	32.92			5.16 ±0.13	4.35	6.10	
Summer	Mg	2.73 ±0.26	2.01	5.68	0.00	Ca/P Ratio	1.76 ±0.03	1.30	1.99	0.21
Winter		4.22 ±0.34	2.04	5.72			1.82 ±0.03	1.61	2.00	

Summer (n=27), Winter (n=19). Significant different at (P≤0.05)

Table 4b. Seasonal variations of plasma micro elements in crossbred cattle with RP

Case	Micro	Mean ±SE (µg /dl)	Min	Max	P-Value	Micro	Mean ±SE (µg /dl)	Min	Max	P-Value
Summer	CO	3.15 ±0.12	2.82	4.58	0.00	Se	0.43 ±0.01	0.360	0.579	0.00
Winter		3.38 ±0.25	2.15	4.65			0.50 ±0.02	0.400	0.660	
Summer	CU	0.52 ±0.05	0.35	1.13	0.00	Fe	6.22 ±0.28	3.68	8.00	0.00
Winter		0.55 ±0.03	0.44	0.86			4.49 ±0.37	2.95	6.94	
Summer	Zn	0.89 ±0.03	0.43	1.00	0.00	Mo	0.05 ±0.01	0.026	0.149	0.00
Winter		1.14 ±0.12	0.59	1.84			0.07 ±0.01	0.023	0.143	
Summer	Mn	0.46 ±0.02	0.20	0.56	0.12	Cd	0.57 ±0.05	0.40	1.11	0.00
Winter		0.31 ±0.04	0.08	0.52			0.57 ±0.08	0.41	1.54	

Summer (n=27), Winter (n=19). Significant different at (P≤0.05)

during spring and summer". There is a fluctuation in the occurrence of RP in the same herd in different years and among herds in the same year (Laven & Peters 1996).

Table 4b illustrated that there was very highly significant (P≤0.01) in plasma concentration between summer and winter in all micro elements except Mn.

The highest percentages of incidence of RP were detected in spring and summer seasons, while the lowest percentage was noticed in autumn. Furthermore, they reported that the causes of the lower incidence in the fall season compared to hot season are due to the environmental and nutritional differences, e.g. temperature and concentrate to forage ration in the diet (Sharma et al. 2017).

Effect of parity on Retained Placenta incidence in cattle

Table 5 showed that plasma concentrations of biochemical components were not significant between parities except in A/G ratio was significant (P≤0.05). Retained placenta was highly significant in pleuriparous than primiparous cows (Sharma et al. 2017). The incidence of retained placenta increased in old cows with parity over fourth (Gabr et al. 2005).

The incidence of RP varies from 4 – 16.1% in cow and it can be much higher in problem herds. It increases during summer with increased parity and following birth of male fetus (El-Malky et al. 2010). Other causes of RP as the age of cows "more frequent in older animals". There is a fluctuation in the occurrence of RP in the same herd in different years and among herds in the same year (Laven and Peters 1996).

Table 6a showed that there was no significant in plasma concentration among parities in all macro elements except in Na was significant (P≤0.05).

The incidence of RP at 1st parity, 2nd parity, 3rd parity, 4th parity, 5th parity and >6th parity were 8.5%, 13.3%, 6.1%, 9.4%, 20% and 28.7%, respectively (Sarder et al. 2010). The incidence of RP in Friesian cows increased significantly from first parity to eighth parity (Gaafar et al. 2010). The rates of retention of placenta in first were equal to second but they were less than third and fifth parity (Azad 2010).

Table 5. Effect of parity on blood biochemical components day in crossbred cattle with RP

Items	Parity	Mean	±SE	Min	Max	P- Value
Cholesterol (mg/dl)	2nd	191.60	±11.26	131.00	231.71	
	3rd	191.38	±6.89	129.50	231.71	0.06
	≥4	163.12	±10.12	128.71	214.55	
	2nd	59.45	±5.76	39.00	78.65	
Glucose (mg/dl)	3rd	51.91	±3.00	38.99	78.65	0.18
	≥4	47.64	±3.43	39.29	72.60	
Total protein (mg/dl)	2nd	6.31	±0.24	5.29	7.42	
	3rd	6.45	±0.14	5.50	7.68	0.64
	≥4	6.20	±0.25	5.00	7.68	
	2nd	5.23	±0.24	4.02	6.28	
Albumin(mg/dl)	3rd	5.14	±0.16	4.02	6.35	0.45
	≥4	4.85	±0.20	4.40	6.35	
	2nd	1.08	±0.12	0.75	1.91	
Globulin (mg/dl)	3rd	1.32	±0.08	0.75	1.89	0.25
	≥4	1.35	±0.15	0.40	2.05	
	2nd	3.79a	±0.75	1.17	6.61	
A/G Ratio	3rd	2.22b	±0.34	1.14	5.33	0.03
	≥4	1.88b	±0.39	1.09	4.77	

Number of 2nd =11, 3rd=23 and ≥4=12. Significant different at (P≤0.05)

Table 6a. Effect of parity on plasma macro elements in crossbred cattle with RP

Parity	Items	Mean (mg/dl)±SE		Min	Max	P-Value	Items	Mean (mg/dl)±SE		Min	Max	P-Value
2nd		146.38[b]	±0.92	140.28	150.00	0.05	Ca	9.03	±0.63	6.45	12.02	
3rd	Na	163.85[a]	±6.42	140.28	215.30			9.00	±0.28	7.45	11.32	0.29
≥4		147.58b	±1.24	140.28	155.01			8.21	±0.32	7.42	10.29	
2nd		18.48	±4.33	5.23	34.60	0.12		5.15	±0.25	4.20	6.40	
3rd	K	25.09	±2.38	5.87	33.42		P	4.93	±0.13	4.18	6.10	0.27
≥4		28.82	±3.07	6.35	36.55			4.73	±0.08	4.40	5.15	
2nd		3.83	±0.52	2.01	5.72	0.16	Ca/P Ratio	1.74	±0.07	1.30	1.99	
3rd	Mg	3.49	±0.32	2.01	5.72			1.82	±0.024	1.61	2.00	0.21
≥4		2.63	±0.41	2.01	5.65			1.73	±0.05	1.51	2.00	

Number of 2nd =11, 3rd=23 and ≥4=12. Significant different at (P≤0.05)

Table 6b. Effect of parity on plasma micro elements in crossbred cattle with RP

Parity	Items	Mean (mg/dl)±SE		Min	Max	P-Value	Items	Mean (mg/dl)±SE		Min	Max	P-Value
2nd		3.68	±0.25	2.82	4.65	0.15	Se	0.50	±0.03	0.39	0.66	0.06
3rd	CO	3.09	±0.19	2.15	4.65			0.46	±0.02	0.37	0.66	
≥4		3.13	±0.19	2.82	4.58			0.41	±0.02	0.36	0.58	
2nd		0.71[a]	±0.09	0.35	1.13	0.004	Fe	5.67	±0.56	3.68	8.00	0.06
3rd	CU	0.50[b]	±0.03	0.35	0.86			4.95	±0.35	2.95	6.95	
≥4		0.44[b]	±0.03	0.35	0.65			6.38	±0.39	3.99	8.00	
2nd		0.88	±0.04	0.66	1.04	0.47	Mo	0.09	±0.02	0.03	0.15	0.08
3rd	Zn	1.00	±0.09	0.43	1.84			0.05	±0.01	0.02	0.15	
≥4		1.07	±0.11	0.68	1.84			0.04	±0.01	0.03	0.13	
2nd		0.40	±0.04	0.20	0.56	0.09	Cd	0.82a	±0.13	0.40	1.54	0.00
3rd	Mn	0.36	±0.04	0.08	0.54			0.52b	±0.05	0.40	1.54	
≥4		0.48	±0.02	0.36	0.56			0.43b	±0.01	0.41	0.49	

Number of 2nd =11, 3rd=23 and ≥4=12. Significant different at (P≤0.05)

Table 6b showed that there was very highly significant ($P \leq 0.01$) in plasma concentration of Cu and Cd between parities, and it was significant ($P \leq 0.05$) in Fe and Se but, there was no significant in Co, Zn, Mn, and Mo. So, the concentration of trace elements decreased with the increased of parity in all trace elements except Zn, Mn and Fe.

CONCLUSION

Retained placenta is failure of expulsion of the placenta and associated membranes within 24 h of calving. Retained placenta, metritis, and endometritis are diseases of immune function in the transition period, which begin at least 2 weeks pre-partum. It is concluded that retention of placenta is the most important factor leading to uterine infection and toxic puerperal metritis occurring during the early postpartum period. Prevention of retained placenta, of course, is the key of economy loss. In conclusion, plasma minerals and biochemical components are different between control and retained placenta groups. In addition, seasons and parities affected the happenings of fetal membrane retention in cattle.

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Antimicrobial and Anti-inflammation Activities of Fraction and Single Peptides Derived from Mare Milk Protein

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ABSTRAK

Kusumaningtyas E, Subekti DT, Fitaningtyas DFL. 2019. Aktivitas antimikroba dan anti-inflamasi fraksi dan peptida tunggal derivat dari protein susu kuda. JITV 24(3): 112-121. DOI: http://dx.doi.org/10.14334/jitv.v24.3.1976.

Susu kuda mengandung peptida bioaktif yang berguna bagi kesehatan manusia dan hewan. Peptida yang terkandung dalam fraksi atau peptida tunggal kemungkinan menunjukkan aktivitas yang berbeda. Tujuan dari penelitian ini adalah untuk mengevaluasi aktivitas antimikroba dan antiinflamasi peptida dalam bentuk fraksi dan tunggal derivat protein susu kuda. Uji antimikroba, fraksi <3 kDa dan peptida tunggal LVNELTEFAK (peptida 1), HPYFYAPELLYYANK (peptida 2), dan LANSLTEFAK (peptida 3) dilakukan terhadap *Escherichia coli* dan *Candida albicans*. Uji antioksidan dilakukan menggunakan metode 2,2'-*azino-bis* (3-*ethylbenzthiazoline*-6-*sulphonic acid* (ABTS) dan 2,2-diphenyl-1-picrylhydrazyl (DPPH). Pengaruh anti-inflamasi dideteksi dengan cara mengukur interleukin 1β (IL- 1β) dan Tumor Necrosis Factor -α (TNF-α) pada mencit setelah pemberian lipopolisakarida (LPS) *Escherichia coli* bersama-sama dengan fraksi atau peptida tunggal. Hasil penelitian menunjukkan bahwa peptida dalam bentuk fraksi memiliki aktivitas antibakteri, antifungi,dan antioksidan terhadap radikal ABTS lebih tinggi daripada semua peptida tunggal (P<0.05). Aktivitas antiinflamasi ditunjukkan oleh peptida 1 dan 2 ditandai dengan penurunan IL-1β yang significant sesudah perlakuan (P<0.05). Berdasarkan hasil tersebut, disimpulkan bahwa aktivitas antimikroba dan antioksidan fraksi <3 kDa lebih baik daripada aktivitas peptida tunggal. Untuk aktivitas antiinflamasi, peptida tunggal menunjukkan aktivitas yang lebih baik dibandingkan fraksi

Kata Kunci: Antimikroba, antioksidan, anti-inflamasi, peptida, susu kuda

ABSTRACT

Kusumaningtyas E, Subekti DT, Fitaningtyas DFL.2019. Antimicrobial and anti-inflammation activities of peptide fraction and purified peptides derived from mare milk. JITV 24(3): 112-121. DOI: HTTP://DX.DOI.ORG/10.14334/JITV.V34.3.1976.

Mare milk protein contains bioactive peptide which is beneficial for human and animal health. Peptides in the fraction and single may show different activities. The objectives of the study were to evaluate antimicrobial and anti-inflammation activities of the fraction and single peptide derived from mare milk protein. Antimicrobial assay was conducted by testing antibacterial and antifungal activities of fraction <3 kDa, single peptide LVNELTEFAK (peptide 1), HPYFYAPELLYYANK (peptide 2), and LANSLTEFAK (peptide 3) against *Escherichia coli* and *Candida albicans*. Antioxidant assay was conducted using 2,2'-*azino-bis* (3-*ethylbenzthiazoline*-6-*sulphonic acid* (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods. Anti-inflammation effect was detected by interleukin 1-β (IL- 1β) and Tumor Necrosis Factor -α (TNF-α) production in mice after administration of *Escherichia coli's* lipopolysaccharide (LPS) and combined with fraction or single peptide. Result showed that peptide in fraction form has higher antibacterial, antifungal activities and antioxidant activities against radical ABTS than all of single peptide (P<0.05). Anti-inflammation activity was showed by peptide 1 and peptide 2 which was indicated by significantly decreasing of IL-1β after treatment (P<0.05). Based on the results, it was concluded that antimicrobial and anti-antioxidant activities fraction <3 kDa was better than single peptide. For anti-inflammation activity, a single peptide showed better activity than fraction.

Key Words: Antimicrobial, antioxidant, anti-inflammation, peptide, mare milk

INTRODUCTION

Mare milk have been consumed by around 30 million people in the world (Potočnik and Gantner 2011), although it is less popular than cow or goat milk. Administration of fermented mare milk, Koumiss, to rat treated with toxic compound showed improvement in kidney and brain (Abdel-Salam et al. 2010). Mare milk

was also reported to have positive effect on patient with cardiovascular disease and promote wound healing (Jastrzebska et al. 2017). In Indonesia, Sumbawa mare milk is commonly used as therapy for cardiovascular, hypertension and gastrointestinal disorder. However, research about beneficial of Sumbawa mare milk on whole milk and fraction were still limited. Milk protein derivative such as peptides and their bioactivity also has not been widely explored and utilized.

Bioactive peptides usually bound in the native protein and will be active after hydrolysis process. The peptides present as a mix peptide in hydrolysate or single after purified. Peptides in single form may show higher or lower activities compared to hydrolysate. Single peptide can be produced by purification from hydrolysate or synthesize the peptide. Both peptide from purification or synthesis have potential to be active. A single synthetic peptide derived from a modified peptide was reported active to various bacteria such as *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa* and *Staphylococcus aureus* (Bormann et al. 2017). In addition, peptide KVISMI derived and purified from whey protein showed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (Pei et al. 2017).

Previous research reveals that hydrolysate and peptide fraction especially fraction <3 kDa of Sumbawa mare milk showed antibacterial and antioxidant activities (Kusumaningtyas et al. 2016). The fraction contains 13 peptides with various physiochemical characteristic such as charge, structure and isoelectric point. Potential of antibacterial activities of these peptides was predicted based on their charge, structure and hydrophobicity. Peptide with high therapeutic index (TI) was predicted to be better as antibacterial peptide compared to the lower one. Peptide LVNELTEFAK and HPYFAPELLYYANK which was predicted to have high antibacterial activities was synthesized and assayed further in laboratory to obtain their real activities.

In addition, peptides are also able to be modified to obtain new peptides with higher activities. In this research, peptide LVNELTEFAK (TI: 28.69) was modified and synthesized to be LANSLTEFAK (TI: 94.91) to increase their therapeutic index which probably increase its bioactivities. Comparison of bioactivities of peptide fraction <3 kDa with single peptide LVNELTEFAK, HPYFAPELLYYANK and LANSLTEFAK were also evaluated.

MATERIALS AND METHODS

Materials

Bacillus thuringiensis was isolated from mare milk from Bogor, Indonesia. *Escherichia coli* (ATCC 25922), *Candida albicans* (BCC F059) were used for antibacterial and antifungal assays. Mare milk was collected from Sumbawa Island, Indonesia. Sumbawa mares are local horses of Indonesia which have a geographic original distribution on Sumbawa Island, West Nusa Tenggara Province that had been stated through the Decree of Minister of Agriculture No 2917/Kpts/OT.140/6/2011, June 17, 2011

Peptide fraction <3 kDa was obtain from hydrolysis of Sumbawa mare milk using *B. thuringiensis* protease and showed high activity against Gram negative bacteria, *Escherichia coli* and *Salmonella Typhimurium*. Purified peptide The sequence of peptide 1 (LVNELTEFAK) and peptide 2 (HPYFAPELLYYANK) was obtained from selected peptide of fraction <3 kDa which had high therapeutic index prediction (Kusumaningtyas et al. 2016), while peptide 3 (LANSTEFAK) was modification of peptide P1 by replacing amino acid valine (V) with alanine (A) and glutamic acid (E) with Serine (S). Those peptides were synthesized by First Base Laboratories, Selangor Malaysia. Molecular weight and purity of the peptides assayed as shown in Table 1.

Fraction preparation

Defatted mare milk was hydrolyzed at 55 °C pH 11 for 30 minutes using crude protease of *Bacillus thuringiensis* (0.67 IU) isolated using ammonium sulfate precipitation according to Kusumaningtyas et al (2016). Hydrolysis was performed at enzyme substrate ratio 1:20 (v/v). The reaction was stopped by freezing immediately at -20^{o}C for 15 min. Lipid and insoluble protein was discarded by centrifugation 11.000 ×g for 15 min. The supernatant was filtered using 0.45 nm membrane (Acrodisc LC 13 mm, 0,45 µm, PVDF, Pall Life Sciences, USA) and then filtered through centrifugal filter MWCO 3k (AMICON Ultra centrifugal units, Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co).

Peptide analysis

Calculation of theoretical physico-chemical properties were conducted using primary structure analysis

Table 1. Molecular weight and purity of the peptides

Peptide	Sequence	Mass (Da)	Purity
Peptida 1	LVNELTEFAK	1162.62	96.72%
Peptida 2	HPYFYAPELLYYANK	1887.92	92.86%
Peptida 3	LANSLTEFAK	1092.58	99.17%
Cecropin A (control peptide)		4003.4	98.77%

Source: data sheet of peptide 1, 2, 3 and Cecropin A

www.expasy.org (Kusumaningtyas et al. 2016) and therapeutic index was analyzed using http://split4.pmfst.hr/split/dserv1/?akcija=run. (Juretić et al. 2009).

Antibacterial assay

Antibacterial assay was conducted according to Keepers et al. 2014 with modification. As much as 100 µl, *Escherichia coli* suspensions of 106 CFU/ mL was mixed to 100 µl peptide fraction <3 kDa, peptide 1 or peptide 2 in ependorf. The 100 µl of mixed suspension was grown onto Mueller Hinton agar plate (BD DifcoTM, Becton Dickinson and Co, USA) and incubated for 24 h at 37°C. The viable colonies were counted. Each treatment was done in three replications. Cecropin A was used as control peptide.

Antifungal assay

Briefly, 100 µl of *Candida albicans* suspensions of 10^6 CFU mL^{-1} was added to the eppendorf containing100 µl peptide fraction <3 kDa, peptide 1 or peptide 2. The 100 µl of mixed suspension was grown onto Sabouraud dextrose agar plate (BD Difco™, Becton Dickinson and Co, USA) and incubated for 24 h at 37 °C. The viable colonies were counted. Each treatment was done in three replications. Cecropin A was used as control peptide (modification from Keepers et al. 2014)

Antioxidant assay

Antioxidant assay was conducted using 2,2'-*azino-bis* (3-*ethylbenzthiazoline*-6-*sulphonic acid* (ABTS) (Sigma-Aldrich, USA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA) according to modification of methods by (Thaipong et al. 2006). Stock solution of ABTS 7.4 mM was mixed with 2.6 mM potassium persulphate and incubated at room temperature in the dark for 18 hours. The solution was adjusted for absorbance 1.1 ± 0.05 at λ= 405 nm by diluted in deionized water. The fresh ABTS was then used for antioxidant assay. Fraction <3 kDa or single peptide 100 µL (1 µg /mL) was added to 200 µL ABTS or DPPH, incubated for 15 minutes for ABTS or 30 minutes for DPPH at room temperature to allow the reaction. Absorbance of the resulting mixture was recorded at λ 405 nm for ABTS and at λ=540 nm using microplate reader (Labsystems, original Multiscan Ex, and Champaign, USA). The scavenging activity of fraction or peptide to ABTS and DPPH radicals was expressed using equation:

$$\text{Scavenging activity (\%)} = 100 \times (A0-A1)/A0$$

Where A0 was absorbance of ABTS/DPPH and A1 was the final absorbance of sample minus initial absorbance. The assays were performed in three replications and the results were presented as means.

Anti-inflammation Assay

Anti-inflammation assay was done according to (Mohamed & Saleem 2014). Mice were treated using: (1) LPS *E. coli* (1 mg/mL, 0.4 mL) as positive control, (2) LPS *E. coli* (1 mg/mL, 0.4 mL) and peptide P1 LVNELTEFAK (1 mg/mL, 0.3 mL), (3) LPS *E. coli* (1 mg/mL, 0.4 mL) and peptide LANSLTEFAK (1 mg/mL, 0.3 mL), (4) LPS *E. coli* (1 mg/mL, 0.4 mL) and fraction <3 kDa (0.1 mg protein/mL, 0.3 mL) (5) NaCl physiologis (0.4 mL). LPS was injected to mice and after 30 minutes, treated mice were injected with fraction, peptide or NaCl physiologis. After 2 hours infection, blood samples were collected for IL-1β and TNF-α cytokines measurement using mouse IL-1β ELISA kit and mouse TNF-α ELISA kit (Sigma Aldrich).

Statistical analysis

Experiment data were statistically analysis using Minitab version 18. Significant differences between samples were analyzed using analysis of variance (ANOVA) and continued with Fisher test.

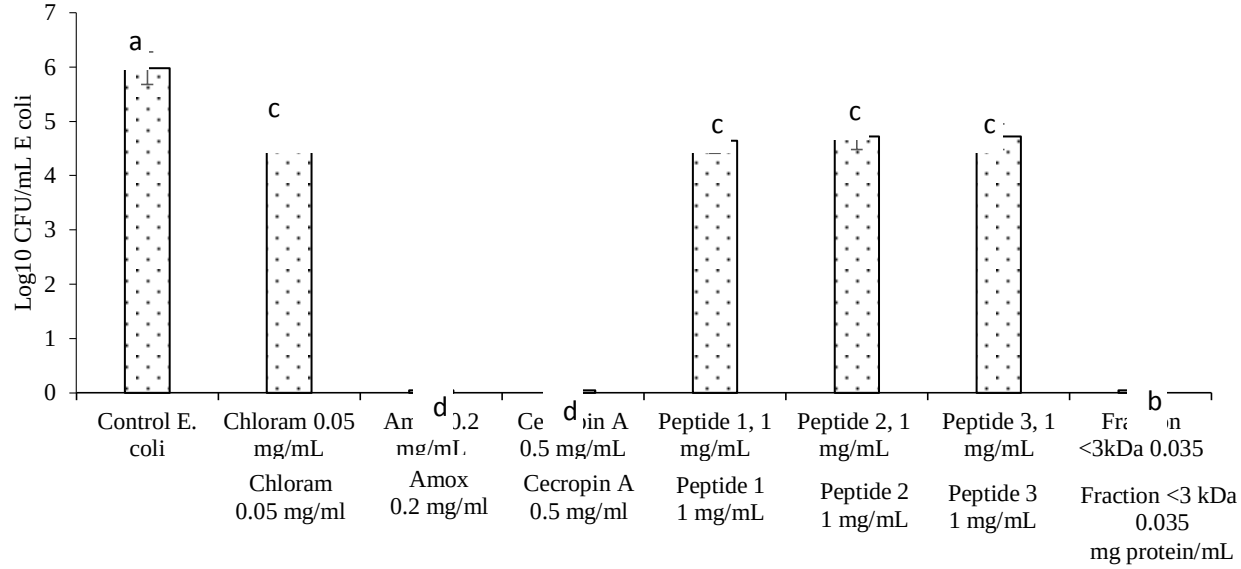

Figure 1. Antibacterial activities of the peptide 1 (LVNELTEFAK), peptide 2 (HPYFAPELLYYANK), peptide 3 (LANSTEFAK) and fraction <3 kDa against *Escherichia coli* ATCC 25922. Chloramphenicol 0.05 mg/mL and Amoxillin 0.2 mg/mL as positive control antibiotic, peptide Cecropin A as a positive control peptide and *E coli* without any treatment as negative control. Different letters indicated a significant different (P<0.05).

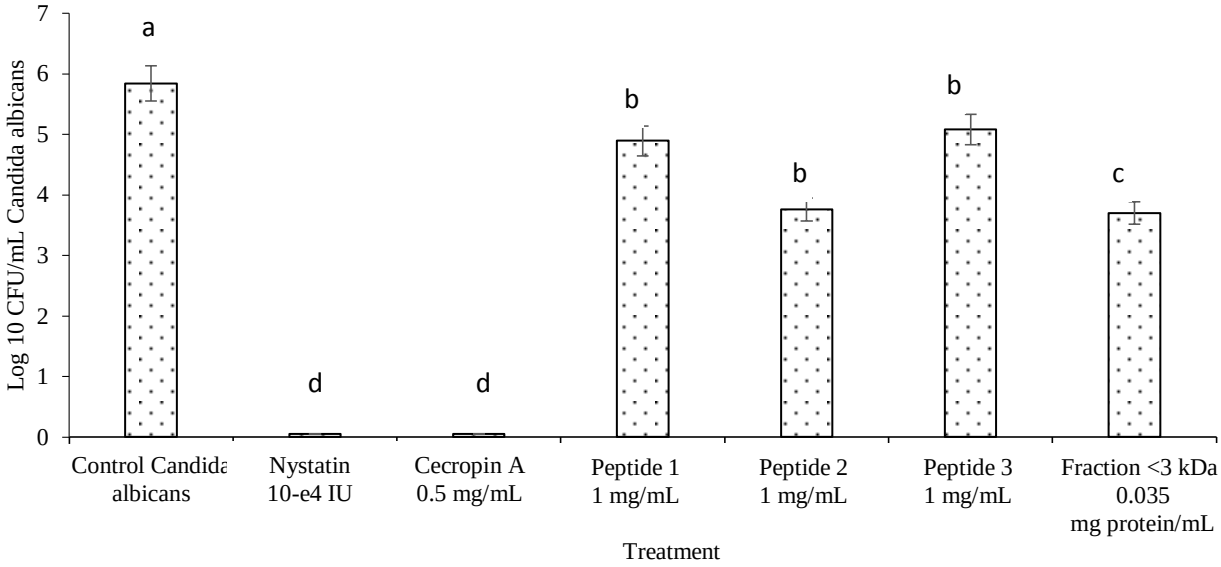

Figure 2. Antifungal activities of the peptide 1 (LVNELTEFAK), peptide 2 (HPYFAPELLYYANK), peptide 3 (LANSTEFAK) and fraction <3 kDa against *Candida albicans* BCC BF059. Nystatin 10,000 IU as positive control of antifungal, peptide Cecropin A as a positive control of peptide and *Candida albicans* without any treatment as negative control. Different letters indicated a significant different (P<0.05).

RESULTS AND DISCUSSION

Antibacterial and Antifungal Activities

Fraction <3 kDa generated from mare milk protein hydrolyzed by *Bacillus thuringiensis* and three of selected peptide derived from these fraction was assayed for antibacterial activities against *Escherichia coli* and antifungal activities against *Candida albicans.* As shown at Figure 1. Fraction <3 kDa was able to inhibit growth of *E coli* up to 6 log cycles similar with Amoxillin 0.2 mg/mL and positive control from commercial peptide, Cecropin A 0.5 mg/mL. The synthetic peptide, peptide 1 (LVNELTEFAK), peptide 2 (HPYFAPELLYYANK), peptide 3 (LANSTEFAK) showed lower antibacterial activities. Similar to result of antibacterial assay, fraction <3 kDa showed higher activity compared to single peptide in antifungal assay. Previously, fractionation of the hydrolysate was able to increase activity. Antibacterial activity of the hydrolysate increased when it fractionated. Minimum bactericidal activity of hydrolysate to *Escherichia coli* and *Salmonella Typhimurium* was >10 mg protein/mL and increased to be 0.035 mg protein/mL in fraction <3 kDa (Kusumaningtyas et al. 2016). Jovanović et al. (2015) was also reported that fractionation of the peptides was able to increase their antimicrobial activity. Fractionation based on the molecular weight of the peptide may concentrate the peptides which have similar activities. However, the antimicrobial activity was decreased when the peptide was purified into single peptide. Almaas et al. (2011) also reported that pure peptide derived from caprine whey showed less antibacterial effect against *Escherichia coli* K12, *Bacillus cereus* RT INF01 and *Listeria monocytogenes* compared to hydrolysates. This suggested that the fraction contains peptides may work synergistically.

Amoxillin was included in beta-lactam antibiotic group which worked by binding irreversibly to transpeptidase enzyme (known as penicillin binding protein= PBPs) (Dowling et al. 2017). The bound caused interference in crosslinking peptidoglycan in bacterial cell wall resulting in weakness and lysis in it. Meanwhile, nystatin bound to ergosterol lipid as a part of membrane of fungal cell induced pore formation in cell membrane (Serhan et al. 2014). This pore caused damage and death of the fungi due to disruption in membrane permeability. Mechanism of action of Cecropin A is by binding to negatively charge of bacterial membrane lipid, therefore form a dense layer leading pore formation in membrane (Silvestro et al. 2000). Complex structure (long peptide sequence) and positively charge of Cecropin A made it able to ion channel into membrane which increased permeability of bacterial or fungal membrane. Consequently, the permeability of bacterial and fungal cell membranes would increase to be very permeable which caused intracellular damage.

Cecropin A works based on the interaction of peptide with negatively charge membrane lipids, therefore electrical charge is a very important factor in the action as antifungal or antibacterial. Positively charge and ability of peptide to form complex with cell membrane might be a key of the effectivity of peptide as an antimicrobial. Positively charge from the peptide will easily bind to negatively charge from bacterial or fungal cell membrane. This is supported by other study which demonstrated that cationic peptides were able to increase cell wall permeability and leading cell membrane damage (Guarna et al. 2006; Manna et al. 2018). Dong et al. (2018) also reported that peptide LK6 or its analogs positively charged +6 or +7 and were able to form alpha helix structures showed high antibacterial activities against *Escherichia coli* and *Staphylococcus aureus*.

Meanwhile, the three peptides tested (peptide 1, 2, 3) have neutral and negative charge therefore their antimicrobial activities were very low. Lee et al (2015) stated that cationic peptides with good antimicrobial activity should be +2 to +7 and consisted of 12 to 100 amino acids. Peptide fraction <3 kDa was a mixture of several peptides with charge -4 to +1, therefore antimicrobial mechanism was not certain. The fraction was effective as antibacterial but less as antifungal. Interaction among peptides in the fraction may contribute to its activity. It may not be only based on positive charge but also other factor such as hydrophobicity, amino acid composition or charge distribution.

Based on the Figure 1, Figure 2 and Table 1, antimicrobial activity of single peptide was determined primary by charge and hydrophobicity. In this research, Cecropin A showed highest antimicrobial activity against *Escherichia coli* and *Candida albicans*, compared to peptide 1, 2, and 3. Cecropin A had therapeutic index prediction only 31.83, lower than peptide 2 and peptide 3, but it showed high charge (+6) and high hydrophobicity +34.74. Yin et al. (2012) reported that balancing of charge distribution and hydrophobicity of the peptide determined antimicrobial activity and peptide toxicity to the mammalian cell. Moreover, antimicrobial activity of the peptide was also determined by amino acid composition in the sequence. Antibacterial peptide commonly was dominated by amino acid L while residue C was dominant in the hydrophobic group of antimicrobial peptides with antifungal activity (Mishra & Wang 2012).

Table 2. Physycochemical analysis and index therapy prediction of assayed peptide

Sequence	Length (aa)	Mass (Da)	Isoelectric Point	Charge	Hidrophobicity (kkal/ml)	Therapeutic index prediction
LVNELTEFAK (Pep 1)	10	1162.62	6.53	-1	+14.89	28.68
HPYFYAPELLYYANK (Pep 2)	15	1887.92	7.47	0	11.74	64.75
LANSLTEFAK (Pep 3)	10	1092.58	6.53	0	+12.68	94.91
KWKLFKKIEKVGQNIRDGIIKA GPAVAVVGQATQIAK (Cecropin A; a commercial peptide)	37	4003.8	10.94	+6	+34.74	31.83

Source: Kusumaningtyas et al. 2016; Cecropin A data sheet

Fraction <3 kDa was composed of 13 peptides which varied in length, charge and hydrophobicity (Kusumaningtyas et al. 2016). High therapeutic index value might increase therapeutic effect and decrease toxicity of the peptide (Tamargo et al. 2015). The peptide with high antimicrobial activity and low toxicity allowed it to penetrate and disrupt bacterial membrane cell without damage host cell membrane. Commonly, peptide was safe for therapy if it had therapeutic index value higher than 10 (Tamargo et al. 2015). Peptide 1 and peptide 2 which were used in this research were selected based on the therapeutic index prediction. The peptides were synthetized and evaluated for their antimicrobial activities against *Escherichia coli* and *Candida albicans*. Peptide 1 was also modified to increase therapeutic index prediction to obtain peptide with higher antimicrobial activity, namely peptide 3. The physicochemical analysis and index therapy prediction of the peptide 1, 2, 3 and Cecropin A are shown in Table 2.

The result also confirmed that high therapeutic index prediction not always represented the antimicrobial activities. Peptide HPYFYAPELLYYANK and LANSLTEFAK which had therapeutic index prediction higher than Cecropin A, had lower activities in laboratory experiment. Therapeutic index in this study was only prediction using statistics calculation based on amino acid sequence, while therapeutic index experimental was determined based on laboratory trials in biological system such as in animal or human cells. Therefore, it was possible that therapeutic index prediction and therapeutic index experimental produced different result.

Antioxidant activity

ABTS (2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) were used to measure scavenging activity of the fraction and peptide 1, 2, and 3. Scavenging activities were determined by reduction of ABTS or DPPH absorbance after addition of peptide fraction or single peptide. Complete scavenging of ABTS or DPPH radical indicated 100 % scavenging activity. Scavenging activity fraction <3 kDa and peptide 1, 2, 3 is shown at Figure 3.

Fraction <3 kDa showed the highest scavenging activity against ABTS and DPPH compare to Peptide 1, 2, 3, although DPPH assay results were not significant different. These indicated that antioxidant activity of peptide in fraction had better activity than in single peptide form. Similar to antimicrobial peptide, it might be due to the peptides in the fraction worked synergistically resulting higher activity. Yousr & Howell (2015) reported that the scavenging activity to DPPH of the selected peptide, YPSPV, 5% antioxidant activity, was lower than their original fraction, EYGF 33 (egg yolk gel filtration fraction 33 which obtained from fraction 33th of egg yolk protein hydrolysate 2 kDa fractionated using sephadex 25) which had scavenging activity of 59.8 %. These data supported that single peptide were not always better than mixed peptides in the fraction.

Anti-inflammation

Inflammation is a component of the innate immune response as a part of host defense against infection and to restore homeostasis in damaged tissues (Manna et al. 2018). Inflammation change to be dangerous if it develops to be excessive and cause organs failure or malfunction. External intervention is needed to maintain the balance of the inflammatory response by organism. LPS is the main activactor of the host defense mechanism (Dong et al. 2018). Aggregated LPS which initially binds with liposaccharide-binding protein (LBP) and the primary LPS receptor CD14; then, LPS-LBP-CD14 complexes are transduced by another membrane protein, Toll-like receptor 4 (TLR4), after which they triggered proinflammatory signaling pathways and induce cytokine

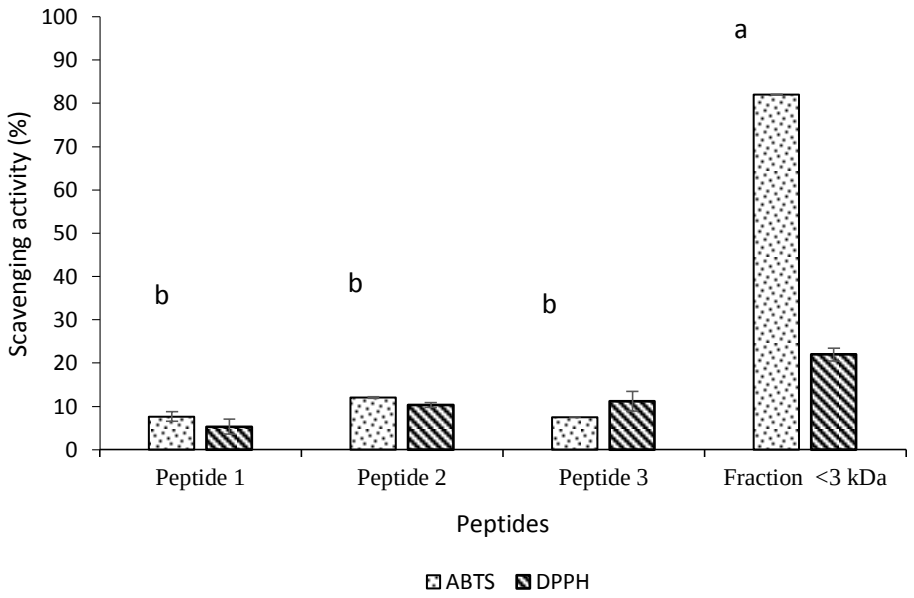

Figure 3. Antioxidant activities of fraction of the peptide 1 (LVNELTEFAK), peptide 2 (HPYFAPELLYYANK), peptide 3 (LANSTEFAK) and fraction <3 kDa to [2, 2'-*azino-bis* (3-*ethylbenzthiazoline*-6-*sulphonic acid*)] (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). Different letters indicated a significant different (P<0.05). Assay using DPPH did not show any significant different (P>0.05)

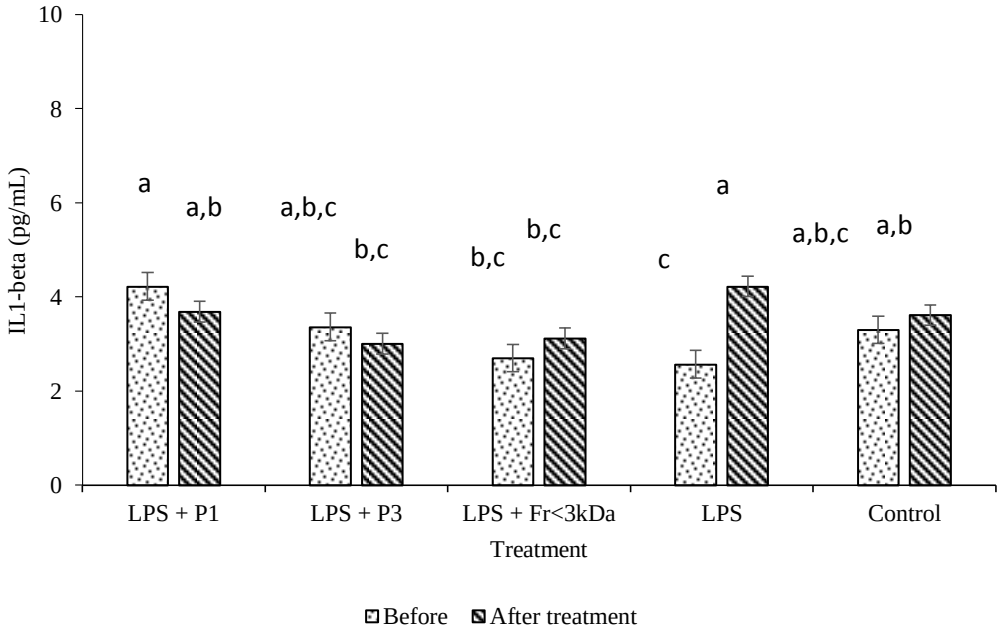

Figure 4. IL 1-β in mice serum before and after treated by lipopolysaccharide *Escherichia coli*, fraction <3 kDa mare milk protein hydrolysate, peptide 1 (LVNELTEFAK) and P3 (LANSLTEFAK). Different letters indicated a significant different (P<0.05).

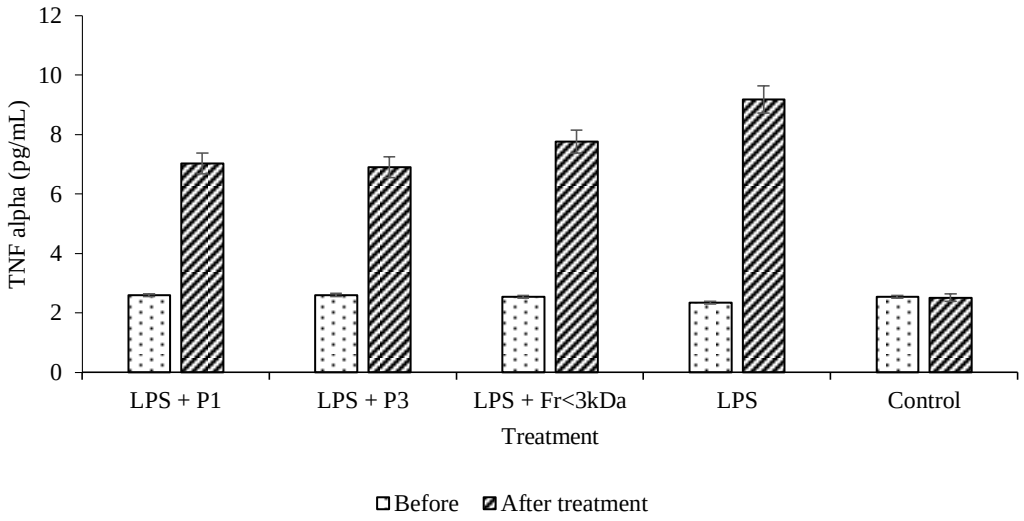

Figure 5. TNF-α in mice serum before and after treated by lipopolysaccharide *Escherichia coli*, fraction <3 kDa mare milk protein hydrolysate, peptide 1 (LVNELTEFAK) and P3 (LANSLTEFAK). TNF alpha, all treatment before or after, did not show any significant different

secretions such as tumor necrosis factor alpha (TNF-α) and interleukins 1β, interleukins 8 and reactive oxygen species (Dong et al. 2018).

Interleukin-1 (IL-1) α/β were proinflammatory cytokines which were considered as key orchestrators of innate immune response (Garlanda et al. 2013). Production of IL-1 was triggered by injury or infection, including microbial ligand and damage associated with molecular pattern (Rock et all 2011). IL-1 induces cytokines, chemokines, growth factors and vascular adhesion molecules IL-1 induces cytokines, chemokines, growth factors and vascular adhesion molecules (Garlanda et al. 2013).

Interleukin-1 β is pro-inflammatory cytokines that exerts pleiotropic on a variety of cells and plays key roles in acute and chronic inflammatory which is implicated in pain, inflammation and autoimmune condition (Ren and Torres 2009). Interleukin-1 (IL-1) is not only a potent regulator of innate immune system important for host defense but is also associated with injury and disease in the brain (Giles et al. 2015). Large production of cytokines proinflammation such as IL-1 beta and TNF-alpha harms to organ and cause mortality.

Antiinflamation assay was conducted by measured proinflammation cytokine concentration IL-1 β and TNF-α. The agent which was able to decrease IL-1β indicated that this agent as anti-inflammatory (Dinarello CA 2011). Lipopolysaccharide (LPS) *E. coli* enhance cytokine IL-1 β production. Treatment with peptide 1(P1: LVNELTEFAK) dan peptida 3 (P3: LANSLTEFAK) indicated decreasing concentration of IL-1 β compared to mice treated LPS. IL-1 β was also increased in mice serum treated with fraction <3 KDa mare milk. TNF-α increase in mice treated with LPS only or peptide P1 and P3, but TNF-α concentration in mice serum treated with peptides P1 and P3 still lower than mice treated with LPS. Slightly decreasing concentration of TNF-α was also observed in mice treated with fraction 3 kDa. Those results showed that peptide P1, P3 or fraction <3 kDa were able to slightly decrease cytokines inflammation.

TNF-α increased in treatment with LPS or peptide, but TNF-α in serum treated with peptide was lower than by LPS alone. In treatment with fraction <3 kDa, TNF-α also decreased although it was still higher than that of single peptide. The result indicated that both fraction or single peptide was able to decrease proinflammation cytokine TNF-α and act as anti-inflammation agent although the results were not significantly different.

According to Sun and Shang (2015), antimicrobial peptide was possible to act as anti-inflammation by neutralization of lipopolysaccharide, killed bacteria and inhibit production of proinflammation cytokines. Decreasing of proinflammation cytokines was also decreasing of inflammation response. Fotschki et al. (2016) reported that mare milk contained immune-modulating properties decreased Ig E and decreased expression of IL-4 had proinflammatory properties in mice sensitized with allergen. It is possible that bioactive peptides in mare milk are able to act as antimicroial as well as anti-inflammation agents. Cationic peptide was

reported to be able to act as anti-inflammation by neutralizing and bounding with LPS (Guarna et al. 2006; Lee E, Shin A 2015; Dong et al. 2018). But in this research, decreasing some inflammatory mediators such as TNFα and IL 1β was able to be triggered by short, negative and positive charge. This indicated that mechanism of bioactive peptide as anti-inflammation did not related to its positive charge. The mechanism has yet not clear.

CONCLUSION

Peptide fraction and purified peptide derived from mare milk protein hydrolyzed using *Bacillus thuringiensis* protease had antibacterial, antifungal, antioxidant and anti-inflammation activities. Generally, fraction <3 kDa had higher antibacterial, antifungal and antioxidant activities compared to single peptide. The result indicated that purification of the peptide in the fraction <3 kDa was not necessary. For anti-inflammation, peptide 1 and peptide 3 were able to reduce IL1-β better than fraction. Therapeutic index based on amino acid sequence should be confirmed by laboratory experiment to determine real antimicrobial activity of the peptide. It is suggested that fraction <3 kDa is a promising agent for future application as antimicrobial and antioxidant

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Characterisation of M2e Antigenicity using anti-M2 Monoclonal Antibody and anti-M2e Polyclonal Antibodies

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ABSTRAK

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Protein Matrik 2 ektodomain (M2e) memiliki sifat lestari dan dianggap sebagai antigen potensial untuk mendeteksi infeksi virus influenza A pada unggas yang divaksinasi (DIVA test). Namun studi yang mempelajari antigenisitas M2 dan respon imun pada manusia atau hewan masih sangat terbatas. Pada studi ini sifat antigenik dari masing-masing tujuh belas M2e peptida dan enam belas protein rekombinan M2e (rM2e) yang memiliki variasi asam amino (aa) pada posisi 10, 11, 12, 13, 14, 16, 18 dan 20 dibandingkan dengan metode western blot (WB) dan enzyme-linked immunosorbent assay (ELISA) menggunakan antibodi monoklonal (mAb) 14C2 dari tikus, dan anti-M2e poliklonal antibody (pAb) yang berasal dari ayam dan kelinci. MAb 14C2 memiliki kekuatan pembeda terbaik dan aa posisi ke-11 merupakan imunodominan paling penting yang mempengaruhi ikatan mAb14C2 hingga tingkat yang terbesar. Perubahan pada posisi 14, 16 dan 18 juga mempengaruhi pengikatan mAb14C2, dan perubahan ini terdeteksi pada semua metode (WB atau ELISA) dan antigen yang digunakan (M2e peptida atau protein rM2e). Untuk anti-M2e pAb dari ayam dan kelinci, aa imunodominan ditemukan pada posisi 10 dan perubahan pada posisi 11 tidak mempengaruhi reaksi antibodi. Pengikatan pAb kelinci juga dipengaruhi oleh perubahan pada aa posisi 14 dan 16, hal ini mengkonfirmasi kontribusi posisi tersebut terhadap antigenisitas M2e. Posisi 10 adalah satu-satunya posisi yang penting untuk pengikatan pAb ayam terhadap M2e. Secara keseluruhan penelitian ini menunjukkan antigenik M2e terletak di antara residu 10 - 18 dan perubahan aa pada posisi 10, 11, 12, 14, 16 dan 18, dapat mempengaruhi ikatan antibodi di dalam protein M2e.

Kata kunci: Virus Influenza A, epitop M2e, antigenisitas

ABSTRACT

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Ectodomain matrix 2 protein (M2e) is a potential antigen for detection of influenza-A-virus infection among vaccinated birds (DIVA test). However, the antigenicity and immune response induced by M2e in either humans or animals are poorly understood. Seventeen M2e peptides and sixteen recombinant M2e (rM2e) proteins with amino acid (aa) changes introduced at position 10, 11, 12, 13 14, 16, 18 and 20 were compared by western blot (WB) and enzyme-linked immunosorbent assay (ELISA) using mouse anti-M2 monoclonal antibody (mAb) 14C2, and chicken- or rabbit-polyclonal antibodies (pAb). The mAb 14C2 had the best discriminating power and aa position 11 was the important immunodominant for mAb14C2, that affected binding to a greatest degree. Changes in the adjacent position 14, 16 and 18 also influenced the binding, and it detected regardless of the method (WB or ELISA), or the antigen used (M2e peptide or rM2e). For chicken pAb and rabbit pAb, the immunodominant aa was position 10 and the antibody reaction was not affected by aa change at 11. The binding of rabbit pAb was also affected by changes at 14 and 16, which confirm the contribution of these positions to the M2e antigenicity. Position 10 was the only important position for the binding of chicken pAb to M2e. Overall, the study showed that the M2e antigenic sites are located between residues 10 – 18 and that aa changes at position 10, 11, 12, 14, 16 and 18 may all affect the antibody binding within the M2e protein.

Key Words: Influenza A Virus, M2e epitope, antigenicity

INTRODUCTION

Highly pathogenic avian influenza subtype H5N1, has continued to be a significant concern for more than a decade globally, and especially in several South East Asia countries where the virus has become endemic in commercial poultry. As fatalities in humans, which have been directly linked to contacts with infected poultry continue to occur, measure that would reduce virus load in the environment, such as vaccination, are

now practiced in several countries. In some countries additional measures are considered such as using the differentiation of infected from vaccinated animals (DIVA) test for use in surveillance of vaccinated flocks in order to estimate the extent of H5N1 challenge in vaccinated flocks.

Ectodomain matrix 2 (M2e) protein is a 24 amino acid long protein located in the Matrix 2 (M2) protein N terminal. It is abundantly express on the cell surface infected by Influenza A virus (IAV), but very few are found in the virion of AIV (Lamb et al. 1985). Previous study, using mouse mAb 14C2 reported that M2e was the most important part for antigenicity of M2 protein as mAb 14C2 could not recognise the M2 lacking the ectodomain (M2e) protein (Zebedee & Lamb 1988). M2e protein is considered to be a potential antigen for DIVA test and high specificity of enzyme-linked immunosorbent assay (ELISA) using synthetic M2e-peptide or recombinant M2e protein has been reported by several studies (Lambrecht et al. 2007; Hemmatzadeh et al. 2013; Tarigan et al. 2015). Although the M2e based DIVA test has been evaluated for possible use in commercial poultry (Lambrecht et al. 2007; Hemmatzadeh et al. 2013; Kim et al. 2010, Tarigan et al. 2015), very little is known about the antigenicity of the M2e and antibody response it induces in infected poultry. Limited studies, however, have been carried on M2e antigenicity and immunogenicity and these studies were mostly focused on the M2e responses in humans, mice, pigs or ferrets (Schotsaert et al. 2009). Those studies concluded that M2e was a poor immunogen and induced antibodies only in a fraction of infected individuals, and antibody titres were low and of short duration (Feng et al. 2006); (Kitikoon et al. 2008); (Bianchi et al. 2001). In addition, antigenic variations in the M2e protein have only been studied using monoclonal antibodies (Zharikova et al. 2005; Zebedee & Lamb 1988).

In chickens, only limited studies have dealt with the immune responses to the M2e protein. An M2e-peptide based ELISA was used as a DIVA test to identify chickens and ducks challenged with HPAI viruses H5N1 and H7N7 (Hemmatzadeh et al. 2013: Lambrecht et al. 2007; Marché et al. 2010). Twenty-four and eighteen amino acid long M2e peptides were recognised in ELISA by immune chicken sera indicating that on the M2e peptide at least some, if not all, antigenic domains are correctly presented. Although antibodies to M2e were detectable, they were not consistently detected and were absent in chicks infected with LPAI isolated from water birds. In another study it was also shown that the M2e-peptide based ELISA was able to detect infection with H9N2 strain in vaccinated commercial poultry (Kim et al. 2010). In these studies the synthetic M2e peptide and recombinant M2e (rM2e) coupled to maltose bonding protein (MBP) were used to assess the suitability of M2e as a DIVA reagent (Lambrecht et al. 2007; Hemmatzadeh et al. 2013), and in another study also as a vaccine candidate (Mozdzanowska et al. 2003). Overall, these studies have indicated that synthetic M2e peptide is suitable for DIVA, and rM2e may both be useful for antigenic mapping of the M2e.

To date, limited report is available regarding M2e antigenicity in chickens or recognition of M2e by sera from various AIV infections (Lambrecht et al. 2007; Marché et al. 2010; Kim et al. 2010). For successful implementation of a DIVA test, it is critical to understand if M2e can be used as a universal detecting agent. Therefore, this study aimed to characterise the antigenic epitopes on M2e and identify critical mutations that influence binding of antisera to the M2e antigen. For this purpose, M2e-MBP recombinant proteins were generated as well as synthetic peptides carrying different mutations in the M2e protein, and used to analyse the M2e antigenic determinants. Two different immunological assays, WB and ELISA, were employed to achieve a comprehensive evaluation of M2e antigenicity using polyclonal antibodies (pAb) generated in chicken and rabbit against the M2eC0 peptide. Monoclonal antibody (mAb) 14C2 was also purchased and used for antigenic characterisation and for comparison with the anti-M2e peptide pAb.

MATERIALS AND METHODS

Production of recombinant M2e proteins (rM2e)

The synthetic M2e gene (M2eC0) was used to generate fifteen M2e mutant genes (M2eC1 to M2eC15) by introducing various mutations at specific sites (Sumarningsih, 2011). These fifteen M2e genes were cloned into pMAL-P4x expression vector and transformed into *E. coli* BL21 DE3 cell (BioLine Pty Ltd, Alexandria, NSW) to express recombinant M2e-MBP proteins, referred to as rM2e (C1 to C15). After cold osmotic shocked, the expressed proteins were purified with amylose resin beads (New England Biolabs, UK), and analysed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) for the size and purity.

Synthetic M2e peptide

All M2e peptides (C1 to C18) were synthetized by GenScript (Piscataway, New Jersey, USA) corresponding to the sequences of M2e mutants (Table 1) with purity varied from 79% to 94%. The M2eC15 peptide was not synthesized because it had homolog sequence to that of M2eC14. The M2eC17 peptide was also not included in this study because the synthesis was unsuccessful even after three different attempts by

GenScript. In initial study, M2eC0 peptide was purchased from Peptide 2.0 (Chantilly, VA, USA). All these seventeen peptides (C0 to C18) were dissolved in sterile ddH_2O (1mg/ml) and diluted in 1:1 with sterile glycerol and stored at -20°C.

Anti-M2 monoclonal antibody 14C2 and anti-M2e polyclonal antibodies

Mouse monoclonal antibody (mAb) 14C2 was purchased from Abcam, (Sapphire BioScience Pty Ltd, NSW) as ascetic fluid. The mAb 14C2 was generated against the M2 protein of Influenza A Virus A/WSN/33 following live inoculation. In this study, mAb 14C2 was optimised by ELISA titration using M2eC3, M2eC4 and M2eC11 peptides to determine the optimal dilution for mAb 14C2 in WB and ELISA.

Anti-M2e polyclonal antibodies (pAb) were produced in chicken and rabbit immunized using 1 mg of M2eC0 peptide in complete Freund's adjuvant, followed by 2 mg, 4 mg and 8 mg of M2eC0 peptide with incomplete Freund's adjuvant (as a second, third and fourth immunisation, in 3 weeks intervals). Chicken and rabbit were bled prior to each immunisation and the pAb were tested in M2eC0 peptide ELISA to determine the titres. The sera were harvested after the third immunisation when the optimum titres of pAb were found.

Western blot (WB)

All sixteen rM2e proteins (C0 to C15) were subjected by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was washed two times using PBS-T (0.5% Tween in PBS) and incubated in blocking buffer (5% skim milk in PBS) for 1.5 hours. All incubations for WB were performed at room temperature. After washing two times, the membrane was incubated with diluted primary antibody for 1 hour, washed five times, and incubated with diluted HRP-conjugated antibody for 1 hour. Different antibody dilution was used for each WB. First WB used dilution at 1/5000 for mAb14C2 and 1/2000 for HRP-rabbit anti mouse IgG. Second WB used dilution at 1/2000 for rabbit pAb and 1/2000 for HRP-goat anti rabbit IgG. Third WB used dilution at 1/1000 for chicken pAb and 1/4000 for HRP-rabbit anti chicken IgG. The antigen-antibody interaction was visualized using Amersham enhanced chemiluminescent (ECL) western blotting detection reagents (GE Healthcare Australia Pty Ltd, Rydalmere, NSW). The membrane was exposed to blue rite autorad film (Astral Pty Ltd, Gymea, NSW) and scanned to measure the band mass by Kodak molecular imaging software.

Value of 100% was given for the band intensity of homologous M2e sequence between antibody and antigen use in each WB. The band intensity for antibody binding to other M2e proteins was then compared to the homologous M2e reaction and expressed as a percentage of binding. Decrease in band intensity of a minimum of 25 to 30% was indicative the reduction in antibody binding.

Enzyme-linked immunosorbent assay (ELISA)

All the procedures for ELISA were performed at room temperature. Both ELISA used antigen concentration at 0.6ng/well for rM2e proteins and M2e peptides, which dissolved in carbonate-bicarbonate buffer (pH 9.6) and coated into each well of microtitre plate (NUNC Maxisorb). After coating for overnight, the plate was washed once with PBS-Tween 0.05% (PBS-T). Then 150µl/well of blocking buffer (5% Newborn Calf Serum in PBS-T) was added and incubated for 2 hours. After washing two times, 100µl/well of diluted antibody was added and the plate was incubated for 1 hour. Similar dilution at 1/2000 was used for all antibodies (mAb14C2, chicken pAb and rabbit pAb) in rM2e ELISA. But the dilution used in M2e-Peptide ELISA was different, which was 1/4000 for mAb14C2 and rabbit pAb; and 1/2000 for chicken pAb. After incubated with antibody, the microplate was washed five times, followed by incubation with 100µl/well of diluted HRP-antibody for 1 hour. The dilution at 1/2000 was used for HRP-rabbit anti mouse IgG and HRP-goat anti rabbit IgG, and at 1/4000 dilution for HRP-rabbit anti chicken IgG. After washing five times, 100µl/well of 3,3' ,5,5'-tetramethylbenzidine (TMB) susbtrate (Sigma Aldrich Pty Ltd, Castle Hill, NSW) was added and incubated for 15 minutes. The reaction was stopped by adding 25µl/well of 2M H2SO4. The binding of antibody to antigen was determined based on the optical density (OD) using microplate reader at a wavelength of 450nm (OD450).

Table 1. Amino acid sequences for the parent and mutant genes of M2e

M2e	Parent [a]	Amino acid sequence
	Mutant [b] , Peptide [c]	
C1	C0 [a]	MSLLTEVETP TRNEWECKCS DSSD
	C1 [b,c]	....G..... ....
C2	C1 [a]	MSLLTEVETP TRNGWECKCS DSSD
	C2 [b,c]	.....L ....
C3	C1 [a]	MSLLTEVETP TRNGWECKCS DSSD
	C3 [b,c]	..... I..... ....
C4	C3 [a]	MSLLTEVETP IRNGWECKCS DSSD
	C4 [b,c]	....K..... ....
C5	C0 [a]	MSLLTEVETP TRNEWECKCS DSSD
	C5 [b,c]	....R.. ....
C6	C5 [a]	MSLLTEVETP TRNEWECRCS DSSD
	C6 [b,c]	....G.G.... ....
C7	C6 [a]	MSLLTEVETP TRNGWGCRCS DSSD
	C7 [b,c]	.....L ....
C8	C6 [a]	MSLLTEVETP TRNGWGCRCS DSSD
	C8 [b,c]	.....H ....
C9	C7 [a]	MSLLTEVETL TRNGWGCRCS DSSD
	C9 [b,c]	..... .K..... ....
C10	C5 [a]	MSLLTEVETP TRNEWECRCS DSSD
	C10 [b,c]	..... I..... ....
C11	C10 [a]	MSLLTEVETP IRNEWECRCS DSSD
	C11 [b,c]	....G...N ....
C12	C1 [a]	MSLLTEVETP TRNGWECKCS DSSD
	C12 [b,c]	....S..... ....
C13	C3 [a]	MSLLTEVETP IRNGWECKCS DSSD
	C13 [b,c]	....R.. ....
C14*	C5 [a]	MSLLTEVETP TRNEWECRCS DSSD
	C14 [b,c]	....G..... ....
C15*	C2 [a]	MSLLTEVETL TRNGWECKCS DSSD
	C15 [b]	.....P .....R.. ....
C16	C16 [c]	MSLLTEVETP TRNEWECKCI DSSD
C18	C18 [c]	MSLLTEVETS TRNEWECRCS DSSD

[a] The M2e parent gene used as a template in mutagenesis
[b] The M2e mutant gene, product of mutagenesis for rM2e protein expression
[c] The M2e sequence for synthetic peptide use in this study
*The sequence of C14 and C15 mutant genes were similar but generated from different parent genes

RESULTS AND DISCUSSION

The M2e based DIVA test has been considered as the test of choice for use in surveillance of vaccinated flocks in Indonesia in order to estimate the extent of H5N1 challenge in vaccinated flocks and to establish zones that are free from H5N1 (Tarigan et al. 2015; Wibowo et al. 2017). Although the M2e based DIVA test has been evaluated for possible use in commercial poultry, very little is known about the antigenicity of M2e and its specificity to detect infections caused by avian influenza virus (AIV).

The M2e protein has been considered as a highly conserved protein among all influenza A virus (IAV) strains and subtypes. For this reason M2e has been studied as a possible candidate for a universal IAV vaccine (Schotsaert et al. 2009) and also as a potential DIVA diagnostic antigen to detect exposure to different AIV subtypes (Kim et al. 2010; Lambrecht et al. 2007). However, in several studies that have focused on M2e from human IV strains, evolution and mutation at some amino acid positions in the M2e have been reported (Furuse et al. 2009; Ito et al. 1991). Also, data obtained following the emergence and spread of H5N1 and additional surveillance in wild birds have further indicated that the M2e protein may also be under the similar selection pressure as are the HA and the NA proteins (Lam et al. 2008). The M2e amino acid variability has also been reported in other studies to occur in the middle part of M2e that potentially could have an affect on its antigenicity (Liu & Chen 2005; Wang et al. 2009). Ito et al. (1991) reported high M2 variation between positions 10 to 28, whereas the first nine amino acids (1MSLLTEVET10) were highly conserved. In this study, the amino acid variation in the M2e protein of different strains of H5N1 and of other frequently isolated AIV subtypes were determined. The most common mutations were identified for generation of recombinant M2e protein to be used for antigenic mapping of the M2e.

The antigenicity of a protein is strongly associated with the hydrophobicity, 17 M2e amino acid sequence were designed (Table 1) based on the hydrophobicity differences, so that each sequence had between one and five selected mutations in comparison to the rM2eC5 sequence (A/Ck/Indo/BL/03 H5N1 strain). These rM2e proteins had different hydrophobicity values, which indicating the potential of each amino acid position contribute to the M2e antigenicity (result not shown). Peptides analogous to these sequences were synthetized and labeled as M2eC0, M2eC1, etc. Antigenic mapping of M2e was performed by testing the binding of polyclonal and monoclonal antibody to each rM2e proteins in WB and ELISA, and to M2e-peptides in ELISA.

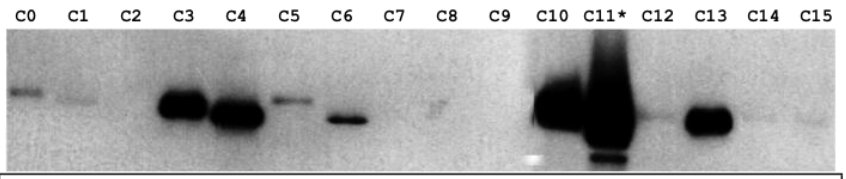
Antigenic mapping using anti-M2 monoclonal antibody 14C2 (mAb 14C2)

Monoclonal antibody 14C2 was used in this study to compare the M2e antigenicity because it was previously shown to specifically recognise amino acid isoleucine at the position 11 (Zebedee & Lamb 1988). The mAb 14C2 was generated against the M2 protein of human influenza virus A/WSN/33/H1N1 strain, and WB result (Figure 1a) showed that mAb 14C2 reacted strongly with rM2eC11 (homologous reaction), which has similar sequence to M2e of A/WSN/33-H1N1 virus. Introduction of two mutation G16E and N20S into rM2eC10 was used to generated rM2eC11, and the WB of rM2eC10 showed that amino acid different in these positions caused a reduction in mAb 14C2 binding to 60%. MAb14C2 also reacted with rM2eC3, rM2eC4 and rM2eC13, that all having isoleucine at the position 11 (11I), but this reaction was lesser in comparison to the homologous reaction, indicating that changes at other positions (E14G, G16E and R18K) could reduce the binding of mAb14C2. The binding to rM2eC4 and rM2eC13 with additional change (E14G) was further reduced to 40% and 35%, respectively. The reduction to rM2eC3, which similar to rM2eC13 (35%), showed that amino acid change at R18K did not affected the binding of mAb 14C2. Additional mutation (I11T) in rM2eC0, rM2eC1, rM2eC14 and rM2eC15 completely abrogated the mAb 14C2 binding to these M2e proteins.

In WB of rM2eC5, to which mAb 14C2 did not bind, when T11I was mutated back generating the rM2eC10, the binding of mAb 14C2 was restored, but only to 60% of binding, its indicating clearly different contribution of positions 11, 16, and 20 to mAb 14C2 binding. Although four constructs rM2eC2, rM2eC7, rM2eC8, and rM2eC9 had additional P10L change, the contribution for this position to M2e antigenicity was not possible to assess because there was no rM2e proteins containing aa differed from rM2eC11 at position 10 only.

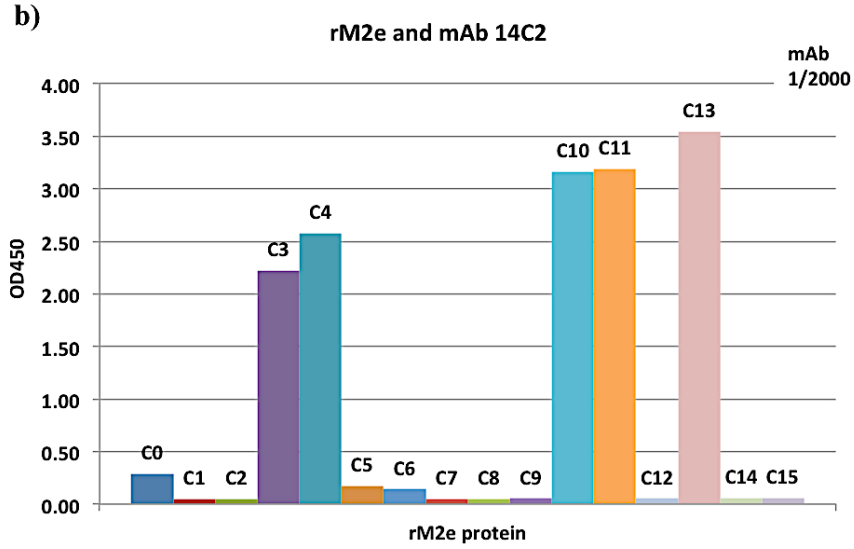
ELISA of mAb 14C2 using rM2e proteins showed similar result as WB (Figure 1.b.). The binding was only found with rM2eC3, rM2eC4, rM2eC10, rM2eC11 and rM2eC13, which contain isoleucine at the position 11 (11I). However, the binding to rM2eC3 and rM2eC4 was reduced in comparison to rM2eC10, rM2eC11 and rM2eC13, indicating that change at position 18 (R18K) could have affected the antibody binding. MAb 14C2 also did not react in ELISA with rM2eC0, rM2eC1, rM2eC5, rM2eC6, rM2eC7, rM2eC8, rM2eC9, rM2eC12, rM2eC14 and rM2eC15, and all these rM2e had amino acid change at position 11 (I11T).

a)



			C0	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11*	C12	C13	C14	C15
% binding			2	1	0	35	40	3	7	0	0	0	60	100	3	34	0	0
Amino acid position	10	P	.	.	L	.	.	.	.	L	H	L	.	.	.	.	.	.
	11	I	T	T	T	.	.	T	T	T	T	T	.	.	T	.	T	T
	12	R	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.
	13	N	.	.	.	.	K	.	.	.	.	.	.	.	S	.	.	.
	14	E	.	G	G	G	G	.	G	G	G	G	.	.	G	G	G	G
	15	W	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	16	G	E	E	E	E	E	E	.	.	.	.	E	.	E	E	E	E
	17	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	18	R	K	K	K	K	K	.	.	.	.	.	.	.	K	.	.	.
	19	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	20	N	S	S	S	S	S	S	S	S	S	S	S	.	S	S	S	S

b)



c)

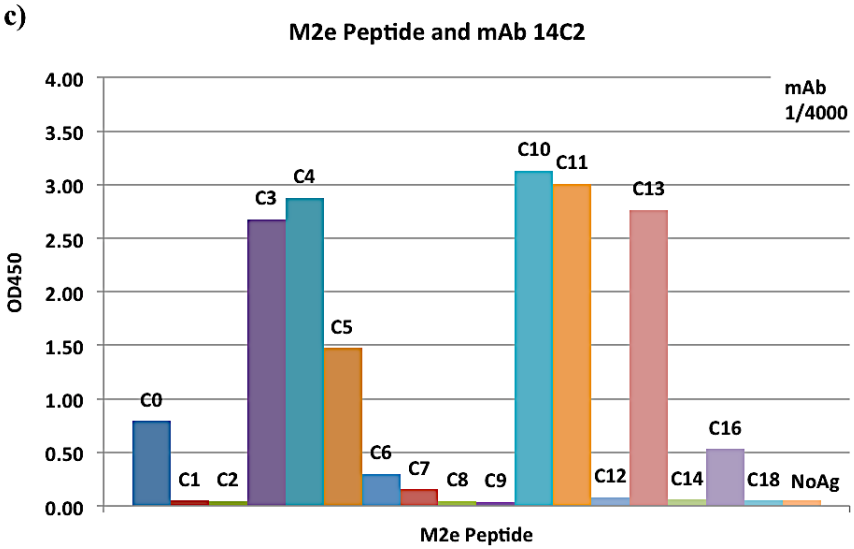


Figure 1. Antibody binding analysis of mAb 14C2 to rM2e proteins in WB (a); mAb 14C2 to rM2e proteins in ELISA (b); and mAb 14C2 to M2e peptides in ELISA (c). Amino acid variations between rM2e proteins to rM2eC11 (*) were shown in the table.

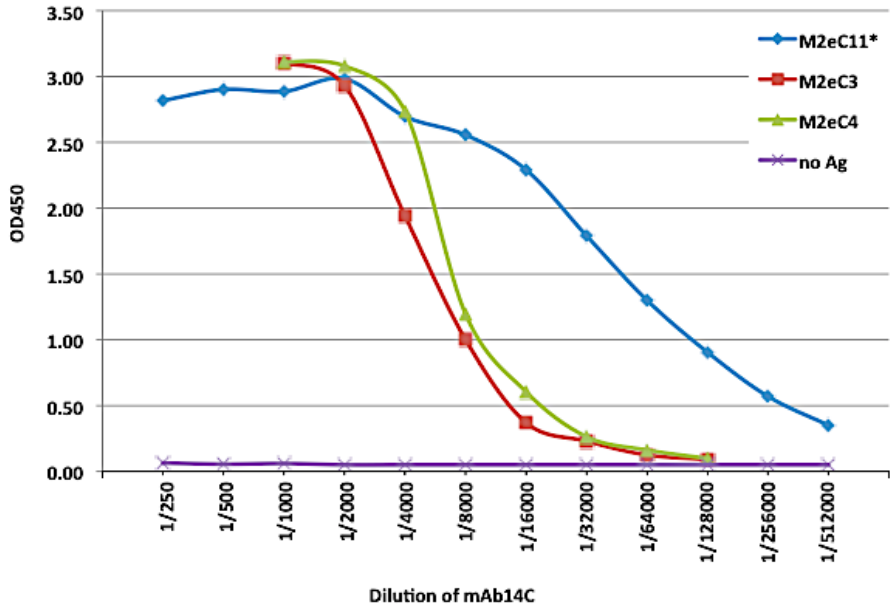


Figure 2. Analysis of mAb 14C2 titre in ELISA using three different M2e peptides (M2eC3, M2eC4 and M2eC11). M2eC11 peptide has the homologous sequence to human influenza A virus A/WSN/33/H1N1 strain used to generate mAb 14C2.

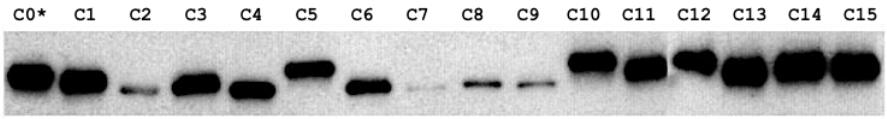
Similar finding was obtained in ELISA with M2e-peptides (Figure 1.c), mAb 14C2 strongly reacted with all five M2e peptides (M2eC3, M2eC4, M2eC10, M2eC11 and M2eC13) that had isoleucine at the position 11, and there was no influence of other amino acid changes to this binding. Positive but low reactions of mAb 14C2 binding was also found with M2eC0 and M2eC5 peptides which had threonine at the position 11 (I11T) and had only one common amino acid change in comparisons to the homologous sequence (rM2eC11) at position 20 (N20S). In this study, mAb 14C2 was titrated on the M2eC11, M2eC3 and M2eC4 peptides (Figure 2) because the binding of mAb 14C2 to M2eC3 and M2eC4 peptide in ELISA differed from the previous result of WB. As shown, there was a difference in binding between these three M2e peptides at the lower concentration of mAb 14C2, indicating that M2eC3 (E14G, G16E, K18R, S20N) and M2eC4 (N13K, E14G, G16E, K18R, S20N) differed antigenically from M2eC11.

Antigenic mapping using anti-M2e rabbit polyclonal antibody

To investigate if there was any different M2e antibody response between species of animal immunized, rabbit polyclonal antibody to the M2eC0 peptide were used in this study to characterise the M2e antigenicity. Strong reactions of rabbit M2e antiserum were detected in WB (Figure 3.a) with rM2eC0 and rM2eC1 (E14G), rM2eC13 (T11I, E14G, K18R), rM2eC14 (E14G, K18R) and rM2eC15 (E14G, K18R) indicating that changes at the position T11I, E14G, and K18R did not influence its binding. The results also showed a complete absence of reaction of rabbit pAb with M2eC2, M2eC7, M2eC8 and M2eC9 that had either P10L or P10H amino acid change. These two changes at position 10 (P10L or P10H) caused the lack of binding equally. It was of interest that amino acid change at position 11 in rM2eC3, rM2eC4, rM2eC10, rM2eC11 and rM2eC13 did not influence the binding of anti-M2e rabbit pAb, nor did the amino acid changes at the position E14G, G16E and K18R and S20N.

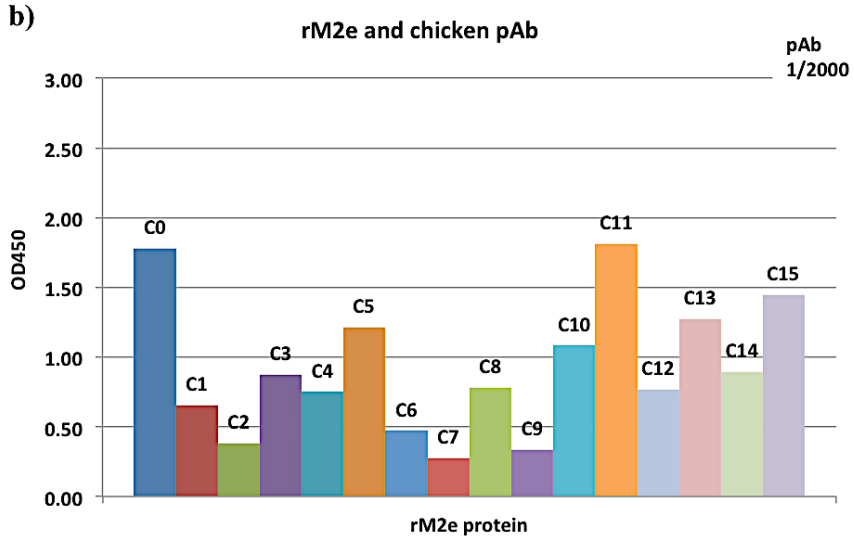
ELISA results using rM2e proteins (Figure 3.b) showed less discriminating value compare to WB. It showed that the highest reaction of rabbit pAb was with the homologous rM2eC0 protein, the binding was reduced to M2eC2, M2eC7 and C9 with changes at position P10L. Result of ELISA M2e-peptide was in agreement with the WB and ELISA rM2e for most peptides (Figure 3.c). Rabbit pAb reacted less with M2eC2, M2eC7, M2eC8, M2eC9 and M2eC18, these all peptides has amino acid different to M2eC0 at position 10 (P10L, P10H and P10S). The differences of antibody binding to M2e were visible only at high dilution (1/4000 concentration) of rabbit pAb indicating the influence of rabbit pAb concentration for differentiation capacity.

a)



% binding			100	90	13	75	67	64	47	0	18	13	79	80	71	107	120	104
Amino acid position	10	P	.	.	L	.	.	.	.	L	H	L	.	.	.	.	.	.
	11	T	.	.	.	I	I	.	.	.	.	.	I	I	.	I	.	.
	12	R	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.
	13	N	.	.	.	.	K	.	.	.	.	.	.	.	S	.	.	.
	14	E	.	G	G	G	G	.	G	G	G	G	.	.	G	G	G	G
	15	W	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	16	E	.	.	.	.	.	.	G	G	G	G	.	G	.	.	.	.
	17	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	18	K	.	.	.	.	.	R	R	R	R	R	R	R	.	R	R	R
	19	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	20	S	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.

b)



c)

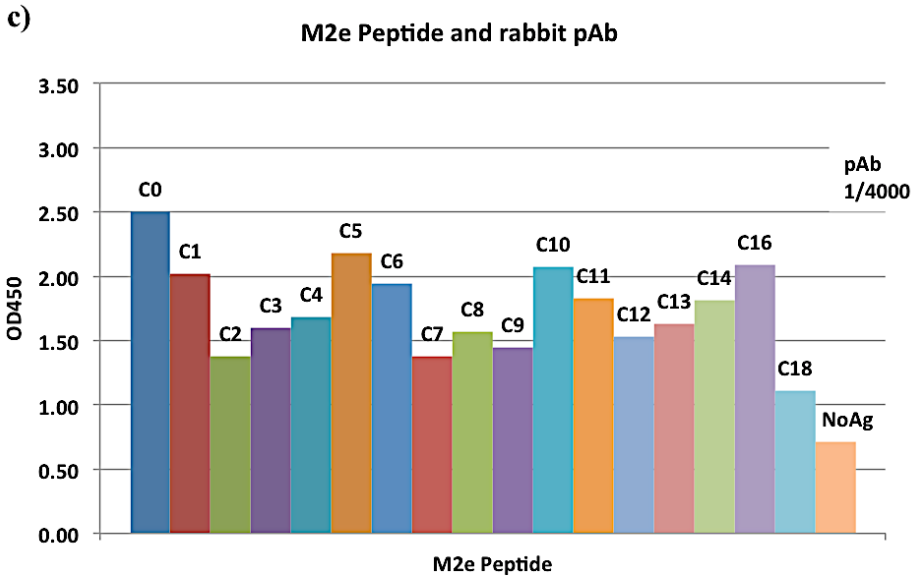


Figure 3. Antibody binding analysis of anti-M2e rabbit pAb to rM2e proteins in WB (a); anti-M2e rabbit pAb to rM2e proteins in ELISA (b); and anti-M2e rabbit pAb to M2e peptides in ELISA (c). Amino acid variations between rM2e proteins to rM2eC0 (*) were shown in the table.

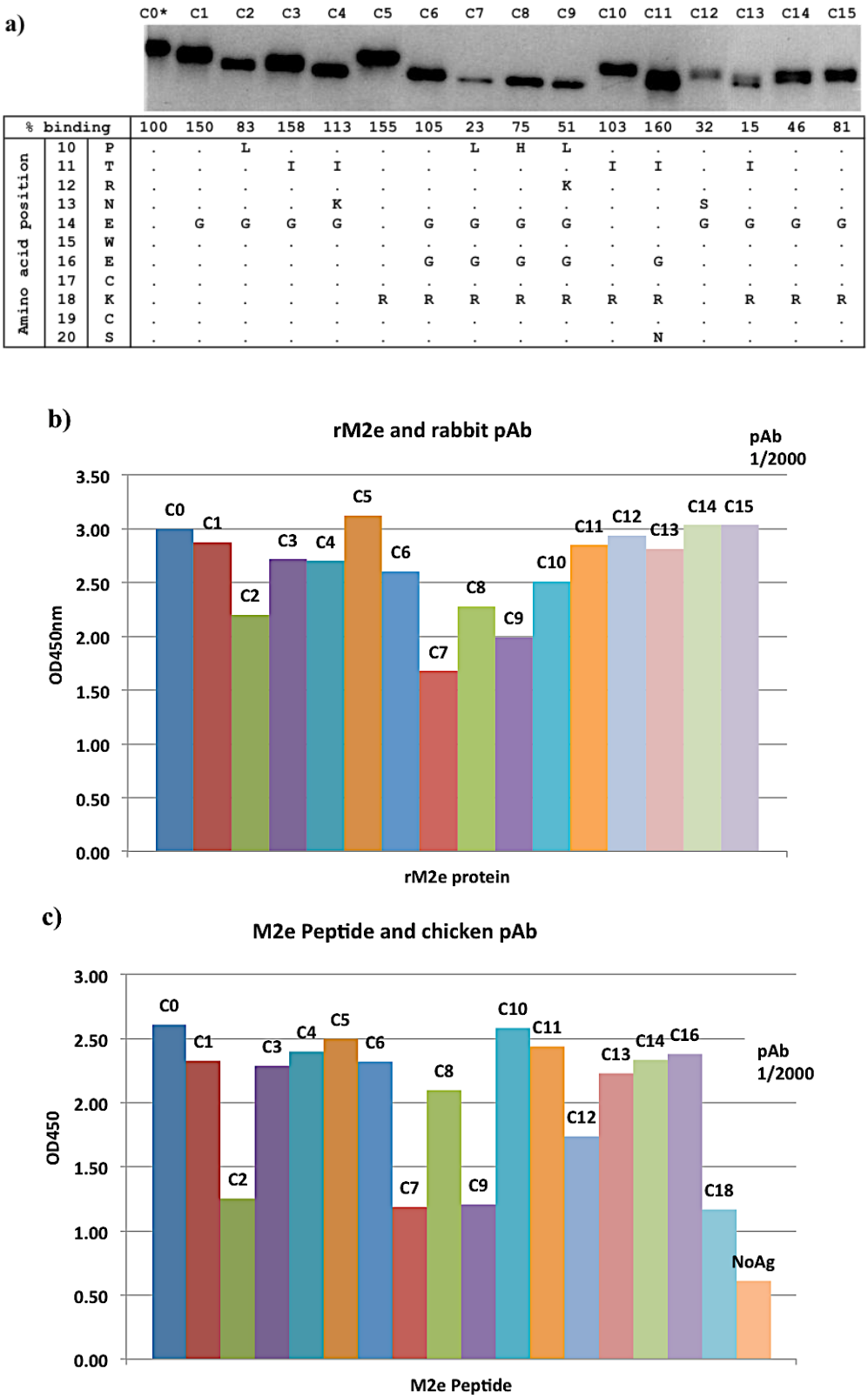


Figure 4. Antibody binding analysis of anti-M2e chicken pAb to rM2e proteins in WB (a); anti-M2e chicken pAb to rM2e proteins in ELISA (b): and anti-M2e chicken pAb to M2e peptides in ELISA (c). Amino acid variations between rM2e proteins to rM2eC0 (*) were shown in the table.

Antigenic mapping using anti-M2e chicken polyclonal antibody

Anti-M2e chicken polyclonal antibody was also used in this study to analyse the M2e antigenicity. WB result showed chicken pAb (Figure 4.a) was strongly reacted with the homologous rM2eC0 and other rM2e proteins, including rM2eC1 (E14G), rM2eC3 (T11I and N13S), rM2eC5 (K18R) rM2eC10 (T11I and K18R) and rM2eC11 (T11I, E16G, E14G, K18R and S20N). This finding suggested that changes at the position T11I, E14G, E16G, K18R and S20N did not influence the binding of chicken pAb. In contrast, chicken pAb was not reacted or weakly reacted with rM2eC7 and rM2eC9 that had amino acid change at the position 10 (P10L), rM2eC12 (N13S & E14G), and rM2eC13 (T11I, E14G and K18R). There was also some reduction of antibody binding to rM2eC2 (P10L and E14G), rM2eC14 and rM2eC15 (E14G and K18R), which indicate that the change at position 13 (N13S) as well as position 14 and 18 (E14G and K18R) also affected the antibody binding when present simultaneously.

Similar result was obtained from rM2e ELISA, it showed the importance of amino acid changes at position 10 (P10L) and 13 (N13S) (Figure 4.b) for chicken pAb binding. The binding of chicken pAb to rM2eC1 (E14G), rM2eC3 (T11I, E14G), rM2eC4 (T11I, R13K, E14G,) rM2eC6 (E14G, E16G, K18R) and rM2eC14 (E14G, K18R) was reduced significantly (>50%), indicating that changes of amino acid T11I, N13S or N13K, E14G, E16G and K18R, had disturbed antigenicity of the M2e, although it has a lesser degree compare to the changes of P10L and N13S.

Analysis using M2e-peptides ELISA (Figure 4.c) was in complete agreement with WB result, it showed significant reduction of chicken pAb binding to M2eC2, M2eC7 and M2eC12 peptide. These M2e peptides had a common amino acid change at position 10 (P10L), 13 (N13S) and 14 (E14G). The antibody binding to M2eC18, which has P10S change, was also abrogated. The M2e peptide ELISA indicated the important amino acid for chicken pAb were located at position 10, 11, 14 and 18.

Table 2. Summary of the important amino acid changes influenced the antibody (mAb 14C2, rabbit pAb, chicken pAb) binding to rM2e in WB, rM2e-ELISA and M2e-peptide ELISA.

Antibody	rM2e-WB	ELISA	
		rM2e	M2e-Peptide
mAb 14C2	P10L, H	P10L, H	P10L, H, S
	I11T	I11T	I11T
	E14G	E14G	E14G
	G16E	G16E	G16E
Rabbit pAb	P10L, H	P10L, H	P10L, H, S
			T11I
			N13K, S
	E14G		E14G
	E16G		E16G
Chicken pAb	P10L	P10L, H	P10L, H
		I11T	I11T
	N13S	N13S	E14G
		E14G	
		K18R	K18R

Anti-M2 mAb 142C and anti-M2e pAb (chicken and rabbit) were used in this study to determine the antigenic epitopes on the M2e protein and the result showed that all three antibodies identified the same immunodominant epitopes, although chicken pAb had broader specificity and recognised additional antigenic epitopes on the M2e which were not recognised by rabbit pAb and mAb 14C2. Overall, all antigenic epitopes identified were located between amino acid position 10 and 18 (Table 2). Single amino acid changes at positions 10, 11, 13 and 18 have affected the binding, with amino acid changes at position 14 and 16 possibly acting co-operatively. It was considered that the entire region between amino acid 10 and 18 form a part of a single epitope in which amino acid changes at the position 10, 11 and 13 effect the binding and lead to the lack of recognition by an antibody. Both chicken and rabbit pAb used in this study contained high titres of anti-M2e antibodies and indicating that the synthetic M2eC0 peptide inoculated with Freund adjuvant was immunogenic and could induced high titre of antibodies recognised by both rM2e proteins and synthetic M2e peptides. High antibody titres (>1/32000) for rabbit and chicken M2e pAb were detected in ELISA (result not shown).

M2e protein has been reported as a poor immunogen and induced low or no detectable antibody titres following live infection in humans (Fiers et al. 2004). The antibodies titres from animals or humans infected with IAV were found to be in low titres and of short duration (Feng et al. 2006). However, it has been shown that the M2e synthetic peptide or recombinant M2e protein could induce high titre of antibody in mice and it can bind to the M2e in the surface of MDCK cells infected by PR8 IAV (Wu et al. 2007). When inoculated as a free peptide, M2e induced low (<100) antibody response in mice (Xia et al., 2011), but this immunogenicity was improved by pairing M2e with certain carrier proteins, such as hepatitis B virus core protein, Freund's adjuvant, the Norovirus P particle and other immunomodulators (Wu et al. 2007; Xia et al. 2011; Li et al. 2011). The number of M2e polyclonal antibodies have been produced and used to study M2e antigenicity (Frace et al. 1999; De Filette et al. 2006).

From direct comparisons of binding to rM2e, two antigenic sites were associated with the positions 10 and 13. Amino acid change at position 10, either P10L or P10S, reduced the antibody binding significantly, indicating that significant proportion of antibodies is directed towards this epitope. Therefore, position 10 is considered as an immunodominant epitope for M2e. At the same position, amino acid change P10H had less impact on antibody binding. It was evident that by introduction of a single mutation P10H in M2eC6 to generate M2eC8, the binding of antibody to the mutant M2eC8 did not change significantly. The amino acid change at the position 13, either N13K or N13S, also reduced antibody binding significantly and could therefore be considered as an immunodominant epitope. When comparisons were made between the parent and the mutant M2e, contribution of other amino acid changes to the antigenicity of the M2e became evident. In particular, the change at the position 14 (E14G) was the most common change between M2e of AIV and caused reduction in antibody binding. In M2e protein with amino acid E14G, additional E16G mutation further reduced antibody binding indicating these two positions might be the part of the same epitope. Another common amino acid change K18R was also reduced the antibody binding, but this was not a consistent finding. In M2e-peptide ELISA using chicken pAb, antigenic differences between proteins were less evident, with amino acid changes P10L and P10S significantly influencing the binding of antibody. Two other amino acid changes that marginally affected the binding of antibody were E14G and K18R. It is consider that the antigenicity differences between rM2e proteins and M2e peptide are due to the higher affinity of anti-M2e pAb for peptide than for rM2e protein, resulting in antibody having high titres and less discriminating ability for minor antigens present in a peptide.

Analysis using rabbit pAb indicated that the only amino acid changes that influence the binding were P10L, P10H or P10S. These changes caused the lack of binding equally, which was contrary to the results with chicken pAb. The reason for lesser discriminating value of rabbit pAb in comparison to chicken pAb obtained by the same immunisation is speculative. Both sera have been obtained by immunisation with the same peptide and adjuvant, and titres were comparable. While antigenic presentation of immunizing M2e peptide should be the same in both rabbit and chicken, it is possible that chicken, as a natural host for AIV, is more capable to recognise minor antigenic differences and mount an effective immune response. Rabbits have been often used to produce antibody against avian pathogen, including against purified antigens, in which case usually it generate broadly reactive antibody response.

Unlike for chicken and rabbit pAb, the dominant epitope detected by mAb 14C2 was isoleucine at the position 11. The highest reaction of mAb 14C2 was found with the M2eC11 (figure 1.a), which has isoleucine at position 11 (11I) and homologous sequence to M2e of A/WSN/33 IAV. However, less reaction was found with other M2e (M2eC3, M2eC4, M2eC10 and M2eC13), which also had isoleucine at position 11. This indicates that changes at other positions (E14G, G16E and R18K) could also have affected and reduced the binding of mAb 14C2 to M2e. Previously, the M2e protein antigenic sites have been

determined to be located in the middle part of M2e, which was in the first ten amino acids at the N-terminal end of M2e (Fu et al. 2009). Also, study using mouse mAb 8C6 suggested that the M2e antigenic determinant was located between amino acid residues 8 to 12 (8-ETPIR14) (Zou et al. 2005). MAb 8C6 was also reported can not recognise 7-mer M2e peptide containing amino acid substitution either at position 9 (T9A), 12 (R12A) or both positions 9 and 12 (T9A; R12A) (Zou et al. 2008). A panel of M2e human recombinant monoclonal antibodies have also been used for M2e antigenic study and showed that different M2e epitopes, SLLTEVETPIRNEWG, LLTEVETPIRNEWG, LLTEVETPIR, and TPIRNE were recognised by monoclonal antibody L66, N547, Z3G1, and C40G1, respectively (Wang et al. 2008). Hence, different results could have been obtained since every monoclonal antibody could only recognise one often-discrete epitope (Zhang et al. 2006).

In the present study, the M2e specific mouse mAb 14C2 was used to analyse the M2e antigenicity. WB and ELISA results showed that amino acid substitution at the position 11 (I11T) could destroy the M2e antigenicity. This result was similar to the earlier study (Zebedee & Lamb 1988) used mAb 14C2 on immunoprecipitation and immunoblot assays, and it showed eight different M2 proteins from heterologous IAV containing different amino acids at the position 11 (I11T) could not be recognised by mAb 14C2. This finding indicates that mAb 14C2 specifically binds to isoleucine at the position 11. The important of isoleucine at position 11 as immunodominant epitope for M2e also reported by study using human mAb 8C6, which specific for the M2e with sequence 5-EVETPIRN-14, it showed that mAb 8C6 weakly reacted with GST-5- EVETPTRN-14 (Liu & Chen 2005). The same study also reported that the residue 10 was the most important amino acid for the M2e antigenicity, that human mAb 8C6 could not bind to GST-EVETLTRN (Liu & Chen 2005). This finding was supported by previous study, which reported the influenza A virus escape mutant with mutation at position 10 (P10L and P10H), found in mice infected using PR8 influenza A virus and treated with mAb 14C2 (Zharikova et al. 2005).

CONCLUSION

In summary, the study showed that the M2e antigenic sites are located between residues 10 – 18 and amino acid changes at these sites may all affect the antibody binding to M2e protein. It also identified that the capacity for antigenic mapping of the M2e protein was different between antibody raised in chicken and rabbit. Isoleucine position 11 is crucial for antibody binding of mAb 14C2 to M2e. However, the critical amino acid changes for rabbit pAb and chicken pAb binding was proline at position 10. Therefore, these positions 10 and 11 can be considered as the important immunodominant epitopes for M2e.

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Identification of Resources in the System of Broiler Farming Business

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ABSTRAK

Amam, Fanani Z, Hartono B, Nugroho BA. 2019. Identifikasi sumber daya dalam sistem usaha peternakan broiler. JITV 23(4): 135-142. DOI: http://dx.doi.org/10.14334/jitv.v24.3.1927.

Aksesibilitas terhadap sumber daya secara teori berpengaruh terhadap pengembangan usaha peternakan broiler di suatu wilayah. Penelitian ini bertujuan untuk merumuskan indikator sumber daya yang berpengaruh terhadap pengembangan usaha peternakan broiler. Penelitian dilakukan pada bulan Agustus 2017 hingga Januari 2018 di Kabupaten Malang, Provinsi Jawa Timur, Indonesia. Jumlah sampel sebanyak 100 peternak ayam broiler. Variabel penelitian terdiri dari sumber daya finansial, teknologi, fisik, ekonomi, lingkungan, sosial, Sumber Daya Manusia (SDM), dan pengembangan usaha peternakan broiler. Data dianalisis menggunakan metode SEM dengan SmartPLS 2.0. Hasil penelitian menunjukkan bahwa pengembangan usaha peternakan broiler dipengaruhi secara langsung dan signifikan oleh sumber daya finansial, fisik, ekonomi, dan SDM, namun tidak signifikan oleh sumber daya teknologi, lingkungan, dan sosial. Pengembangan usaha peternakan broiler secara tidak langsung melalui SDM dipengaruhi secara signifikan oleh sumber daya finansial, teknologi, fisik, dan ekonomi. Kesimpulan dari penelitian ialah sumber daya teknologi berperan penting walau tidak secara langsung karena harus didukung oleh SDM peternak dalam pengembangan usaha peternakan broiler.

Kata Kunci: sumber daya usaha ternak, pengembangan bisnis, SDM, dan ayam pedaging.

ABSTRACT

Amam, Fanani Z, Hartono B, Nugroho BA. 2019. Identification of resources in the system of broiler farming business. JITV 23 (4): 135-142. DOI: http://dx.doi.org/10.14334/jitv.v24.3.1927.

Accessibility of resources in theory can affected the development of broiler farming in a region. This research was conducted with the objectives to formulate indicators of resource wich is influence to the development of broiler farming business. The research was conducted in August 2017 up to January 2018 in Malang District of East Java Province, Indonesia. The number of sample is 100 respondents of broiler farmer was participated in this research. The observed variables consist of: (a) financial resources, (b) technology resources, (c) physic resources, (d) economy resources, (e) environmental resources, (f) social resources, (g) human resources, and (h) business development. The data was analyze used by SEM with SmartPLS 2.0 analysis tool. The results indicate that the development of broiler farming business is directly influenced with significant value by the financial, physic, economic, and the human resources, but not affected by technology, environmental, and social resources. The development of broiler farming business is indirectly influenced through quality of human resources is affected by the financial, technology, physic, and economy resources. The conclusion of this research is that technology resources play an important role indirectly, because it must be supported by human resources in the model development of broiler farming business.

Key Words: livestock resources, livestock business development, human resources, and broiler farming business

INTRODUCTION

The high growth of Indonesian population (1.38 per year) is directly proportional to the increasing public demand for chicken meat which reaches 9 kilograms per capita per year. This shows that the poultry industry in the broiler sector is an industry that has great potential to be developed in developing countries such as Indonesia. The potential is seen from several advantages of the poultry sector: (a) short harvest period, (b) land efficiency, (c) small capital, and (d) availability of industry from upstream to downstream which is a unity of agribusiness and agroindustry systems, so as to absorb many of the workforce as tangible assets (David 2009; Hunger & Wheelen 2003; Pearce & Robinson 2013).

The poultry industry grows faster along with the increase of meat consumption of 7.75 kg/capita/year from chicken meat of 3.80 kg (49%), while from beef is only 0.36 kg (0.05%), and the rest comes from other

livestock meat. Increased consumption of chicken meat in the future leads to an increase in water use.

The role of government in efforts to promote the poultry industry in Indonesia, especially broiler has been arranged in the form of business partnership based on: (1) Government Regulation Number 44 of 1997 on partnership, (2) Decree of the Minister of Agriculture Number 940/Kpts/OT.2010/10/97 on guidelines of partnership of agricultural enterprises, and (3) Law Number 9 of 1995 concerning small businesses. Partnerships are business partnerships between small and medium-sized businesses or large businesses by demonstrating the principle of mutual need, mutual strengthening, and mutual benefit. (Gocsik et al. 2015) stated that the broiler breeding sector has the best perspective in the short and medium term for market development. How to overcome market failure, increase adoption, productivity, and welfare is done with a business partnership system (Ragasa et al. 2018). Contract farming is a sales arrangement between a farmer and a firm, agreed before production begins, which provides the farmer with resources or services. Many governments and donors promote contract farming as part of agricultural development policies (Ton et al. 2018).

The objectives of developing agriculture and livestock sectors are: (1) increasing revenues, (2) balancing business, (3) increasing group resources, (4) increasing business scale, and (5) improving business ability, making it strong and self-reliant. Lambrecht and Ragasa et al. (2018) argue that agricultural partnerships are one of the private-led strategies to improve market coordination and smallholder welfare. This is because according to Huh et al. (2012) the price of the contract is determined at the beginning of the season when the market price is still uncertain. Currently, many family farms are closing down, being rented out or sold outside the family, and also in European (here termed non-family farm transfer) (Joosse & Grubbström 2017).

The development of livestcok farming business is inseparable from the role of farmer resources (Amam et al. 2019a). Livestock farming business resources include financial, technology, and physic resources (Amam et al. 2019b). Livestock farming business resources also consist of economy, environmental, and social resources (Amam et al. 2019a). The greater the farmer's access to resources, the greater the farmer's chances of developing their livestock farming business. This research aims to formulate resource indicators that influence the development of broiler farming business in Malang District.

MATERIALS AND METHODS

The Research was conduted in August 2017 up to January 2018 in Malang District of East Java Province, Indonesia. 100 respondents as broiler farmer was participated in this study who was determined by purposive sampling. The Respondents were chosen by Animal Husbandry and Animal Health Departmen of Malang District. The exogenous and the endogenous variables are in Table 1.

Data collection used observation and survey methods with interview and questionnaire techniques. The questionnaire uses a likert scale of +1 to +5. The data were analyzed by SEM (Structural Equation Modeling) with SmartPLS 2.0 analysis tool. The model was estimated through partial least squares with SmartPLS (Küster et al. 2016).

The new indicator test results from the outer model value, that is the specification of the relationship between the latent variables and the indicator, also called the outer relation or measurement model, which explains the characteristics of latent variables with the indicator or variable menifest (Willy & Jogiyanto 2015; Wiyono 2011; Sholihin & Ratmono 2013).

The reflective indicator model, the equation is written as follows:

$$X_i = \lambda_{x1}\,\xi_i + \delta_i\,;\; Z_i = (\lambda_{zi}\,\eta_i) + \varepsilon_i\,;\;\; Y_i = (\lambda_{Yi}\,\eta_i) + \varepsilon_i$$

The hypothesis in this research (based on Figure 1) was that the financial resources (X_1), technology resources (X_2), physic resources (X_3), economy resources (X_4), environmental resources (X_5), and social resources (X_6) have an effect on the human resources (Z) and the development of broilers farming business (Y) in Malang district.

RESULTS AND DISCUSSION

Indicator Test

Indicator test of X_1, X_2, X_3, X_4, X_5, X_6, Z, and Y used PLS (Partial Least Square) methods is a test that can directly eliminate invalid and ineligible indicators. Indicators that meet the requirements are having an outer loading value >0.500, whereas if the outer loading value <0.500 then the indicator is invalid and does not meet the requirements. Indicator testing results on Table 2 showed that all indicators are >0.500, means that all of them can be used as factors.

Table 1. Exogenous and endogenous variables and indicators used in the experiment

Exogenous Variables	Indicators	
Financial Resources (X_1)	Primary income	$X_{1.1}$
	Income from broiler farming business	$X_{1.2}$
	Side income from non-farm business	$X_{1.3}$
	Income from other livestock farming	$X_{1.4}$
	Amount of saving	$X_{1.5}$
	Broiler population	$X_{1.6}$
Technology Resources (X_2)	Post harvest marketing	$X_{2.1}$
Physic Resources (X_3)	Mastery of information facility	$X_{3.1}$
	Use of household electricity	$X_{3.2}$
Economy Resources (X_4)	Number of family member's involvement	$X_{4.1}$
	Use of leisure time to recreation	$X_{4.2}$
	Credibility of broiler farmer	$X_{4.3}$
Environmental Resources (X_5)	Utilization of manure for fertilizer	$X_{5.1}$
	Utilization of agricultural waste for broiler feed	$X_{5.2}$
Social Resouces (X_6)	Role in social organization	$X_{6.1}$
	Relationship with village official	$X_{6.2}$
	Relationship with health workers	$X_{6.3}$
	Relationship with livestock service	$X_{6.4}$
	Relationship with feed suppliers	$X_{6.5}$
	Relationship with Day Old Chicken (DOC) suppliers	$X_{6.6}$
Endogenous variables		
Human Resources (Z)	Total worker	Z_1
	Number of harvests per year	Z_2
Business Development (Y)	Income increases	Y_1
	Broiler population increases	Y_2
	Worker increases	Y_3
	Pen of production increases (on farm)	Y_4

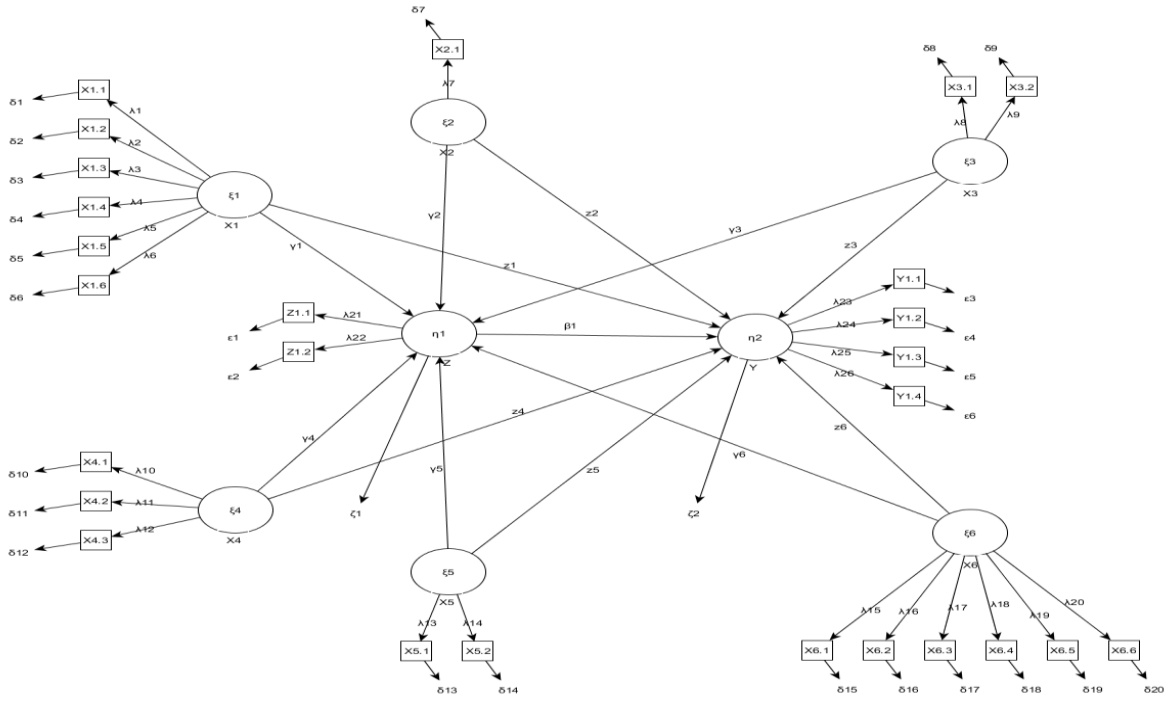

Figure 1. Variable relationship model ued in the experiment

Structural test

SEM (Structural Equation Model) analysis with PLS (Partial Least Square) methods can not be separated from model testing or outer model results consisting of convergent validity, discriminant validity, value of AVE (Average Variance Extracted), value of CR (Composite Reliability), and value of CA (Cronbach's Alpha). Test model of the development of broiler farming business consist of value of AVE, CR (Composite Reliability), and (CA) Cronbach's Alpha is as presented in Table 3.

Factors affecting development of broiler farming business

The result showed that X_1, X_3, X_4 and Z also have significantly affect to the Y (Table 4) at the level of α 5%. The path coeficiens from the influence of them to the Y respectively are 18.8%, 8.6%, 23.8% and 45.8%, meaning there were positive influence of X_1, X_3, X_4 and Z on the Y. The greater the role of them, the greater the Y. Otherwise, the result showedthat X_2, X_5 and X_6 do not significantly affect the Y at the level of α 5%. It shows that the data does not support the research, meaning that the X_2, X_5 and X_6 do not affect the Y.

The Financial resources affect the development of broiler farming business. In general, high income from broiler farming, side income from non-farming businesses, income from other livestock farming, amount of savings, and the broiler population led to the development of broiler farming business increased by 0.188 (18.8 %). This shows that the more access the farmers in obtaining financial resources, the higher the development of broiler farming business. Indarsih et al. (2010) said that contract broiler farming was chosen because risk sharing 27.6% and financial credits 25,8%, while Septiani et al. (2017) said that the difference in production costs with risk and the total production cost without risk was about 8% to 10%.

The Technology resources does not affect the development of broiler farming business. In general, knowledge of post-harvest marketing did not lead to the development of broiler farming; due to low knowledge of DOC selection the production alternative deals with selecting good breeds of chicks, low knowledge of broiler feed technology, low knowledge of broiler health and mooring management, ignorance of body weight and FCR appeared to be most eficient for small or/and large projects (Sherif & Al-Kahtani 1999). Indarsih et al. (2010) said that contract broiler farming was chosen because the guarantee of marketing 23.3%.

Table 2. Matrix indicators used in the experiment

Indicators	Outer Loading	Result	Indicators	Outer Loading	Result
$X_{1.1}$	0.597	valid	$X_{5.2}$	0.827	valid
$X_{1.2}$	0.673	valid	$X_{6.1}$	0.569	valid
$X_{1.3}$	0.798	valid	$X_{6.2}$	0.728	valid
$X_{1.4}$	0.669	valid	$X_{6.3}$	0.696	valid
$X_{1.5}$	0.680	valid	$X_{6.4}$	0.741	valid
$X_{1.6}$	0.798	valid	$X_{6.5}$	0.784	valid
$X_{2.1}$	0.998	valid	$X_{6.6}$	0.630	valid
$X_{3.1}$	0.761	valid	$Z_{1.1}$	0.945	valid
$X_{3.2}$	0.917	valid	$Z_{1.2}$	0.587	valid
$X_{4.1}$	0.789	valid	$Y_{1.1}$	0.798	valid
$X_{4.2}$	0.724	valid	$Y_{1.2}$	0.939	valid
$X_{4.3}$	0.543	valid	$Y_{1.3}$	0.936	valid
$X_{5.1}$	0.830	valid	$Y_{1.4}$	0.931	valid

Table 3. Value of outer model used in the experiment

Variables	AVE	CR	CA	R *Square*
Y	0.816	0.946	0.925	0.564
Z	0.619	0.755	0.451	0.705
X_1	0.499	0.855	0.807	
X_2	1.000	1.000	1.000	
X_3	0.711	0.829	0.612	
X_4	0.481	0.731	0.442	
X_5	0.686	0.814	0.544	
X_6	0.462	0.835	0.768	

Table 4. Value of inner model in farming business development

Test	t statistic (t table=1.660)	Path Coeficient
X_1. Financial resources → Y. Business development	2.421	0.188
X_2. Technology resources → Y. Business development	0.558	-0.064
X_3. Physic resources → Y. Business development	1.821	0.086
X_4. Economy resources → Y. Business development	2.445	0.238
X_5. Environmental resources → Y. Business development	1.207	0.104
X_6. Social resources → Y. Business development	1.051	-0.100
Z_1. Human resources → Y. Business development	2.904	0.458

Table 5. Value of inner model in human resources

Test	t statistic (1.660)	Path Coeficient
X_1. Financial resources → Z. Human resources	2.629	0.169
X_2. Technology resources → Z. Human resources	6.092	0.561
X_3. Physic resources → Z. Human resources	2.308	0.206
X_4. Economy resources → Z. Human resources	2.312	0.199
X_5. Environmental resources → Z. Human resources	1.514	0.110
X_6. Social resources → Z. Human resources	1.604	-0.155

The Physic resources affect the development of broiler farming business. In general, the control of information facilities and control of household electricity led to the development of broiler farming business increased by 0.086 (8.6%). It shows that the more access farmers in obtaining physic resources, the higher the development of broiler farming business, so the capital is not the main reason to work with the integrators (Indarsih et al. 2010).

The economy resources affect the development of broiler farming business. In general, family labor, the opportunity to use leisure time for recreation, and the credibility of farmers led to the development of broiler farming business increased by 0.238 (23.8%). This shows that the more access farmers in obtaining economy resources, the higher the development of broiler farming business.

The environmental resources does not affect the development of broiler farming business. In general, the use of sewage for fertilizers and the use of agricultural waste for animal feed did not lead to the development of broiler farming business is increased due to the high level of air pollution, the level of soil contamination, water pollution, and sound pollution. The government involvement was needed to encourage poultry industry growth and legislation on maintaining environment (Indarsih et al. 2010).

The social resources do not affect the development of broiler farming business pattern, due to low relationships with other farmers which resulted in low information and minimal role. So new investors should be encouraged to overcome instability price (Indarsih et al. 2010). Male herders were more experienced, received more benefits, showed greater interest in discussions on topics related to farming, followed information from TV and radio, and received more services offered by veterinary clinics, which proved more beneficial for them (Aldosari 2018).

The result (Table 5) showed that X_1, X_2, X_3 and X_4 significantly affect the intervening variable that is the Z. t arithmetic shows their number greater than the value of t table that is 1.660 at the level of α 5%. It shows the hypothesis that there are influence are accepted, so that X_1, X_2, X_3 and X_4 significantly affect the Z. The coefficient of parameters from the influence of them to the Z, respectively are 16.9%, 56,1%, 20,6% and 19,9%. Meaning there are positive influence of them on Z. The greater the role of them, the greater the quality of Z.

The opposite of that, there are two indicators that X_5 and X_6 which have t arithmetic smaller than the value of t table was 1.660 at the level of α 5%. It shows the hypothesis was rejected, so the X_5 of Z. It shows that the data does not support the research, meaning that the X_5 and X_6 do not affect the quality of Z.

Factors affecting human resources

Financial resources affect the human resources of broiler farming business. In general, the high income of farmers from broiler farming and non-farm side business accompanied by the fulfillment of daily family necessities caused the quality of human resources increased by 0.169 (16.9%). It shows that the more access farmers in obtaining financial resources, the higher the quality of broiler farmer. Huang et al. (2018) says that contract farming has been increasingly found to benefit smallholders in developing countries, yet much less is known about its role in the poultry industry where economies of scale could be more prominent, but direct experience with producer contracting allowed cooperatives to evade institutional and ideological lock-in (Hogeland 2015).

The technology resources affect the human resources of broiler farming business. In general, knowledge of post-harvest marketing led to human resources of broiler farming business increased by 0.561 (56.1%). This shows that the more acces farmers in obtaining technology resources mastery, the higher the quality of broiler farming business. Pivoto et al. (2018) Smart Farming (SF) involves the incorporation of information and communication technologies into machinery, equipment, and sensors for use in agricultural production systems, so this can only be achieved through increased use of emerging technologies and automated systems (Føre et al. 2018)

such as geographical and farm related risk factors for Newcastle disease (Wiseman et al. 2018).

Physic resources affect the human resources of broiler farming business. In general, the mastery of information facilities and the mastery of household electricity causes the human resources of broiler farming business increased by 0.206 (20.6%). This shows that the more access farmers in obtaining physic resources, the higher the quality of of broiler farming business, so modified farming has generated new opportunities and new forms of added value to the available resources (Pasmans & Hebinck 2017).

The economy resources affect the human resources of broiler farming business. In general, family labor, the opportunity to use leisure time for recreation, and credibility of farmers causes the human resources of broiler farmers increased by 0.199 (19.9%). This shows that the more access farmers in obtaining economy resources, the higher the quality of broiler farming business. Women's off-farm work in particular is frequently cited as a source of empowerment for farm women. However, little attention is paid to the joint strategies of how both men and women together challenge the dominant narrative of gender on the family farm (Cush et al. 2018).

The environmental resources do not affect the human resources of broiler farming business. In general, the utilization of manure for fertilizers and the use of agricultural waste for animal feed does not cause the human resources of broiler farming business to increase due to high levels of air pollution, soil pollution levels, noise pollution, and water pollution, because water quality problems as a way to prevent diffuse agricultural pollution (Vincent & Fleury 2015) so livestock farming is one of the most environmentally threatening industries worldwide (Rivero & Daim 2017). So Hu et al. (2017) said that driven by the growing demand for food products of animal origin, industrial livestock and poultry production has become increasingly popular and is on the track of becoming an important source of environmental pollution.

The social resources do not affect the human resources of broiler farming business. Social Farming (SF) engages groups at risk of social exclusion in agricultural activities with the aim of including them in society, providing them with job opportunities, and empowering them (Guirado et al. 2017). In general, rule in social organization, relationships with village officials, relationships with health workers, relationships with livestock services, relationships with feed supplier, relationships with DOC suppliers, and relationships with financial institutions, and relationship with marketer company (postharvest) do not lead to poor human resources of broiler farmers due to low relationship with farmers others that resulted in low information of farmers who accompanied the lack of role in community organizations, so the bargaining power of farmers is low, especially in broiler sale price that closely related to livestock marketers (Khan et al. 2018) said that the people of fish consumption and preference is high in the study area and people prefer fish more than chicken and meat for consumption purposes.

CONCLUSION

The model of broiler farming business development directly was influenced by financial resources 18,8%, physic resources 8,6%, economy resources 23,8%, and human resources 45,8%. The model of broiler farming business development indirectly through human resources was influenced by financial resources 16,9%, technology resources 56,1%, physic resources 20,6%, and economy resources 19,9%. This research showed that technology resources play an important role indirectly, because it must be supported by human resources in the model development of broiler farming business.

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e. Author with same family name is written consecutive: (Dawson J 1986; Dawson M 1986).
f. Several different authors are written consecutive: (Kannan et al. 2000; Grandin 2007; Santosa et al. 2012).
g. Institution: CSA (2011).....

11. **Table:**
a. Standard word used is Times New Roman with 1 space distance and 11 of font size.
b. Title is simple, clear, and understable sentence without reads the manuscript.
c. Each column from table should has heading. Its unit separated from title by comma, in parentheses, or at its bottom.
d. Table description is written under the table with 1 space distance and 11 of font size. Data source is written under the table or in the table in own header.

Dividing line is made in form of horizontal.

12. **Figure and graphic:**
a. Title uses Times New Roman with 1 space distance and 11 of font size. It is a simple and clear sentence which is laid under the figure or graphic.
b. Line in graphic should show clearly difference of one and others, if there is more than one curve.
c. Clear contrast figure with proportionate size and high resolution to present the best performance.

Write figure or graphic source under the title.

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