EFFECT OF AGE, PREGNANCY AND LACTATION ON ERYTHROCYTE OSMOTIC FRAGILITY AND MEMBRANE PHOSPHOLIPIDS OF HOLSTEIN FRIESIAN COWS

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ABSTRACT

Apparently, healthy 105 cows were used in this study to investigate the effect of age, pregnancy and lactation on osmotic fragility of Holstein Friesian cow's erythrocyte and its membrane phospholipids. These healthy cows were classified according to age and their physiological status (pregnancy and lactation) into 7 groups. Blood samples were collected from different groups for determination of osmotic fragility by osmotic fragility test and phospholipids fractions by thin layer chromatography. The present study revealed that the fragility was increased with advanced age. Moreover, the pregnant cows showed a significant decrease in the fragility of erythrocytes as compared with the lactating ones. Regarding to phospholipids, the results showed that increasing age caused a significant decrease in total phospholipids. While, age, pregnancy and lactation caused non-significant changes in sphingomyelin and phosphatidylserine percentages. Concerning the phosphatidylcholine and phosphatidyl-ethanolamine, the results revealed that increasing age caused significant decrease in phosphatidylcholine and a significant increase in phosphatidylethanolamine percentages. Regarding the pregnancy and lactation, pregnant cows had significantly higher percentages of phosphatidylcholine than lactating cows of the same age. From this study, it is concluded that maintenance percentages of phospholipids fractions of erythrocyte membrane is essential for retaining the normal lifespan of the cell. Whenever distortion in percentages of phospholipids occurs, the cells undergo lysis. Thus, it is recommended that old and lactating cows should be supplemented by antioxidant to increase the resistance of erythrocytes to improve the productivity and health care during these critical physiological periods.

Keywords: cows, erythrocytes, osmotic fragility, phospholipids.

(BVMJ-27(2): 104-115 , 2014)

1. INTRODUCTION

The main function of the RBCs is to provide the tissue with oxygen and remove carbon dioxide and protons produced in metabolic processes; therefore, the structure of RBCs is subordinate to their tasks (Andrzej et al., 2002). The physiological activities (pregnancy and lactation) are considered as metabolic stresses sufficiently causing a considerable instability of the organism's hematological findings (Maria and Monika, 2010). In addition, the age of the animal is considered as an important physiological factor affecting some of blood constituents (Devi and Kumar, 2012) in dairy cattle. Erythrocyte membranes are important models for studying the structure of natural lipid bilayers (Roelofsen and Zwaal, 1976). Surprisingly no direct information is available regarding the lipid asymmetry in bovine erythrocytes and its generation and maintenance in the face of a quite different plasma lipid composition. So, this study was carried out to define these aspects of the biology of bovine erythrocytes as influenced by age, pregnancy and lactation.

2. Materials and methods

2.1. The farm and housing:

The blood samples were collected from a private sector of animal production in Ismailia government for production of Holstein- Friesian cows. Apparently healthy 105 cows were used in this study. These healthy cows were classified into 7 groups (immature calves of 3-6 months of age, pregnant cows of 2-3 years of age, lactating cows of 2-3 years of age, pregnant cows of 3-5 years of age, lactating cows of 3-5 years of age, pregnant cows above 5 years of age and lactating cows above 5 years of age). The animals were housed in open yard system with moderate animal intensity. Cows were housed on a free stall barn with special feeders and drinking water available *ad libitum*. Apparently health animals with no history of appreciable health problems were chosen and kept under identical zoohygienic conditions and feeding program.

2.2. Feeding program:

The rations in the farm were three types according to the age of animals and their reproductive state (pregnancy and lactation): 1) ration for young calves, 2) ration for pregnant cows (late stage of gestation), and 3) ration for early lactating cows.

2. 3. Climate data:

The study was done during a period extended from October to February with an average dry temperature of 15.47 ± 1.84°C and relative humidity of 82.75 ± 3.98% which represent a cold season.

2. 4. Experimental animal design:

Grouping of cows according to age and reproductive status (pregnant or lactating) as shown in Table 1

2. 5. Blood sampling:-

Blood samples were collected via jugular vein puncture (using minimum restrain) into heparinized bottles for obtaining whole blood samples for determination of osmotic fragility of red blood cells and phospholipids' fractions in the bovine erythrocyte membrane.

2. 6. Determination of erythrocyte osmotic fragility:

Osmotic Fragility was done according to Faulkner and King (1970).
The percent haemolysis was then calculated using the formula according to (Faulkner and King, 1970).

$$\text{Percent haemolysis} = \frac{\text{Optical density of test}}{\text{Optical density of standard}} \text{ X } 100$$

Erythrocyte osmotic fragility curve was obtained by plotting percent haemolysis against the sodium chloride concentrations. The preparation of different concentrations of hypotonic solution in osmotic fragility test as shown in Table 2

2. 7. Determination of the phospholipids percentages in erythrocyte membrane:

Determination of the phospholipids percentages in erythrocyte membrane by extraction of membrane phospholipids according to Folch et al. (1957). For analysis of phospholipids, Phospholipids were identified by simultaneous chromatography of reference phospholipids, and comparison with previously published data using the same densitometry with CAMAG TLC (Thin Layer Chromatograph) Scanner 4 and Win CATS software in absorption mode at 360 nm using a deuterium lamp, 420 or 720 nm using a tungsten lamp. Alternatively video densitometry (Video scan) of image took under white light. Evaluation was via peak height or area, polynomial regression (Murray, et al., 2007). Four phospholipids classes were determined in the erythrocyte membrane of cows: 1-phosphatidylcholine (PC), 2-phosphatidylserine (PS), 3-phosphatidylethanolamine (PE), 4-sphingomyelin (SM),

2.6. Statistical analysis of data:

The data were expressed in terms of mean ± standard error. One- way ANOVA at 5% level of significance followed by Fishers Least Significant Difference test (LSD)

(Fisher, 1935) was employed in order to determine Significant Difference among different groups. When appropriate, Duncan multiple tests (at 5%) (Duncan, 1959) were applied to evaluate the differences among means. The statistically homogenous means were denoted by similar alphabets. All analyses were performed using SPSS 16.0 version for Windows.

Table 1: Grouping of cows according to age and reproductive status (pregnant or lactating).

Age	Reproductive status	Grouping number
3-6 months	Immature calves after weaning age	I
2-3 years	Pregnant (late stage of pregnancy)	II
	Lactating (early lactation)	III
3-5 years	Pregnant (late stage of pregnancy)	IV
	Lactating (early lactation)	V
Aged (more than 5 years)	Pregnant (late stage of pregnancy)	VI
	Lactating (early lactation)	VII

Table 2: The preparation of different concentrations of hypotonic solution in osmotic fragility test.

The tube	Blood volume	Nacl 1% volume (ml)	D.W volume (ml)	Final total volume (ml)	Nacl %
1	20 µL	0.2	1.8	2	0.1%
2	20 µL	0.4	1.6	2	0.2%
3	20 µL	0.6	1.4	2	0.3%
4	20 µL	0.8	1.2	2	0.4%
5	20 µL	1	1	2	0.5%
6	20 µL	1.2	0.8	2	0.6%
7	20 µL	1.4	0.6	2	0.7%
8	20 µL	1.6	0.4	2	0.8%
9	20 µL	1.8	0.2	2	0.9%
10	20 µL	0	2	2	0%

D.W→ Distilled water

3. Results

3. 1. Effect of age, pregnancy and lactation on hemolysis percentage (%) of Holstein cows erythrocytes:

Table 5 showed that there were non significant changes in hemolysis percent of erythrocytes at NaCl concentration of 0.1, 0.3, 0.6, 0.7, 0.8 and 0.9 %. Meanwhile, at concentration 0.2 % NaCl, there was significant decrease in hemolysis percent in pregnant cows of 2-3 years old compared to other groups. The same table also showed that, at concentration of 0.4 % NaCl there was a significant increase in hemolysis % in cows 2-3 years old in both pregnant and lactating cows compared to other groups. Also at concentration of 0.4% NaCl, there was a significant increase in hemolysis % in lactating cows aged 3-5 years old compared to pregnant cows of the same age and 2-3 years old and immature calves. All aged groups above 5 years old and 3-5 years old lactating cows showed significant increase in hemolysis percent compared to immature calves and 2-3 years old at concentration of 0.4% NaCl. At concentration 0.5 %, there was significant decrease in hemolysis percent in immature calves and pregnant and lactating cows 2-3 years old compared to other groups (3-5 years old and aged cows over 5 years old groups). Also at the same concentration, lactating cows 3-5 years old showed significant increase in hemolysis % compared to other groups. Table 5 revealed that aging did not cause significant change in hemolysis % at concentration 0.2% NaCl except pregnant cows 2-3 years old showed significant decrease compared to all other groups, whereas at concentration, 0.4% NaCl aging caused significant increase in hemolysis % in aged groups over 5 years old and 2-5 years old lactating cows compared with other groups, however, 2-3 years old groups showed significant decrease compared to other all groups. At concentration 0.5% NaCl aging caused significant increase in hemolysis % in 3-5 years old pregnant and lactating cows compared to immature calves and 2-3 years old cows, at the same concentration aged group's cows above 5 years showed significant increase compared to immature and 2-3 years old cows. Table 5 also revealed that pregnancy caused significant decrease of hemolysis % in 2-3 years old cows and non significant decrease in 3-5 years old and aged cows above 5 year old at concentration 0.2% NaCl, whereas at concentration 0.4 and 0.5% NaCl pregnancy caused significant decrease in hemolysis % in 3-5 years old cows and non significant decrease in 2-3 years old cows and above 5 years old cows.

3. 2. Effect of age, pregnancy and lactation on phospholipids concentrations of Holstein cow's erythrocytes membrane:

Table 6, The results of the present study revealed that increasing age caused significant decrease in total phospholipids while lactating cows showed non significantly decrease in total phospholipids comparing with pregnant cows of the same age group. As observed in table 6, it was found that the sphingomyelin fraction is the most abundant in all animals studied, followed by phosphatidylcholine. In addition, table 6 illustrated that age, pregnancy and lactation caused non-significant changes in sphingomyelin and phosphatidylserine percentages while concerning to the phosphatidylcholine, the presented data showed that cows of 3-6 months age and pregnant cows of 2-3 years age had the highest significant percentage of phosphatidylcholine comparing to all the other groups. Moreover, lactating cows of 2-3 years age, showed significant higher percentage of phosphatidylcholine than lactating cows of 3-5 Years age and aged lactating cows. Regarding to the pregnancy and lactation, there were non-significant differences between pregnant and lactating cows during the different ages in phosphatidylcholine percentage. Whereas, pregnant cows had none significantly higher percentage of phosphatidylcholine

Table 3: Effect of age, pregnancy and lactation on haemolysis percentages (%) of Holstein cows erythrocyte (mean±SE) (n=15):

NaCl concentrations Animal groups	0.1%	0.2%	0.3%	0.4%	0.5%	0.6%	0.7%	0.8%	0.9%
3-6 months	100±0.00[a]	96.57±0.57[ab]	91.71±0.68[a]	36.75±1.44[b]	1.73±0.22[c]	0.00	0.00	0.00	0.00
2-3 Y pregnant	100±0.00[a]	93.00±1.33[c]	89.86±1.06[a]	15.75±1.25[c]	1. 78±0.09[c]	0.00	0.00	0.00	0.00
2-3 Y lactating	100±0.00[a]	97.14±0.74[ab]	91.14±1.08[a]	17.00±0.08[c]	1.79±0.06[c]	0.00	0.00	0.00	0.00
3-5 Y pregnant	100±0.00[a]	96.57±0.70[ab]	93.29±1.03[a]	38.25±1.17[b]	4.43±0.26[b]	0.00	0.00	0.00	0.00
3-5 Y lactating	100±0.00[a]	98.14±0.53[a]	93.14±0.70[a]	69.00±1.30[a]	6.58±1.01[a]	0.00	0.00	0.00	0.00
above 5 Y pregnant	100±0.00a	95.57±0.84b	89.43±1.36a	62.25±1.82a	4.58±0.79b	0.00	0.00	0.00	0.00
above 5 Y lactating	100±0.00a	96.29±0.64ab	92.00±1.70a	64.00±1.16a	4.83±0.12b	0.00	0.00	0.00	0.00

Means with different letters in the same column are significantly different ($p < 0.05$).

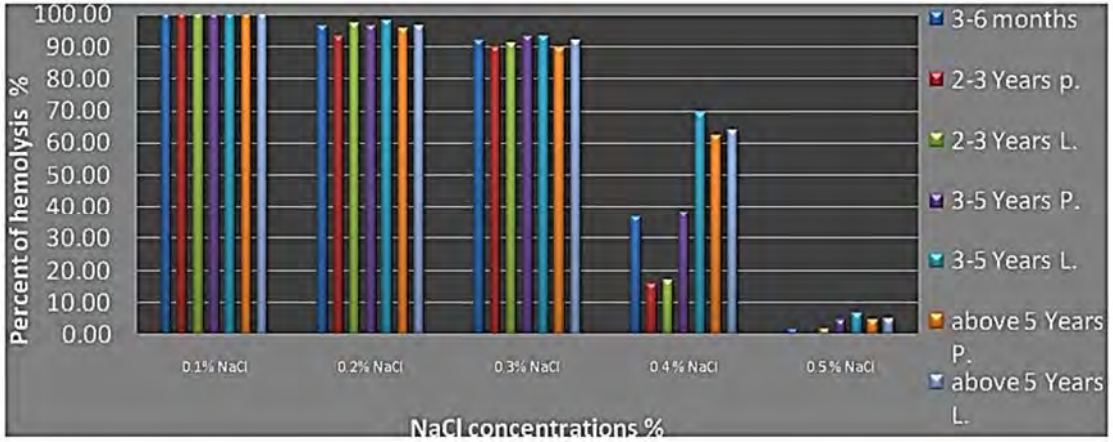

Figure 1: Effect of age, pregnancy and lactation on haemolysis percentages (%) of Holstein cow's erythrocyte.

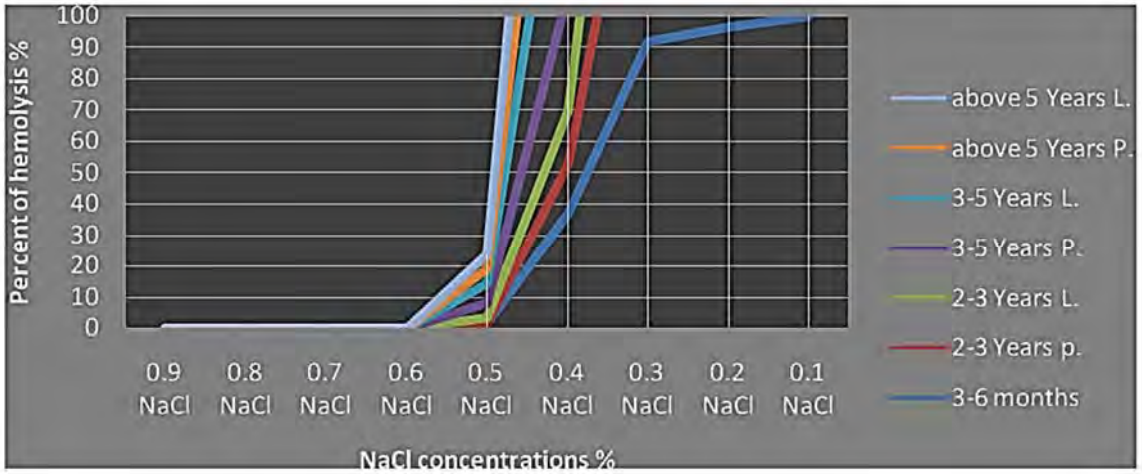

Figure 2: Effect of age, pregnancy and lactation on fragility hemolysis curve of Holstein cow's erythrocyte.

Table 4: Effect of age, pregnancy and lactation on phospholipids (%) of Holstein cows erythrocyte membrane (mean ±SE) (n=3):

Parameters / Groups	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylserine	Sphyingomyelin	Total(µg/mg)*
3-6 months	16.77±0.50^{a}	24.65±0.35^{c}	23.99±0.98^{ab}	34.60±0.98^{a}	27.41±0.98^{ab}
2-3 Y pregnant	16.99±0.25^{a}	25.21±0.61^{c}	22.88±0.87^{b}	34.93±0.99^{a}	28.11±0.99^{a}
2-3 Y lactating	15.74±0.70^{ab}	25.62±0.90^{bc}	23.32±0.97^{ab}	35.39±0.46^{a}	24.47±0.29^{abc}
3-5 Y pregnant	14.87±0.87^{bc}	26.84±0.43^{ab}	24.44±0.50^{ab}	33.77±0.43^{a}	23.69±0.21^{bc}
3-5 Y lactating	12.09±0.46^{c}	28.02±0.64^{a}	25.88±0.41^{a}	34.05±0.99^{a}	22.54±0.54^{c}
above 5 Y pregnant	13.78±0.91^{bc}	27.04±0.62^{ab}	24.50±0.38^{ab}	35.59±0.99^{a}	21.56±0.63^{c}
above 5 Y lactating	13.43±0.59^{c}	28.20±0.73^{a}	25.01±0.80^{ab}	33.46±0.68^{a}	22.55±0.54^{c}

Means with different letters in the same rows are significantly different ($p < 0.05$). * Total phospholipids expressed in µg/mg protein.

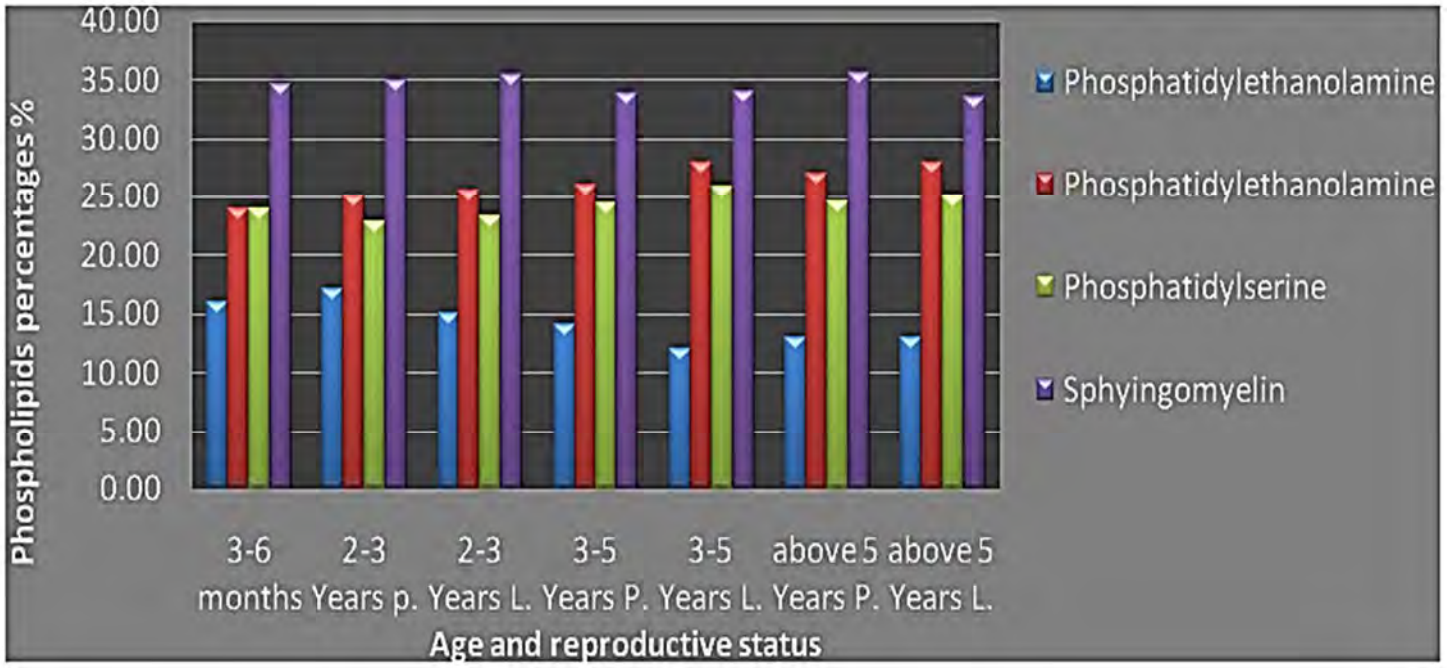

Figure 3: Effect of age, pregnancy and lactation on phospholipids (%) of Holstein cows erythrocyte membrane.

than lactating cows of the age at different tested ages. Concerning to phosphatidylethanolamine, the cows of 3-6 months age and pregnant cows of 2-3 years age had significantly lower percentage of phosphatidylethanolamine than cows of 3-5 years age (pregnant and lactating) and aged cows (pregnant and lactating) but had none significantly lower percentage than lactating cows of 2-3 years. Moreover, pregnant cows had non significantly lower percentage of phosphatidylethanolamine than lactating cows at different ages. Regarding to phosphatidylserine, there were no significant differences between different groups in phosphatidylserine percentage except lactating cows of 3-5 years age, which had significant higher percentage of phosphatidylserine than pregnant cows of 2-3 years.

4. Discussion

The obtained results showed that the osmotic fragility of Holstein cow's erythrocytes was significantly increased with age. Similar results obtained by Srour et al. (2000), Meurs et al. (2005), Tiffert et al. (2007) and Arun Kumar (2011) who found increase fragility with age in human and Davies and Goldberg (1987) in rabbits.

The mechanism by which the fragility increased with age was explained by Droge (2002) and Arun Kumar (2011) who indicated that as animals advance in age, the increased fragility contributed to damage of erythrocytes membrane by oxygen radicals in the form of lipid peroxidation and protein degradation with decreased antioxidants levels caused by imbalance in redox signaling and generation of free radicals at rates that cannot be matched by endogenous antioxidant such as glutathione and superoxide dismutase (Oyagbemi et al., 2009) which has an influence on erythrocyte membrane integrity and makes the cells more fragile and labile to damage. However, Tiffert et al. (2007) reported that increased osmotic fragility of erythrocyte by aging is due to reduction in the number and activities of calcium mediated potassium channels in the erythrocyte membrane.

The obtained results from the present study revealed that the pregnant cows had higher resistance than lactating cows. This result is in consistence with the findings obtained by Miller et al. (1993), Formigoni et al. (1997) and Ronchi et al. (2000) revealed that the erythrocyte membrane fragility increased during the early lactation phase in Holstein cows. Also these results are in agreement with the results of Lurie (1993) and Kim et al. (2002) and in pregnant women and rabbits respectively. The higher resistance (lower fragility) of erythrocytes of pregnant cows with respect to non-pregnants is attributed to hemodilution (Mulei and Daniel, 1988) which resulting in increased production of immature erythrocytes which called reticulocytes which more resist than mature erythrocyte (Brecher and Stohlman, 1961). While the lower resistance (higher fragility) of lactating cows could be attributed to an imbalance of the oxidative status, oxidative stress and formation of lipid peroxidation process during the early lactation phase (Miller et al., 1993; Formigoni et al., 1997 and Ronchi et al., 2000).Finally, from this study it is concluded the higher resistance of erythrocytes in pregnancy may has important function during pregnancy to withstand the microcirculation of the placenta and increase the survival of them during pregnancy to compensate hemodilution and low PCV.

The results of the present study revealed that increasing age caused significant decrease in total phospholipids while lactating cows showed non significantly decrease in total phospholipids comparing with pregnant cows of the same age group. These results are in agreement with those given by Kanti and Syed (2010) who reported that the advanced ages were associated with a loss of cholesterol and phospholipids of human erythrocytes membrane. These results of total

phospholipids could be explain the increase in osmotic fragility of erythrocyte with advanced ages and lactation that was observed in the present study. The significant decrease in total phospholipids in advanced ages, pregnancy and lactation which act as physiological stressors could be attributed to increased lipid oxidation of cell membranes associated with a loss of cholesterol and phospholipid (Kanti and Syed, 2010) due to decreased the activities of membrane ATPases in stress (Kakimoto et al., 1995). Also, the results of the present study revealed that increasing age caused significant decrease in phosphatidylcholine and significant increase in phosphatidylethanolamine percentage. The increased phosphatidylethanolamine percentage with increased age and lactation could be attributed to the increased lipid peroxidation resulted from these physiological stressors of old ages and lactation resulted in a significant decrease in the content of Phosphatidyl-ethanolamine Polyenoic fatty acids and an increase in incorporation of palmitic acid into Phosphatidyl-ethanolamine (Tiurin et al., 1996). Regarding to the pregnancy and lactation, pregnant cows had significantly higher percentage of phosphatidylcholine than lactating cows of the same age. Moreover, pregnant cows had none significantly lower percentage of phosphatidylethanolamine than lactating cows of the same age.

Acknowledgements

The authors are particularly grateful to the central lab, faculty of veterinary medicine, Benha University, Egypt, for assistance in laboratory tests.

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كلية معتمدة 2013

تأثير العمر، الحمل والرضاعة على هشاشة خلايا الدم الحمراء لأبقار الفريزيان الهولندي ونسبة الفوسفوليبيدات في غشاء هذه الخلايا

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** شعبة الهندسة الوراثية والبيوتكنولوجي-كيمياء حيوية-المركز القومي للبحوث بالدقي

الملخص العربي

قد تم استخدام عدد 105من الابقار ذو حالة صحية جيده ظاهريا في هذه الدراسة لمعرفة تأثير العمر، الحمل والرضاعة على هشاشة خلايا الدم الحمراء لأبقار الفريزيان الهولندي ونسبه الفوسفوليبيدات (الدهون الفوسفورية) في غشاء هذه الخلايا. وقد تم تقسيم الأبقار الي مجموعات علي حسب العمر والحالة التناسلية (الحمل والرضاعة) الي سبعة مجموعات. ولقد تم تجميع عينات الدم من المجموعات المختلفة لقياس الهشاشة الاسموزية باستخدام اختبار الهشاشة الاسموزية وكذلك لقياس نسبة انواع الفوسفوليبيدات باستخدام جهاز الفصل الكروماتوجرافي. لقد أثبتت هذه الدراسة ان هشاشة خلايا الدم الحمراء زادت مع تقدم العمر. علاوة على ذلك، الابقار العشار فقد كان هناك نقص معنوي في هشاشة خلايا الدم الحمراء مقارنة بالأبقار الحلاب. بالنسبة للفوسفوليبيدات، لقد اوضحت النتائج ان زيادة العمر قد تسبب في نقص معنوي في الفوسفوليبيدات الكلية. بينما العمر، الحمل والرضاعة لم يتسبب في اي تغيرات معنوية في نسبة الأسفينجوميالين والفوسفاتيديل سيرين. بالنسبة للفوسفاتيديل كولين والفوسفاتيديل ايثانولامين، لقد أثبتت النتائج ان تقدم العمر قد تسبب في نقص معنوي في نسبة للفوسفاتيديل كولين وزيادة معنويه في نسبة الفوسفاتيديل ايثانولامين. فيما يتعلق بالحمل والرضاعة، الأبقار العشار سجلت زيادة معنوية نسبة الفوسفاتيديل كولين بالمقارنة بالأبقار لحلاب في نفس العمر. من هذه الدراسة، من الممكن ان نستخلص ان الحفاظ على نسبة انواع الفوسفوليبيدات في غشاء خلايا الدم الحمراء يعتبر ضروريا للحفاظ علي العمر الطبيعي لهذه الخلايا. عندما يحدث اي تغيير في نسبة الفوسفوليبيدات يؤدي الي تكسير مبكر في الخلايا ومن ثم تمت التوصية بإضافة مضادات اكسدة لأبقار الحلاب والكبيرة في العمر لزيادة مقاومة خلايا الدم الحمراء مما يحسن الإنتاجية والرعاية الصحية اثناء هذه الفترات الفسيولوجية الحرجة.

(مجلة بنها للعلوم الطبية البيطرية: عدد 27(2):104-115 , ديسمبر2014)