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INFLUENCE OF LITTER SIZE ON ULTRASOUND ESTIMATED FETAL GROWTH CURVE, MATERNAL STEROIDS, OXIDATIVE STRESS AND SERUM FREE RNA IN GOATS

Influencia del tamaño de la camada en la curva de crecimiento fetal estimada por ultrasonido, esteroides maternos, estrés oxidativo y ARN libre en suero en cabras

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ABSTRACT

This study aimed at evaluating the litter size influence on fetal growth (marked by biparietal diameter), steroid hormones (estradiol and progesterone), oxidative stress markers [Total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase (CAT), Glutathione peroxidase (GPx), and malondialdehyde (MDA)], total proteins, and serum-free RNA. Goats (n=150) were blood sampled and assessed ultrasonographically during the mid-stage of pregnancy (6th to 14th week) and were classified into non-pregnant (n=64), single (n= 55) twine (n= 25), and triple (n= 6) pregnancy according to a number of feti. The correlation coefficient of caprine fetal growth was R^2 = 0.9609, 0.9418, and, 0.928 in single, twin, and triple feti, respectively. The area under the curve of the fetal growth was 286.2, 282.1, and 263.4 for single, twin, and triple caprine fetuses. The mean reduction rate in fetal growth compared to singleton pregnancy was 1.65±1.03 and 8.32±2.41 % in twine and triple feti, respectively. Estradiol significantly (P< 0.05) decreased, while progesterone (P< 0.01) and serum-free RNA (P< 0.001) increased in pregnant animals compared to non-pregnant. TAC and MDA increased in multiple pregnancies compared to non-pregnancy in association with the decrease of SOD and catalase activities. GPx activity and total proteins substantially decreased in triple pregnancy than non-pregnancy. Cell-free RNA negatively correlated with estradiol, CAT, GPx, and total proteins, and positively correlated with P4, TAC, and MDA. In conclusion, litter size greatly impacted fetal growth, maternal steroids, and serum-free RNA, and preload to oxidative stress-mediated health disorders in pregnant goats.

Keywords: Goat, Litter size, Oxidative stress, Serum-free RNA, Steroids, Ultrasound

RESUMEN

El objetivo fue evaluar la influencia del tamaño de la camada en el crecimiento fetal (marcado por el diámetro biparietal), hormonas esteroides (estradiol y progesterona), marcadores de estrés oxidativo [capacidad antioxidante total (TAC), superóxido dismutasa (SOD), catalasa (CAT), glutatión peroxidasa (GPx) y malondialdehído (MDA)], proteínas totales y ARN libre en suero. Se tomaron muestras de sangre de cabras (n = 150) y se evaluaron ecográficamente durante la etapa media de la gestación (semana 6 a 14) y se clasificaron en no gestantes (n = 64), gestación simple (n = 55), doble (n = 25) y triple (n = 6), de acuerdo con el número de fetos. El coeficiente de correlación del crecimiento fetal con la gestación simple, doble y triple fue R^2 = 0,9609, 0,9418 y 0,928, respectivamente. El área bajo la curva del crecimiento fetal fue 286,2, 282,1 y 263,4 para gestación fetal simple, doble y triple. La tasa de reducción media en el crecimiento fetal en comparación con la gestación simple fue de 1,65 ± 1,03 y 8,32 ± 2,41% en gestación doble y triple, respectivamente. El estradiol disminuyó significativamente (P <0.05), mientras que la progesterona (P <0.01) y el suero libre de ARN (P <0.001) aumentaron en animales gestantes en comparación con los no gestantes. El TAC y MDA aumentaron en gestaciones múltiples en comparación con los no gestantes en asociación con la disminución de las actividades de SOD y catalasa. La actividad de GPx y las proteínas totales disminuyeron sustancialmente en el embarazo triple que en no gestantes. El ARN libre de células se correlacionó negativamente con estradiol, CAT, GPx y proteínas totales, y se correlacionó positivamente con P4, TAC y MDA. En conclusión, el tamaño de la

camada afectó en gran medida el crecimiento fetal, esteroides maternos y ARN libre en suero, y los trastornos de salud mediados por el estrés oxidativo en cabras preñadas.

Palabras clave: Cabra, Tamaño de la camada, Estrés oxidativo, ARN libre en suero, Esteroides, Ultrasonido

INTRODUCTION

Goats are polytocous animals, and most the animal producers prefer to rear those animals for this peculiarity (multiparous vs. uniparous) to increase their economic returns. Nevertheless, management of pregnant goats with ignoring the consequences of multiple fetuses on animal lifetime, dystocia, milk production, lactational weight losses, and postpartum uterine involution/ovarian cyclicity would interfere with goat production/reproduction development (Menchaca and Ungerfeld, 2017).

Pregnancy is a physiological period during which various metabolic pathways are altered, resulting in modification of energy substrates consumption, increase in oxygen consumption, and high metabolic placental demands (Illsley et al., 2010).

Predictions of the number of fetuses would allow appropriate nutritional management of females at late gestation that would prevent pregnancy toxemia (Ford, 1983), minimize pre-kidding feeding costs, optimize birth weight, weaning weight, and survivability of lambs and reduce the incidence of dystocia (Gearhart, 1988), and predict the adverse pregnancy outcome (Abd El Hameed et al., 2018).

Estrogen and progesterone are among steroid hormones produced from the ovary and uterus and are essential for pregnancy maintenance (Kumar and Magon, 2012). Small ruminants that show multiple ovulations produce more progesterone than those who had one ovulation (Boscos, 2003; Gür et al., 2011), in association with the increased number of fertilized ova, and the developed corpora lutea (Bradford, 1985).

An increased placental metabolic demand during the gestation period to meet the fetal growth needs is a preload to the increased reactive oxygen species (ROS) and the cumulative oxidative stress (Myatt, 2010). Goats that carry multiple fetuses are at a major risk for pregnancy toxemia that is strongly associated with oxidative stress (Olfati et al., 2013). Estimating blood enzymatic antioxidants (catalase, glutathione peroxidase, and superoxide dismutase) together with malondialdehyde (MDA) values may give information that is more valid on the level of oxidative stress (Celi et al., 2010).

Ribonucleic acid (RNA) is one of the placental-derived nucleic acids that can pass the mother's bloodstream and other body fluids (Chim,2008). The levels of cell-free nucleic acid in maternal circulation is of value for monitoring placental function and prenatal diagnoses of pregnancy-associated disorders (Malarmathi et al., 2016).

To the authors' knowledge, scarce work cares about the influence of litter size on the fetal growth, endocrine milieu, oxidative stress, and serum-free RNA in animals with special emphasis on goats (multiparous animal model). Therefore, the current study aimed to verify the effect of litter size on intrauterine fetal growth and pregnant dam healthy status through an ultrasound monitoring of fetal growth rate as well as the evaluation of the levels of circulating estradiol, progesterone, total antioxidant capacity (TAC), reactive oxygen scavenger enzymes (SOD, CAT, GPx), MDA and cell-free RNA in singleton, twin and triple pregnancy as compared with non-pregnant status.

MATERIAL AND METHODS

Animals

The present study was conducted on a total number of150 goats admitted to the Veterinary Clinic of Meet Kenana, Qalyubia, for pregnancy diagnosis. All goats were investigated at least three times ultrasonographically during the 2nd trimester of pregnancy (6-14 weeks of gestation) and were classified according to the number of feti into pregnant bearing a single fetus (n =55), twin fetuses (n=25), and triple fetuses (n=6) and non-pregnant animals (n=64). The mean ±SE of age (years) and body weight (kg) were 2.80±0.27 and 46.00 ±1.64, 3.32±0.19 and 43.24±1.09, 3.65±0.65 and 43.33±0.96, for the goats had single, twin, and triple feti, respectively.

Ultrasound imaging examination

Animals were scanned ultrasonographically in standing and/or laying positions through a transabdominal approach using a real-time B- mode scanner (Sonoscape A5, China) equipped with a 3-8MHz trans-rectal linear transducer according to Kandiel et al. (2008). A contact ultrasound gel (carboxymethyl-cellulose gel) was applied to the acoustic window on the right flank above the udder after removing hair whenever possible to improve the image quality. Then, the transducer was placed at 5.0 cm in front of the rear leg and 2.5 cm above the teat. Pregnant goats were examined for determining litter size and were evaluated for gestational age using biparietal diameter.

Blood sampling and assessment

Non-hemolyzed blood samples (5ml) were collected from each goat before ultrasound examination through jugular veinpuncture into plain vacutainer tubes. Blood samples were centrifuged at 2500 rpm for 15 min and the sera were separated and stored at – 20 °C until being assayed.

Steroid hormonal assessment

Serum estradiol (E2, pg/ml) and progesterone (P4, ng⁄ml) concentrations were estimated quantitively by Enzyme-Linked Immunosorbent assay (ELISA) using commercial ELISA kits (Chemux BioScience, South San Francisco, USA) and ELISA microplate reader (BioTek ELx800, Vermont, USA) at 450 nm wavelength.

Measurement of antioxidants and total protein in serum

The level of total antioxidant capacity (TAC) was determined colorimetrically via commercial kit (TAC TA2513, Bio Diagnostic, Egypt) at 505 nm as described by Koracevic et al. (2001). The activity of SOD, CAT, and GPx was estimated

colorimetrically using commercial kits (SOD SD2521, CAT CA 2517, and GPxGP2524, Bio Diagnostic, Egypt) at 560 nm,520 nm, and 340 nm, respectively according to Nishikimi et al. (1972), Góth (1991) and Paglia and Valentine (1967), respectively. The MDA (MD2529, Bio Diagnostic, Egypt) and total proteins (TP 2020, Bio Diagnostic, Egypt) levels were measured using a commercial kit according to the method of Ohkawa et al. (1979) and Gornall et al. (1949) at 532 nm and 550 nm, respectively

Cell-free RNA evaluation

Cell-free total RNA was measured according to Nagy (2019). In brief, a purified cell-free total RNA was prepared from 200 µl of thawed serum samples on ice using a miRNeasy serum/plasma kit (Qiagen) according to manufactures protocol. During purification steps, 3.5 µl of lyophilized C. elegans miR-39 miRNA mimic (miRNeasyserum Spike-In Control) were added at concentration 1.6×108 copies/µl (working solution). To elute RNA, 14 µl of RNase-free water was added to the ecenter of the spin column membrane. Afterward, the purified cell-free total RNA was kept at -80 °C. The concentration of total RNA was checked by Nano-drop 2000/c (Thermo Fisher Scientific, Wilmington, USA), and the RNA integrity was evaluated by denaturing agarose gel electrophoresis and ethidium bromide staining (Thermo Fisher scientific, USA).

Statistical analysis

Data were expressed as mean ± SE and graphically presented. Analysis of significance between means was done non-parametrically using Krause wells statistical test. Correlation between tested parameters was analyzed using Spearman's correlation coefficient. P value was set at 0.05 to test the significant differences.

RESULTS

Fetal growth rate

The changes in fetal growth marked by BPD in goats beard single, twine and triple feti is presented in fig. 1A and their represented growth curve is shown in fig. 1B. The slope and correlation represented the fetal growth was Ys = -0.5714X2 + 9.4143X + 13.1; R^2 = 0.9609, YTw = -0.6554X2 + 9.6161X + 12.95; R^2 = 0.9418, and YTr= -0.6482X2 + 10.257X + 8.63; R^2 = 0.928 in single, twine and triple feti, respectively (Fig. 1B). The differences between the slopes were not significantly (F = 0.33, P=0.73) varied.

The area under the curve (signified the overall growth rate) was 286.2, 282.1, and 263.4 for single, twin, and triple fetuses' pregnancy in the examined goats. The mean reduction rate in fetal growth to singleton pregnancy was 1.65±1.03 and 8.32±2.41 % in twine and triple feti, respectively (Fig. 1C).

Maternal steroid hormones

The mean E2 hormonal levels were 21.98 ± 1.38, 14.19 ± 1.10, 13.58 ± 090, and 13.59 ± 1.14 pg/ml in non-pregnant, singleton, twine, and triple pregnancy, respectively. Statistically, E2 levels were significantly (P< 0.05) lower in pregnant animals, regardless of the fetal number, than non-pregnant ones (Fig. 2 A).

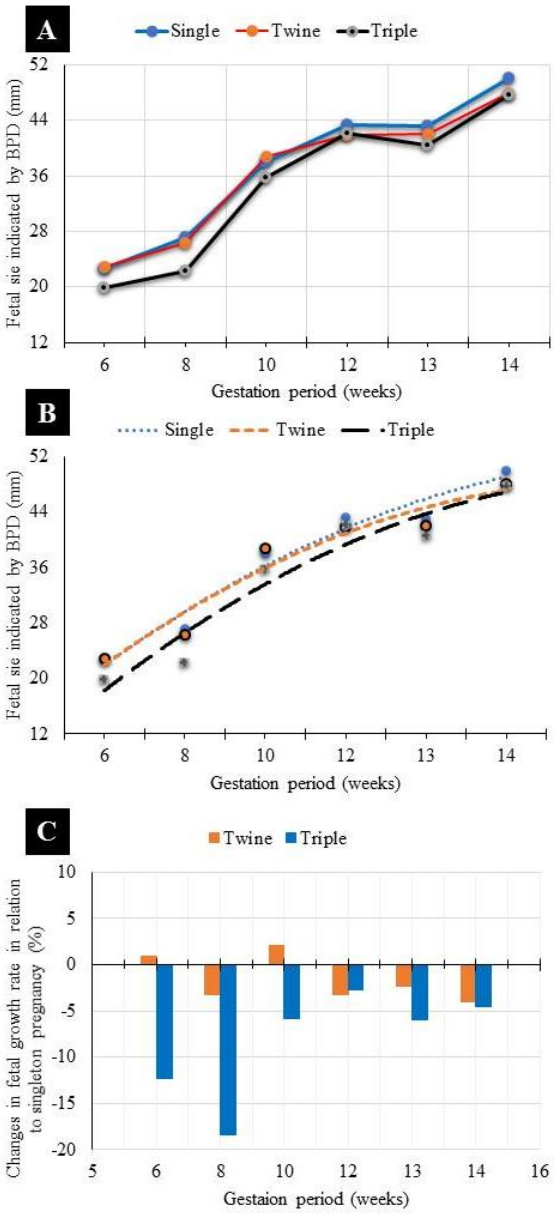

Figure 1. Changes in does fetal growth rate marked by biparietal diameter measurement. A. Represented biparietal diameter in single, twine and triple fetuses. B. Linear regression correlation between fetal growth and gestation period (weeks). C. Changes in fetal growth rate in twin and triple fetuses relation to single pregnancy (%).

The mean P4 hormonal levels were 1.46 ± 0.08, 2.40 ± 0.06, 3.20 ± 0.08, and 4.26 ± 0.12 ng/ml in non-pregnant, singleton, twine, and triple pregnancy, respectively. The levels P4 increased stepwise with the fetal number and were significantly (P< 0.01) higher in pregnancy compared to non-pregnant status (Fig. 2 B).

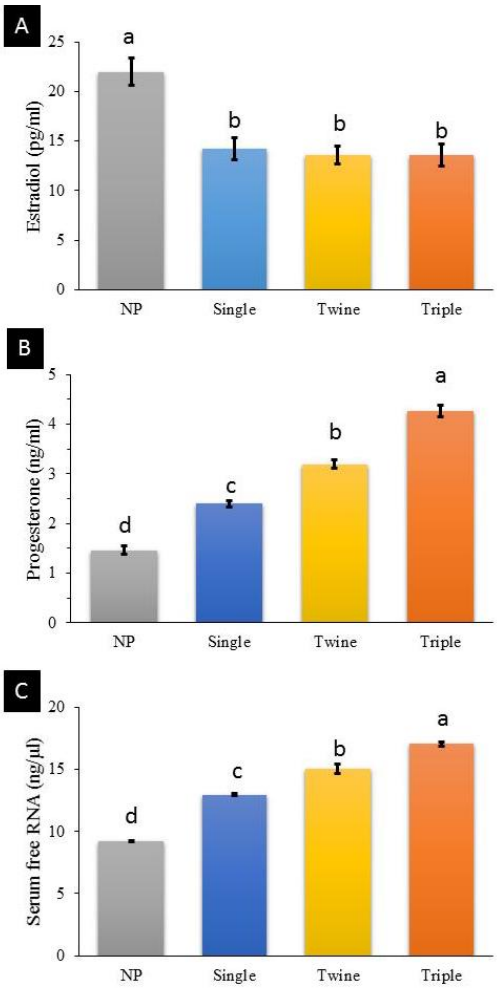

Figure 2. Serum concentration of estradiol (A), progesterone (B) and serum free RNA (C) in non-pregnant (NP), single, twine and triple does fetuses. Values (mean ± SEM) with different letters were significantly different at p< 0.05.

Oxidative stress indices, lipid peroxidation, and total proteins

Total antioxidant capacity (TAC) values were generally higher (P<0.05) in twins and the triple bearing does than that reported in non-pregnant animals (11.93±1.15 vs 11.20±1.06 vs 5.64±0.45mMol/L).

Superoxide dismutase (SOD) levels were low in twin (P< 0.05) and triple bearing does (P< 0.01) compared with non-pregnant (8.52±0.48 vs 7.93±0.30 vs 12.50±0.77 U/ml). Catalase (CAT) activity was significantly low in twins (P< 0.05) and the triple bearing does(P<0.005) than that reported in non-pregnant animals (8.80±0.75 and 8.00±0.74 vs18.00±1.90 U/L).

Glutathione peroxidase levels were generally low in triple bearing does(P<0.001) while and tended (P = 0.06) to be lower in twin bearing does than non-pregnant animals (triple 24.00±0.69 and 27.00±1.90 vs non-pregnant 40.00±2.60mU/ml). Lipid peroxidation (MDA) values were higher in twin (P<0.05) and triple (P< 0.01) pregnancy than in non-pregnant animals (50.0 ± 4.9 and 52.0 ± 4.4. vs 26.0 ± 2.3 mMol/ml). Total proteins markedly (P< 0.001) declined in triple pregnancy compared to non-pregnant status (6.70 ± 0.18 vs 7.2 ± 0.30 g/dl).

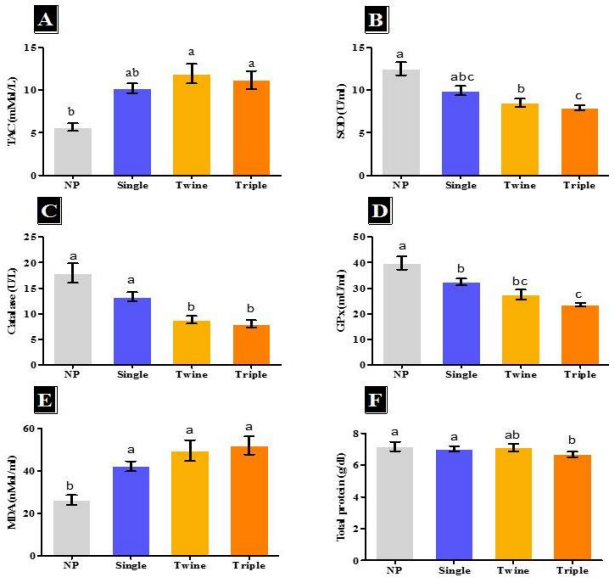

Figure 3. Serum concentration of total antioxidant capacity (TAC, A), Superoxide dismutase (SOD, B), Catalase (C), Glutathione peroxidase (GPx, D), Malondialdehyde (MDA, E), and total protein (F) in non-pregnant, non-pregnant (NP), single, twine and triple does fetuses. Values (mean ± SEM) with different letters were significantly different at p< 0.05.

Maternal serum-free RNA

The mean serum free RNA levels in maternal circulation were 9.22 ± 0.04, 12.98 ± 0.07, 15.03 ± 0.37, and 17.04 ± 0.15 ng/µl in non-pregnant, singleton, twine, and triple pregnancy, respectively (Fig. 2C). The levels of serum-free RNA augmented stepwise with a fetal number and were significantly (P< 0.001) higher in pregnancy compared to non-pregnancy.

Correlation between fetal growth rate, biochemical parameters, and serum-free RNA

Correlation between estimated biochemical parameters (total proteins, GPx, CAT, MDA, SOD, TAC, E2, and P4) in does is presented in table (1). RNA was negatively correlated with total proteins, GPx, CAT, E2, and positively correlated with MDA, TAC, and P4. Total proteins were negatively correlated with MDA, TAC, and P4, and positively correlated with GPX, CAT, and SOD. GPx was negatively correlated with MDA, TAC, P4, and positively correlated with CAT, SOD, and E2. CAT was negatively correlated with MDA, TAC, P4, and positively correlated SOD and E2. MDA was negatively correlated with SOD and positively correlated with P4. SOD was negatively correlated with TAC and P4 and positively correlated with E2. TAC was negatively correlated with E2 and positively correlated with P4. E2 was negatively correlated with P4.

Table 1. Correlation between estimated biochemical parameters in does.

	RNA	TP	GPX	CAT	MDA	SOD	TAC	E2	P4
TP	-0.67**								
GPX	-0.89**	0.66**							
CAT	-0.87**	0.48*	0.74**						
MDA	0.78**	-0.73**	-0.66**	-0.71**					
SOD	-0.88**	0.78**	0.87**	0.72**	-0.76**				
TAC	0.58**	-0.28	-0.69**	-0.57**	0.38	-0.55*			
E2	-0.64**	0.42	0.73**	0.58**	-0.41	0.77**	-0.73**		
P4	0.96**	-0.69**	-0.91**	-0.81**	0.74**	-0.86**	0.67**	-0.67**	
BPD	-.12	0.12	0.05	0.19	-0.28	0.15	0.36	-0.33	0.10

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION

Poor incomes farmers try to invest in goat rearing and breeding due to their high prolificity. The average litter size of goats varies from 1.6 to 2.0 (Amoah et al., 1996), with the rate of multiple births reaches up to 68.39% (Haldar et al., 2013). Nevertheless, scarce work is available concerning the effect of litter size on fetal growth and maternal steroid hormones, oxidative stress, and serum-free RNA in goats. Our data showed that the fetal number affected progesterone levels and disturbed the dam oxidative status (through decreasing antioxidant enzymes) that are reflected in the increase of total antioxidant capacity, lipid peroxidation, and serum-free RNA.

Farmers in the first ultrasound examination of their animals need to know if they are pregnant or not. Following this, they requested to affirm if there are single and multiple pregnancies. However, they ignore the influence of the fetal number on their growth rate, which will consequently impact the reproductive wastage and the post-natal survival rate in association with poor feed supplementation of the pregnant does (Osuagwuh and Aire, 1990). The evaluation of fetal growth and development can be accomplished primarily through fetal biometry (Manning, 1999), specially biparietal diameters as one of the basic biometric parameters that can be measured during mid-and late gestation (Gouda et al., 2021). In the current study, we noticed slight numerical differences in the growth rate between single, twin, and triple feti during the mid-gestation period. Such difference might be attributed to the limitation of uterine size compared to the continuous fetal growth and/or size. This is in agreement with Reichle and Haibel (1991), who showed that the difference in ovine fetal growth rate based on breed, sex, or litter size is minimal during the first half of gestation. Perhaps such variation might reach a statistically significant level if the animals were examined during the late gestation period as the inconsistencies in size between kids were not present until late in the pregnancy. On the other hand, we noticed up to a 10% reduction rate in fetal growth in twine and triple feti to a singleton pregnancy. Likewise, Mellado et al. (2011) showed that the litter weight moderately increases in goats at a reduced rate with increases in fetal number.

Concerning the influence of litter size on maternal E2 and P4 levels during mid-gestation, the present data showed that E2 levels significantly decreased with pregnancy regardless of the litter size, while P4 levels increased significantly ($P< 0.01$) stepwise with a fetal number. Manalu et al. (1996) reported that maternal serum progesterone and estradiol concentrations can be used as a strong indicator for the selection of goats with higher litter size. Castagnino et al. (2015) conveyed that circulating E2 levels increased with the advance of pregnancy but were not affected by the type of pregnancy. Yazici et al. (2018) reported that Day 51 of gestation is the earliest time for the detection of a significant difference in serum P4 concentration between single and twin pregnant cases. On the contrary, during early pregnancy, from Day 7-51 of gestation, there was no effect of type of pregnancy (single or twin fetuses) on plasma P4 concentration (Singh et al., 2019).Concomitantly, former studies declared the positive correlation between P4 concentration, the litter size (Castagnino et al., 2015; Abd El Hameed et al., 2018), number of lambs or kids born (Boscos et al., 2003), number of CL (Samartzi et al. 1995) and CL diameter (Gür et al. (2011).

Pregnancy is well-known to increase oxidative stress, a phenomenon generated as consequence of high amounts of circulating ROS and the imbalance between pro-and antioxidant processes (Nawito et al.,2016). Oxidative stress can cause damage to body tissues/organs and predispose to several diseases/disruptions over time. Such status represents a constrain against successful goat reproduction. In the current study, TAC (a marker of oxidative stress) and MDA (a marker of lipid peroxidation) increased significantly with the decrease of antioxidant enzymes (SOD, CAT, GPx) in multiple pregnancies than single and non-pregnant cases. TAC is an indicator of the antioxidant capacity of the body to counteract oxidative damage. Lipid peroxidation (MDA level) is credited in association with an increased oxygen requirement and circulating lipids (Gur et al., 2011). Abdel-Ghani et al. (2016) found that multiple bearing goats had high TAC values during the 3rd and 4th months of pregnancy. Antioxidant enzymes play an essential role in reducing the excess of the liberated ROS. However, this mechanism was not enough with the stress of multiple pregnancies (Ognik et al., 2015; Nawito et al., 2016), and might predispose to the decrease of fetal viability in favor of the developed oxidative stress.

Maternal cell-free RNA is considered as a new non-invasive marker for the diagnosis of prenatal disorders and pregnancy-associated diseases (Tsui et al., 2002; Lo et al., 2007). The present study showed that the levels of serum-free RNA increased stepwise with a fetal number and were significantly higher in pregnancy compared to non-pregnancy. Ge et al. (2011) found that several circulating micro RNAs in maternal plasma were validated that remarkably changed in twin pregnancy, and they suggested that miRNAs might involve the process of pregnancy such as the generation of twin pregnancy. These data also suggested the specific miRNAs could be used as potential biomarkers for the prognosis of pregnancy status and helps in establishing the therapy for pregnancy complications such as preeclampsia.

CONCLUSIONS

In conclusion, the fetal number significantly affected the fetal growth rate indices and circulating progesterone levels and serum-free RNA in goats and is responsible for the pregnancy-associated with oxidative stress. It is highly recommended to diagnose the litter size early enough during the gestation period to avoid oxidative stress-related disorders during late pregnancy e.g., pregnancy toxemia which impacts dam health and fetal viability and decreases post-natal newborn survival.

Conflict of interest

No conflict of interest

Author contributions

Mohamed M.M. Kandiel, Karima Gh. M. Mahmoud and Omnia. M.Z. El-sayed designed and carried out the study. Sally Ibrahim analysed the data, Mahmoud E.A. Abou-El-Roos aided in the interpretation of the results. Mohamed M.M. Kandiel and Omnia. M.Z. El-sayed wrote the manuscript. All authors approved the final version of the paper.

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REFERENCES

1. Abdel-Ghani MA, El-Sherry T M, HayderM, Abou-Khalil NS. Profile of peroxidative injury and antioxidant indicators in singleton, twins and multiple bearing goats throughout pregnancy. Asian Pacific J Reprod. 2016; 5(5):400-405.
2. Abd El Hameed A R, Mahmoud K Gh M, Nawito M F, Sosa ASA, Ezzo O H. Progesterone concentration, oxidants and antioxidants indices in singleton and multiple bearings ewes and does. Bios Res.2018; 15(4):3881-3887.
3. Amoah EA, Gelaye S, Guthrie P, Rexroad CE. Breeding season and aspects of reproduction of female goats. J Anim Sci. 1996; 74:723–728.
4. Boscos CM, Samartzi FC Lymberopoulos AG, StefanakisA, Belibasaki S. Assessment of progesterone concentration using enzymeimmunoassay, for early pregnancy diagnosis in sheep and goats. Reprod Domest Anim. 2003; 38:170–174.
5. Bradford GE. Selection for litter size. In: R.B. Land and D.W. Robinson (Eds). Genetics of Reproduction in Sheep, Butterworth, London, 1985; pp. 3- 17.
6. Castagnino DS, Härter CJ, Rivera AR, Lima LD, Silva HGO, Biagioli B, Resende KT, Teixeira IAMA. Changes in maternal body composition and metabolism of dairy goats during pregnancy. Revista Brasileira de Zootecnia. 2015; 44(3), 92-102.
7. Celi P, Di Trana A, Claps S. Effects of plan of nutrition on oxidative stress in goats during the peripartum period. Vet J. 2010;184: 95-99.
8. Chim SS, Shing TK and Hung EC. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem.2008; 54: 482-490.
9. Ford EJH. Pregnancy toxemia. Diseases of Sheep. Martin, W.B. (Ed.). Blackwell Scientific Publications, London,1983; pp.147-151.
10. Ge Q, Li H, Yang Q, Lu J, Tu J, Bai Y. Sequencing circulating miRNA in maternal plasma with modified library preparation. ClinChim Acta.2011; 412(21): 1989-1994.
11. Gearhart M A, Wingfield W E, Knight A P, Smith J A, Dargatz D A, Boon J A, Stokes C A. Real-time ultrasonography for determining pregnancy status and viable fetal numbers in ewes. Theriogenology.1988; 30:323–337.
12. Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. J Biol Chem. 1949;177(2):751-766.
13. Góth L. A simple method for determination of serum catalase activity and revision of reference range. ClinChim Acta.1991; 196(2-3): 143-151.
14. Gouda AH, Agag MA, Kandiel MMM. Ultrasound guided fetometry in Egyptian sheep and goats. Benha Veterinary Medical Journal.2021;40 (1): 49-53.
15. Gür S,Türk G, DemirciE, Yüce A, Sönmez M, Ozer S, Aksu EH. Effect of pregnancy and foetal number on diameter of corpus luteum, maternal progesterone concentration and oxidant/antioxidant balance in ewes. Reprod Domest Anim.2011; 46: 289–295.
16. Haldar A, Pal SK, Chakraborty S, Hazorikaa M, Pan S, Majumdarc D, Biswas CK, Patra A, Mirmahmoudid R, Prakash BS. Endocrine markers for identifying prolificacy potential and predicting fetal number in goats. Anim Reprod Sci. 2013;140: 54–61.
17. Illsley NP, Caniggial, Zamudio S. Placental metabolic reprogramming: do changes in the mix of energy-generating substrates modulate fetal growth?.Int. J Dev Biol.2010; 54 (2-3): 409-419.
18. Kandiel MM, Watanabe G, Li JY, Manabe N, El Azab AI, Taya K. Physiological roles of inhibin in regulation of FSH secretion and follicular development during early pregnancy in goats. Domest Anim Endocrinol.2008; 35(2):157-163.
19. Koracevic D, Koracevic G, Djordjevic VS, Andrejevic S, Cosic VV. Method for the measurement of antioxidant activity in human fluids. J ClinPathol.2001; 54: 356–361.

20. Kumar P, Magon N. Hormones in pregnancy. Nigerian Medical Journal: Journal of the Nigeria Medical Association,2012; 53(4): 179–183.
21. Lo YM, Lun FM, Chan KC, Tsui NB, Chong KC. Digital PCR for the molecular detection of fetal chromosomal neuploidy. Proc Natl Acad Sci USA.2007;104(32): 13116-13121.
22. Malarmathi M, Vadivoo VS, Muthuramalingam T, Senthil Kumar TM A, Palanisammi A. Bovine prenatal sex determination using cell free fetal nucleic acid present in maternal plasma. Biolife Journal.2016;4(3):431-436.
23. Manalu W, Sumaryadi MY, Kusumorini N. Effect of fetal number on concentrations of circulating maternal serum progesterone and estradiol of does during late pregnancy. Small Rumin Res.1996; 23: 117–124.
24. Manning FA. Fetal biophysical profile. Obstet Gynecol Clin North Am.1999; 26(4):557-577.
25. Mellado M, Meza-Herrera CA, Arévalo JR, De Santiago-Miramontes MA, Rodríguez A, Luna-Orozco JR, Veliz-Deras FG. Relationship between litter birth weight and litter size in five goat genotypes. Anim Prod Sci.2011; 51(2): 144-149.
26. Menchaca A, Ungerfeld R. Reproductive Strategies for Goat Production in Adverse Environments. In: Simões J., Gutiérrez C. (eds) Sustainable Goat Production in Adverse Environments: Volume I. Springer, Cham,2017;pp. 71-88.
27. Myatt L. Reactive oxygen and nitrogen species and functional adaptation of the placenta. Placenta.2010;31(Suppl.): S66- S69.
28. Nagy B. Cell-free nucleic acids in prenatal diagnosis and pregnancy-associated diseases. Int. Fed Clin Chem J.2019; 30(2): 215-223.
29. Nawito MF, Abd El Hameed AR, Sosa ASA, Mahmoud KGM. Impact of pregnancy and nutrition on oxidant / antioxidant balance in sheep and goats reared in South Sinai, Egypt. Veterinary World.2016;9: 801-805.
30. Nishikimi M, Rao A , Yagi K .The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys ResCommun.1972;46: 849–854.
31. Ohkawa H,Ohishi W, Yagi K. Assay for lipid peroxides in animal tissues by Thiobarbituric acid reaction. Anal Biochem. 1979;95: 351–358.
32. Ognik K, Patkowski k, Gruszecki T, Kostro K. Redox status in the blood of ewes in the perinatal period and during lactation. Bull Vet InstPulawy.2015;59:557-561.
33. Osuagwuh AI, Aire TA. Intrauterine growth rates of the west African dwarf goat and some foetal organs in relation to strategic feed supplementation during pregnancy. Zentralbl Veterinarmed A. 1990;37(3):198-204.
34. Olfati A, Moghaddam G, Bakhtiari M. Diagnosis, treatment and prevention of pregnancy toxemia in ewes. Int J AdvBiolBiomRes.2013; 1(6): 1452-1456.
35. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med. 1967;70: 158-169.
36. Reichle JK, Haibel GK. Ultrasonic biparietal diameter of second trimester pygmy goat fetuses. Theriogenology.1991;35: 689-694.
37. Samartzi F, Belibasaki S, Vainas E, Boscos C. Plasma progesterone concentration in relation to ovulation rate and embryo yield in Chios ewes superovulated with PMSG. AnimReprodSci.1995; 39: 11–21.
38. Singh SP, Ramachandran N, Sharma N, Goel AK, Gururaj K, Kharche SD. Temporal changes in plasma profile of pregnancy-associated glycoprotein, progesterone and estrone sulfate associated with fetal number during early- and mid-pregnancy in goats. AnimReprodSci.2019;205:115–125.
39. Tsui NB, Ng EK, Lo YM. Stability of endogenous and added RNA in blood specimens, serum, and plasma. Clin Chem.2002;48(10), 1647- 1653.
40. Yazici E, Ozenc E, Celik HA, Ucar M. Ultrasonographic foetometry and maternal serum progesterone concentrations during pregnancy in Turkish Saanen goats. Anim Reprod Sci. 2018;197: 93–105.