Changes in Serum Cortisol Level During Pregnancy in Ewes and The Effect of Fetal Number

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Abstract

This study was carried out to monitor serum cortisol level in pregnant ewes and comparing it with the non-pregnant values. Also, cortisol levels were monitored in single-fetus bearing and twin-fetus bearing ewes. This study was carried out during the breeding season on 31 pregnant and 5 non-pregnant ewes. Pregnancy and fetal numbers were determined through transrectal ultrasonography. Blood samples were collected every two weeks starting at day 30 of pregnancy until day 135 of pregnancy. Blood samples were collected in parallel from non-pregnant ewes as a control group. Cortisol was measured using ELISA.The results showed that serum cortisol levels increased gradually in pregnant ewes until day 90 of pregnancy reaching the highest level at day 135. Serum cortisol level was 4.26 ± 0.16 ng/ml at day 135 of pregnancy, while its level in non-pregnant ewes was 1.85 ± 0.15 ng/ml. Cortisol level was little bit higher in primiparous than pluriparous ewes, however, the difference was not significant. Regarding the effect of fetal number on cortisol level, twin-fetus bearing primiparous ewes had the significantly highest level. In addition, twin-fetus pulriparous ewes had a higher cortisol level than single-fetus bearing ewes.

In conclusion, cortisol level increased gradually during pregnancy until day 90 reaching the highest level at day 135 of pregnancy. In addition, cortisol level was significantly higher in twin-fetus bearing ewes than single-fetus bearing ewes. This indicates that twinning increases stress on the pregnant ewes especially in primipara which need more care and attention.

Keywords: Cortisol, Ewes, Pregnancy

Introduction

Cortisol is a glucocorticoid hormone produced by the adrenal cortex. It is released in response to stress and low level of blood glucose. Circulating concentrations of cortisol are elevated in many mammalian species including humans during pregnancy (**Carr *et al, 1981***). Pregnancy may represent a physiological stress which increases maternal plasma cortisol ***(Keller-Wood, 1996 & 1998)*** in pregnant ewes to double values in late gestation ***(Bell et al, 1991).***

The secretion of cortisol is ultimately controlled by the central nervous system. Stress induces the hypothalamus to release corticotrophin resealing hormones which stimulates the anterior pituitary to release adrenocorticotrophic hormone (ACTH). ACTH travels through the blood stream to adrenal cortex and increases synthesis and release of cortisol. Numerous studies have demonstrated the importance of this endocrine axis as a central feature of the fetal stress response, as well as for coordinating readiness for birth with the timing of birth ***(Challis et al, 2001; Whittle et al, 2001& Wood, 2005).*** In the late phase of gestation the fetus triggers the onset of parturition through increased activity of hypothalamic-pituitary-adrenal axis. There are increases in the concentration of ACTH followed by a rise of cortisol secretion in the fetal circulation. The increase of adrenal cortex sensitivity to ACTH also induces placental estrogen production.

Cortisol is rapidly synthesized and secreted in response to ACTH; this is part of a response to stress cortisol is a regulator of glucose in ruminants, which acts to increase gluconeogenesis from amino acids. In starving ruminants the gluconeogenesis is maintained by elevated levels of glucocorticoids ***(Azab and Abdel-Maksoud, 1999)***. In lactating ruminants the rate of hepatic gluconeogenesis and the relative concentrations of glycogenic precursors regulate the level of milk production ***(Huntington, 1990).***

Common challenges met by grazing pregnant sheep are under nutrition leading to a loss of body reserves, may be due to poor pasture quality and growth during the winter months and lack of adequate nutritional supplementation. Pregnancy is a metabolically demanding physiological state and could increase the animals' vulnerability to environmental challenges.

Challenges that threaten energy homeostasis, such as cold exposure, will normally lead to activation of the hypothalamic-pituitary-adrenal axis resulting in the release of cortisol ***(Chrousos, 2009; Sapolsky et al, 2010).*** Cortisol facilitates the mobilisation of energy substrates and supports energy homeostasis. However, hypothalamic-pituitary-adrenal axis responses to stressors are attenuated during pregnancy ***(Brunton et al, 2008),*** which could have implications for pregnant animals exposed to cold challenges.

Previous studies demonstrated that the ovine fetus is adversely affected when maternal cortisol concentrations are decreased to non-pregnant concentrations in late gestation; fetal growth rate is reduced, with reduced blood pressure and an increased incidence of hypoxia ***(Jensen et al, 2002 & Jensen et al, 2005).***

Adrenocortical activity is involved during physical ***(Ferlazzo and Fazio, 1997)*** and psychological ***(Fazio et al, 2008 & 2009)*** challenges in domestic animals through the release of cortisol. Although homeostatic mechanisms function to keep substrates in the blood at comparatively constant concentrations, some changes in adrenocortical and biochemical values are likely to occur in different species, like camel, sheep and goat ***(Alila-johansson et al, 2003; Nazifi et al, 2003; Yildiz et al, 2005 & Saeed et al, 2009).***

Remarkable neuroendocrine changes occurs during pregnancy, optimizing fetal growth and development, protecting the fetus from adverse exposures, and preparing the mother for timely parturition. More specifically, gestation dramatically affects the maternal hypothalamic-pituitary-adrenal axis, leading to increased basal levels of corticotropin-releasing hormone, adrenocorticotropin (ACTH), cortisol, in human plasma ***(Lindsay and Nieman, 2005).***

The hypothalamic-pituitary-adrenal axis is one of the major systems involved in stress response and its regulation. The hypothalamic-pituitary-adrenal axis is activated during stress and threat ***(Weinstock, 2005)*** and studies have looked at the concentrations of its end product, cortisol, as an endocrinological marker of stress and anxiety ***(Weinstock, 2008).*** It has been suggested that the activation of the hypothalamic-pituitary-adrenal axis is one of the main biological mechanisms underlying the effects of prenatal stress ***(Huizink et al, 2004; Talge et al, 2007).***

Fetal growth is metabolically constrained ***(Vatnick et al, 1991 & Gluckman et al, 1992)*** and increasing fetal size in late gestation places greater demands on maternal metabolism and transplacental transfer of nutrients ***(Schneider, 1996).*** Poor maternal weight gain, fetal growth restriction and maternal fasting have all been identified as factors associated with premature delivery in humans and domestic species ***(Silver 1990, Ott 1993; Hediger et al, 1995 & McMillen et al, 1995).***

Placental hormones are required for the establishment and maintenance of pregnancy, adaptation of the maternal organism to pregnancy, growth and well-being of the fetus, as well as development of the endocrine mechanisms involved in parturition ***(Challis et al, 2001 & Whittle et al, 2001).***

The interaction between estradiol and the fetal hypothalamic-pituitary-adrenal axis might function as a positive feedback loop that enables an increase in concentrations of estradiol and cortisol before birth ***(Whittle et al, 2001; Pasqualini, 2005 & Wood, 2005).***

Physical and psychological Stressors also activate the hypothalamo-pituitary-adrenal axis and lead to marked and persistent increase in serum concentrations of glucocorticoids ***(Guillaume et al, 1992 & Komesaroff and Funder, 1994).*** The augmented glucocorticoid secretion induced by stress may contribute to the reduction in fertility commonly associated with stress.

In farm animals, plasma cortisol has become a widely used parameter for measuring stress. However, only few data dealing with the effect of fetal number on cortisol level were traced. The aim of the present study was to monitor changes in serum cortisol level in ewes during pregnancy. In addition, the effect of fetal number on cortisol level was also studied.

Materials and methods

Animals and experimental design:

This study was carried out on 36 apparently healthy ewes on a private farm under field condition in AL-Bayda city, north Libya during the breeding season starting from September 2013 until February 2014. Al Bayda city (32° 45' N, 21° 44' E) found in the El- Gabal Akhdar in which the highest point of the green mountain is about 850 meters above the sea level.

The age of the ewes were ranged from 1.5 to 7 years old and their body weight ranged from 30 to 50 kg. All animals were kept under the same environmental and nutritional conditions during the period of study. Animals were fed on natural grazing in addition to concentrates (1 kg for each ewe).

Ewes were separated from males and estrus was synchronized by injecting each ewe with 0.5 ml Estrumate i.m. (125 µg Cloprostenol, Schering- Plough, Animal Health, UK). After estrus detection, ewes were allowed to be mated using fertile males except 5 ewes to be used as a non-pregnant control. Pregnancy and fetal numbers were diagnosed using transrectal ultrasonography starting at day 25 post-mating according to ***Medan et al (2010).*** This study was designed as follow:

- Group 1: Primiparous and single-fetus bearing (10 ewes),
- Group 2: Primiparous and twin-fetus bearing (4 ewes).
- Group 3: Pluriparous and single-fetus bearing (10 ewes).
- Group 4: Pluriparous and twin-fetus bearing (7 ewes).
- Group 5: non-pregnant (5 ewes).

Blood samples and cortisol analysis:

Blood samples were collected from jugular vein into vacutainer tubes without anticoagulant from all animals in the examined groups in the morning before going out for natural grazing. Blood samples were collected every two weeks starting at day 30 until day 135 of pregnancy. Serum was separated and stored at – 20 ºC until assay.

Cortisol was measured by a solid phase competitive ELISA. The commercial ELISA kits were used to measure concentration of cortisol according to the manufacturer's instructions.

Statistical analysis:

The obtained data were statistically processed and expressed as mean ± standard error of mean of each analyzed group. The differences between the obtained values were statistically analyzed by Student's t-test in case of comparing between two groups or one-way ANOVA in case of comparing between more than two groups and using Duncan's multiple range test as a posthoc. The differences between groups were considered significant when $P < 0.05$. SPSS statistical package software was used for statistical analysis (SPSS, 2012).

Results

Mean serum cortisol level in pregnant and non-pregnant ewes:

The mean serum cortisol levels in non-pregnant and pregnant ewes during the period of study are shown in Fig. 1. There were no clear changes in cortisol level in non-pregnant ewes except a minor increase at day 105 (during December) and 120 (during January). However, cortisol level was significantly higher in pregnant ewes compared with the non-pregnant. In pregnant ewes, there was a gradual increase in cortisol level from day 30 of pregnancy until day 75. On the other hand, there was an abrupt increase at day 90 of pregnancy and continued until day 135. The mean serum cortisol level was significantly (P<0.01) higher 4.26 ± 0.16 ng/ml in pregnant ewes at day 135, while it was 1.85 ± 0.15 ng/ml in non-pregnant ewes.

Effect of fetal number on cortisol level in pregnant ewes:

The data presented in table 1 showed that serum cortisol level in single-fetus bearing and twin-fetus bearing ewes. Also, changes in cortisol levels in primiparous and pluriparous pregnant ewes are shown in Fig. 2.

Serum cortisol level was significantly higher in primiparous twin-fetus bearing ewes compared with other groups (Table 1). Also, mean serum cortisol level was higher in pluriparous twin-fetus bearing ewes than single-fetus bearing ewes.

As shown in Fig.2, the mean cortisol levels were slightly higher in pluriparous ewes than primiparous ewes except at days 60 and 90 of pregnancy. However, the differences between them are not significant.

Cortisol levels in solitary cases of abortion in ewes:

Figure 3 shows cortisol levels in four cases of abortion occurred during the present study. Three ewes carrying twin-fetus and one case carrying single fetus. The level of cortisol were higher in aborted cases in comparison with non-aborted cases, however, the exact cause of abortion was not investigated. Abortion occurred at day 105 of pregnancy in three cases and at day 120 in one case.

Table 1: *Cortisol level (mean ± SE) during pregnancy in primiparous (single-bearing & twin-bearing) and pluriparous (single-bearing & twin-bearing) ewes.*

Day of pregnancy	Primiparous ewes		Pluriparous ewes	
	Single-bearing (n=10)	Twin-bearing (n=4)	Single-bearing (n=10)	Twin-bearing (n=7)
30	1.95 ± 0.08 [a]	2.63 ± 0.24 [b]	1.87 ± 0.06 [a]	1.97 ± 0.12 [a]
45	2.18 ± 0.22 [b]	3.08 ± 0.18 [c]	1.97 ± 0.07 [ab]	2.50 ± 0.23 [b]
60	2.47 ± 0.36 [ab]	3.60 ± 0.21 [c]	2.61 ± 0.24 [abc]	2.10 ± 0.29 [bc]

75	2.56 ± 0.26 [a]	3.97 ± 0.17 [b]	2.52 ± 0.16 [a]	2.93 ± 0.38 [a]
90	3.50 ± 0.20 [a]	5.15 ± 0.43 [b]	4.09 ± 0.25 [a]	4.43 ± 0.43 [ab]
105	3.25 ± 0.31 [a]	6.15 ± 0.27 [b]	3.33 ± 0.17 [a]	4.17 ± 0.65 [a]
120	3.37 ± 0.14 [a]	5.76 ± 0.32 [b]	3.41 ± 0.07 [a]	4.12 ± 0.68 [a]
135	3.99 ± 0.21 [a]	5.75 ± 0.22 [b]	4.12 ± 0.23 [a]	4.14 ± 0.32 [a]

[a,b,c] Means with different superscripts are significantly different ($P<0.05$) within the same row.

**Indicates significant differences ($P<0.01$) between mean cortisol values in pregnant and non-pregnant ewes

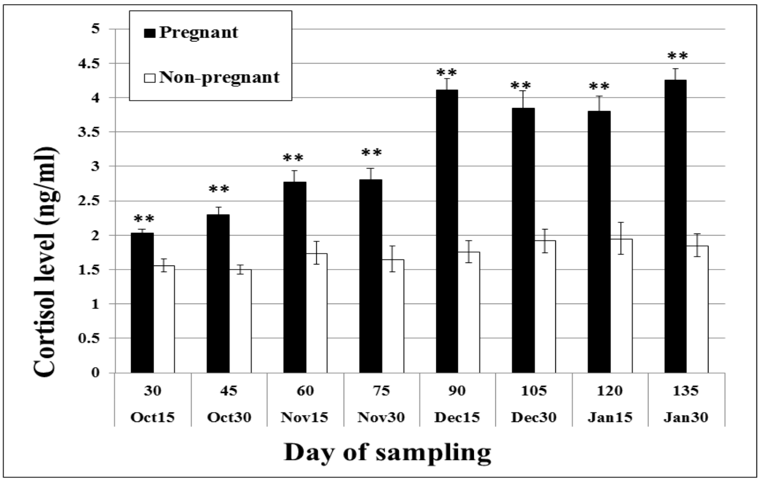


Fig 1: *Changes in serum cortisol level (mean ± SE) in pregnant and non-pregnant ewes.*

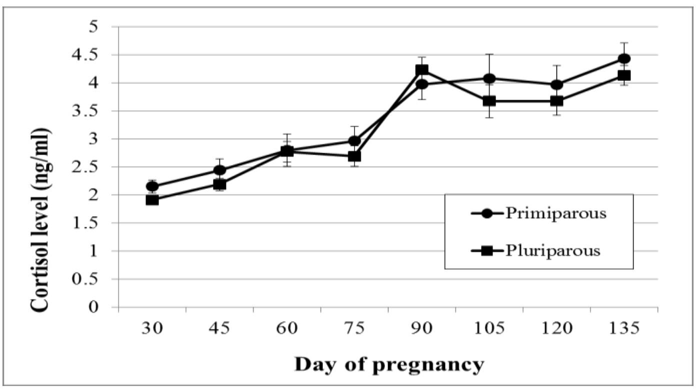
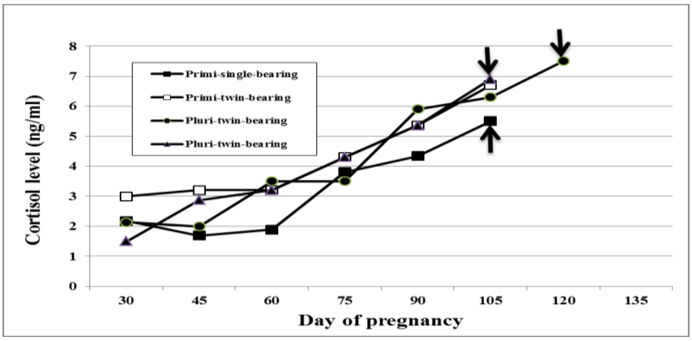


Fig 2: Serum cortisol level (mean ± SE) in primiparous and pluriparous pregnant ewes.



Arrows indicate days of abortion.

Fig 3: Serum cortisol level (mean ± SE) in solitary cases of abortion in ewes.

Discussion

It is well established that the secretion of stress hormone (cortisol) increases during stress ***(Barglow et al, 1985).*** Previous study had shown that in the sheep, the prepartum increase in circulating cortisol is required for the differentiation and maturation of fetal organs such as the fetal lung, liver, kidney, and brain and for the normal timing of parturition and the successful transition to extrauterine life ***(Challis et al, 2000). Forhead et al (2002)*** reported that in the sheep fetus, plasma cortisol and leptin concentrations increased in parallel and were positively related between 130 and 140 days gestation and that fetal adrenalectomy resulted in lower plasma leptin concentrations after 136 days. Fetal cortisol levels, however, can be elevated prematurely during late gestation by adverse intrauterine conditions, such as cord compression, hypoxemia, and undernutrition ***(Gardner et al, 2001 & 2002).*** In addition, raising fetal cortisol levels by exogenous infusion before term lowers placental delivery of glucose to the fetus and alters placental enzyme activities and hormone synthesis ***(Clarke et al, 2001 & Ward et al, 2004).***

The prepartum rise in fetal cortisol had an effect on increased P450 C17 activity in ovine placenta. As a result, C21 steroids (pregnenolone, progesterone) reaching the placenta could be metabolized to C19 steroids decreasing placental progesterone production. When the progesterone:estradiol-17β ratio is decreased, pregnancy is threatened ***(Challis et al, 2001 & Weems et al, 2007).***

Circulating cortisol concentrations obtained in the present study were in the physiological range reported in the previous studies in ewes. However, cortisol level obtained in this study was high in the twin-fetus bearing ewes, which indicate that pregnancy in twins may be a physiological stress on the mother and needs special attention and care.

In pregnant ewes, endogenous cortisol increases fetal glycogenesis when secreted during late pregnancy and increases as part of normal signaling prior to parturition ***(Fowden, 1990).*** However, there is a species variation regarding cortisol changes during pregnancy especially in mares. Previous results reported in mares and Jennies revealed a different trend of cortisol levels, since lower concentrations were detected in pregnant mares and Jennies compared with non-pregnant ***(Gill et al, 1985 & Fazio et al, 2011).*** There was a progressive decrease of circulating cortisol concentrations from the 3rd to the 10th month of pregnancy in mares, as reported by ***Flisińska-Bojanowska et al (1991)*** these results could be related to the negative correlation reported for equine species ***(Asa et al, 1983)*** between plasma cortisol and estrogen levels, which increase from the 3rd to the 7^{th} 8th months in pregnant mares ***(Nett et al, 1975 & Noden et al, 1978).*** The higher cortisol levels in the 11th month of pregnancy could be related to equine fetus production at the end of the pregnancy, according to the decrease of maternal progesterone concentrations. However, fetal adrenocortical glands are rich in 17α-hydroxylase, which modifies pregnenolone in cortisol only after the 310th day of pregnancy ***(Chavatte et al, 1995; Ousey, 2004 & Ousey et al, 2005).***

During mammalian pregnancy, except rodents, the concentration of cortisol in mother's blood is much higher than that in the fetus ***(Whittle et al, 2001).*** In sheep, 90% of cortisol in the fetal circulation is of maternal origin at the time when fetal adrenals begin cortisol production, i.e. close to term, and then fetus becomes the primary source of circulating glucocorticoids ***(Burton and Waddell, 1999).*** Between the mother and her fetus, only placenta is a barrier apart from uterus which prevents excess of maternal cortisol crossing into the fetus ***(Whittle et al, 2001).*** At the end of pregnancy cortisol predominates, which is explained by the important biological role of this hormone during the prenatal period ***(Alfaidy et al, 2003).***

Previous studies mentioned that there was a relationship between serum cortisol and glucose level. The studies indicated that low glucose uptake were accompanied by a fall in the transplacental glucose concentration and an increase in placental glucose consumption, which probably were secondary to activation of fetal glucogenesis ***(Barbera et al, 1997& Ward et al, 2004).*** The hypothesis that cows suffering from stress and/or painful diseases have elevated blood glucose levels due to an increase in serum cortisol ***(Forslund et al, 2010).***

In the present study, cortisol level increased abruptly to a higher level after day 90. This abrupt elevation in cortisol level may be attributed to contribution from the fetus. In support of this hypothesis, ***Challis et al (2001)*** *and* ***Whittle et al (2001)*** have demonstrated that the fetal hypothalamic-pituitary-adrenal is not responsive until around day 60 of gestation.

Increased cortisol level not only affects pregnancy, but also affects ovarian function in non-pregnant animal. A previous study indicated that infusion of sheep with stress-like concentrations of cortisol suppress ovarian function and block or delay follicular development and the preovulatory surge of LH ***(Daley et al, 1999).*** Other studies indicated that one sign of the stress response is the close association of reproductive suppression and activation of the hypothalamo-pituitary-adrenal axis leading to enhanced glucocorticoid secretion ***(Sapolsky et al, 2000 & Tilbrook et al, 2000).***

Cortisol which is used as an indicator of stress, is an anti-inflammatory agent that act to modulate the production of cytokines and prostaglandins required for ovulation, embryo implantation, fetal growth and placental development ***(Andersen, 2002).*** Animal studies also provide good evidence that prenatal stress can have long-lasting effects on the offspring ***(Weinstock, 1997& 2005).***

A previous study has shown that loss of the late pregnancy rise in maternal cortisol production in pregnant ewes, impairs fetal growth and higher cortisol concentration is required for fetal growth ***(Jensen et al 2011)***. In the present study, cortisol level increased in late pregnancy and this is important for normal fetal growth. However, the cortisol level was higher in the aborted cases recorded in this study. Although the exact cause of abortion is not investigated, it is clear that cortisol level increased before abortion.

In the present study, there was a minor elevation of cortisol in the non-pregnant ewes during December and January (cold months in the El- Gabal Akhdar, the area of the present study). That Increase in serum cortisol in non-pregnant ewes could be explained by cooling of the weather. Cooling of weather may create some discomfort and stress and increases cortisol secretion ***(Damjanovic, 2008).***

In conclusion, this study demonstrated that during pregnancy in ewes, there was a progressive increasing in serum cortisol. In addition, cortisol level was significantly higher in twin-fetus bearing ewes than in single-fetus bearing ewes. Cortisol level was highest in primiparous ewes carrying twins. This indicates that twining is considered as a physiological stress on the mother especially in primipara and needs more attention and care.

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التغيرات في مستوي هرمون الكورتيزول في سيرم الأغنام العشار و تأثير عدد الأجنة

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الملخص العربي

أجريت هذه الدراسة لرصد مستوى الكورتيزول في السيرم للنعاج العشار و مقارنتها بمستواه في النعاج الغير عشار تحت الظروف الحقلية. أيضا مستويات الكورتيزول تم رصدها في النعاج التي تحمل في توأم أو حمل فردي إضافة إلى النعاج التي تحمل لأول مرة (بكرية) و متكررة الولادات.أجريت هذه الدراسة أثناء موسم التناسل على عدد ٣١ نعجة عشار و ٥ نعاج غير عشار. تم تشخيص الحمل و تحديد عدد الأجنة بإستخدام الموجات فوق الصوتية عبر المستقيم. تم تجميع عينات الدم مرة كل أسبوعين إبتداء من اليوم ٣٠ من الحمل و حتى اليوم ١٣٥. تم تجميع عينات دم موازية من نعاج غير عشار كمجموعة ضابطة و فصل السيرم و حفظه مجمدا عند درجة حرارة ٢٠ تحت الصفر حتى إجراء التحليل.أظهرت النتائج إرتفاع تدريجي في مستوى الكورتيزول أثناء الحمل حتى اليوم ٩٠ و وصلت إلى أعلى مستوى عند اليوم ١٣٥ من الحمل. متوسط مستوى الكورتيزول عند اليوم ١٣٥ من الحمل كان ٤,٢٦ ± ٠,١٦ نانو جرام/ مل مقابل ١,٨٥ ± ٠,١٥ نانو جرام/ مل في النعاج الغير عشار. كان مستوى الكورتيزول أعلى قليلا في النعاج البكرية عن مستواه في النعاج ذات الولادات المتكررة و لكن الفرق غير معنوي. بالنسبة لتأثير عدد الأجنة على مستوى الكورتيزول، أظهرت النعاج البكرية التي تحمل في توأم أعلى المستويات. إضافة إلى أن المجموعة متكررة الولادات و تحمل توأم أظهرت إرتفاعا في مستوى الكورتيزول عن النعاج التي تحمل حمل فردي.

الخلاصة: مستوى الكورتيزول إزداد تدريجيا أثناء الحمل حتى اليوم ٩٠ و وصل إلى أعلى مستوى عند اليوم ١٣٥. إضافة إلى أن مستوى الكورتيزول كان مرتفعا معنويا في النعاج ذات الحمل التوأم عن النعاج ذات الحمل الفردي. هذا يشير إلى أن الحمل في توأم يزيد من الإجهاد و خاصة على النعاج البكرية و تحتاج رعاية و إهتمام أكثر من النعاج ذات الحمل الفردي.