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Assessing Progesterone Levels in Awassi Ewes: A Comparison between Pregnant and Non-Pregnant, Bearing Twins, and Singletons During the First Trimester

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THIS study aimed to measure the concentration of progesterone (P4) in pregnant, mated/non-pregnant, and anestrous controlled Awassi ewes as well as in ewes carrying singletons and twins. A total of 35 ewes were used, which were subdivided into two groups: control (5 ewes) and treated (30 ewes). The estrus was synchronized in the treated group using P4-intravaginal sponge with eCG. All ewes were examined by ultrasound in different periods to confirm pregnancy status and fetal number. Blood samples were collected from ewes in both groups at four different time dates (15, 25, 35, and 50 days) post-insemination (PI). Results showed that P4 levels were significantly elevated ($P<0.05$) in pregnant (ranging from 4.90±0.21 - 7.55±0.23 ng/mL) compared to mated/non-pregnant ewes (ranging from 2.52±1.62 - 1.112±1.2 ng/mL) and control ewes (ranging from 0.53±0.15 - 0.579±0.3ng/ mL) during all four experimental periods. Additionally, the P4 concentrations of ewes with twin fetuses were significantly ($P<0.05$) elevated compared to ewes carrying only one fetus at days 35 (7.36±0.24 Vs 5.68±0.11) and 50 (8.89±0.13 Vs 7.36±0.24) PI, but not significantly before that. This study concluded that a threshold value of >4.9 ng/mL can be used to differentiate between pregnant and empty ewes from day 25 onwards. The human P4 kit was precise and showed significant differences between pregnant, mated/non-pregnant, and anestrous ewes (control) during all experimental periods. Furthermore, P4 concentrations were detected to be significantly higher in twin-bearing ewes compared to a singleton at day 35 PI and beyond.

Keywords: Progesterone, Pregnant, Twin, Singleton, Awassi ewes.

Introduction

Awassi is an Iraqi local breed ewes having good reproductive performance, conception rate and litter size [1]. Several proteins, minerals, electrolytes, enzymes and blood biochemical profile had changing during gestation in sheep [2,3].

Placenta is paracrine and endocrine organ, in ruminant, the main function of placenta is steroid hormones, proteins and growth factors production. Progesterone is main steroid hormone produced by placental cells to support the pregnancy in sheep after gestational day 50, before that, the pregnancy depend on P4 of CL (CL expressed autosomal genes were associated with P4 formation) [4]. Subsequently, the placenta becomes the fundamental source of P4 and its receptor [5], therefore, it's considered a nonspecific marker for pregnancy. During the

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early stages of pregnancy the P4 output varies depend on number of CL, number of embryos, size and embryos sex [6].

Plasma P4 profiles was mentioned by Ganaie et al. [7], the mean plasma P4 concentration increased from 1.4 ng/mL in period after ovulation until day 6 to 4ng/mL between 2-4 weeks for gravid ewes, while, the P4 level declined < 1ng/ mL in mated/non pregnant ewes. After this period, the P4 level increased progressively between the intervals 15-30 to 60–75, and reach a plateau level <14 ng/mL at the fourth month of gestation. Lastly, by the progress gestation, the P4 level declined slightly until lambing. The peripheral P4 levels reach to lower concentration within one

The P4 levels rise gradually through first two months when corpus luteum made a considerable to the total P4 produced by the mother through a large part of gestation, while placenta secretes little P4 (6.7-7.75 ng/ mL in 1st and 2nd months, respectively), then, the placenta could be make a major contribution to total P4 production in the pregnant ewes, therefore the P4 level steadily increased to platue (15.71 and 24.91 ng/ mL in 3rd and 4th months, respectively), after that, it decline gradually from 11.7 ng/ mL toward lambing [8].

Although, ultrasonography used practically to diagnosis pregnancy, fetal number and some physiological and pathological changes in ewes [9-12]. The P4 assay is a very valuable method for pregnancy determining in early, mid and late gestational stages [8,13]. On day 17 post conception, the pregnant ewes had P4 concentration ≤ 1.0, 1.5, 2.5 and 4.0 ng/mL. Predictive positive and negative values, sensitivity, accuracy of pregnancy diagnosis by progesterone assay on day 17 post breeding were higher with moderate specificity in threshold >4 ng/ml [14].

Progesterone concentration measurement at interval 17-21 post breeding considered a remarkable method for pregnancy determination in local Iraqi ewes. The finding of Mohammed et al. [15] illustrated that accuracy for P4 assay method was 88.5% in Iraqi local ewes at threshold 4.5 ng / mL at day 21 for pregnant ewes and lower than 1.9 ng/ mL for non-pregnant ewes. Rahman et al. [16] tested the accuracy of pregnancy detection in different pregnancy intervals (15 to 30 days, 31 to 45 days, 46 to 60 days and 61 to 75 days) depended on P4 level, their finding demonstrated that accuracy were ranged from 80% to 85% in conjunction with pregnancy progression.

Regarding the advantage for plasma P4 analysis in determining pregnancy, it also use to determine the fetal numbers in sheep. Many researches pointed out that plasma P4 level in multiples embryos carrying ewes were superior than ewes had single pregnancy from 3rd week three until last 10 days of gestation. A significant positive correlation was observed between P4 concentration and the number of lambs borne, the mean P4 concentration of the ewes carrying twins (12.8 ± 1.4 ng/ mL) was higher in comparison with those carrying single foetuses (11.3 ± 1.1 ng/ mL) at day 19 after sponge removal (day 16 post conception) [14]. Despite the fact that P4 assay had very high Se for gestation prediction, but it recorded low Serum progesterone (Sp) because its nonspecific for pregnancy before day 50.

The human P4 kit is a readily available tool that can be used for detecting pregnancy in veterinary research, and it has many advantages such as cost-effectiveness and the ability to produce accurate results in ewes [17]. Samples such as blood, milk and feces are used to monitor P4 levels in pregnant ewes [18,19]. Thus, this study was conducted to assess the peripheral plasma P4 levels in controlled, non-pregnant, and pregnant Awassi breed during the first trimester of pregnancy using the human P4 kit. Furthermore, the objective of this study was to determine a value of serum progesterone (P4) concentration, assessed using an enzyme-immunoassay (EIA), for the early distinction between pregnant and non-pregnant ewes.

Material and Methods

Ethical approval

The experiment was carried out under the conditions of Ethics Committee, Faculty of veterinary medicine, University, Alfallujah University.

Animal preparation and sample processing:

Thirty five multiparous Awassi ewes with an average age between 3-4 years were studied. All animals were housed in one flock with five breeding rams were used for heat detection and breeding with ratio 1:6. The experiment was conducted at Veterinary College (Fallujah University), latitude 33.37 and longitude 43.74. The experiment span extended from February to October 2022.

All experimental ewes were checked by 6.5 MHz trans-rectal and 4.5 MHz transcutaneous ultrasound (Chison ECO2/China), and it was

confirmed that they were empty. Subequently, the animal divided into two groups; control (5n) and treatment groups (30n). The estrus synchronized and pregnancy was inducted by using synchronization protocol; P4 (Intravaginal sponge) was inserted for 14 days+ PMSG 500 IU at time of P4 withdrawal and mated by the five breeding rams with a very high semen quality (concentration, motility as well as dead and live percentage) after two-three days from P4 sponge withdrawal.

All control ewes were periodically checked by ultrasonography (15, 25, 35, and 50 MHz) to ensure that they were not pregnant. Also, post-mating ewes were examined by transcutaneous ultrasonography on different periods to confirm the pregnancy status and litter size.

Progesterone assay

Blood collection

Five mL of blood was collected from control ewes at days (15, 25, 35, and 50) natural mating and from treatment group ewes at days (15, 25, 35, and 50) days post-mating. The blood was evacuated into gel tube (SABA/Jordon) and centrifuged immediately for 10 min and the plasma aspirated and kept at freezer until P4 assayed.

Plasma P4 concentration determination

The P4 level in the plasma was determined using the enzyme immunoassay (EIA) technique via Cobas E411 Analyzer (Roche/Switzerland) using human P4 enzyme immunoassay kits P4 III Reagent (Roche/Switzerland) with sensitivity >99%.

Statistical analysis

The data analysis was achieved via SAS (2018) (v9.6) [20]. The Least significant differences (LSD) and one way ANOVA were done to estimate the significant variations among means.

Results and Discussion

Measurement of progesterone in pregnant, non-pregnant and control ewes

The present study observed blood P4 levels in studied ewes corresponds to the pregnancy status. The mean plasma P4 concentration non significantly increased from 4.90±0.21 ng/ mL at day 15 to 7.55±0.23 ng/ mL at day 50 PI in pregnant ewes. The P4 levels were significantly rise in pregnant compared with same bred; natural breeding/non-pregnant (ranged 2.52±1.62-1.112±1.2 ng/ mL) and control ewes (ranged 0.579±0.3-0.53±0.15 ng/ mL) for the four experimental periods (15, 25, 35 and 50). The results showed a significant elevation in P4 levels for bred/non-pregnant in period 15-25 PI, and non-significant rise in days 35 and 50 PI compared to control. Generally, no significant differences were observed in P4 fluctuations for each bred/ pregnant, bred/non-pregnant and control ewes through the experimental period (Table 1).

As mentioned by Boscos et al. [14], the mean P4 concentration of ewes proven pregnant was significantly higher than that of non-pregnant ewes in days 19 after sponge removal (day 17 PI). There is satisfactory agreement between the present study and Gür et al. study [6], which showed that P4 concentrations in pregnant ewes were found significantly higher ($p < 0.001$) than in non-pregnant ewes (2.8 Vs 5-9 ng/ mL).

Alexander et al. [21] has already noted that mean P4 levels increased from less than 0.3 ng/ mL around ovulation to more than 3 ng/ mL on day 7 in both bred and non-bred ewes. The P4 level reach greater than 3 ng/ mL in pregnant ewes

TABLE 1. The mean levels (±SE) of P4 concentrations in pregnant/non pregnant ewes at different gestational periods

	Period/days	Pregnant (No=25)	Non Pregnant (No=5)	Control (No=5)
1	**15th**	A4.90±0.21^{c}	B2.52±1.62^{a}	C0.57±0.33^{b}
2	**25th**	A5.50±0.24^{c}	B2.24±1.95^{a}	C0.59±0.04^{b}
3	**35th**	A6.29±0.19^{a}	B1.16±1.08^{b}	C0.88±0.61^{b}
4	**50th**	A7.55±0.23^{a}	B1.11±1.20^{b}	C0.53±0.15^{b}
LSD		1.01		

Means with a different small letter in the same column are significantly different (P<0.05)

Means with a different capital letter in the same row are significantly different (P<0.05)

and less than 0.4 ng/ mL in all non-pregnant ewes at day 19 PI. Then, the level increased gradually to 7 ng/ mL on day 50 in pregnant ewes. Kose et al. [22] recorded that P4 level for pregnant ewes in day 15 around 4 ng/mL, then slightly decrease until day 25 PI, while, it rapidly dropped to less than 1 ng/mL in early embryonic mortality.

The present study agreed with DeNicolo et al. [23], who observed that mean P4 concentration reach to 5 ng/mL in pregnant ewes at day 14 PI, while decline from 3 to 1 ng/ mL between day 14-16 PI, a further decrease occur (< 1 ng/ mL) after day 16 in spring. These deviations between P4 concentrations of pregnant ewes and between the proposed discriminatory levels as compared with other authors' results may be attributed to small differences of the reference range existing between RIA and EIA [24], although both methods have been proved accurate for P4 determination.

Unlike control ewes, the P4 mean level for bred/non-pregnant and still elevated period 15 PI, because two ewes had elevated P4 concentrations (< 4 ng/ mL), two showed (< 2.5 ng/ mL) and one (< 1 ng/ mL) beyond day 15, After that, one ewes of high P4 concentration (< 4 ng/ mL) displayed slightly decline in P4 level, but still elevated along the experimental period (< 3.0 ng/ mL), while, the other one showed decrease in P4 level below 1 ng/ mL on day 35 PI. The other three ewes showed sharp decline to > 1.0 ng/ mL on days 25 to 50 PI. Control ewes failed to display elevated P4 concentrations (< 1.0 ng/ mL). There are several possible explanations for this findings; early embryonic mortality, failure of fertilization and persist CL in case of endometritis/pyometra may happened. Embryonic mortality may occur in the two ewes that had P4 level < 4 ng/ mL, one of them the P4 level decline to basal level in day 25(embryonic mortality may occur after day 15), and the another ewes not decline below 3 ng/ mL along experimental period, it may due to occurrence of embryonic death and have persist CL and/or endometritis/pyometra after day 25 PI. Also, the hormone level deviations might also exist because of differences in the ewes age, sampling and environmental factors (like climate or season).

This concurs well with previous findings of DeNicolo et al. [23] which revealed that five mated/non-pregnant ewes had elevated P4 concentrations beyond Day 16, the cause was explained by the occurrence of early embryonic death. High incidence of early embryonic death recorded in ewes, ranging from 5-22% in FGA-intravaginal sponges treatments [25] and about 20% from total pathological lesions of the ewes experienced embryonic and fetal loss [26].

Another possible reason for this is related to fertilization failure accompanied with sampling occur before luteolysis (day 15); 3 out of 5 in bred/non pregnant ewes recorded low P4 concentration (n=2 (< 2.5 ng/ mL) and n=1 (< 1 ng/ mL)) beyond day 15, fertilization failure is more sense in this case. The high level of P4 in day 15 PI (< 1 ng/ mL) may be due to several reasons, the most important of which is ovulation not occur at starting of estrus, and luteolysis happen during or after sampling (luteolysis occur during or after day 15). The timing of LH surge after estrus onset 10 hr (range 4-20 hr) in FGA+ eCG protocol, ovulation occur after 18 from onset of estrus [27,28]. While, the ovulation of natural estrus occurs about 36 hr after the onset of oestrus [29]. Additionally, the luteolysis started on day 15.3 -15.7 and complete in day 15.9 -16.3 in different ewes breed caused drop in P4 level [30].

Fertilization failure may occurs in experiment due to frequent ejaculation during a short period of time during use of estrus synchronization protocols. Some rams prefer to breed only one or two ewe several times over the course of the estrus phase, which leads to decrease sperm concentration, depletion of mature sperm and an increase in the percentage of immature sperm. There is satisfactory agreement between repeated ejaculation and poor semen quality, Kaya et al. [31] reported that progressive motility rates, sperm concentration decreased and abnormal sperm morphology increased with regards to elevated semen collection frequency. Additionally, Mandiki et al (1998) [32] mentioned that frequent ejaculations lowered semen quantity and this effect varied among breeds throughout the seasons.

Another pathological lesions may cause conception failure, like endometeritis, cystic endometrial hyperplasia and tumours. More recent evidence proposes that incidence of reproductive disorders in ewes were 4.14% and cystic endometrial hyperplasia and embryo loss were the most frequent pathological disorders [26]. Also, lower ovulation rate and failure to mate with the ram increased fertilization failure [33].

One bred/non pregnant ewe failed to show premature drop in P4 level (< 1 ng/ mL), due to short luteal phase, because the experiment out of season (late winter and spring), ewes did not display estrus again. These matches with DeNicolo et al. [23] results, which find that concentration decreased at day 12, reach < 1 ng/ mL in day 16 and remained in basal values from 16 to 18 PI in mated non pregnant ewes during spring, also, the ewe failed to display estrus.

Progesterone based determination of twin and singleton

After confirmed the pregnancy type by echography data, the ewes were divided into two categories; Singleton and twin embryo/ foetus pregnancy. Again, from the total of 25 confirmed pregnancies, 16 ewes exhibited single and nine exhibited twin pregnancy, no triplets were recorded. By comparing the peripheral P4 concentrations for both groups for the all periods; the results demonstrated that P4 levels of the animals with twin-foetus pregnancy were significantly ($P<0.05$) elevated than in ewes bearing one fetus only in days 35 and 50 PI. Additionally, non-significant variance was observed in P4 levels for twin fetus pregnancy during all experimental periods, same findings was detected for single fetus pregnancy (Table 2).

The determined lack of significant variance in the peripheral plasma levels of P4 between ewes with one and two embryos on the 15th and 25th gestation day is in partially agreed with the results by Gür et al.[6], which find out that a significantly increment ($p < 0.01$) in pregnant ewes bearing twin embryo/fetuses than in pregnant ewes bearing a single embryo/fetuses in day 30 and 76 PI. Similarly, Yotov [34] study, illustrated that no significant differences were observed in the P4 levels of twin and single carrying ewes on days 20 and 40 PI, after that, the variance became significant (day 60). Additionally, Boscos et al. [14] recorded no significant variance between the mean P4 concentrations of the ewes bearing twins compared with single bearing ewes in day 17 PI. Eastwood et al. (1976) [35] mentioned that many factors affect the P4 level such as breed, embryo weight as well as the CL size and number, that explained why the diagnoses of litter size based on peripheral P4 levels are unlikely to be efficient in early pregnancy.

Moreover, Pasciu et al. [17] mentioned that the significant difference between ewes carrying twins versus singletons were only recorded the second half of pregnancy. Furthermore, this study is in agreement with Hussain et al., who reported that P4 concentration was significantly greater in does carrying twin than single does in both 1st and 2nd months of gestation.

In apposite, this findings were in contrast with Nessim et al.[36], who reported that the higher P4 concentration was detected in ewes carrying twins than single lamb at earlier period of gestation; days 10 ($P<0.05$) and 20 ($P<0.01$) of pregnancy. Muller et al. (2003) [37], also argued that it can distinguish between single-foetus and multifoetus pregnancy on the 19th day PI in cross breed ewes.

The majority of bearing twin embryo/fetuses had two corpora lutea, therefore, the steroidogenic output from ovarian tissue increased, this explains the increase in the peripheral blood level of P4 in bearing twin ewes in first trimester. This opinion came in constant with Gür et al. study [6], which found that sex bearing twin ewes possess two corpora lutea.

TABLE 2. The progesterone concentration in single and twin foetus pregnancy

	Period/days	Singleton	Twin
1	Day 15 PI	A4.72±0.30^{b}	A5.25±0.26^{b}
2	Day 25 PI	A5.18±0.26^{b}	A5.92±0.50^{b}
3	Day 35 PI	A5.68±0.11^{b}	A7.36±0.24^{a}
4	Day 50 PI	A7.36±0.24^{b}	A8.89±0.13^{a}
	LSD	0.75	

Means with a different small letter in the same column are significantly different ($P<0.05$)

Means with a different capital letter in the same row are significantly different ($P<0.05$)

The controversy between the references may be due to the difference between the breed, age, weight, nutritional, as well as genetic factors, Yotov [34] study proved that there are significant differences in P4 concentration between the Pleven Blackhead and Trakia Merino breed at different stages of pregnancy.

Conclusion

The present study find out a significant elevation in P4 levels in pregnant ewes compared to control anestrous and mated/non-pregnant ewes. A cut off value of >4.9 ng/ mL can be utilized to differentiate between gravid and non-gravid ewes from day 25 onwards. Furthermore, P4 concentrations were found to be significantly higher in ewes carrying twins or singles at day 35 PI and beyond. Finally, human P4 kits are reliable and precise for determining pregnancy status and litter size.

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Conflict of interest

There are no conflicts of interest to be declared.

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Author contributions

Conceptualization, study design, sample collection, and Ultrasonography: Younis Laith.

Data analyses, Manuscript drafting, and Manuscript finalization: Hatif, Saad.

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تقييم مستويات البروجسترون في النعاج العواسي: مقارنة بين النعاج الحوامل وغير الحوامل ، ذوات التوائم ، والمفردة خلال الأشهر الثلاثة الأولى من الحمل

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هدفت هذه الدراسة إلى قياس تركيز هرمون البروجسترون في النعاج الحامل والمتزاوجة / غير الحوامل والنعاج العواس الخاضع للرقابة وكذلك في النعاج التي تحمل اجنة مفردة وتوائم. تم استخدام عدد ٣٥ نعجة تم تقسيمها إلى مجموعتين: مجموعة (٥ نعجة) ومجموعة المعاملة (٣٠ نعجة). تمت مزامنة الشبق في المجموعة المعاملة باستخدام الإسفنجات المهبلية بروجستيرون مع ECG. تم فحص جميع النعاج بواسطة الموجات فوق الصوتية في فترات مختلفة للتأكد من حالة الحمل وعدد الاجنة. جمعت عينات الدم من النعاج في كلا المجموعتين في أربعة تواريخ مختلفة (١٥ ، ٢٥ ، ٣٥ ، ٥٠ يوم) بعد التلقيح. أظهرت النتائج أن مستويات البروجستيرون كانت أعلى بكثير ($P<$ ٠,٠٥) في الحوامل (تراوحت بين ٤,٩٠ ± ٠,٢١-٧,٥٥ ± ٠,٢٣ نانوغرام / مل) مقارنة بالنعاج المسفدة / غير الحوامل (تتراوح بين ٢,٥٢ ± ١,٦٢-١,١١٢ ± ١,٢ نانوغرام / مل).) ونعاج السيطرة (تتراوح من ٠,٥٣ ± ٠,١٥-٠,٥٧٩ ± ٠,٣ نانوغرام / مل) خلال جميع الفترات التجريبية الأربعة. بالإضافة إلى ذلك، كانت تركيزات البروجستيرون للنعاج ذات الأجنة المزدوجة مرتفعة بشكل ملحوظ ($P<$ ٠,٠٥) مقارنة بالنعاج التي تحمل جنينًا واحدًا فقط في الأيام ٣٥ (٧,٣٦ ± ٠,٢٤ مقابل ٥,٦٨ ± ٠,١١) و ٥٠ (٨,٨٩ ± ٠,١٣ مقابل ٧,٣٦ ± ٠,٢٤) PI ، لكن ليس بشكل ملحوظ قبل ذلك. خلصت هذه الدراسة إلى أنه يمكن استخدام قيمة حدية أكبر من ٤,٩ نانوغرام / مل للتمييز بين النعاج الحامل والفارغة من اليوم ٢٥ فصاعدًا. كانت مجموعة P٤ البشرية دقيقة وأظهرت تمايزًا معنويًا بين النعاج الحامل والمتزاوجة / غير الحوامل والنعاج (مجموعة المقارنة) خلال جميع فترات التجربة. علاوة على ذلك ، تم اكتشاف أن تركيزات البروجستيرون أعلى بشكل ملحوظ في النعاج الحاملة للتوائم مقارنة بالنعاج المفرد في اليوم ٣٥ بعد التسفيد وما بعده.

الكلمات الدالة: البروجسترون ، النعاج, الحوامل ، التوأم ، الفردي ، العواس.