

EFFECT OF DIETARY UREA ON OVARIAN STRUCTURES IN SAIDI EWES DURING FOLLICULAR AND LUTEAL PHASES

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SUMMARY

The aim of this study was to record the number of ovarian follicles, to evaluate the quality of oocyte and to determine serum concentrations of luteinizing (LH) and progesterone (P_4) hormones in Saidi ewes fed on urea in relation to follicular and luteal phase. Eighteen clinically healthy ewes of initial body weight 33.8 ± 2.71 kg and about 4 years age were randomly assigned to three groups; the control group (G1) was given basal control diet which met energy requirements for body weight maintenance and the other two groups were fed on basal diet with 1% (G2) and 1.5% (G3) urea on dry matter (DM) basis. The duration of this study was lasted for 3.5 months. The estrous cycles of the ewes were synchronized using two injections of prostaglandin ($PGF_{2\alpha}$). In each group, three animals were slaughtered 40 hrs after the second injection of $PGF_{2\alpha}$ (follicular phase) whereas the remaining three animals were slaughtered 10 days after the second injection of $PGF_{2\alpha}$ (luteal phase). Upon slaughtering, blood sample and reproductive systems were collected. Stages of estrous, weight of ovaries in addition to the numbers of follicles were recorded. Follicles were categorized according to their diameter into; <2, 2-4 and >4 mm. Follicles (2-4 and >4 mm) were aspirated for recovery of oocytes and recording their quality. Oocytes were graded into cumulus enclosed; partially cumulus enclosed and denuded oocytes. Ovulation rate was recorded through the number of corpora lutea or albicantia. Concentrations of P_4 and LH hormones were determined.

Differences in response to synchronization among groups were observed. Numbers and diameters of follicles were decreased ($P > 0.05$) in G2 and ($P < 0.05$) G3 compared to G1. Numbers of aspirated follicles, and quality of oocytes decreased in ($P < 0.05$) G2 and G3 compared to G1. Ovulation rate showed no significant difference among experimental groups. Concentration of P_4 decreased ($P < 0.05$) in the G3 compared to G1 during luteal phase.

It could be concluded that dietary urea had a negative effect on the ovarian follicle numbers and diameters, oocyte quality and serum P_4 concentrations.

Keywords: Ewes, urea, ovary, follicles, oocytes, quality, progesterone

INTRODUCTION

Nutrition and metabolic status have several effects on reproduction and fertility (Robinson 1996 and O'Callaghan and Boland, 1999). Dietary intake can affect oocytes' morphology developmental capacity and embryo production (O'Callaghan *et al.*, 2000). The way nutrition influences embryo production remains not fully known characterized. It is not known whether the effect occurs before fertilization, early embryonic development or through uterine environment. Low protein in diet of mouse leads to behavioral and cardiovascular abnormalities in offspring (Watkins *et al.*, 2008).

Dietary urea level (1 & 1.5%) in ewes' diet was reported to be associated with significant elevation in serum urea nitrogen and decrease in glucose concentrations were (Ziyadah *et al.*, 2010). High concentration of plasma urea nitrogen decreases embryo viability of cattle embryos collected seven days post-insemination (Roads *et al.*, 2006) and mice (Mohammed and Attaai, 2011). This may be due to alternation of

uterine pH (Elrod and Butler, 1993; Elrod *et al.*, 1993; Rhoads *et al.*, 2004) which resulted from the concentrations of urea, Mg, K, P and Zn in uterine fluid (Jordan *et al.*, 1983). In addition, Blanchard *et al.* (1990) demonstrated that percentage of fertilized and transferable embryos collected from super-ovulated cows was greater when cows fed a lower level of degradable protein compared to those fed excess rumen degradable protein. Elevation of plasma urea nitrogen reduces ovine embryo viability and development under *in vivo* and *in vitro* conditions (Bishonga *et al.*, 1996; McEvoy *et al.*, 1997a,b). Despite these observations, the timing and mechanism(s) underlying the deleterious effects of excessive urea nitrogen on fertility remain unclear. While previous studies have examined the effect of urea on oocyte maturation and embryo development *in vitro* (De Wit *et al.*, 2001; Ocon and Hansen, 2003), up to our knowledge non have addressed the ovarian activity and oocytes quality during follicular and luteal phase in Saidi ewes.

The aim of this study was to investigate the effect of feeding Saidi ewes on diet containing urea on the ovarian activity, quality of oocytes, luteinizing and progesterone hormones concentrations in relation to follicular and luteal phase.

MATERIALS AND METHODS

The present study was carried out in the experimental farm of the Department of Animal Production, Faculty of Agriculture, Assiut University throughout the period from January to April, 2009.

Animal and management:

A total number of 18 clinically healthy Saidi ewes (adapted for urea feeding) of about 4 years of age and their live weight average 33.8 ± 2.17 kg were randomly divided into three groups (n=6 each). The control group (G1) was given basal diet which met energy requirements (NRC, 1985) and the other two groups were fed on basal diet with 1% (G2) and 1.5% (G3) urea on dry matter (DM) basis.

Experimental ewes were housed individually in cement floor pens and fed daily one kilogram of experimental diet for 90 days. Water was made available at all times. Ration was composed basically on corn, un-decorticated cottonseed cake, wheat straw, molasses, lime stone and minerals. Rations (G1, G2 & G3) were approximately similar in crude protein (9.49, 9.40 & 9.49%) and metabolizable energy (1858, 1848, 1736 kcal/kg).

Estrous cycle synchronization:

Experimental ewes received two intramuscular injections of $125 \mu\text{g}$ of prostaglandin (Estrumate; Bayer) to synchronize the estrous cycle, with interval of 10 days according to Beck *et al.* (1993).

Blood sampling:

Blood samples (10 ml) were obtained from the experimental ewes just before slaughter using jugular vein puncture. Blood samples were allowed to clot at room temperature before centrifuging at 400 r.p.m. for 15 min. Upon centrifugation, serum was decanted into clean and dry Eppendrofe tubes and stored at -20°C until subsequent analysis of progesterone and luteinizing hormones.

Determination of follicular and luteal phases:

Experimental ewes were divided randomly to two equal groups (n=3 each) to be slaughtered after the 2nd dose of $\text{PGF}_{2\alpha}$ injection. The first group was slaughtered 40 h (follicular phase), while the 2nd after 10 days (luteal phase). After slaughtering of ewes, reproductive tracts were

removed and were put in sealed plastic bag, and they placed in container contains worm (37.5°C) physiological saline, then transferred to the laboratory within 20 minutes.

Evaluation of ovarian structures and oocytes:

Collected ovaries were examined to characterize follicular and luteal phases. Numbers of follicles and corpora lutea and albicantia were counted. Follicles were categorized according to their diameters using vernier caliper into three classes; <2, 2-4 and >4 mm. Oocytes from follicles of < 2 mm diameter were aspirated from follicles $\geq 2\text{mm}$ using 18-gauge needle and syringe. The oocytes were counted and classified into three classes based on the cumulus cells and homogeneity of the cytoplasm according to Tornera *et al.* (2003), as follow:-

- Grade 1: Oocytes were completely invested with cumulus cell layers (good oocytes).
- Grade 2: Oocytes were surrounded with scanty cumulus cell layers (fair oocytes)
- Grade 3: Naked (denuded) oocytes.

Determination of serum progesterone (P4) and luteinizing hormones (LH):

Serum samples were analyzed for LH and progesterone hormones using the DPC IMMULITE 1000 chemiluminescent immunoassay system (Diagnostic Products Corporation, Los Angeles, USA). Analytical sensitivity of LH was 0.1 mIU/ml and progesterone was 0.2 ng/ml. Intra- and interassay coefficients of variations were 5.7 % and 12.3 % for LH and 9.5 % and 9.9 % for progesterone.

Statistical analysis

Data were analyzed using General Linear Model (GLM) procedure of SAS (SAS institute, 1998) according to the following model. Duncans multiple range test was used to compare among means of the control and treated groups. The model was as follows:

$$Y_{ij} = \mu + T_i + E_{ij}$$

Where: μ = the mean,

T_i = the effect of treatment (1 control, 2 fed 1% urea and 3 fed 1.5% urea),

E_{ij} = the standard error.

RESULTS AND DISCUSSION

1. Estrous synchronization:

Response of G1, G2 and G3 to $\text{PGF}_{2\alpha}$ treatment is presented in Table (1) on Effects of dietary urea level (1% & 1.5%) on estrous synchronization upon injection with prostaglandin are presented in table 3. Two third (66.6%; 2/3) of control animals were in the proper stage of estrus; follicular or luteal stage whereas 33.3%-100% of animals fed dietary urea

were in the proper stage of estrus. Responses of ewes to estrus synchronization are variable with urea level. It seems as urea 1% accelerate responses to prostaglandin injection whereas urea 1.5% slow the response. This might be explained through the increase in serum urea concentration. Dietary urea increased serum urea concentration (Ziyada *et al.*, 2010) in Saidi ewes. Butler (1998) found that increase of serum urea level altered pH of uterine environment, which enhanced secretion of PGF_{2α}. The increase of uterine PGF_{2α} inhibits corpus luteum (CL) growth (Koziorowski *et al.*, 1989; Davies, 2005). Small luteal cells are insensitive to PGF_{2α}, while large luteal cells contain PGF_{2α} receptors (Fitz *et al.*, 1982). This explains that ewes fed on urea ration respond to PGF_{2α} lesser than free urea ones.

Because the response of ewes to estrous synchronization upon PGF_{2α} injection was differed among groups, therefore, the following data of ovarian weights, follicle size and numbers and oocyte quality were statistically valid to compare G1 to G2 during follicular phase and G1 to G3 during luteal phase.

2. Effects of dietary urea level on ovarian weights and follicle sizes:

Effects of dietary urea level (G2 & G3) on ovarian weights and follicular number and sizes are presented in table (2). The non significant differences in the weight of ovaries among the experimental groups might be related to differences in follicle or corpus luteum sizes. Corpus luteum weights on days 3 and 14 of the natural estrous cycle (Fields and Fields, 1996) were 0.47 and 4.7g, respectively. Osman and Shehata (2005) and Mohammed (2009) found that the corpus luteum represents 30.1% of the ovarian weight in ruminants.

The numbers of follicles decreased ($P < 0.05$) in urea treated ewes whereas they were not differed during the stage of estrous cycle. Dietary urea level in G2 and G3 had a negative effect on ovarian follicle size during follicular or luteal phase. Hammon *et al.* (1997) found that ammonia N concentrations in follicular fluid are influenced by dietary protein intake in cattle and are negatively associated with ovarian follicle size. Furthermore, recent studies in cattle demonstrate that the number of antral follicles is highly variable among animals (Murasawa *et al.*, 2005). Cushman *et al.* (2009) concluded that antral follicle count in beef cows and heifers is influenced by birth weight and age but not by stage of the estrous cycle.

3. Effects of dietary urea level on quality of recovered oocytes:

Effects of dietary urea level (G2 and G3) on the quality of recovered oocytes are presented in table (3). The numbers of ovarian follicles (< 2, 2-4 & > 4 mm) decreased upon urea feeding

during the follicular and luteal phases. Dietary excesses of rumen degradable protein given in discrete feeds lead to elevated concentrations of ammonia in follicular fluid. The adverse effect on the oocyte is likely to involve inhibition in the growth and metabolism of the oocyte-supporting granulosa cells (Rooke *et al.*, 2004). It also appears to be follicle stage and size specific with pre-antral and medium-sized follicles being most affected. Since medium-sized follicles can be induced to ovulate by giving gonadotrophins, this may also explain why the adverse effect seems to be more prevalent in gonadotrophin-stimulated than spontaneously ovulating animals. The results indicated that oocytes recovery rate of punctured follicles was about 60-75.0% and decreased ($P < 0.05$) in urea groups compared to control. Recovery of oocytes might be related to follicle size which decreased ($P < 0.05$) in urea groups. Scott *et al.* (1989) found that recovery rates were significantly higher in 18- to 20-mm follicles ($P < 0.01$) and lower in those ≤ 11 mm ($P < 0.001$).

4. Effects of dietary urea level on ovulation rate:

Effects of dietary urea level (G2 and G3) on ovulation rate are presented in table (4). Data were presented in five animals (five out of six) of each group because of confusing corpora albicantia of estrous cycles (last and previous ones) which led to impaired detection of ovulation rate. Non –significant differences in ovulation rate in the three groups come in agreement with the findings of Bishonga *et al.* (1996) who studied the effect of excess dietary urea on ovulation of sheep. Ewes were given a basal control diet (C) which met energy requirements for body weight maintenance. Other treatments were basal diet plus 24 g of urea/day (low urea, L) or plus 48 g (high urea, H)/day. There were no significant differences in ovulation rates among the three groups.

5. Effect of dietary urea on serum luteinizing (LH) and progesterone hormones:

Effects of dietary urea level (G2 & G3) on serum luteinizing (LH) and progesterone hormones during luteal and follicular phases respectively are shown in table (5). The results indicated that the differences between the serum LH concentration in ewes of G2 and G1 were not significant. These results agreed with those of Bishonga *et al.* (1996) who found that serum LH concentration in ewes fed on urea ration was not significantly differed than those fed untreated one. Additionally, they added that the LH surge onset time and amplitude were not correlated to ovulation rate and were not affected by treatment. The result also revealed that progesterone concentration was lower ($P < 0.05$) in ewes of G3 than G1. The present results

disagree with of Bishonga *et al.* (1996) who reported that there were no significant differences in serum progesterone levels of ewes fed on urea diet and those fed control one. Reduction of serum progesterone concentration may be referred to that dietary urea leads to elevate plasma urea nitrogen (Ziyadah *et al.*, 2010), the later causes alteration in uterine pH which results in increase of uterine luminal PGF2 α which depresses corpus luteum.

In conclusion, dietary urea had a negative effect on the ovarian follicle numbers and diameters, oocyte quality and serum P₄ concentrations. Therefore, it is recommended to avoid elevation of serum urea level during the breeding season.

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Table 1. Estrous synchronization response (%) of ewes fed control ration (G1), 1% urea (G2) and 1.5% urea (G3) slaughtered after forty hours (follicular phase) and ten days (luteal phase) of the second injection with PGF_{2α}

Treat	Time of slaughter after 2 nd injection of PGF _{2α}	No. slaughtered animal	Actual ovarian phase		
			Follicular Phase	Luteal phase	Reponse % (No.)
G1	Forty hours	3	2	1	66.6 (2/3)
	Ten days	3	1	2	66.6 (2/3)
	Total	6	3	3	
G2	Forty hours	3	3	0	100 (3/3)
	Ten days	3	2	1	33.3 (1/3)
	Total	6	5	1	
G3	Forty hours	3	1	2	33.3 (1/3)
	Ten days	3	0	3	100 (3/3)
	Total	6	1	5	

Forty hours after second injection with PGF_{2α} ; expected follicular phaseTen days after second injection with PGF_{2α} ; expected luteal phase**Table 2. Weight of ovaries, number of follicles and their diameters during the follicular and luteal phase of urea treated ewes**

Estrous stage	Group	Weight Ovary	No follicles	Follicle diameter		
				< 2 mm	2-4 mm	> 4 mm
Follicular phase	G1	1.8± 0.8	15.3 ^a ±10.1	7.0 ^a ±6.0	6.3 ^a ±3.2	2.0 ^a ±1.0
	G2	1.4± 0.1	9.0 ^b ± 5.0	4.6 ^b ±3.2	3.0 ^b ±1.4	1.4 ^b ±0.9
Luteal phase	G1	2.0± 0.3	15.6± 2.3	10.3 ^a ±1.5	4.0±1.0	1.3±0.57
	G3	2.2± 0.7	11.8±9.7	7.6 ^b ±7.0	3.2±1.6	1.0 ± 0.7

a, b: Values with the different superscripts in the same column differ significantly (P<0.05)

Table 3. Numbers of aspirated follicles (2 - > 4 mm), recovered oocytes their quality during the follicular and luteal phases of urea treated animals

Estrous stage	Group	Aspirated Follicles	Recovered Oocytes	Oocyte quality		
				Good	Fair	Denuded
Follicular phase	G1	8.3 ^a ± 4.1	5.0 ^a ± 2.6	3.3 ^a ± 1.7	1.3 ^a ± 0.5	0.3 ± 0.5
	G2	4.4 ^b ± 2.2	3.2 ^b ± 1.5	2.0 ^b ± 1.2	0.8 ^b ± 0.4	0.4 ± 0.5
Luteal phase	G1	5.3 ± 1.5	4.0 ^a ± 1.0	3.0 ^a ± 1.0	0.6 ± 0.5	0.3 ± 0.5
	G3	4.2 ± 2.3	2.8 ^b ± 1.3	1.6 ^b ± 1.1	0.8 ± 0.4	0.4 ± 0.5

a, b: Values with the different superscripts in the same column differ significantly (P<0.05)

Table 4. Ovulation rate during the follicular and luteal phases of urea treated ewes

Treat	No. animals	Ovulation rate (No copora lutea)
G1	5	1.2 ± 0.44
G2	5	1.2 ± 0.44
G3	5	1.2 ± 0.44

Table 5. Luteinizing and progesterone hormone (mean ± SE) concentrations (ng/m) measured during the follicular and luteal phases of Saidi ewes fed basic ration (G1), basic ration contain 1% urea (G2) or 1.5% urea (G3)

Treat	Follicular phase	
	Luteinizing hormone	Progesterone hormone
G1	40.14 ± 0.69	3.21 ^a ± 0.05
G2	39.81 ± 0.80	2.37 ^b ± 0.07

a,b: Values with the different superscripts on the same column significantly (P<0.05)

تأثير عليقة اليوريا علي تركيب المبيض في الأغنام الصعيدية أثناء الطور الحويصلي والليوتيني

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الهدف من البحث هو تقدير عدد الحويصلات المبيضية وتقييم جودة البويضات وكذلك تقدير تركيزات هرموني البروجستيرون والتبويض تحت تأثير مستوى اليوريا في عليقة الأغنام الصعيدية أثناء الطور الحويصلي والليوتيني. أجريت هذه الدراسة على ١٨ من النعاج السليمة صحياً ، عمرها حوالي ٤ سنوات ومتوسط أوزانها ٣٣.٨١ كجم. تم تقسيم النعاج عشوائياً إلى ثلاثة مجموعات، حيث اشتملت كل مجموعة على (٦ نعاج) غذيت مجموعة الكنترول على عليقة خالية من اليوريا وتغطي احتياجات الطاقة اللازمة لحفظ الحيوان. بينما تم إضافة اليوريا بنسبة ١% ، ١.٥% من المادة الجافة للمجموعتين الثانية والثالثة على التوالي. استمرت التجربة ٣.٥ أشهر. لتوحيد دورة الشبق عند النعاج قيد التجربة، تم حقنها بهرمون البروستاجلاندين ($PGF2\alpha$) مرتين بينهما عشرة أيام. تم ذبح ثلاثة حيوانات من كل مجموعة للطور الحويصلي بعد ٤٠ ساعة من الحقنة الثانية للبروستاجلاندين في حين أن الثلاثة حيوانات الأخرى ذبحت للطور الليوتيني بعد ثلاثة أيام من الحقنة الثانية للبروستاجلاندين. أخذت عينات الدم قبل الذبح مباشرة. تم تقدير وزن المبايض وعدد الحويصلات ذات القطر أقل من ٢ مم، ٢-٤ مم، أكبر من ٤ مم. شُفطت الحويصلات الأكبر من ٢ مم لجمع البويضات وتقدير جودتها. تم تقدير جودة البويضات التي بويضات محاطة كلياً بخلايا الركام ، بويضات محاطة جزئياً بخلايا الركام وبويضات غير محاطة بخلايا الركام. قدر معدل التبويض بعدد الأجسام الصفراء أو البيضاء في المبيض. قدرت تركيزات هرموني البروجستيرون والتبويض في عينات السيرم. أظهرت النتائج اختلافات في الاستجابة للحقن بالبروستاجلاندين بين المجموعات. زاد عدد الحويصلات وقطرها وجودة البويضات معنوباً في مجموعة الكنترول عن المجموعات المغذاة علي عليقة اليوريا أثناء الطور الحويصلي والليوتيني. لم يتغير معدل التبويض بين المجموعات. زاد تركيز هرمون البروجستيرون معنوباً لمجموعة الكنترول في حين لم يتغير تركيز هرمون التبويض بين المجموعات. نستنتج مما سبق أن عليقة اليوريا لها تأثير سلبي علي عدد البويضات وقطرها وجودة البويضات وكذلك تركيز البروجستيرون.