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## EXCESS DIETARY UREA INTAKE IN EWES AND ITS EFFECT ON OVULATION RATE AND EMBRYO DEVELOPMENT.

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### ABSTRACT

The effect of excess dietary urea on ovulation and early embryo development of sheep was studied. Thirty Border Leicester x Scottish Black face ewes randomly assigned to three treatments were given a basal control diet (C) which met energy requirements for body weight maintenance. Other treatments were basal diet plus 24g of urea/day (low urea, L) or plus 48g (high urea, H)/day.

The reproductive cycles of the ewes were synchronized using a single injection of prostaglandin (PGF<sub>2α</sub>) and progesterone by an intravaginal controlled internal drug release (CIDR) device for 12 days. Ovulation was induced by the use of pregnant mare serum gonadotrophin (PMSG). Ewes were inseminated approximately 52 hours after CIDR device removal using a laparoscopic technique. Embryos were recovered at Day 4 or Day 11 after insemination from half of the ewes from each treatment group.

There were no significant differences in ovulation rates among the three groups. The embryo recovery rates were not affected by day of recovery. At embryo recovery on Day 4, 7/13 in C, 3/6 in L and 0/7 in H embryos were morulae. After 72 hours of *in vitro* culture 6/10 in C, 2/3 in L and 0/4 in H embryos developed to the blastocyst stage. Pregnancies sustained were C 6/8, L 5/7 and H 1/3 of the autotransfers. Throughout the experiment plasma urea levels were significantly affected by diet ( $p < 0.01$ ). Plasma ammonia levels in the H group were significantly higher than those in the C and L groups ( $p < 0.05$ ) for 4 hours after each feed. There was no treatment effect on plasma progesterone concentration. The luteinizing hormone (LH) surge onset time and amplitude were not correlated to ovulation rate and were not affected by treatment. It is concluded that high circulating concentrations of plasma urea

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and ammonia have an adverse effect on early embryo development. This effect was independent of any alterations in progesterone and LH concentrations.

Key Words: dietary urea, embryo development, ewes

#### INTRODUCTION

In sheep production systems, low producing animals that are solely dependent on low quality forages are often given dietary supplements of urea or other sources of non-protein nitrogen (NPN) to alleviate the protein shortage arising from a low yield of microbial protein<sup>20</sup>. The diets of high producing animals, in contrast, are supplemented with high quality protein that is largely undegradable (UDP) in the rumen, or their diets include high quality forage with a level of rumen degradable protein (RDP) that is grossly in excess of that needed by rumen microbes for maximum microbial protein synthesis. Whether or not the level and quality of protein (undegraded versus readily degradable) has any detrimental effect on reproduction is uncertain. Although there are reports of normal fertility of cows on protein diets, there are other studies in which excess dietary protein has been associated with a reduction in fertility in cows<sup>3,6,7,11,15</sup>. Where adverse effects have been noted, they have on occasion been ascribed to RDP<sup>3,9</sup>. The reported variation in fertility in various studies<sup>3,4,6,11</sup> has been attributed to suboptimal reproductive management (such as treatment of uterine disorders and estrus detection programs) during the postpartum period and not to dietary protein<sup>4</sup>. Elrod<sup>6</sup> concluded that the adverse effect on fertility manifests itself regardless of the source and degradability of the protein when crude protein is fed in excess of animal requirements. He asserted that the effect is brought about by changes in uterine pH. However, the mechanisms by which this pH change is modulated by excess dietary protein has not been identified. Despite the claim that excess dietary protein can reduce fertility, there is little or no evidence that fertility was reduced in dairy cows fed a diet with a protein level as low as 13.2% or as high as 20%<sup>4,11</sup>. The fertility criteria measured in these studies were days to first ovulation, number of services per conception, conception rate to first service, pregnancy rate and calving interval. There is however, a lack of similar studies in sheep.

The objective of the present study was to investigate the effect of varying levels of dietary urea (RDP) during and after the preovulatory priming period on ovulation and the early embryo development in sheep.

#### MATERIALS AND METHODS

*Thirty* 2–6 years old Greyface (Border Leicester x Scottish Blackface) ewes were brought indoors during late April with their lambs and maintained on an artificial photoperiod of 12 hours of light, 12 hours of darkness each day. At four weeks the

lambs were weaned. After weaning the lambs, a week later the ewes were placed on the experimental diets. The body weights were measured at the start and end of the experimental period. The ewes were ranked in ascending order of their initial weights, and each consecutive trio was randomly allocated to the three treatments. The treatments were C: basal diet 1600g, L: basal diet 1600g plus 24g urea (low urea), and H: basal diet 1600g plus 48g urea supplement. All diets were weighed separately for each ewe and supplemented with minerals and vitamins. Urea supplements were mixed into the basal diet at the time of feeding. Sodium sulphate was added as source of sulphur in the ratio of 14:1 by weight, urea:sulphur. The diet was fed at the rate of 800 g/feed twice daily at 08:00 and 16:00 hours. The diet was fed in the period before, during and after progesterone priming and up to 30 days after intrauterine artificial insemination. Water was available at all times. The basal diet composition in g/kg was: hay 832, molasses 147, dicalciumphosphate 10, sodium chloride 10 and minerals and vitamins 1. Hay had 89.62% dry matter and the nitrogen on fresh basis was 0.834%. Ewes were housed and fed in individual concrete-floor pens. After one week on these diets each ewe received a single intramuscular injection of 150  $\mu$ g of PGF<sub>2 $\alpha$</sub>  (Cloprostenol; Estrumate, Cooper Animal Health, Crewe, UK). Estrus was synchronized by using a CIDR device containing 0.3g progesterone (InterAg Hamilton, New Zealand). This was inserted into the vagina at 40 hours after the PGF<sub>2 $\alpha$</sub>  injection and remained in position for a period of 12 days. Ovulation was induced with an intramuscular injection of 800 IU PMSG (Intervet UK, Ltd., Cambridge) given at the time of CIDR withdrawal. The ewes were then exposed to a vasectomized ram at 2-hour intervals beginning 24 hours after CIDR removal for the determination of the time of estrus onset. Estrus was defined as the time at which ewes stood to be mounted by the vasectomized ram.

Throughout this experiment, fresh semen was used for insemination. It was collected from the same Suffolk ram by an artificial vagina and immediately diluted 1:1 with phosphate buffered saline (PBS pH 7.4) kept at 37°C. Intrauterine artificial insemination (IUI) at 52 hours after CIDR device removal using a laparoscopic technique was carried out as described by Scudamore<sup>23</sup>. Approximately 100x 10<sup>6</sup> of semen was deposited into each uterine horn.

#### *Embryo recovery*

Anesthesia was induced and maintained after intubation using halothane, nitrous oxide and oxygen. At laparotomy, the number of corpora lutea were counted and the embryos were recovered by retrograde flushing of each oviduct at Day 4 after IUI<sup>12</sup>. At Day 11 after IUI at laparotomy, embryos were recovered by flushing both the uterine horns and oviducts as described by Wallace<sup>26</sup>. Embryos were collected in ovum culture media supplemented with 10% v/v heat-treated fetal calf serum (FCS). In all recoveries at Day 4 and Day 11, one embryo was returned (autotransfer) when one or more embryos were recovered. At Day 4 a fine glass pipette attached by

rubber tubing to a 1ml syringe was used to suck up the ovum from collection dish. The pipette was inserted through the puncture wound created by an 18 gauge blunted needle about 2 cms caudal to the utero-tubal junction. For Day 11 embryos, a thick glass pipette (0.5 cm diameter drawn to a blunted point, 0.3 cm diameter) was used. This was inserted into the opening in the uterine horn that had been used previously to recover the embryos. Embryos transferred back to the donor (autotransfer) were considered viable if the ewe was diagnosed as pregnant at Day 18 from the time of IUI. Pregnancy was diagnosed by determining the plasma progesterone concentration in Day 18 blood samples.

Embryos recovered at Day 4 and not autotransferred were cultured for 72 hours in tissue culture media 199 (TCM-199) supplemented with 10% FCS at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. Every 24 hours, individual ova were assessed at 200x for development and quality based on the criteria described by Lindner and Wright<sup>16</sup>.

#### *Blood sampling*

Blood samples were collected by jugular vein puncture using a vacutainer into plastic tubes with heparin starting just before insertion of the CIDR device and then once daily until Day 30 after insemination. The two-hourly samples were collected over a twenty four hour period starting 24 hours after CIDR device removal. These samples were analysed for LH, plasma urea and plasma ammonia concentration.

#### *Plasma urea and plasma ammonia*

Plasma urea and plasma ammonia concentrations were determined on an autoanalyzer by a method based on that described by Talke and Schubert<sup>24</sup>.

#### *Progesterone*

Progesterone content was determined by radioimmunoassay following extraction as described by Djahanbakhch<sup>5</sup>. The lower limit of detection of the assay was 0.15 g/ml. Since the progesterone measurements were all done in a single assay, only the intra-assay coefficient calculated for 3 quality control pools was measured in duplicate, and was 9%. The mean recovery of progesterone in the extraction was ( $\pm$ SEM)  $61.5 \pm 1.0\%$  or  $N=150$ .

#### *Plasma luteinizing hormone*

Plasma LH concentration was measured in duplicate by a direct double antibody radioimmunoassay technique described by McNeilly<sup>19</sup>. Only samples taken at two hourly intervals between 24 and 48 hours after the CIDR device removal were assayed for LH to determine the onset and magnitude of the LH surge. Three tubes in duplicate were set up for quality control. The intra-assay coefficient of variation was 10.5%.

#### *Statistical Analysis*

The body weights of the ewes, the plasma urea and plasma ammonia concentrations, progesterone levels, LH surge peak and time intervals were compared using the Student's t-test. Chi-square analysis was applied to investigate treatment differences

in the proportion of the ewes with and without embryos recovered from them at Day 4 and Day 11 after insemination. Where the numbers were too small, the data are presented without statistical analysis.

## RESULTS

### *Body weights*

There were no treatment effects in the final body weights of the ewes. During the period of study, all ewes maintained their body weights ( $\text{kg} \pm \text{SEM}$ : initial weight  $65.5 \pm 0.9$ , final weight  $65.6 \pm 1.1$ ).

### *Progesterone*

There were no significant differences in the mean progesterone concentration ( $n=10$ ) for each treatment just before CIDR device withdrawal ( $p>0.10$ ). The mean ( $\pm$  SEM) concentrations were  $1.86 \pm 0.19$ ,  $1.48 \pm 0.14$  and  $1.59 \pm 0.12 \text{ ng/ml}$  for the control, low urea and high urea groups respectively. There was no evidence that urea supplementation altered progesterone concentrations (Fig. 1).

### *LH*

All 30 ewes exhibited a surge during the sampling period with individual LH surges occurring between 28 and 44 hours after CIDR device removal. The timing of LH surge was not significantly different between the treatment groups ( $p>0.10$ ).

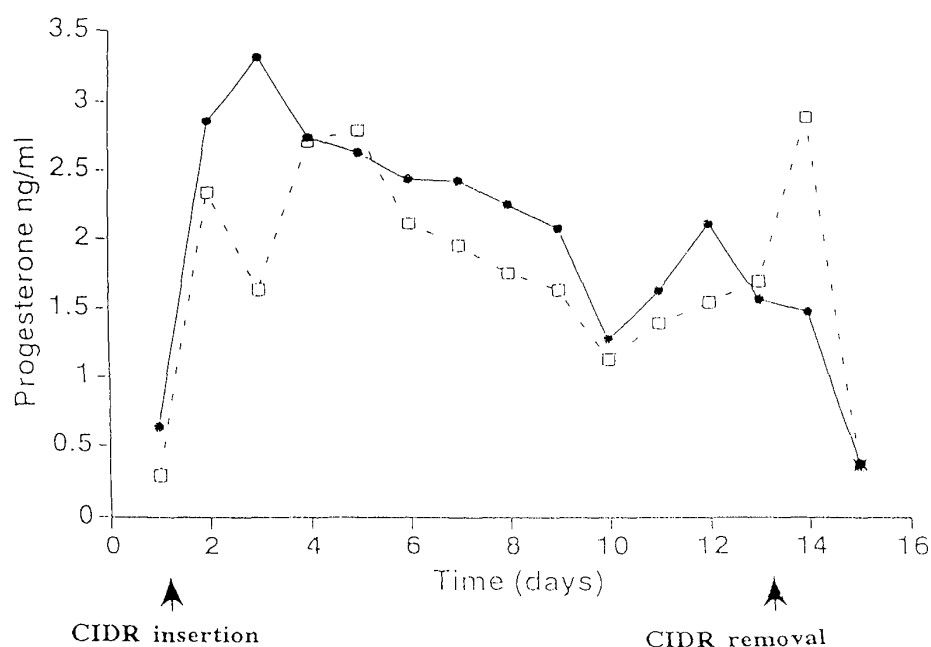


Fig. 1. Progesterone profiles for 2 ewes from the extreme treatments a control (□) and high urea (•) treatment ewe from immediately before CIDR insertion to after CIDR removal.

The time of LH peak onset for the high urea group was (35.0 h) slightly later compared with the low (33.0 h) and the control (33.0 h) groups. There was a significant difference ( $p < 0.01$ ) between the low urea and the high urea groups in the LH surge peak heights (Table 1). There was no difference between the low urea and control groups. Although the control group had a 20% higher LH amplitude than the high urea group, this difference was not significant due to the high variability for the control group (18.5–128.2 ng/ml).

#### *Plasma urea*

Over the 24 hour period in which the blood samples were taken 2 hourly, plasma urea concentrations were significantly different between treatments ( $p < 0.01$ ). The initial (approx. 08:00hrs) prefeeding urea levels were significantly different ( $p < 0.01$ ) for all treatments. Figure 2 provides a clear overview of the treatment profiles in relation to feeding times. In the control group, the prefeeding level was the highest recorded during the 24 hour period. In contrast, the low and high urea groups exhibited peak concentrations 4 hours after feeding. In all three groups, the level of plasma urea tended to decline throughout the 24 hour period with the 16:00 hour feed causing little more than a transitory arrest in this decline. Since dry matter intake was constant, feeding times were standardized, and no refusals were recorded, it was assumed that the daily plasma profiles for urea and ammonia were consistently the

Table 1. The effect of treatment on periovulatory events and their timing.

No of ewes	Control	Low urea	High urea
	( $\pm$ SEM)	( $\pm$ SEM)	( $\pm$ SEM)
	10	10	10
LH surge amplitude (ng/ml)	44.6 $\pm$ 10.7 <sup>ab</sup> )	47.2 $\pm$ 2.9 <sup>a)</sup>	29.0 $\pm$ 4.2 <sup>b)</sup>
Area under LH surge (mm <sup>2</sup> )	74.0 $\pm$ 19.6	81.0 $\pm$ 30.6	58.0 $\pm$ 21.1
LH surge onset to IUA1 interval (hrs)	19.4 $\pm$ 0.8	19.1 $\pm$ 1.3	17.0 $\pm$ 1.5
Interval from CIDR device removal to estrus onset (range. hrs)	28.0 $\pm$ 1.0 (24–32)	27.0 $\pm$ 1.1 (26–34)	30.0 $\pm$ 1.4 (24–38)

<sup>ab)</sup> Values with different superscript differ significantly ( $P < 0.01$ ).

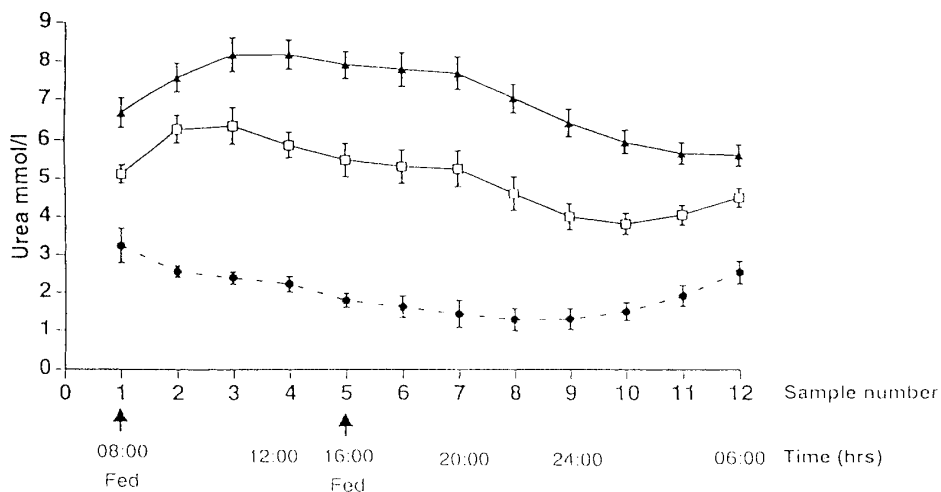


Fig. 2. Twenty-two hour profiles for plasma urea in control (•), low urea (□) and high urea (△) fed ewes. Each value is the mean  $\pm$  sem for 10 ewes.

same throughout the experimental period.

#### *Plasma ammonia*

The average concentrations for plasma ammonia over the 24 hour sampling period showed that there was a significant difference ( $p < 0.01$ ) between the control and the high urea groups, but no significant difference between the control and low urea groups. The difference between the high urea and low urea groups was also significant ( $p < 0.05$ ) as shown in Figure 3. The levels did not remain elevated throughout the 24 hour sampling period. Up to 4 hours after the afternoon feed, the high urea group was significantly different from the control ( $p < 0.01$ ) and the low urea group ( $p < 0.02$ ), while the low urea group was not significantly different ( $p > 0.10$ ) from the control except at 2 hours after the morning feeding. There was a positive correlation between plasma urea and plasma ammonia ( $r = 0.75$ ;  $p < 0.05$ ).

#### *Estrus onset*

All the ewes exhibited estrus before they were inseminated. The interval between CIDR device withdrawal and estrus onset was not significantly different ( $p > 0.10$ ) among the groups (Table 1).

#### *Ovulation rate and embryo recovery rate*

The ovulation rates among the groups were not significantly different ( $p > 0.05$ ) (Table 3). Similarly, the number of embryos recovered per ewe was not significantly different between treatment ( $p > 0.05$ ) (Table 2). The high urea group had the lowest recovery rate. The high recovery rate in the control group at day 4 was attributed to



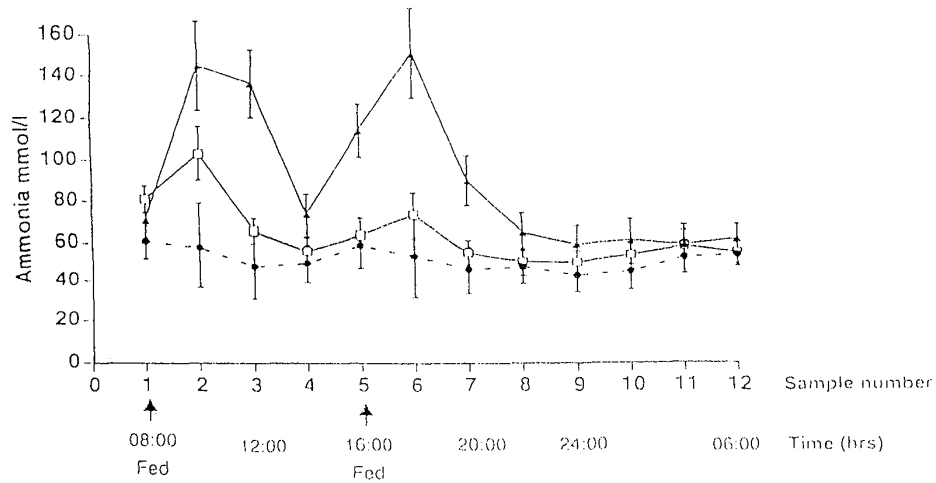


Fig. 3. Twenty-two hour profiles for plasma ammonia in Controls (•), low urea (□) and high urea (△) fed cwes. Each value is a mean  $\pm$  sem for 10 ewes.

Table 2. Treatment mean values for embryo recoveries on Day 4 and Day 11 after insemination (in percentage).

Day of recovery	No of ewes per group	Treatment		
		Control ( $\pm$ SEM)	Low urea ( $\pm$ SEM)	High urea ( $\pm$ SEM)
4	5	56.0 $\pm$ 14.0 <sup>a)</sup>	29.0 $\pm$ 14.4	39.0 $\pm$ 16.7
11	5	44.0 $\pm$ 17.8	51.0 $\pm$ 20.8	10.0 $\pm$ 10.0 <sup>a)</sup>

<sup>a)</sup> Values with same superscript are significantly different ( $P < 0.05$ ).

one ewe which showed a typical superovulatory response. The recovery rate in the control group at Day 4 was significantly different from the recovery at Day 11 in the high urea group ( $p < 0.05$ ).

Day of recovery (hence method of recovery) had no effect on the number of embryos recovered ( $\chi^2 = 1.04$ ). One ewe from the control exhibited a superovulatory response. There is no reason to believe that this was anything other than a chance result. If the data of that ewe are removed from the mean, the ovulation rates become  $3.4 \pm 0.7$  for the control,  $2.9 \pm 0.5$  for the low and  $2.4 \pm 4.2$  for the high urea groups. Two ova from a ewe on the high urea treatment were retarded, and were without spermatozoa on the zona pellucida.

Table 3. The effect of treatment on Ovulation rate, embryo recovery/ewe and pregnancy rate.

		Control	Low urea	High urea
No. of ovulations ( $\pm$ SEM)		4.1 $\pm$ 0.9	2.9 $\pm$ 0.5	2.4 $\pm$ 0.4
(range)		(1–10)	(1–5)	(1–5)
No. of embryos recovered/ewe ( $\pm$ SEM)		2.2 $\pm$ 0.7	1.1 $\pm$ 0.3	0.8 $\pm$ 0.4
(range)		(0–8)	(0–3)	(0–4)
No. of Pregnancies/ No. autotransferred	Day 4	3/3	2/3	0/3
	Day 11	3/5	3/4	—*

\* One pregnancy was obtained without prior autotransfer.

### *Embryo development*

Although the numbers of embryos were small, in general, the embryos of ewes receiving urea particularly at the high level were less well developed than those from the control ewes. The total number of embryos recovered at Day 4 according to stages of development were: <16 cells, 2 from the control, 1 from the low urea and 3 from high urea group. 16–32 cells: control 4, low urea 2 and high urea group 4. At morula stage: control group 7, low urea 3 and 0 from the high urea group. The embryos that were not autotransferred were cultured *in vitro*. Following the 72-hours *in vitro* culture, 6/10 embryos in the control group, 2/3 in the low urea group and 0/4 embryos in the high urea group developed to the blastocyst stage. The Day-11 recovered embryos were all at hatched blastocyst stage except one ovum from the control group which was developmentally retarded. All embryos recovered on Day 11 were considered viable *in vivo*.

### *Pregnancy rates*

The control group sustained the highest number of pregnancies followed by the low urea group and the high urea group had the least (Table 3). One ewe from the high urea group with no embryo recovery at Day-11 was found pregnant on Day 18 and this was confirmed by ultrasound scanning on Day 45.

## DISCUSSION

The lack of treatment effect on the final body weight of the ewes was expected. The diets were calculated to supply the ewes' maintenance energy requirements, and the small deficit in the rumen degradable protein in the control diet was unlikely to influence weight changes. Both the supplemented diets had an excess of RDP; low

urea to the extent of 50 to 60 g/day and the high urea to the extent of over 120 g/day.

Plasma urea concentrations largely reflect protein intakes<sup>10</sup>). Plasma urea is a product of ammonia metabolism by the liver. The plasma urea and plasma ammonia in this study was positively correlated ( $r=0.75$ ). The plasma urea concentration observed in the low and high urea groups were comparable to or slightly higher than those reported in dairy cows<sup>2,3,7,8,11,14</sup>) and in heifers<sup>7,8</sup>) fed high levels of dietary protein that resulted in a reduction in fertility.

In the studies in which high dietary protein was fed to cows, the reduction in fertility observed was ascribed to ammonia<sup>13</sup>). Although its mechanism of action is not known, ammonia is a substance toxic to tissue<sup>24</sup>). It has been suggested that ammonia, through some unknown mechanisms, alters the uterine secretory activity. The alteration cause changes in the ionic concentrations<sup>14</sup>), and consequently pH in the uterine environment during luteal phase of the oestrus cycle<sup>8</sup>).

Adequate circulating concentrations of progesterone prior to ovulation are required for subsequent normal development of the embryo<sup>1</sup>). There is evidence that the progesterone concentrations can be influenced by dietary factors such as energy levels<sup>17</sup>). The high dietary urea intake in this study did not alter the circulating progesterone concentration in the priming period. This is in agreement with the findings of Elrod and Butler<sup>7</sup>) and Blauwiekel et al<sup>2</sup>) in cattle. It is concluded that the effect of high dietary urea intake on embryo development is not mediated via changes in circulating concentrations of progesterone.

There was no evidence that the treatments influenced the mean interval to LH surge onset from the time of CIDR device withdrawal, or the interval between LH surge and IUAI. To the author's knowledge, these are the first observations to test whether or not blood ammonia concentrations influence pituitary function in ewes. Correlations between the various parameters of the LH surge and ovulation rate were not significant in the present study. These correlations therefore add further support to the hypothesis that the parameters of LH surge play little if any role in controlling the number of follicles that ovulate<sup>22</sup>).

In the present study, the exact time of ovulation was not determined. There were no significant differences between treatment groups in the LH surge timing. Hence it is argued that differences in the stage of development of embryos recovered from ewes on Day 4 after IUAI were unlikely to be attributed to differences in the time of ovulation and therefore of the age of oocyte at fertilization. Treatment differences were considered to reflect (a) effect on oocyte maturation brought about by high blood urea and ammonia concentration but not mediated through alteration in preovulatory progesterone concentration or (b) a direct effect of the urea and/or ammonia on the oviductal environment during the early cell division stages of the embryo. In the present study, it was difficult to separate the pre- and post-ovulatory

influences on embryo development.

Absence of spermatozoa on zona pellucida on some ova implies that the high urea treatment may have led to a hostile uterine and/or oviductal environment that compromised sperm viability.

In view of treatment effects on progesterone levels and periovulatory endocrine events, the observation that there were less pregnancies established and even fewer pregnancies sustained in ewes on the high urea supplemented diet suggests that urea supplementation caused early embryo mortality. The results indicate that the effect was manifest by Day 4 post-insemination. Thus, the general picture from this study is that an excess of RDP in the form of dietary urea is detrimental to the establishment of pregnancy in sheep. The detrimental effects manifest right through the pre-implantation stages. These findings, albeit generated from a fairly extreme experimental model, may well have implications for current grassland husbandry practices in which dairy cows in particular graze spring pastures that are very high in rumen degradable proteins.

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