

# Ultrasonographic examinations of embryonic-fetal growth in pregnant Akkaraman ewes fed selenium supply and dietary selenium restriction

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## SUMMARY

The objective of this study was to evaluate embryonic and fetal growth by ultrasonographic measurements in pregnant Akkaraman ewes fed a maintained diet either given or not given supplemental selenium (Se) and vitamin E (vit E). Thirteen pregnant ewes were allotted to two groups, a group of 5 ewes supplemented with Se and vit E and a group of 8 ewes not supplemented. The supplemented group received barley plus straw and 0,1 ppm Se per kg -15 IU vitE per kg DM from 9 months prior to breeding season until 40 day after parturition. The other group was fed with the same protocol without Se and vit E supplementation. The different structures of ovine fetuses were measured by ultrasonography from day 15 until day 130 of gestation. Suitable regression models were used for each group at the advanced pregnancy, through taking the biparietal diameter (BPD), diameters of orbita, stomach, heart, trunkus, abdomen, femur, humerus, radius-ulna, tibia-fibula and countable caruncle and relationship between the period of pregnancy and fetal growth. The mean serum Se levels of Se and vit E unsupplemented and supplemented groups were 120.6 ng per ml (SE 3.7) and 211.3 ng per ml (SE 11.5), respectively. BPD, diameters of fetal heart, abdomen, trunk, stomach, orbit diameter, the length of extremities and caruncle measurements were not affected by maternal serum Se level. All ultrasonographic measurements in both groups exhibited linear or exponential increase as correlated with the advancement of pregnancy. The results obtained from this study indicated that maternal serum Se concentration from 112.9 ng per ml to 235.1 ng per ml during pregnancy had neither negative nor positive effect on embryonic and fetal growth.

**Keywords:** Embryo, Fetal growth, Sheep, Se vitE.

## RÉSUMÉ

### Suivi échographique de la croissance embryonnaire et foetale chez les brebis Akkaraman gravides recevant une ration supplémentée ou restreinte en sélénium

L'objet de cet étude était d'évaluer par échographie la croissance embryonnaire et foetale chez des brebis gravides Akkaraman alimentées avec une ration d'entretien et recevant ou non une supplémentation en sélénium (Se) et vitamine E (vitE). Treize brebis gravides ont été réparties en deux groupes, un groupe de 5 brebis supplémentées en Se et vitamine E et un groupe de 8 brebis non supplémentées. Le groupe supplémenté a reçu de l'orge et de la paille et 0,1 ppm Se par kg plus 15 IU vit E par kg DM pendant 9 mois avant la saison sexuelle et jusqu'au quarantième jour après la parturition. L'autre groupe a reçu la même ration sans supplémentation en Se et vit E. La taille des différentes structures embryonnaires et foetales a été évaluée par échographie du quinzième au cent trentième jour de gestation. Des modèles de régression adaptés ont été utilisés pour chaque groupe à un stade avancé de gestation, pour analyser la relation entre différents paramètres de la croissance foetale: le diamètre bipariétal (BPD), les diamètres des orbites, de l'estomac, du coeur, du tronc, de l'abdomen, les longueurs du fémur, de l'humérus, du radius-ulna, du tibia-fibula et la taille des caroncules et le stade de gestation. Les concentrations sériques moyennes en Se des groupes supplémenté et non supplémenté en Se et Vit E ont été de 120.6 ng par ml (SE 3.7) et 211.3 ng par ml (SE 11.5), respectivement. Le diamètre bipariétal et les diamètres du coeur, de l'abdomen, du tronc, de l'estomac et des orbites ainsi que les longueurs des membres et la taille des caroncules n'ont pas été affectés par les concentrations maternelles en Se. Toutes les mesures échographiques des deux groupes ont présenté une augmentation linéaire ou exponentielle au cours de la gestation. Les résultats obtenus dans cette étude suggèrent que des concentrations maternelles en Se comprises entre 112.9 et 235.1 ng par ml au cours de la gestation n'ont pas d'impact sur la croissance embryonnaire et foetale.

**Mots clés :** Embryon, Croissance foetale, Moutons, Vitamine E-Se.

## Introduction

The Akkaraman sheep represent 41.3% of the sheep population of Central and Southeastern Anatolia. They are fat-tailed sheep, considered resistant to diseases and adapted to marginal production condition. Their breeding season ranges from September to November [34]. Animals have been affected

from Se deficiency for many years and different parts of this problem have been studied in Turkey [1,4,24].

Selenium (Se) is an essential element in the diet of animals, which is involved in immune response and thyroid function, and is necessary for growth and fertility [11,25,32,33]. Supplementation with 0.1-0.3 mg Se/kg dry matter (DM) in ewes is considered adequate for preventing deficiency in

sheep [21]. The metabolic function of Se is closely linked to vitamin E. There is a complex nutritional interrelationship between Se and vit E so that each can spare or alter the requirement of the other, but not completely replace each other [20].

There is a strong relationship between the fetus and dam concerning Se metabolism during pregnancy. The dams seem to sacrifice their Se level in order to maintain the fetal Se disposition [7]. According to human studies, selenium concentrations are decreased by 10-30% during pregnancy due to active transportation of selenium to the fetus and fetus has the ability to compensate partially for low maternal selenium [14].

In many circumstances, selenium supplementation before mating enhances conception rate in ewes by both increasing sperm motility and reducing early embryonic death. Furthermore, selenium status is important for multiple births in ewes [11]. A single administration of vitamin E-selenium complex to pregnant ewes during the last trimester of gestation has been shown to prevent Nutritional Muscular Dystrophy (NMD) disease in their lambs [5].

There is limited information about the effect of the selenium restricted diet before pre-mating and during pregnancy on the development of embryo and fetus in ewes. Therefore, this study aimed to monitor embryonic and fetal development using ultrasonography (USG) in Akkaraman ewes fed with selenium-vit E restricted diet.

## Materials and Methods

### ANIMALS AND DIETS

The experiment was carried out with 12-13 month- old 16 Akkaraman ewes at their first breeding season. Three ewes in the supplemented group were eradicated from the study due to dermatitis, abortion and negative pregnancy. The remaining 13 ewes were assigned with one of the groups according to dietary Se treatments. Eight ewes in unsupplemented group were fed with maintained diet without selenium and vitamin E supplement from 9 months prior to the breeding season until 40 days after lambing. Each ewe in this group received daily 700 g barley and 300 g straw for 9 months prior to mating. Barley and straw were increased to 900 g and 400 g respectively, during pregnancy and 40 days after parturition. 5 ewes in supplemented group (the control group) were fed with the same diet protocol supplemented with 15 IU Vit E/kg DM and 0.1 ppm Se/kg DM based on the National Research Council's Criteria [21]. Selenium concentrations in barley and straw specimens were ranged from 0.07 to 0.12 ppm and 0.11 to 0.17 ppm, respectively. All ewes were housed in sheds with two sections, which was allowed for group feeding and were kept under natural day light and ambient temperature during the experiment. Access to water was *ad libitum*. In addition, all ewes were dewormed with antiparasitic control programme. The pregnancy of ewes has been tested using ultrasound since day 15 of gestation.

### SAMPLE COLLECTION AND LABORATORY ANALYSIS

The blood samples were collected into tube with lithium

heparin via jugular venipuncture. Serum samples were kept at  $-20^{\circ}\text{C}$  until analysed. Two blood samples for Se levels were taken from all ewes; the first blood samples were obtained in the breeding period, the second sample collected from each animal was taken place 3 weeks before the expected lambing date. Also, barley and straws samples were taken for Se analysis.

The content of selenium in the feeds and serum concentration of Se were analysed by wet ashing and flourometric detection of the 2,3-diaminonaphthalene derivative. The procedure was essentially same with the method of KOH and BENSON [17].

### OESTRUS SYNCHRONIZATION

The oestrus signs of animals in both groups were monitored by putting a teaser ram into the ewe flock twice a day (in the morning and in the evening) at first four days of the mating season. Ewes showed oestrus signs were recorded. Ewes did not showed oestrus signs in this period were given a total dose of 0.18 mg cloprostenol per ewe (Estrumate<sup>®</sup>, 0.26 mg/ml SANOFI – DIF) on the 5<sup>th</sup> day. Hand breeding was applied for all of the ewes at the following oestrus cycle.

### REAL TIME – ULTRASOUND MEASUREMENTS

B – Mode real time ultrasonography (Shimadzu<sup>®</sup> SDL 32, Japan) equipped with a 5.0 MHz linear probe was used for ultrasonographic examinations. The fetal structures were measured on the monitor and their photos were printed (Sony<sup>®</sup> 890 CE).

Each ewe was restrained for transrectal linear scanning as described by KÄHN [12]. The ultrasound probe was firstly lubricated and then introduced to the rectum through the anus. Then it was pushed forward cranially for about 15 cm. The probe was advanced slowly after identifying the urinary bladder. Then the probe was rotated laterally through  $45^{\circ}$  in both directions until the uterus was observed.

The transcutaneous ultrasonography was performed while the ewe was standing or in dorsal recumbency. For transcutaneous sonography, the probe was applied to the groin area cranial to udders as described by KÄHN [12].

Embryonic vesicle, embryo and fetal heartbeat were evaluated in ewes, between days 15 and 37 of gestation by using transrectal linear scanning. BPD (Biparietal Diameter) and diameters of abdomen, trunk, caruncle, femur, humerus, radius– ulna, tibia - fibula, heart, stomach and orbit were measured in the ewes by transcutaneous ultrasonography after the 37<sup>th</sup> day of gestation. The fetal growth was examined at 7 day intervals between the 15<sup>th</sup> and 37<sup>th</sup> days of gestation. Fetal structures were examined at 10 day intervals after the 37<sup>th</sup> day of gestation.

Symmetrical images of fetal head (biparietal diameter) were frozen on the monitor and were measured by using electronic callipers and real - time ultrasound imaging system as described by HAIBEL *et al.* [9,10]. For measuring the diameter of orbita, the largest sonographic section of the sharp line of demarcation between the hyperechoic orbita

and hypoechoic eyeball were chosen [13]. The size of long bones such as femur, humerus, radius – ulna and tibia – fibula were measured according to the zones of intensive ossification which appeared as hyperechoic [13]. Abdominal cavity was determined as the widest transversal section at the level of liver. The longitudinal diameter of stomach was recorded in the abdominal cavity. At the trunk, measurements comprised the transversal diameter at the level of the last rib and the fetal stomach as described by GONZALEZ DE BULNES *et al.* [8]. The largest transversal intra – luminal diameter was chosen for the heart's size. Caruncles were measured as described by KELLY *et al.* [16]. Caruncle was identified and frozen on the monitor at its maximum dimensions. Caruncle scanning was continued until 10 caruncles were measured.

## STATISTICAL ANALYSIS

Type of the study was randomized controlled trial. All statistical analyses were performed by SPSS 14.01 (serial: 9869264). Ultrasonographic monitoring of fetal growth was analysed to test best fitting data the polynomial (i.e. linear ( $y = a + bx$ ) or the quadratic regression ( $y = a + bx + cx^2$ ). The appropriate regression equations were determined based on results from polynomials for unsupplemented group and supplemented group, where;  $y$  = the parameter involved (dependent variable),  $a, b, c$  = parameters,  $x$  = the days of gestation,  $x^2$  = square of gestation days (independent variable). Independent samples T-test was used to compare continuous variables of the groups. P-value of 0.05 was used to determine the level of significance.

## Results

### SERUM SE LEVELS

In the present study, the mean Se concentration was  $120.6 \pm 3.7$  ng/ml in unsupplemented group (with a minimum of 112.9 ng/ml and maximum of 128.2 ng/ml). The serum Se levels were found  $211.3 \pm 11.5$  ng/ml (with a minimum of 187.5 ng/ml and maximum of 235.1 ng/ml) in supplemented group. Serum selenium concentrations were not significantly different between first samples and second samples taken at advanced pregnancy in both groups.

### THE ULTRASONOGRAPHIC FINDINGS BETWEEN 15<sup>TH</sup> AND 37<sup>TH</sup> DAYS OF GESTATION

Spherical anechoic embryonic vesicle was first observed on day 15 of gestation. The mean diameter of the vesicle measured 8 mm on the first day of detection. The embryo had a C-shape in midsagittal section of embryonic vesicle and had a length of 6 mm on day 23. The embryonic heart-beat was first noticed on day 24.

### THE ULTRASONOGRAPHIC FINDINGS AFTER 37<sup>TH</sup> DAY OF GESTATION

All of measurements were correlated with the period of

pregnancy. The highest correlation coefficient was 0.99 for BPD measurement in both groups and the lowest correlation coefficient was 0.67 for femur measurement in supplemented group (Table 1). Placentoms and skeletal bones were first determined on the 37<sup>th</sup> day of pregnancy.

The growth pattern of BPD in supplemented and unsupplemented groups showed exponential and linear increase, respectively. A linear increase was determined for fetal orbit diameter in both groups (Table I). However, no significant differences were observed between supplemented and unsupplemented groups in the measurements of fetal BPD and orbita.

The internal diameter of stomach was showed reasonably good structure for ultrasonographic measurements after 55<sup>th</sup> day. While the stomach and the heart diameters were presented a linear increase during the advanced period of the pregnancy, measurements of fetal abdomen and placentoms were exhibited an exponential increase in both groups (Table I). Considerable differences was statistically not observed between the groups. An exponential increase was obtained from the fetal truncus in unsupplemented group, and a linear increase was obtained from fetal truncus in supplemented group (Table I). However, the difference between this parameters was not statistically significant between the groups.

The humerus and femur length were determined as early as on day 37 of gestation whereas the length of tibia – fibula and radius – ulna on day 43. While the lengths of fetal femur, radius – ulna and tibia – fibula demonstrated a linear increase in pregnancy in both groups, the humerus length displayed an exponential increase in unsupplemented group and a linear increase in supplemented group (Table I). There were no significant differences between two groups for the length of humerus, femur, radius-ulna and tibia-fibula. The growth rate of the femur length during pregnancy was greater in unsupplemented group compared with supplemented group (Figure 1).

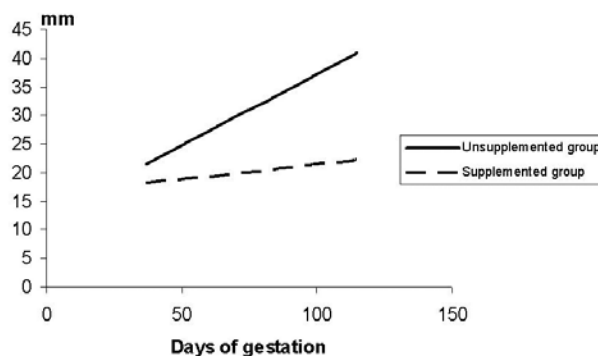


FIGURE 1 : The length of femur in both groups ( $p > 0.05$ ).

## Discussion

The mean concentration of Se in unsupplemented group ( $120.6 \pm 3.7$  ng/ml) was significantly lower ( $p < 0.05$ ) during gestation when compared to the mean values of Se in serum of the supplemented group ( $211.3 \pm 11.5$  ng/ml). Serum Se

	Fetal structure		Equation	Regression equation	Time interval of data points (days)	Coefficient of determination ( $r^2$ )
1	Biparietal diameter	Unsupplemented group	Exponential	$y = -12.75 + 0.633 x - 0.001 x^2$	(37 – 128)	0.99
		Supplemented group	Linear	$y = -9.69 + 0.484 x$		0.99
2	Abdomen	Unsupplemented group	Exponential	$y = -27.94 + 1.103 x - 0.003 x^2$	(35 – 130)	0.98
		Supplemented group	Exponential	$y = -23.28 + 0.975 x - 0.002 x^2$		0.99
3	Trunk	Unsupplemented group	Exponential	$y = 23.63 - 0.438 x + 0.006 x^2$	(43 – 130)	0.95
		Supplemented group	Linear	$y = -3.49 + 0.368 x$		0.90
4	Femur	Unsupplemented group	Linear	$y = 12.37 + 0.248 x$	(37 – 115)	0.79
		Supplemented group	Linear	$y = 13.61 + 0.210 x$		0.67
5	Humerus	Unsupplemented group	Exponential	$y = -28.45 + 0.945 x - 0.003 x^2$	(37 – 130)	0.92
		Supplemented group	Linear	$y = -6.88 + 0.368 x$		0.90
6	Heart	Unsupplemented group	Linear	$y = -3.33 + 0.219 x$	(55 – 130)	0.88
		Supplemented group	Linear	$y = -1.14 + 0.182 x$		0.86
7	Stomach	Unsupplemented group	Linear	$y = -18.86 + 0.459 x$	(55 – 129)	0.93
		Supplemented group	Linear	$y = -18.83 + 0.466 x$		0.92
8	Orbit diameter	Unsupplemented group	Linear	$y = -2.70 + 0.206 x$	(45 – 113)	0.92
		Supplemented group	Linear	$y = -10.07 + 0.282 x$		0.94
9	Radius-ulna	Unsupplemented group	Linear	$y = -10.00 + 0.413 x$	(43 – 112)	0.98
		Supplemented group	Linear	$y = -19.68 + 0.597 x$		0.91
10	Tibia-fibula	Unsupplemented group	Linear	$y = -19.843 + 0.548 x$	(43 – 115)	0.97
		Supplemented group	Linear	$y = -17.80 + 0.547 x$		0.96
11	Caruncle	Unsupplemented group	Exponential	$y = -20.78 + 1.124 x + 0.006 x^2$	(37 – 129)	0.75
		Supplemented group	Exponential	$y = -31.92 + 1.370 x - 0.007 x^2$		0.88

$y =$  the parameter involved (dependent variable),  $a, b, c =$  parameters,  $x =$  the days of gestation,  $x^2 =$  square days of gestation (independent variable).

TABLE I : Relationship of fetal structures to age of gestation.

concentrations have been directly related to dietary Se intake in many animal species. The reflection of Se supplementation was seen in increasing serum Se values. The normal selenium concentrations in blood of sheep ranged from 120 to 150 ng/ml [29]; from 0.08 to 0.20 ppm [19] and 10-20 ng/ml [27]. It is suggested that ovine blood selenium concentrations below 0.08 mg/l [2] or < 0.04 ppm [19] or <10 ng/ml [27] are considered as a marker of deficiency. There is a discrepancy in reports about the adequate level of Se when compared with reference levels. Therefore, the results obtained in both groups were within the reference levels according to North American and European researchers, however, were significantly higher according to New Zealand researchers (approximately a 10- fold difference).

In this study, Se deficiency was not evidenced in ewes which were fed with a basal maintenance diet without Se and Vit E supplement over one year. WHITE *et al.* [31] reported a 0.05 mg/Se kg supplemented diet was insufficient to maintain the Se status of the ewe in the final stages of gestation, on the other hand, some reports indicated that grazing animals may accumulate more available organic Se over many years [3].

In the present study, BPD, diameters of fetal heart, abdomen, trunk, stomach, orbit, the length of extremities and caruncle measurements were not affected by maternal serum Se level, and also, all ultrasonographic measurements in both

groups exhibited linear or exponential increase as correlated with the advancement of pregnancy.

NEVILLE *et al.* [22] reported that fetal body weight, curved crown-rump length, and heart girth were not affected by different Se treatments (0.1 ppm; 3 ppm and 15 ppm) from 50<sup>th</sup> to 134<sup>th</sup> day of gestation. These researchers indicated that fetal tissue cellularity, particularly in intestine, liver, and kidney, is responsive to maternal Se supplementation. Otherwise, fetal metabolic hormones are not affected by supranutritional Se status in dam [30] and the administration of Se and vitamin E had no significant effect on oestrus, fertility [6].

KUPLULU *et al.* [18] reported in Akkaraman ewes that the embryonic vesicle was in the shape of a smooth anechoic vesicle in 5 mm diameter on 17<sup>th</sup> day, and the embryo was measured as 8 mm on 21<sup>st</sup> day of pregnancy. They also showed that heart beat, placentoms and ossification of bones could be measured as early as on 28<sup>th</sup>, 35<sup>th</sup> and 40<sup>th</sup> days of pregnancy, respectively. In contrast, we showed the anechoic vesicle was in 8 mm diameter on 15<sup>th</sup> day and length of embryo was 6mm on 23<sup>rd</sup> day of gestation whereas heart beat was first detected on 24<sup>th</sup> day of gestation, and placentomes and ossification of fetal bones were observed on 37<sup>th</sup> day of gestation. All ultrasonographic measurements for fetal structures in advanced period of gestation in this study were consistent with previously reported studies in Akkaraman ewes [18].

HAIBAL and PERKINS [9] reported that fetal head width could be determined clearly at the early stage of gestation in Suffolk sheep, but measurement of such parameters became a fairly time consuming processes at advanced pregnancy (last trimester). In another study, measurement of fetal BPD was easier at early period of pregnancy (49<sup>th</sup> – 84<sup>th</sup> days) [26], whereas it was difficult between 100<sup>th</sup> – 110<sup>th</sup> days as pregnancy advanced in Merinos ewes [15]. In our study, the measurement of fetal skull was easily performed after the 37<sup>th</sup> day. It was rarely difficult to take images between 95<sup>th</sup> and 115<sup>th</sup> days of pregnancy since the fetal head remained in the abdomen. It was necessary to determine the suitable position of foetus in order to take a symmetric image. Excessive artefacts due to intensive calcification should also be taken into account because they could be associated with inadequate or poor image quality.

OSGERBY *et al.* [23] reported that the femur length of fetus in pregnant ewes fed with deficient diet in the ratio of 30% was longer at day 90 of gestation in comparison with fetus in pregnant ewes fed with normal diet. In our study, Se-vit E supplementation had no affect on fetal femur length (p: 0.762), but the linear increase rate of fetal femur in unsupplemental group was higher than other group during pregnancy (Fig.1).

Considerable part of the growth of placenta has been completed approximately on 70<sup>th</sup> day of pregnancy [28]. The effect of maternal malnutrition may be associated with damages on placenta and cause a delay on the ovine fetal growth [23]. It was unable to determine any transformation on the dimension of such type of placenta in any group although there were increase on the number of D – Type placenta in ewes fed with deficient diet in the ratio of 15 % before the mating season and during the first 70 days of pregnancy [28]. The number of A – Type placenta decreased in ewes fed with a deficient diet, and the weight and diameter of such type of placenta decreased morphologically [28]. In the current study, however, a slight increase in the size of caruncle was observed in unsupplemented group in comparison with supplemented group, but this increase was not statistically significant. Decrease in the average diameter of placenta may be related to the randomly selected and measured caruncles.

In conclusion, the pregnant Akkaraman ewes had serum Se concentrations ranging from 112.9 ng/ml to 235.1 ng/ml, which were 10 times higher than reference concentrations according to New Zealand researchers and did not show alterations in fetal growth restriction at ultrasonographic measurement. Maternal serum Se concentrations obtained in this study were tolerable levels for fetus. Also, our findings indicated that Akkaraman ewes adapted to maintenance diet should be tested for serum Se concentrations prior to Se-vit E supplementation.

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