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Research Article

Changes in Lipid Peroxidation, Glutathione and Fertility in Tuj Sheep After Combined Administration of Vitamin A and E and Passive Immunization with Testosterone Antibodies [1]

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Abstract

This study investigated the effect of testosterone antibodies and a combination of vitamin A and E on reproductive performance and lipid peroxidation in Tuj sheep during the oestrus period. Two castrated Tuj rams were used to produce an ovine testosterone antibody. To perform the experiment, 30 clinically healthy adult Tuj sheep were divided into three groups, in each group had 10 sheep. The Control group were given a placebo, Group I was injected with the testosterone antibody alone and Group II was injected with testosterone antibody plus a combination of vitamins A and E in Freund's incomplete adjuvant. The testosterone antibody and vitamin combination were administered at synchronization and 1 week before synchronization. To synchronize the sheep, 2.5 ml GnRH was injected to sheep in Control, Group I and Group II. Control, Group I and II were subsequently given 600 IU PMSG with 2 ml $PGF_{2\alpha}$ at 5th day of synchronization. Progesterone levels were higher in the two treatment groups than in the control group as pregnancy progressed. Plasma malondialdehyde levels were higher during initial drug application and prior to mating but were lower in the experimental groups than in control during pregnancy and after parturition. Erythrocyte glutathione levels remained significantly higher in experimental groups than in Control during pregnancy. The number of offspring and the lambing rates in Group I and Group II was higher than the Control. There were no stillbirths in Group I. The number of non-pregnant sheep was lowest in Group II. In summary, injections of testosterone antibody and a combination of vitamins A and E led to an increased incidence of multiple pregnancies in sheep and a greater number of lambs were born. These data indicate that the immunoneutralization of testosterone combined with a reduction in free radicals via the antioxidant activities of vitamins led to increased rates of conception and twinning. Also, it is thought that to allow the growth of the herd in a shorter time, testosterone antibody and combination of vitamins A and E can be applied.

Keywords: *Testosterone antibody, Vitamin A, Vitamin E, Tuj sheep, MDA, GSH, Progesterone, Fertility*

Testosteron Antikoru ile Pasif İmmunizasyon ve A-E Vitamini Kombinasyonu Uygulanmış Tuj Koyunlarında Döl Verimi, Glutatyon ve Lipid Peroksidasyonda Meydana Gelen Değişikler

Özet

Bu çalışmada Tuj koyunlarına östrus döneminde testosteron antikoru ile yapılan pasif immunizasyonun ve A ve E vitamini kombinasyonu uygulamalarının üreme döneminde döl verimi ve oksidatif stres üzerine etkileri araştırıldı. Bu amaçla, 30 Tuj koyunu her grupta 10 koyun olmak üzere 3 gruba ayrıldı. İlk grup Kontrol grubu olarak değerlendirildi ve senkronizasyondan 7 gün önce plesebo uygulandı. Grup I'deki koyunlara testesteron antikoru (AnT), Grup II'deki koyunlara AnT ve Freund's adjuvant incomplate içinde A-E vitamini kombinasyonu uygulandı. Vitamin ve AnT uygulamaları senkronizasyon günü ve senkronizasyondan bir hafta önce yapıldı. Hayvanları senkronize etmek için, Kontrol, Grup I ve Grup II'deki koyunlara 2.5 ml GnRH enjekte edildi. Kontrol, Grup I ve Grup II'deki koyunlara senkronizasyonun 5. günü 600 IU PMSG ile 2 ml $PGF_{2\alpha}$ uygulandı. Deney gruplarının plazma progesteron düzeyleri gebelik süresince kontrol grubuna göre yüksek olarak belirlendi. Deney gruplarının Plazma malondialdehit düzeyleri ilk ilaç uygulamaları yapıldığında ve koç katımından önce yüksekken, gebelik süresince ve doğumdan sonra kontrol grubuna göre düşük olarak tespit edildi. Deney gruplarının, kontrol grubuna göre yüksek eritrosit glutatyon düzeylerini gebelik döneminde ve doğumdan sonra koruduğu gözlemlendi. I. ve II. Grupların bir batında doğan yavru sayılarının ve kuzulma oranının kontrol grubuna göre daha yüksek olduğu gözlendi. I. Grupta hiç ölü doğum olmazken, II. Grupta gebe kalmayan hayvan sayısı diğer gruplardan daha düşüktü. Sonuç olarak, testosteron antikorunun serbest testosteron düzeyini düşürmesi ve vitaminlerin antioksidan etkileri ile serbest radikal düzeylerinin azalmasının koyunlarda gebelik performansını ve bir batında doğan yavru sayısını arttırdığı tespit edilmiştir. Ayrıca, testosteron antikoru ve testosteron antikoru ile A ve E vitamin kombinasyonlarının büyük sürülerde uygulanması ile işletmelerde daha kısa zamanda sürülerin büyütülmesinin mümkün olabileceği düşünülmektedir.

Anahtar sözcükler: *Testosteron antikoru, Vitamin A, Vitamin E, Tuj koyunu, MDA, GSH, Progesteron, Fertilite*

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INTRODUCTION

The number of offspring per parturition is the most important factor in terms of managing the productivity of sheep. To manipulate the number of offspring, sheep are selectively crossed with others, using techniques such as flashing and a variety of different synchronisation protocols. Earlier studies have described the development of effective ways to improve fecundity in sheep as well as the number of offspring. These include both active [1,2] and passive [3] immunization against a variety of steroid hormones. Immunization against specific steroids represents a useful management option to increase reproductive performance in sheep via a relatively simple treatment. Increased levels of fertility were reported by many authors following passive immunization against testosterone [4-6]. Immunization against testosterone leads to changes in the concentration of biologically active progesterone as a result of cross-reacting with antibodies. Antibodies are known to bind efficiently with their appropriate endogenous circulating steroid [2,3]. The effect of immunization upon receptivity and fertility is via reductions in the amount of unbound and biologically inactive hormones [7]. In addition, sheep that were passively immunized against testosterone showed induced changes in the secretion of gonadotrophins [3,8]. Furthermore, this procedure leads to increased rates of ovulation and lambing [1,5].

Recent research [9-16] has aimed, first, to develop reproductive technologies to produce high-yielding lambs in large numbers and, second, to create supplementation strategies to protect sheep and embryos from oxidative damage by free radicals. If the antioxidant system is impaired, reactive oxygen species (ROS) can initiate lipid peroxidation and DNA damage, leading to cell death [9]. Therefore, excessive oxidative stress during the mating and gestation periods of sheep can be controlled by the administration of antioxidants [10]. Within the prepartum period, the administration of vitamin A and E, scavengers of free radicals, can protect oocytes and embryos from oxidative damage during gestation [11-14]. Furthermore, levels of antioxidant enzymes, such as glutathione, glutathione peroxidase, superoxide dismutase, can be elevated via the combined administration of vitamin A and E, leading to reduced levels of lipid peroxidation and ROS generation in oviductal and follicular fluid [13,15,16].

Vitamin A and E play important roles in a variety of biological processes, including fertility, the regulation of embryonic growth and cell differentiation. In addition, vitamin A and E play key roles in the patterns of cellular differentiation occurring during embryonic and foetal development and are responsible for proximodistal patterning, limb development and regeneration, neural differentiation and axon outgrowth [15-17]. Micronutrient deficiencies have also been associated with major reproductive risks, ranging from foetal structural defects to infertility. The periconceptional period consists of a number of critical stages, including preconception, conception, implantation, placentation and embryo organogenesis. These phases are critical in determining successful foetal development and health and can be influenced by maternal nutrition, particularly imbalances in micronutrients [13]. Embryonic and foetal development, implantation and placentation are particularly vulnerable to maternal micronutrient levels. Micronutrient supplementation may also play a role in altering development of the placenta, a structure that is critical for nourishing the foetus throughout pregnancy. In addition, evidence indicates a role for micronutrient supplementation in preventing some pregnancy disorders [5]. In fact, despite initial normal growth cycles, foetuses may develop impaired growth during the second part of gestation as a result of nutrient deprivation occurring early in gestation. Furthermore, sheep in poor body condition are typically less productive and the supplemental injections of vitamin A and E have been shown to increase viability of embryos and lambs [18].

The present study investigated the influence of supplementary injections of testosterone antibody and a combination of vitamin A and E, upon reproductive performance, lipid peroxidation, glutathione and progesterone levels in Tuj sheep.

MATERIAL and METHODS

Animal Treatment

Thirty clinically healthy, weighing average 55±5 kg, 3-5 years of age Tuj sheep were randomly divided into three groups. Applications were initially made during estrus period of ewes. The first group was used as the Control (n=10) and were given a placebo, Group I (n=10) was injected with testosterone antibody alone, whereas Group II (n=10) was injected with testosterone antibody and a combination of 100.000 IU of vitamin A [31.58 mg all-trans retinol (Sigma®, R2500, USA) dissolved in 0.5 ml Freund's adjuvant incomplete (Sigma®, F5506, USA)] and vitamin E [18.22 mg DL-α-Tocopherol acetate (Sigma®, T3376, USA)] dissolved in 0.5 ml Freund's adjuvant incomplete (Sigma®, F5506, USA)]. Study design in experimental groups are shown in *Fig 1*. Testosterone antibodies and the vitamin combination were administered 1 week before synchronization (-7th day) and at the point of synchronization (premating). To synchronize the sheep, Control, Group I and Group II were given an IM injection of 2.5 ml GnRH (0.004 mg Bucerelin acetate, Receptal®, MSD, Turkey). Control, Group I and II were subsequently administered with 600 IU PMSG (Chronogest/PMSG, 6000 IU, MSD, Turkey) with 2 ml PGF 2α (5 mg Dinoprost, Dinolytic®, Zoetis, Turkey) at 5th day of synchronization. Rams were then added to the sheep enclosures and oestrus monitored in the sheep for 6 days *(Fig. 1)*. The rams used in the study were examined andrologically and macroscopic

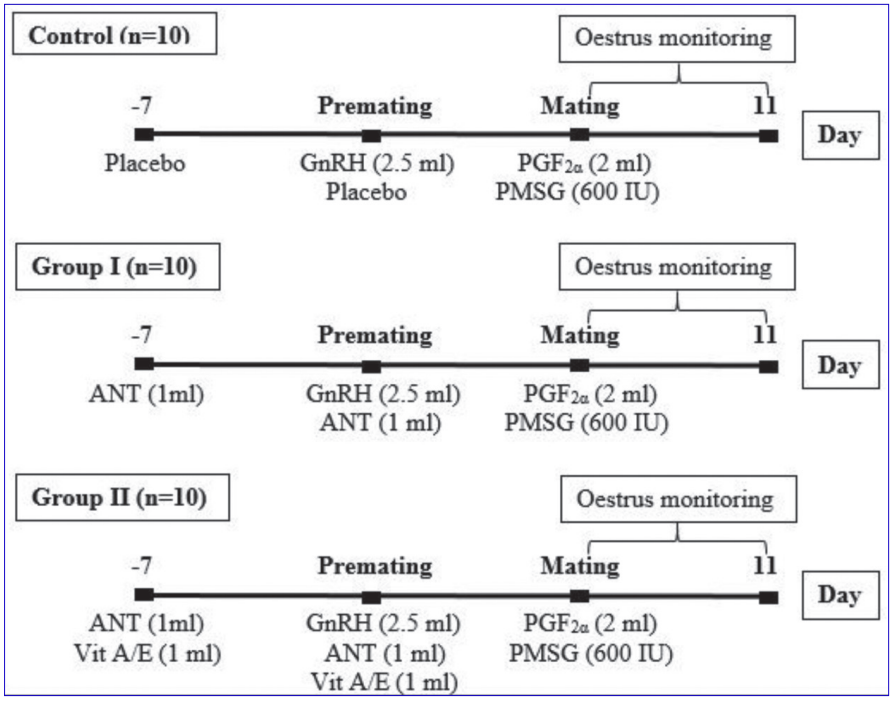


Fig 1. Study design in experimental groups

and microscopic sperm examinations were performed at the same time. Sheep were assessed for pregancy after 35 days by ultrasonography (Sonosite, Vet 180 Plus, USA).

Antibody Production

Two castrated healthy Tuj rams were used to produce ovine anti-testosterone antiserum. Rams were given five injections with an interval of 3 weeks between each injection. For the first injection, 5 mg of testosterone-3-carboxymethyloxime-bovine serum albumin (T-3-CMO-BSA, Sigma®, T3392, USA) conjugate in 2.5 ml of non-ulcerative complete Freund's Adjuvant (Sigma®, F5881, USA) was injected into different areas of dorsal skin in an intra-cutaneous manner. After 3 weeks, a booster dose of 3 mg of the batch of conjugate in incomplete Freund's adjuvant (Sigma®, F5506, USA) was injected via the same route and blood samples taken from the jugular vein 7 days later. Samples were taken from the rams every 2 weeks when antibody titres were appropriate. Testosterone antibody levels were determined by ELISA. Plasma was separated by centrifugation at 4°C and 3000 × *g* for 10 min and frozen at -20°C.

Sample Collection

To measure levels of progesterone and lipid peroxidation in the plasma, blood samples were obtained from the jugular vein either at synchronization or 1 week previously, before and after mating, and once a month during pregnancy and after giving birth. Blood was sampled using heparinized vacutainer tubes. Plasma was then separated by centrifugation (3000 × *g*, for 10 min at 4°C) and frozen (-20°C) to await further analysis.

Analytical Procedures

Lipid peroxidation contents were assessed by measuring thiobarbituric acid reacting substance (TBARS) in plasma according to the method of Placer et al.[19]. TBARS was determined in terms of malondialdehyde (MDA) content, which served as a standard of 1,1,3,3-tetraethoxy-propane (Sigma Chemical Company, T9889, USA). The values of MDA reactive material were expressed in terms of TBARS (nmol/ml plasma). Glutathione (GSH) levels of haemolysed red blood cells were measured spectrophotometrically using Ellman's reagent [20]. Haemoglobin concentration in lysed erythrocytes was also determined by the cyanmet haemoglobin method [21].

Progesterone Measurements

Progesterone levels in blood samples were determined by radioimmunoassay (RIA) using commercial kits (Immunotech®, France). Intra- and inter-assay coefficients for these kits were 6.5% and 7.2% respectively.

Statistical Analysis

Data were analysed by analysis of variance (ANOVA) using SPSS 16.0 software. Tukey's test was used to separate and compare mean data. Pregnancy and lambing rates were compared with the chi-square test. All results were expressed as the mean ± standard deviation (SD). P value <0.05 was considered to be statistically significant.

RESULTS

Plasma Progesterone Levels

Changes in plasma progesterone levels of the sheep are shown in *Fig. 2*. Progesterone levels began to increase after mating. Levels of progesterone were higher in treatment groups than the control group after this time (P<0.05) and increased as pregnancy progressed (P<0.001). In the first month of pregnancy, progesterone levels were highest in Group II (P<0.001), whereas those were higher in the Group I than in the other groups during the 3rd and 4th month of pregnancy (P<0.001).

Plasma MDA Levels

Changes in plasma MDA levels of the sheep are

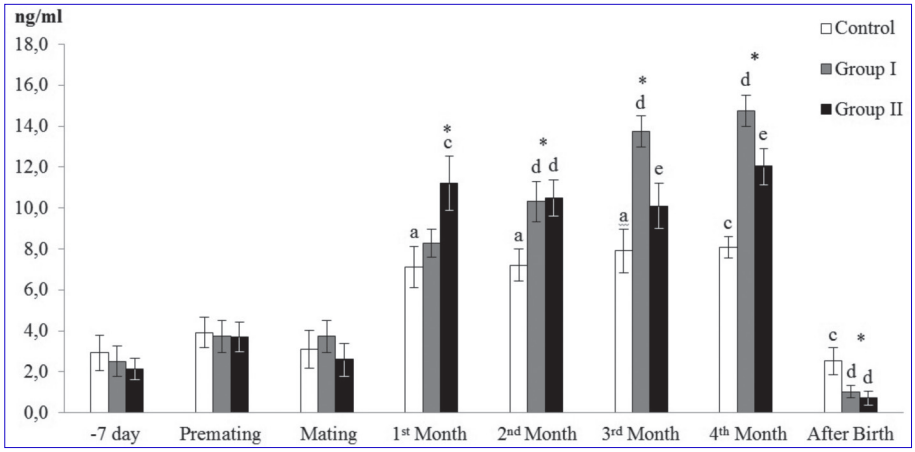
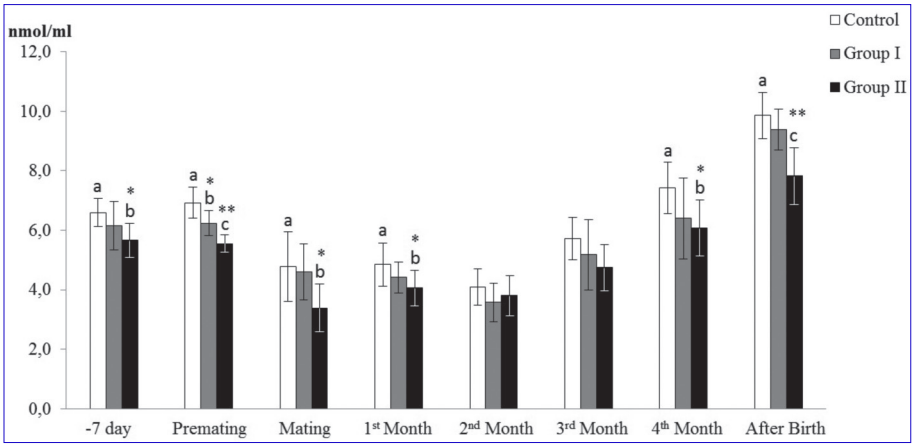


Fig 2. Levels of plasma progesterone (ng/ml) before and during pregnancy and after parturition in Tuj sheeps injected with Vitamin A-E combination and AnT. **Group I:** AnT alone, **Group II:** AnT + Vitamin A-E combination. a, b, c, d, e = Different letters indicate significant differences between groups, * P<0.001

Fig 3. Levels of Plasma MDA (nmol/ml) before and during pregnancy and after parturition in Tuj sheeps injected with Vitamin A-E combination and AnT. **Group I:** AnT alone, **Group II:** AnT + Vitamin A-E combination. a, b, c, d, e = Different letters indicate significant differences between groups, * P<0.05, ** P<0.01



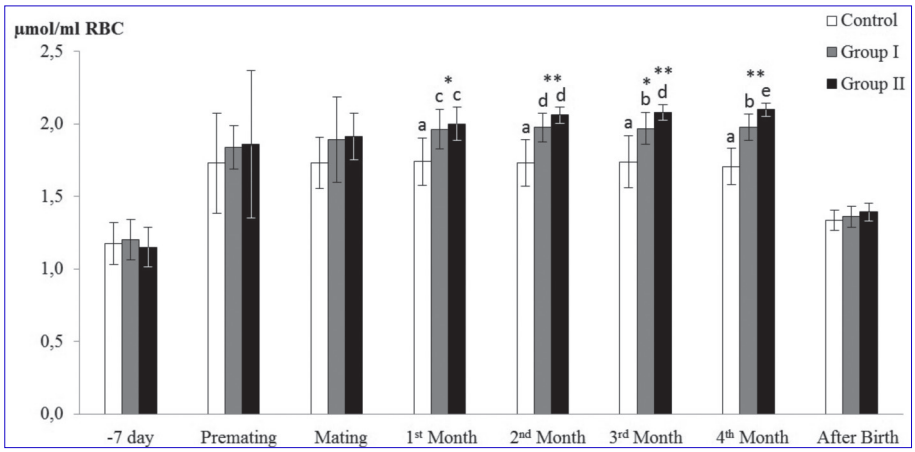


Fig 4. Levels of Plasma GSH (µmol/ml RBC) before and during pregnancy and after parturition in Tuj sheeps injected with Vitamin A-E combination and AnT. **Group I:** AnT alone, **Group II:** AnT + Vitamin A–E combination. a, b, c, d, e = Different letters indicate significant differences between groups, * P<0.01, ** P<0.001

shown in *Fig. 3*. Plasma MDA levels were high during drug application and prior to mating (P<0.01) but were lower in the experimental groups than in the control group during the 1st and 4th month of pregnancy (P<0.05) and after parturition (P<0.001). Although the levels of MDA in the plasma were higher in the control group than in the experimental groups, no significance was observed during the 2nd and 3rd month of pregnancy.

GSH Levels in Erythrocytes

Changes in the GSH levels of erythrocytes isolated from blood samples are shown in *Fig. 4*. Levels of erythrocyte GSH levels did not change following injections in the experimental groups, although a significant increase was observed during pregnancy (P<0.01). Erythrocyte GSH levels remained significantly higher in experimental groups than in the control group during pregnancy (P<0.001) and levels increased as pregnancy progressed.

Reproductive Performance

The effect of a combination of vitamins A and E and AnT injections on the reproductive performance of sheep

Table 1. *Effect of vitamin A-E combination and AnT injections on the reproductive performance of sheep*

Determined Measurements	Control (n=10)	Group I (n=10)	Group II (n=10)	P-value
Rate of Lambing (%)	66	125	100	-
Number of singlelambs	4	7	7	-
Number of tweens	-	-	1	-
Number of triplet	-	1	-	-
Number of offsprings	4	10	9	-
Number of stillbirths	2	-	1	-
Number of non-pregnant sheep	4	2	1	-
Ram joining - start of estrus (h)	67.20	64.44	62.29	-
First estrus response (%)	70^{b} (7/10)	90^{a} (9/10)	90^{a} (9/10)	a:b: 0.001
Pregnancy rate (%)	60^{b} (6/10)	80^{ac} (8/10)	90^{a} (9/10)	ac:b: 0.003; a:b: 0.001
Fecundity rate	0.7 (7/10)	1.2 (12/10)	1.0 (10/10)	

Oestrus rate *= number of sheep showing estrus × 100/total number of sheep;* ***Pregnancy rate*** *= number of pregnant sheep × 100/total number of sheep;* ***Lambing rate*** *= number of foetuses × 100/number of pregnant sheep;* ***Fecundity rate*** *= number of foetuses/total sheep number*

and formula for determining key fertility indices are given in *Table 1*. The number of offspring in Group I and Group II were higher than that in the Control group. In addition, there were no stillbirths recorded for Group I. The number of non-pregnant sheep was lowest in Group II. As shown in *Table 1*, the number of lambs and the rate of lambing in Group I and Group II were 125% and 100% higher, respectively, than those in the Control group. In addition, twins and triplets were seen in Group I and Group II. The time when the ram joined the sheep at the start of estrus, first estrus response and pregnancy rate were higher in Group II than in the other groups. However, fecundity rate was higher in Group I than in other groups.

DISCUSSION

Several authors have shown that gonadal hormones can be manipulated by both active and passive immunization methods and thus increase fertility in sheep [3,6]. However, the responses of sheep to active immunization against a variety of steroids have tended to be variable and have resulted in reduced rates of conception. Other studies have shown that passive immunization against testosterone leads to excessive ovarian stimulation and increased secretion of steroids into the ovarian vein and the follicullar fluid of ewes [7,22]. It is possible that the removal of biologically active local androgens using a testosterone antibody may result in an increased ovulation rate and improved fecundity in both sheep and cattle [3,23]. In the present study, we used passive immunization to testosterone, along with a combination of vitamin A and E to improve reproductive traits in sheep. Lambing rate and the number of live lambs were higher in experimental groups than in control groups. We also achieved a high conception rate and increased the proportion of twins and triplets, indicating that the use of testosterone antibody, with a vitamin combination, can markedly improve reproductive capacity in sheep.

Pregnancy involves anabolic states that are directed via hormones to produce nutrients in the maternal tissues and their transfer to the developing foetus via the placenta. Nevertheless, reproductive loss during pregnancy is the most significant problem in sheep breeding and it is known that progesterone plays a key role in the establishment and maintenance of pregnancy. As the hormone of pregnancy, progesterone stimulates maintenance of the early uterine environment and also development of the placenta that takes over progesterone production after week 5-8 of gestation and causes the smooth muscle of the uterus to relax. Studies of the application of vitamins A and E [24,25] and AnT [4,26] have previously shown an effect upon plasma levels of progesterone, embryonic viability and twinning rate [3,22]. However, to date, there has not been any research focussed upon the precise effects of their use together, or actually direct comparison of their actions. The present results suggest that the combination of vitamin A and E along with AnT significantly increased plasma progesterone level after mating until the 1st month of gestation. After the 2nd month of pregnancy, the application of AnT increased and maintained high progesterone levels. This may be due to the synergistic effect of antioxidants with AnT.

In pregnancy, the synthesis of hormones and changes in the partial pressure of oxygen in the placenta, leads to the formation of ROS in both the placenta and foetus. Furthermore, the multiplication and proliferation of cells, and their high rates of metabolism, cause the formation of ROS after electrons escape from mitochondria within the embryo and foetus [15]. Oxidative stress via the release of ROS, and lipid peroxidation, would be highly detrimental to the viability of both the mother and foetus. Stores of

vitamins and minerals in gestating females protect the mother and foetus from ROS fluxes and lipid peroxidation, which is essential to create an imbalance between ROS production and scavenging activity [11,27]. A study indicated that higher levels of antioxidants such as superoxide dismutase (SOD), catalase (CAT) activities or total antioxidant power (TAP) and lower levels of oxidative stress markers such as lipid peroxidation (LPO) in the endometrial secretions were associated with successful in vitro fertilization outcome [28]. During specific states of pregnancy, vitamin A and E deficiency can lead to a failure to express some genes and is also followed by increased release of MDA and of 8-oxo-7, 8-dihydro-2′-deoxyguanosine (a marker of DNA peroxidation) and by a reduction in mitochondrial GSH content [29]. Also, Nawiota et al.[16] suggested that pregnancy constituted the most oxidative stress and lipid peroxidation facing the grazing and concentrated diet feed sheep and goats under arid and saline conditions. In this study, achieved increase in oxidative stress and decrease in GSH levels during mating and pregnancy reduced by application of the combination of vitamin A-E and ANT.

Vitamin A and vitamin E have a synergic effect upon ROS trapping and the placental transport of vitamins A and E between the mother and foetus is sufficient enough to protect them from the destructive affects of lipid peroxidation. Inhibition of lipid peroxidation and the trapping of ROS via the actions of vitamin A and E have been reported to protect the integrity of mitochondria in the placenta and thus prevent extensive oxidative degradation [30]. Reduction in nutrient intake and mineral and vitamin requirements, especially vitamin A and E, from 28 to 78 days of gestation are highly likely to reduce growth and development of the ovine foetus [9]. Reports have stated that it is therefore necessary to provide supplements during the mating period of sheep in Autumn months, when the quality of grass declines and the vitamin requirements of grazing sheep increases [24,25,31]. Thomas and Kott [31] also concluded that unsupplemented ewes on rangeland lost a significant amount of weight during early to mid-gestation and that even after supplementation during late gestation, the health of their lambs was compromised. In addition, a recent report indicate that daily supplementation of vitaminE during the last 6-7 weeks before lambing decreases the stillbirth rate of ewes [14]. Low levels of maternal vitamin A and E has been shown to be associated with intrauterine growth retardation of both the embryo and foetus [24,32]. In a similar fashion, Johansson et al.[33] showed that cows in organic dairy production can fulfill their requirements of vitamins A and E without any supplementation of synthetic vitamins, except at the time around calving, when the requirements are high. Collectively, these findings support the need for vitamin supplementation during mating and pregnancy to protect both the mother and offspring from the deleterious effects of lipid peroxidation and to maintain a healthy pregnancy. Moreover, researchers offer many programs for nutrition and reproduction support to enhance reproductive performance, on the basis that increased nutritional requirements can support foetal growth and development, as well as improve ROS trapping and antioxidant levels [3,11,22,30]. The present research investigated the application of vitamins and E alone, or in combination with AnT, upon lipid peroxidation and GSH activity, when the quality of the pasture deteriorated during times of short day length. Data showed reductions in both lipid peroxidation and MDA levels, along with increased activity of GSH, an enzyme associated with ROS trapping.

Consequently, our present data indicate that the application of AnT and a combination of vitamin A and E increased fertility by reducing stress generated by mating and pregnancy in sheep. The passive immunization procedure is already progressing to farm trials. The present study suggests that the combination of AnT and vitamins A and E may result in further improvements in reproductive performance. Further research should aim to establish optimized ways of applying such techniques under practical conditions.

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