Effects of ß-carotene supplementation and age on the oxidative status, production and reproductive performance of grazing ewes

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ABSTRACT

Context. There is some evidences that physiological stages such as breeding, pregnancy, parturition and lactation may trigger oxidative stress. It is also observed in several species, including sheep, that age affects their vulnerability to oxidative stress. Aim. This study investigated the effects of supplemental ß-carotene and age on the oxidative status of grazing ewes around breeding, pregnancy, parturition and early lactation as well as on their production and reproduction performance. Methods. Hundred and four ewes were divided into two broad age groups (young=1-3 years and old =4-6 years). Within age groups, ewes of similar age were randomly assigned to four treatment groups (A1, A2, A3, C) using a completely randomized block design. The young (n=13) and old sheep (n=13) in groups A1, A2 and A3 were daily drenched with 100 mg, 75 mg and 50 mg ß-carotene, respectively. Group C was unsupplemented (Control). Supplementation lasted 28 days before oestrus synchronization; 14 days during oestrus

synchronization (CIDR) and hand-mating; 18 days post-hand-mating; 30 days before lambing and 15 days after lambing. All animals were grazed on Kikuyu grass (*Pennisetum clandestinum*). Six ewes from each group (total = 24) were sampled for blood to measure oxidative and hormonal status during the experiment. Key results. Supplemental ß-carotene ≥75 mg/day may act as a pro-oxidant in ruminants under oxidatively stressed conditions such as parturition. Age did not affect the ewes' oxidative status. The supplemental ß-carotene did not affect bodyweight, oestrus parameters, corpus luteum size, conception rate and litter size. Except for a tendency of lambs from supplemented ewes to have heavier weight at 15 days old (P = 0.080), age group and supplemental β-carotene did not affect the lamb mortality rate or weight at birth or 15 days old. Older ewes showed earlier and longer duration of oestrus than younger ewes. Conclusions. Supplementation of grazing ewes with ß-carotene should not be ≥75 mg/day especially at parturition period as it may work as a pro-oxidant. The older ewes seem to have earlier oestrus with a longer duration than the younger ones. Implications: Caution is urged when administering high doses of β-carotene to ewes during the peri-parturient period.

Keywords: sheep, hydroperoxides, CIDR, DMPD, oestrus parameters, litter size

Introduction

Oxidants such as the reactive oxygen species (ROS) help the cell to produce energy, transduce signals, express genes and maintain immunity (Bayr 2005; Valko *et al.* 2007). Conversely, when antioxidants in the body are overwhelmed by oxidants, ROS may alter the reduction-oxidation state, causing damage to DNA, protein, lipid and cell components (Ames *et al.* 1993; Miller *et al.* 1993; Bayr 2005). The damage occurs when oxidants remove hydrogen atoms or electrons from the target molecule or add oxygen, leaving it unstable and ready to attack nearby molecules

(Lykkesfeldt and Svendsen 2007). The state in which oxidants exceed antioxidants is called oxidative stress, which is often the case during pathophysiological conditions (Sies 1997). However, in ewes (Kamiloğlu *et al.* 2006; Piccione *et al.* 2008; Mohebbi-Fani *et al.* 2012; Ognik *et al.* 2015; Lotfollahzadeh *et al.* 2016; Santarosa *et al.* 2021) as well as in goats (Di Trana *et al.* 2006; Celi *et al.* 2008; Jozwik *et al.* 2010; Zobel *et al.* 2015) and cows (Drackley 1999; Castillo *et al.* 2005) there are some evidence that normal physiological stages such as breeding, pregnancy, parturition and lactation may trigger oxidative stress. It has also been observed in several species, including sheep, that age affects their vulnerability to oxidative stress (Ashok and Ali 1999; Andriollo-Sanchez *et al.* 2005; Salar-Amoli and Baghbanzadeh 2010). Oxidative stress affects animal production and reproduction performance. In females, oxidative stress may reduce litter size (Stier *et al.* 2012) and lead to granulosa cell apoptosis (Tilly and Tilly 1995), polycystic ovary syndrome, endometriosis, unexplained infertility, recurrent pregnancy loss, spontaneous abortion and preeclampsia (Agarwal *et al.* 2012). Thus, antioxidant supplementation during oxidatively stressed conditions is suggested as a tool to improve production and reproduction performance. Recently, there has been a growing interest in using natural antioxidants to meet such objectives. One of the most studied and utilized natural sources of antioxidants is β-carotene (Choe and Min 2006; Lucas *et al.* 2020).

β-carotene is a red-orange pigment and one of a large group called carotenoids (Smith 1998). In animals, β-carotene functions primarily as pro-vitamin A (Smith 1998; Nagarajan *et al.* 2017) and as a scavenger of free radicals, especially singlet state oxygen (Stahl and Sies 2005). However, some authors consider the ß-carotene effects to come from its impact on biochemical systems and not from its antioxidant properties (Pryor *et al.* 2000). They argue that ß-carotene can not donate a hydrogen or an electron to stabilise oxidants. For example, both vitamin E and vitamin C have a

hydroxyl group that is attached to an aromatic system, while ß-carotene has only a hydrocarbon compound (Pryor *et al.* 2000). Nonetheless, the use of ß-carotene supplementation as therapy for oxidative stress is controversial (Lykkesfeldt and Svendsen 2007). For example, some authors reported a positive impact following β-carotene supplementation (Peirce 1954; Salem *et al.* 2015), while others reported no effect on the production and reproduction performance (Brozos *et al.* 2007; Meza-Herrera *et al.* 2011; Köse *et al.* 2013; Meza-Herrera *et al.* 2014; Gore 2016; Meza-Herrera *et al.* 2017). Besides others, two of the reasons for the inconsistent results might be the animal physiological stage at which the ß-carotene was supplemented and the age of the animals used. It is hypothesized that supplementation of ewes with ß-carotene may improve oxidative status during various stages of reproduction and thereby improve their reproduction and production outcomes. The objective of the present study was to evaluate the effect of ß-carotene supplementation and age on the oxidative status of grazing ewes during breeding, pregnancy, parturition and early lactation as well as on their production and reproduction performance.

Materials and methods

Materials

The ß-carotene product (10% ß-carotene) was obtained from Pennville (Pty) Ltd, Pretoria, South Africa. The controlled internal drug releasing device (CIDR) and eCG hormone were obtained from RAMSEM (PTY) LTD, Bloemfontein, South Africa. N, N-Dimethyl-p-phenylenediamine dihydrochloride (DMPD) and Iron (III) chloride hexahydrate were purchased from Sigma-Aldrich (Ltd) (USA).

Animals and treatments

The study ethical approval was obtained from the University of Pretoria Animal Ethics Committee (Reference (EC056-17)). The trial was conducted at the experimental farm of the University of

Pretoria, South Africa from September 2017 to May 2018. The farm is located at latitude 25°44'30" south and longitude 28°15'30" east at an altitude of 1360 m (Van Niekerk *et al.* 2009). Hundred and four South African Mutton Merino ewes were divided into two broad age groups (young=1-3 years and old =4-6 years). Within each age group the ewes were further arranged according to their actual age. Thereafter, four ewes with similar ages were picked at a time and randomly allocated to four groups. The four groups in turn received randomly one of the beta-carotene levels (A1, A2, A3 and C) as an experimental treatment in a completely randomized block design (CRBD). The ß-carotene product was dissolved in water to give a concentration of 25 mg/ml. A 100 mg (20,000 RE), 75 mg (15,000 RE) and 50 mg (10,000 RE) ß-carotene were daily drenched to 13 young and 13 old sheep in the A1, A2, and A3 groups, respectively. No application was made to the control group (C). All animals were grazed on Kikuyu grass (*Pennisetum clandestinum*) during the day (8-9 h) and were penned at night. No wool shearing was performed on the ewes during the experimental period.

The administered doses of β-carotene used in this study are above the vitamin A requirement. The vitamin A requirement for the ewes (63.3 ± 0.8 kg) used in this study is equivalent to 9.94 mg of β-carotene/day during breeding and early lactation, 14.40 mg/day during late gestation and 16.93 mg during lactation (NRC 2007). The treatments were administered to the ewes by using a drenching gun with the animals standing in a sheep chute right before grazing. The supplementation period was 105 days, which was divided into two periods: 60 days and 45 days. The first period lasted 28 days before oestrus synchronization; 14 days during oestrus synchronization and hand-mating; and 18 days post-hand-mating. The second period covered 30 days before lambing and 15 days after lambing.

Blood sample collection

Blood samples of six ewes randomly chosen from each group (a total of 24 ewes) for blood sampling were collected (Table 1). Blood was sampled using an 18 G BD vacutainer® needle from the jugular venipuncture into heparinised BD vacutainer® tubes. The samples were collected on day zero (onset of supplementation), day 14, day 28 (day of CIDR insertion), day 40 (day of CIDR removal), day 41, day 42 (day of hand-mating), day 43, day 54 (day of CL measuring) and day 60 from the first supplementation period. During the second supplementation period, the sampling was done on day zero of supplementation, day 10, day 20, day 30 (day of lambing) and day 45 (15 days after lambing). Te collected blood samples were centrifuged for 10 minutes at 1000g (20 °C) to recover plasma aliquots, which were stored at - 20 °C for subsequent analysis.

Table 1. Mean, minimum and maximum statistics of age and litter size of ewes selected for blood sampling

Item	Expermental groups (β-carotene supplementation)			
	100 mg (n = 6)	75 mg (n = 6)	50 mg (n = 6)	Control (n = 6)
Age (years)	3.5 (1-6)	3.1(1-5)	3.3(1-6)	3.3(1-6)
Litter size	1.6 (1-3)	2(1-4)	1.7(1-3)	1.6(1-2)

Bodyweight of ewes and lambs

Bodyweights of the ewes were recorded on day zero, day 28 (day of CIDR insertion), day 40 (day of CIDR removal) and day 60 from the first supplementation period. During the second supplementation period, weighing was done on day zero (30 days before lambing), day 20 (10 days before lambing), day 30 (day of lambing) and day 45 (15 days after lambing). The bodyweights of the lambs were measured on the day of birth and at 15 days old. The animals were weighed using a one decimal point electronic weighing balance (TAL-TEC, South Africa).

Ultrasonographic examinations

Ultrasonographic examinations were performed using B-mode real-time ultrasound scanner (Aloka 500 SSD, Japan), connected to a transrectal probe with a 7.5 MHz linear array transducer (model UST-660-7.5). The examinations were done to measure the size of the corpus luteum on day 12 post-hand mating and to confirm pregnancy on day 35 post-hand mating (Mogase *et al.* 2016; Gore and Lehloenya 2020).

Oestrus synchronization and hand mating

After the first 28 days of supplementation with ß-carotene, all ewes oestrus cycles were synchronized with progesterone using controlled internal drug release devices (CIDR) which were inserted and remained intravaginally for 11 days. At CIDR withdrawal, the ewes were intramuscularly injected with 350 IU eCG hormone. Ewes were teased for three days (two times per day) with vasectomised ram after the withdrawal of CIDR. Oestrus response (%), the onset of oestrus (h) and Oestrus duration (h) were measured. Ewes were hand-mated twice at 48 and 60 h after CIDR withdrawal with rams of proven fertility. Ewe in oestrus was manually introduced to a ram in an isolated pen. Breeding was supervised and the ewe was permitted to mate with the ram for two ejaculations, then the ewe was manually removed and sequentially replaced. After the second-hand mating, the rams were left with ewes for a joining period of approximately two oestrus cycles (34 days).

Oxidative status

The plasma oxidative status/capacity was assessed as mmol ferric equivalent (mM FE/L) using the DMPD-based method modified by Mehdi and Rizvi (Mehdi and Rizvi 2013). The assessment of the oxidative status measured hydroperoxides, which are formed by the oxidation of lipids,

peptides, and amino acids (Verde *et al.* 2002). A detailed description of the method used in this study is given elsewhere (Ahmed *et al.* 2021).

Estrogen and progesterone Analysis

Plasma samples were analysed for Estrogen and Progesterone concentrations using an enzyme immunoassay (EIA) performed on microtiter plates at the endocrine laboratory of the University of Pretoria. Details of the two EIA's, are described by Schwarzenberger *et al.* (1996) and Palme and Möstl (1993). The coefficient of variance for the intra-assay variance ranged from 6.66% to 7.59% (progesterone) and 5.42 to 7.75% (estrogen), respectively. The coefficient of variance for the inter-assay variance ranged from 7.49% to 7.56% (progesterone) and 5.42% to 7.75% (estrogen), respectively. The sensitivity of the Progesterone (VUW) EIA used was 320 pg/mL plasma and the Estrogen (Estrone (E1) and Estradiol (E2) (VUW)) EIA used was 8 pg/mL plasma. Both tests were carried out in duplicates for each sample.

Statistical analyses

Statistical analyses were carried out using the integrated RStudio tool (RStudio 2021.09.1+372 for Windows). The procedure of general linear mixed model analysis of variance was conducted for bodyweight, oxidative status and hormonal status, with treatment, sampling time and age as fixed effects and animal ID as random effects. The statistical model used was:

Y *ijkl* = μ + Animal *i* + Treat *j* + Sample *k* + Age *l* + (Treat *j* * Sample *k* * Age *l*) + E *ijkl*

Where: Y *ijkl* = response variable. μ = general mean. Animal *i* = random effect of the *i*th animal ID. Treat *j* = effect of the ß-carotene treatment. Sample *k* = effect of sampling time. Age *l* = effect of the age. Treat *j* * Sample *k* * Age *l* = interactions. E *ijkl* = experimental error.

Similarly, an ANOVA was conducted for lambs' bodyweight at birth and 15 days old, with treatment, sex and litter size as fixed effects, and animal ID as random effects. The statistical model used was:

Y *ijkl* = μ + Animal *i* + Treat *j* + Sex *k* + Lsize *l* + E *ijkl*

Where:

Y *ijkl* = response variable. μ = general mean. Animal *i* = random effect of the *i*th animal ID. Treat j = effect of the ß-carotene treatment (1-4). Sex *k* = effect of the sex of the lamb (1-2). Lsize *l* = effect of the litter size (1-4) of the lambs. E *ijkl* = experimental error.

The general linear mixed model with a Poisson distribution fitted was used for the analysis of litter size with treatment and animal age as fixed effects, and weight at zero time as random effects. The package lme4 (Package lme4 version 1.1-27.1) was used to create those mixed models (Bates *et al.* 2014). The general linear model (GLM) (Package stats version 4.1.3) procedure was used to analyze the effect of treatment and age on the oestrus duration, oestrus onset and corpus luteum size using the following model:

Y *ij* = μ + Treat *i* + Age *j* + E *ij*

Where: Y ij = response variable, μ = general mean, Treat j = effect of the ß-carotene treatment (1-4), Age *j* = effect of the age of the ewe (1-2) and E *ij*= experimental error.

A binomial logistic regression was used to evaluate the effects of ß-carotene supplementation and the age of the ewes on conception rate and oestrus response. Similarly, the effects of ß-carotene supplementation, age, sex and litter size on lamb mortality were also assessed using the binomial logistic regression. When found significant, variables and interactions were pairwise compared using Tukey contrast (Package emmeans version 1.7.1-1). Data were presented as means ± standard error (SE).

Results:

Effect of ß-carotene supplementation, physiological stage and age on the bodyweight of ewes

Fig. 1 shows the effect of ß-carotene supplementation, physiological stage and age on the bodyweight of ewes. The results show that ß-carotene supplementation had no significant effect on the bodyweight (p = 0.978) in both young and old ewes. A significant difference in bodyweight was recorded between old and young ewes. Older ewes were heavier than their younger counterparts ($p < 0.001$). The physiological stages significantly affected bodyweight of ewes during the experimentation period ($p < 0.001$). Ewes gained weight gradually and reached their peak during the last month of pregnancy. After lambing, the bodyweight of ewes significantly decreased for two weeks.

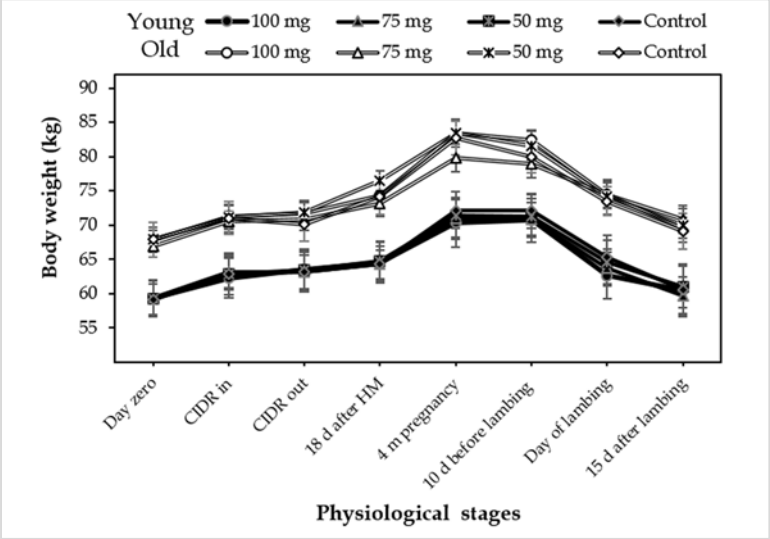

Fig. 1. Effect of ß-carotene supplementation, age and physiological stage (day zero, CIDR in, CIDR out, 18 days after hand mating (HM), 4 months of pregnancy, 10 days befor lambing, day of lambing and 15 days after lambing) on body weight (kg) of ewes

Effect of ß-carotene supplementation, physiological stage and age on oxidative status of ewes

Our results indicated that ß-carotene supplementation and the physiological stage of ewes, as well as the interaction between both, significantly influenced ($p < 0.001$) their oxidative status (Fig. 2). The results showed that pre-mating or during pregnancy, ß-carotene supplementation did not affect the oxidative status of the ewes. At parturition, however, the group supplemented with 100 mg and 75 mg ß-carotene showed higher ($P < 0.001$) oxidative status than the control group. However, the observed significant differences faded 15 days later after lambing. The oxidative status of the group supplemented with 50 mg ß-carotene did not differ significantly from their counterparts at parturition. Moreover, oxidative status on the day of parturition was significantly higher than that of pre-pregnant and pregnant ewes in groups supplemented with 100 mg and 75 mg ß-carotene. Then, 15 days later after lambing the oxidative status of both groups declined back to levels similar to those during pregnancy or pre-pregnancy. The oxidative status for control and 50 mg ß-carotene groups did not significantly change during the various physiological stages. Although it appears that old ewes had a higher value of oxidative status than young ones (332 ±11.9 and 312 ± 12.5 mmol FE/L, respectively), our results showed no significant difference between the two groups ($p = 0.213$).

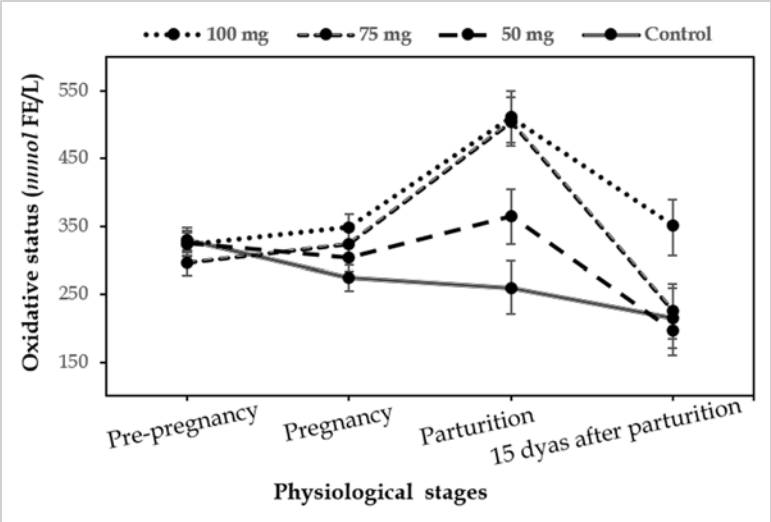

Fig. 2. Effect of interaction between ß-carotene supplementation and physiological stage (pre-pregnancy, pregnancy, parturition and 15 days after parturition) on oxidative status of ewes

Supplementation with ß-carotene did not affect (p = 0.353) the oxidative status of the ewes during oestrus synchronization (Fig. 3). However, the oxidative status of the ewes was significantly affected (p = 0.002) by oestrus synchronization (Fig. 3). Compared to CIDR insertion (285 ± 18.1 *mmol* FE/L), the removal showed a non-significant increase (341 ± 18.1 *mmol* FE/L) in the oxidative status of the ewes. After maintaining this oxidative status for 48 h (340 ± 20.9 *mmol* FE/L), it significantly declined at 72 h (259 ± 18.4 *mmol* FE/L) to a level similar to that before the CIDR removal. Our results suggest that oxidative status may slightly increase (hydroperoxides increase) during the 72 hours after CIDR removal.

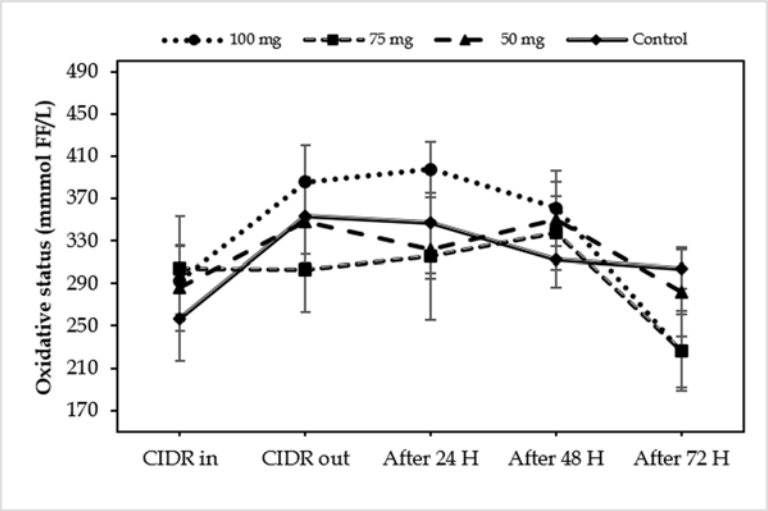

Fig. 3. Effect of between ß-carotene supplementation and oestrus synchronization (CIDR in, CIDR out, 24 H, 48 H and 72 H after CIDR removal) on the oxidative status of ewes

Effect of ß-carotene supplementation, time of sampling and age of ewes on progesterone and estrogen concentrations

Fig. 4 shows the effect of ß-carotene supplementation, time of sampling and age on the progesterone and estrogen concentrations in ewes. Supplementation with ß-carotene did not affect plasma progesterone (P4) concentration ($p = 0.506$). The time of sampling ($p < 0.001$) and age ($p = 0.0137$), as well as the interaction between both ($p = 0.037$), significantly influenced the progesterone concentration in the ewes. The interaction results show a significant difference between the old and young ewes on day zero. This difference, however, became non-significant on the day of mating and 12 days after mating. Old and young ewes had higher progesterone concentrations 12 days after mating than they did on the day of mating ($p < 0.001$). Supplementation with ß-carotene tended to affect the estrogen concentration of ewes ($p = 0.079$). Although with a tendency to higher level estrogen concentration on day zero did not differ

significantly (p = 0.083) from that on the day of CIDR removal. The concentration of estrogen in older ewes did not also differ significantly (p = 0.259) from that of young ewes.

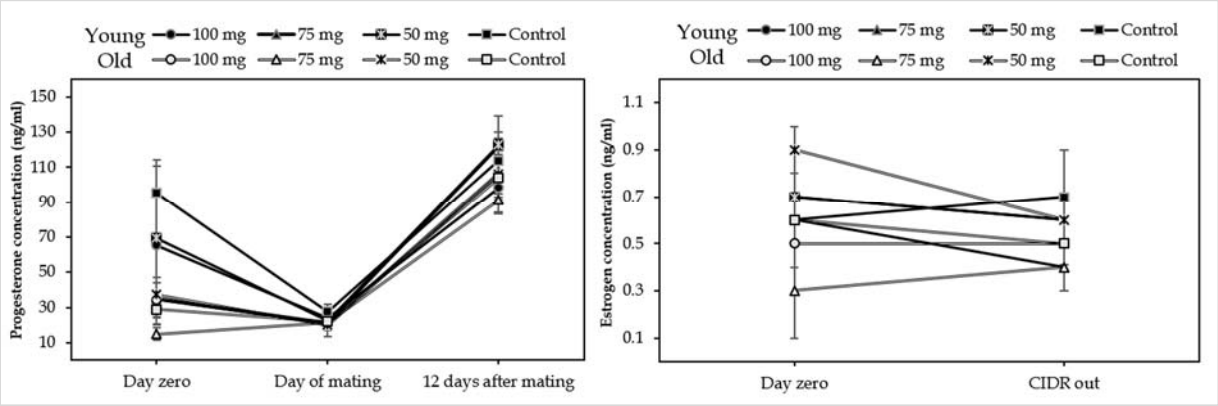

Fig. 4. Effect of ß-carotene supplementation, age and sampling time on progesterone and estrogen concentrations in ewes

Effect of ß-carotene supplementation and litter size on the weight of lambs

Fig. 5 presents the effect of ß-carotene supplementation of ewes and litter size on the weight of lambs at birth and 15 days old. The supplementation of ewes with ß-carotene did not affect the birth weight of their lambs (p = 0.750). However, lambs from ewes supplemented with ß-carotene tended to have a higher weight at 15 days old (p = 0.080). The litter size significantly affected the weight of the lambs at birth and 15 days old (p < 0.001). Single lambs were the heaviest both at birth and 15 days old compared to twins, triplets or quadruplets. At birth and 15 days old, twins were heavier than triplets and quadruplets. No significant difference in weight was observed between triplets and quadruplets at birth or 15 days old.

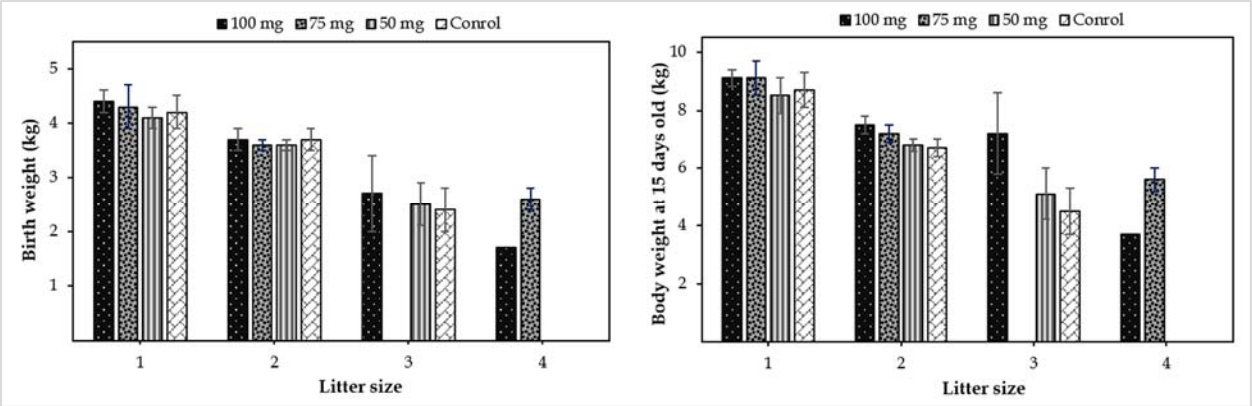

Fig. 5. Effect of ß-carotene supplementation and litter size on body weight (kg) of lambs at birth and 15 days old

The results showed a tendency for the male lambs to be heavier than females at birth ($p < 0.072$) and 15 days old ($p < 0.074$). At birth, the weight means were: 3.33 ± 0.13 and 3.13 ± 0.13 kg, while at 15 days old were: 6.82 ± 0.21 and 6.40 ± 0.22 kg for male and female lambs, respectively.

Effect of ß-carotene supplementation and age of ewes on the litter size of lambs

Table 2 presents the litter size incidence ratios with 95% confidence intervals (95% CI) across various levels of β-carotene supplementation and ewes age. The ß-carotene supplementation and ewe age did not significantly affect the litter sizes. However, quadruplets were found in the 100 mg (4.2%) and 75 mg ß-carotene groups (11.1%), but not in the control or 50 mg ß-carotene groups. Frequencies of singles, twins, triplets and quadruplets for young ewes were: 47.8, 41.3, 4.3 and 6.5 %, while for old ewes were: 38.2, 52.7, 7.3 and 1.8 %, respectively.

Table 2. Effect of β-carotene supplementation and age of ewe on the incidence rate ratio of litter size with associated 95% confidence intervals (CI)

Item	Incidence rate ratio	95% CI	*P*- value
Intercept	1.74	1.25 - 2.42	0.001
Treatment: 100 mg/day	0.92	0.59 - 1.42	0.702
Treatment: 75 mg/day	1.03	0.69 - 1.55	0.887
Treatment: 50 mg/day	0.93	0.61 - 1.42	0.741
Age: Old	1.03	0.76 - 1.39	0.859

Effect of ß-carotene supplementation, age of ewes, sex and litter size on the mortality rate of lambs

Table 3 displays the odds ratios with 95% CI for mortality rate up to 15 days across various levels of β-carotene supplementation, ewe age, lamb sex, and litter size. With the exception of litter size all other variables were statistically not significant. The results suggest that ß-carotene supplementation, age of ewe and sex of the lamb do not affect lamb mortality up to 15 days. Lambs from quadruplet litters had 43.51 times higher odds ($P = 0.004$) and lambs from triplet litters had 31.43 times higher odds ($P = 0.005$) of mortality compared to lambs from single litters (Table 3). In addition, lambs from litter size of two had 4.04 times higher odds of mortality compared to lambs from single litters but was statistically non significant ($P = 0.212$). The results indicate that larger litter sizes (> 2) were associated with higher lamb mortality up to 15 days, while smaller litters sizes indicated markedly lower odds of mortality.

Table 3. Odds ratios of lamb mortlity rate (15 days old), oestrus response and conception rate with associated 95% confidence intervals (CI) by different variables

Item	Odd ratio	95% CI	*P*- value
Mortality rate			
Intercept	0.02	0.00 - 0.18	0.001
Treatment: 100 mg/day	2.33	0.86 - 12.7	0.309
Treatment: 75 mg/day	0.61	1.13 - 5.62	0.626
Treatment: 50 mg/day	0.59	0.82 - 2.93	0.554
Litter size: 2	4.04	1.07 - 32.94	0.212
Litter size: 3	31.43	1.24 - 353.73	0.005
Litter size: 4	43.51	1.25 - 500.34	0.004
Sex: Female	0.42	0.58 – 1.30	0.141
Age: Old	1.95	0.67 – 7.19	0.321
Oestrus response			
Intercept	0.99	0.97 - 1.01	0.964
Treatment: 100 mg/day	1.00	0.99 - 1.01	0.991
Treatment: 75 mg/day	0.96	0.89 - 1.04	0.891
Treatment: 50 mg/day	1.00	0.99 - 1.01	0.998
Age: Old	1.02	0.98 - 1.06	0.917
Conception rate			
Intercept	0.99	0.96 - 1.01	0.955
Treatment: 100 mg/day	0.96	0.88 - 1.04	0.876
Treatment: 75 mg/day	1.00	0.99 - 1.01	0.997
Treatment: 50 mg/day	1.00	0.99 - 1.01	0.997
Age: Old	1.03	0.98 - 1.08	0.898

Effect of ß-carotene supplementation and age on oestrus parameters, conception rate, and corpus luteum size in ewes

The odds ratios with 95% CI for oestrus response and conception rate by β-carotene supplementation and ewe age are displayed in Table 3. Supplemental ß-carotene and ewe age had no effect on oestrus response and conception rate. The effect of age and β-carotene supplementation on oestrus onset, oestrus duration and corpus luteum size is presented in Table 4. Supplemental ß-carotene had no effect on oestrus onset (p = 0.900), oestrus duration (p = 0.959) and corpus luteum size (p = 0.935). Although age did not affect the corpus luteum size (P = 0.553),

older ewes significantly showed earlier onset ($p < 0.001$) and longer duration of the oestrus ($p < 0.001$) than the younger ewes (Table 4).

Table 4. Effect of β-carotene supplementation and age of ewe on oestrus duration, oestrus onset, and corpus luteum size at 12 d after hand-mating (Mean ± SE)

Item	Oestrus onset (hrs)	Oestrus duration (hrs)	Corpus luteum size (mm)
Treatments			
100 mg	19.4 ± 1.9	43.6 ± 2.7	9.9 ± 0.8
75 mg	20.0 ± 1.8	45.3 ± 2.6	9.2 ± 0.8
50 mg	18.2 ± 1.8	44.4 ± 2.6	9.9 ± 0.8
Control	18.7 ± 1.9	45.4 ± 2.6	9.8 ± 1.0
p-value	0.900	0.959	0.935
Age			
Young	23.2 ± 1.4	38.2 ± 2.0	10.0 ± 0.6
Old	15.0 ± 1.2	51.2 ± 1.8	9.4 ± 0.6
p-value	<0.001	<0.001	0.553

Discussion:

The lack of significant effect of ß-carotene supplementation on bodyweight agrees with previous reports in sheep (Brozos *et al.* 2007) and goats (Arellano-Rodriguez *et al.* 2009). The significant difference in bodyweight between old and young ewes observed in our result was previously documented by some authors (Croker *et al.* 1990; Oddy *et al.* 2018). Ewes gained weight gradually and reached their peak during the last month of pregnancy. The observed gradual increase in weight is due to gestation, with maximum weight gain occurring during the last month, accounting for approximately one-third of total pregnancy weight gain (Thiruvenkadan *et al.* 2008). After lambing, the ewes' weights may have decreased further during the early days of lactation because of the high demand for energy and protein as milk production is given priority over maintaining weight.

Researchers have reported ewes to be prone to oxidative stress pre, during and post-parturition (Mousaie *et al.* 2017; Dunière *et al.* 2021; Moradi *et al.* 2021; Yaqub *et al.* 2021). Our study

assessed plasma oxidative status by measuring hydroperoxides, which are intermediate products resulting from the oxidation of lipids, peptides, and amino acids. Interestingly, as shown, supplementation of ewes with 100 mg and 75 mg ß-carotene increased the hydroperoxides levels in the plasma. Although ß-carotene's antioxidant properties are to quench singlet oxygen, scavenge oxyradicals, and terminate other free radical reactions, it is evident that its overall physiological action is complex (Black 2002). Under oxidative stress conditions, biochemical studies have shown that carotenoids may act as a pro-oxidant (Zhang and Omaye 2001; Alija *et al.* 2005; Siems *et al.* 2005). When carotenoids are exposed to reactive species under mild oxidative stress, products with high molecular weight, such as apo-carotenals, will be formed (Ribeiro *et al.* 2018). However, under heavy oxidative stress, supplemented carotenoids degrade into high levels of short-chain carbonyls, aldehydes, and epoxides. For example, short-chain products such as β-cyclocitral, β-ionone, ionene, 5,6-epoxi-β-ionone, dihydroactinidiolide and 4-oxo-ionone are consistently found after oxidation of β-carotene by various oxidizing agents (Siems *et al.* 2005). Being highly reactive pro-oxidants, these breakdown products may lead to cell oxidative damage (Siems *et al.* 2005; Ribeiro *et al.* 2018). The elevated levels of hydroperoxides observed with higher ß-carotene supplementation (75 mg and 100 mg/day) could be related to the accumulation of these broken-down products due to parturition-induced oxidative stress. Our results indicate that ß-carotene may work as pro-oxidants under oxidatively stressed conditions, especially at high levels of supplementation. As far as we know, this is the first in-vivo study in ruminants that shows ß-carotene may work as a pro-oxidant under high oxidative stress conditions. In this context, the results may indicate that parturition is one of the most oxidatively stressed conditions during the female reproduction cycle (Bernabucci *et al.* 2005; Ali 2011; Dunière *et al.* 2021). The ß-carotene in the 100 mg and 75 mg groups might have acted as the first line of defence, interacting

excessively with the oxidants and shielding the rest of the antioxidants, resulting in a higher concentration of hydroperoxides than in the control or 50 mg groups. However, the control group oxidative status showed stable status from pre-mating through to 15 days after lambing and was not elevated during parturition. It is crucial to note that the animals in this study were grazing, meaning they might have received a pasture-dependent antioxidant combination (Luo *et al.* 2019; Ford *et al.* 2021). The combination of antioxidants may have been able to block the oxidative stress effects without raising plasma hydroperoxide concentrations. Nevertheless, further research in grazing ewes measuring and specifying carotenoid breakdown products and oxidative damage markers across different physiological states and β-carotene supplement doses could substantiate if and when carotenoids become pro-oxidative.

Our results suggest that oxidative status may slightly increase during the 72 hours after CIDR removal (Fig. 3). Salinas-Rios *et al.* (2016), reported similar results in terms of lipid peroxidation, where CIDR removal caused a numerical non-significant increase in peroxidation and then declined gradually thereafter. At CIDR removal, ewes in this study were injected with eCG. With its FSH-like activity, the eCG may initiate preovulatory events by increasing endogenous gonadotrophin levels (Abecia *et al.* 2012). The slight increase in oxidative status following CIDR removal could be due to the hormonal changes caused by CIDR removal and eCG injections. These changes may increase metabolic activity and thus oxidative status during this period of oestrus synchronization. The slight increase in oxidative status may also be partially due to vaginal irritation caused by the removal of CIDR (Kuru *et al.* 2018).

Although it appears that old ewes had a higher oxidative status than young ewes, our results showed no significant difference between the two groups. However, Martino *et al.* (2012) reported that reactive oxygen species were significantly higher in oocytes from aged donors as compared

to those from young ones. Nevertheless, in their study, Martino *et al.* (2012) used different age groupings (young: 2-5 y and old: 7-10 y) than those in our study, which may explain the difference in results.

Our results are in line with the previous studies in sheep (Smith *et al.* 1976) and cows (Kaewlamun *et al.* 2011; Hye *et al.* 2020), in which plasma progesterone was not affected by ß-carotene supplementation. Multiple factors influence the development, regression, and secretory activities of the corpus luteum (Hye *et al.* 2020). Therefore, supplementing with ß-carotene alone may not be sufficient to promote luteal development and progesterone production (Hye *et al.* 2020). Nevertheless, other studies indicated that β-carotene supplementation increased progesterone concentration when injected at oestrus synchronization in cows (Çelik *et al.* 2009) and in goats when orally supplemented pre-and during estrus synchronization (Arellano-Rodriguez *et al.* 2009; Gore and Lehloenya 2020). The differences between studies might be due to the season, species, breed, age, ß-carotene content in the feed, and level of ß-carotene supplementation used.

A higher level of progesterone in old ewes than in younger ewes at day zero may be the result of an active corpus luteum, whose activity was neutralized later by CIDR of oestrus synchronization. Similar progesterone concentrations in young and old ewes seen in current and previous studies (Smith *et al.* 1976; Michels *et al.* 1998) may reflect similar luteal function in young and old sheep. The increased progesterone concentration 12 days after mating in both old and young ewes is probably due to the formation of the corpus luteum.

Celik *et al.* (2009) studied the effect of ß-carotene injection (1 mg/kg) on repeat breeder cows. They performed an Ovsynch program with two injections of GnRH (10µg), 7 d before and 48 h after the injection of PGF2α (25 mg). Our results agree with their findings where ß-carotene injection did not affect serum estrogen levels on the day of the first GnRH injection or the day of

the PGF2α injection. However, they observed an increase in estrogen level on the day of the second GnRH injection. Their results may indicate that the ß-carotene effect on estrogen was only observed during the late proestrus or oestrus stage when estrogen usually peaks. Those later periods were not included in our study as estrogen concentration was measured at the very beginning of proestrus (at CIDR removal). No statistically significant effect of ß-carotene supplementation on estrogen concentration has also been observed in goats (Gore and Lehloenya 2020). The observed lack of significant effect of age on estrogen concentration in ewes, in the present study, agrees with earlier reports (Smith *et al.* 1976; Gonzalez-Bulnes *et al.* 2004).

In this study, the supplementation of ewes with ß-carotene did not affect the birth weight of their lambs. Similarly, it has been reported that ß-carotene supplementation did not influence the birth weight of goats kids (Gore 2016) and calves (Kaewlamun 2010). However, lambs from ewes supplemented with ß-carotene tended to have a higher weight at 15 days old. This encouraging tendency for higher weight gain at 15 days suggests opportunities to further research underlying mechanisms and optimal supplementation levels using larger experimental scales for better experimental power.

Our results may indicate that the difference in weight at birth between singletons and twins may persist as the lambs grow. In their study, Corner *et al.* (2007) reported that singletons were heavier ($P < 0.05$) than twin lambs at birth, 42 and 94 days old. The persistence of the difference may be due to competition for dam's milk in the case of twins, as opposed to no competition in the case of singletons (Boujenane and Diallo 2017).

The results showed a tendency for the male lambs to be heavier than the female ones at birth and 15 days old. It was documented in many studies that male lambs are born heavier and may grow faster than females (Morel *et al.* 2008; Boujenane and Diallo 2017). However, in some studies, the

effect of sex on the birthweight of lambs was not detectable (Abdullah 2010; Simeonov *et al.* 2014).

Our results suggest that ß-carotene supplementation does not affect lamb survival. According to our knowledge, no such investigation has been conducted in ewes. Nevertheless, unlike in the current study, a positive effect on the survival of calves was reported in ß-carotene injected cows (Zubova *et al.* 2021). The differences between studies might be due to species, feed ß-carotene content, and level of ß-carotene supplementation used. While some prior studies align with our findings regarding the insignificant impact of ewe age (Barazandeh *et al.* 2012) and lamb sex (Boujenane *et al.* 2013) on lamb survival, others have reported significantly higher survival rates for female lambs compared to males (Zapasnikienė 2002; Binabaj *et al.* 2013; Tesema *et al.* 2020) and for lambs from older ewes compared to younger ones (Barazandeh *et al.* 2012; Ceyhan and Kozaklı 2023). The disparities in findings across studies may be attributed to variations in the timing of lamb survival assessments. For instance, (Aktaş *et al.* 2015) found no effects of ewe age on survival at 75-90 days, consistent with our study. However, they observed lower survival at 120 days for lambs from younger ewes. Similarly, (Boujenane *et al.* 2013) detected no lamb sex effects at 90 days, while (Barazandeh *et al.* 2012) and (Ceyhan and Kozaklı 2023) reported elevated male mortality in the first week. The effect of the litter size on the mortality of lambs may be closely related to the effect of the litter size on the birth weight and weight increase in the lambs (Morris *et al.* 1999; Corner *et al.* 2007). As demonstrated previously (Fig. 5), lambs from triplet and quadruplet litters were significantly lighter than those of one and two, which typically results in lower survival rate.

The lack of significant effect of ß-carotene supplementation on litter sizes in ewes was also reported elsewhere (Ozmen *et al.* 2022). Our findings are similar to those found in goats (Gore

and Lehloenya 2020) and cattle (Wang *et al.* 1987; Wang *et al.* 1988; Hye *et al.* 2020), where ß-carotene supplementation did not affect the oestrus response, onset, duration, or conception rate. No effect on the corpus luteum size was also reported in cows (Hye *et al.* 2020). In disagreement with the current study, β-carotene supplementation increased oestrus response in ewes (Salem *et al.* 2015) and the conception rate in cows (Aréchiga *et al.* 1998; Bhateshwar *et al.* 2021). Besides differences between species, the inconsistencies between studies may be due to variability in β-carotene content in the feed and levels of β-carotene supplementation used. For example, in Salem *et al.* (2015) study, no green forages were offered, indicating the diet likely did not meet the ewe β-carotene requirement before supplementation.

Our results indicate that older ewes may exhibit more intense oestrus symptoms than younger ones. This indication was observed elsewhere (Simitzis *et al.* 2006; Pascual-Cordova *et al.* 2009). No significant effect of age on corpus luteum size was also reported (Berlinguer *et al.* 2012). In disagreement with the present results, Berlinguer *et al.* (2012) reported no effect of age on oestrus duration. However, Berlinguer *et al.* (2012) compared adults (4-6) and very old ewes (12-14 y), while in this study, we compared young (1-3 y) and old ewes (4-6 y), which may explain the differences in findings.

Conclusions

In the literature, the role of β-carotene supplementation as a therapy for oxidative stress in animals is unclear. Besides others, two of the reasons for the inconsistent results might be the physiological stage at which the ß-carotene was supplemented and the age of the animals used. This study is the first to report for sheep grazing pasture, supplemental ß-carotene may act as a pro-oxidant during parturition. The study raises concern over high supplemental β-carotene exacerbating oxidative stress during periods of significant physiological stress. However, these effects do not appear to

manifest in lower survival or additional stress for the ewe and lamb. Interestingly, quadruplet lamb litters emerged only in groups receiving higher β-carotene doses. This observation might require further investigation on the effect of ß-carotene supplementation before and during breeding, especially in flok with low fertility.

Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of Interest. None of the authors has any conflict of interest to declare.

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