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Temporal profiles of indicators of oxidative status in plasma and mammary cells during regenerative involution of dairy goats

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Research Article

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Abstract

Regenerative involution is crucial for renewing the mammary gland and maximizing milk production. However, the temporal profiles indicators of oxidative status during this phase are still unclear. In this study, Experiment 1 aimed to investigate the dynamic changes in indicators of oxidative status in plasma during regenerative involution. The dairy goats were dried off at 8 weeks (wk) before kidding (−8 wk, $n = 14$) or −12 wk ($n = 6$). The blood samples taken at −8, −7, −6, −5, −4, −3, −2, −1 wk, on the day for kidding (0 wk) and the first week after kidding (+1 wk, milk production 1.28 ± 0.31 kg per day). Experiment 2 aimed to investigate the dynamic changes in indicators of oxidative status in mammary cells. Seven selected goats were biopsied for tissue collection and cell isolation at −8, −4, −1, +1 wk (milk production 1.28 ± 0.31 kg per day), respectively. Plasma analysis in Experiment 1 showed an increase in reactive oxygen species (ROS) levels, peaking at −4 wk ($P < 0.01$). No significant differences were observed between the dry-off treatments ($P = 0.36$). The activity of superoxide dismutase (SOD) in plasma remained stable from −7 wk to the first week after kidding (+1 wk), while glutathione peroxidase (GSH-Px) activity peaked at −4 wk. An increased catalase activity was observed at +1 wk ($P < 0.01$), indicating its response to lactation. In Experiment 2, an increase in ROS levels in isolated mammary cells was observed at −4 wk, while SOD, GSH-Px, and malondialdehyde levels in tissue homogenates rose around kidding ($P < 0.01$). The dynamic change of the oxidative status suggests that targeted antioxidant strategies would be helpful for regenerative involution of mammary gland in ruminants.

Introduction

The involution of the mammary gland in dairy ruminants is crucial for maximizing milk yield in subsequent lactation. In nonpregnant mammals, drying off leads to extensive tissue remodeling and loss of alveolar structure, which marks the initiation of involution in the dry period. However, animals being pregnant during the late lactation when milking is terminated results in an overlap of mammary involution and pregnancy in dairy livestock. Consequently, the cell proliferation stimulated by pregnancy contradicts the cell death stimuli for mammary involution during the dry period (Capuco and Ellis 2013). The period of mammary growth and involution between successive lactations is referred to regenerative involution in dairy livestock (Capuco and Choudhary 2020; Capuco and Ellis 2013). During regenerative involution, the rate of DNA synthesis in mammary tissues is 80% higher in dry cows compared to lactating cows, emphasizing the importance of mammary cell renewal during this period (Capuco and Akers 1999; Capuco et al. 1997). Understanding the complex processes involved in regenerative involution could help shorten the dry period.

Reactive oxygen species (ROS) are byproducts of aerobic metabolism. The high levels of ROS may damage lipids, proteins, and DNA, resulting in a condition referred to as oxidative stress (Sies et al. 2022). Superoxide dismutase (SOD), a key enzymatic antioxidants, plays a crucial role in neutralizing increased levels of ROS (Diebold and Chandel 2016; Gurer-Orhan et al. 2018). The SOD converts superoxide into hydrogen peroxide (H_2O_2), which can then be further converted to water (H_2O) by glutathione peroxidase (GSH-Px) and catalase (CAT). Beyond oxidative status, ROS also have reactivity towards various biological targets and contribute to redox signaling and biological functions in mammals, which is referred to redox biology. Within the mammary gland development, ROS are suggested as an indicator of signaling morphogenesis, and homeostasis (Kannan et al. 2014; Niki 2016). Increased ROS levels are involved in the formation of lumen and are associated with the activation of luminal progenitor cells in rodent mammary gland (Kannan et al. 2014; Schafer et al. 2009). The genetic deletion of enzymatic antioxidant glutaredoxin 3 hinder mammary development (Pham et al. 2017), suggesting a role of ROS in mammary organogenesis.

During mammary gland involution, ROS are suggested signals for the death of ROS-sensitive mammary epithelial-secretory cells (Baratta et al. 2018; Zaragozá et al. 2010). Experiments *in vitro* showed that introduction of exogenous ROS can induce this involution (Thomas et al. 2011). Although previous studies has demonstrated increased ROS levels in transition of dairy cows (Bernabucci et al. 2002; Spaans et al. 2022; Z et al. 2024), the temporal profiles of oxidative status indicators during the entire regenerative involution have not been thoroughly investigated *in vivo*. To address this gap, we conducted a comprehensive study in dairy goats. Our findings revealed an increase in ROS levels at 4 weeks (wk) before kidding (−4 wk), followed a significant decrease around kidding, both in plasma and mammary cells. The observed alterations in oxidative status suggest a critical role of ROS associated with mammary gland development.

Materials and methods

Animals and experimental design

The experimental procedures were approved by the Animal Care Committee of Zhejiang University (Hangzhou, China) and were conducted in accordance with the Guidelines for Animal Research of Zhejiang University. In Experiment 1, we aim to systematically assess the temporal profiles of the ROS and enzymatic antioxidants in plasma during regenerative involution. We selected 20 dairy goats (3-year old, multiparous, second lactation, 90-day pregnancy with estrus synchronization, weight 56 ± 1.5 kg, averaged milk production in previous lactation 1.48 ± 0.28 kg per day) for collection of plasma samples. Among these goats, six were dry-off at 12 wk before the kidding (−12 wk) and the remaining 14 were dried off at 8 wk before the kidding (−8 wk). B-ultrasound was employed to determine the pregnancy status and all the selected goats were pregnant. Before drying off, the goats were milked twice a day. They were treated with a standard antimicrobial therapy to protect against intramammary infections (penicillin at 20 mg/kg every 12 h for 7 days) at drying off. All goats had blood samples taken once a week at −8, −7, −6, −5, −4, −3, −2, −1 wk, on the day for kidding (0 wk) and the first week after kidding (+1 wk, milk production 1.51 ± 0.26 kg per day). Blood samples were collected from the jugular vein.

In Experiment 2, we aim to assess the oxidative status of the mammary cells. For this experiment, another seven Saanen dairy goats (3-year old, second lactation, 90-day pregnancy with estrus synchronization, weight 55 ± 2.1 kg, averaged milk production in previous lactation 1.34 ± 0.23 kg per day) were selected for the collection of blood and mammary tissues. These seven goats were dried off at −8 wk. The mammary gland tissues were biopsied and collected at −8, −4, −1, +1 wk (milk production 1.28 ± 0.31 kg per day). All the goats were sampled at each of these times. Blood samples were collected from the jugular vein before the biopsy. For both experiment, the diets fed to the goats remain unchanged from −8 to +1 w. The diets were changed for the lactation stage after the last blood sample or tissue collection. The nutrient compositions of diets for the dry period and lactation are described in Tables S1 and S2 in the Supporting file.

Sample collection and processing

All the plasma and mammary tissue samples were collected first or second day each week. Selected dairy goats were fasted on the morning of sampling. Approximately 3 mL of blood samples were collected from the jugular vein using an anticoagulation tube containing heparin sodium. The samples were gently mixed and then centrifuged at 3,000 × *g* for 15 min at 4°C to separate the plasma, which was then frozen at −80°C until further analysis.

Mammary tissues were obtained from each of the seven goats through surgical procedures at different time points: −8, −4, −1, +1 wk by a professional veterinarian. The operation method of biopsy was referred to the previous procedures with modification (Cai et al. 2020; Farr et al. 1996). The surgical sites for these four time points were selected from the middle of the right-lateral udder, left-lateral udder, right-caudal udder, and left-caudal udder of the goats, respectively. The goats were anesthetized with an intramuscular injection of Sedazine II (xylazine hydrochloride injection). An area of the middle udder was chosen to avoid major blood vessels. The skin surface of the area was shaved and followed disinfection with iodine and 75% ethanol solution. Local anesthesia was performed by subcutaneous injection of procaine hydrochloride at the biopsy site. The collected tissues, approximately 1 g each, were immediately washed and trimmed in sterile physiological saline. The mammary tissue was then collected into a DMEM/F12 medium containing 10% fetal bovine serum (FBS) and stored at 4°C. Fresh tissue samples were dissociated for cell isolation within 2 h. After obtaining the sample, the biopsy incision of mammary gland was closed with absorbable sutures. Penicillin and streptomycin were injected intramuscularly. During the lactation stage, the goats were milked by hand every day for the next week until all the blood clots in the udder were removed (Hu et al. 2024).

Plasma parameters

The concentrations of ROS in plasma were determined immediately on the day of sampling using a kit (Jiangsu Enzyme-labeled Biotechnology Co. Ltd., Suzhou, China). Briefly, plasma ROS attach to a probe of the kit, converting dichlorodihydrofluorescin to a product that fluoresces. All 96-well microtiter plates (Thermo Fisher Scientific, Waltham, MA) were read using a Fluoroskan Ascent apparatus (Thermo Fisher Scientific). Fluorescence was determined at 480 nm of excitation and 530 nm of emission. A standard curve was created at various concentrations. Thus, fluorescence is proportional to the amount of ROS in the sample. The kits for assay of triglyceride (A110-1-1), nonesterified fatty acid (NEFA, A-042-2-1) and β-hydroxybutyrate (BHBA, E030-1-1) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The levels of malondialdehyde (MDA), SOD activity, GSH-Px activity, CAT activity, and total antioxidant capacity (T-AOC) in plasma were analyzed using an Auto Analyzer 7020 instrument (Hitachi High-Technologies Corporation) with commercial colorimetric kits (Ningbo Medical System Biotechnology Co. Ltd.) referring to previously description (Gu et al. 2022; Luo et al. 2022, 2024; Hu et al. 2024). To ensure the assay validity and specificity, each kit was tested for cross-reactivity with closely related analytes. According to the manufacturer, no significant cross-reactivity was observed. All sample concentrations fell within the linear range specified by the kit (e.g., T-AOC: 0.1–5 mmol/L; GSH-Px: 5–500 U/mL), as confirmed by standard curves with $R^2 > 0.99$. To further ensure the reliability of our own measurements, all samples were analyzed in duplicate, and the mean value was used for statistical analysis. For each batch, standard curves and internal controls were run in parallel to validate the consistency of the assay. All samples were randomized across assay batches to eliminate systematic errors and batch effects.

Mammary tissue digestion

To generate single-cell suspensions, the mammary gland tissues were digested using a mix enzyme system (Jia et al. 2024). First, the samples were finely minced and then incubated in a digestion solution consisting of 1X Collagenase/Hyaluronidase (#07912, Stemcell Technology, Vancouver, BC), EpiCult-B (#05611, Stemcell Technology), and 5% FBS. This incubation took place at 37°C in a shaking incubator for 2 h. After incubation, the dissociated cells underwent red blood cell lysis. They were then treated with 1 U/mL dispase (#07913, Stem Cell Technologies) and 0.1 mg/mL DNase (#07470, Stem Cell Technologies) for five minutes. Finally, the extracted cells were re-suspended in PBS containing 0.3% BSA and counted using Trypan Blue. For the assessment of ROS, 100,000 isolated mammary cells were used in each sample.

Oxidative status indicators in mammary cells

The intracellular ROS concentration in the mammary cells was measured using a cellular ROS assay kit (Abcam, ab113851). This kit use the cell permeant reagent 2',7' – dichlorofluorescin diacetate to semi quantitatively assess ROS in live cell samples. The detection process followed the manufacturer's instructions precisely (https://www.abcam.cn/products/assay-kits/dcfda–h2dcfda-cellular-ros-assay-kit-ab113851.html). In brief, the probe, dichlorodihydro-fluorescein diacetate, was diluted with serum-free culture medium at a ratio of 1:1000 and mixed with the cells at a concentration of approximately 100,000 per milliliter. The cell solutions were then incubated at 37°C for 20 min. Afterward, the cells were washed three times with PBS and resuspended for ROS detection using flow cytometry (FACSVerse, BD Company, NJ, USA). Fluorescent intensity was excited by the 488 nm laser and detected at 535 nm. Fluorescent intensity is proportional to the amount of ROS in the samples. The detection methods for MDA concentration and the activity of SOD, GSH-Px, CAT, and T-AOC were followed the manufacturer's instructions precisely. Briefly, tissue lysates are prepared by a dounce homogenizer homogenization. Homogenize 100 mg of frozen tissue in 1 mL of chilled 1X cell extraction buffer. Incubate on ice for 20 minutes. Centrifuge at 18,000 × *g* for 20 min at 4°C. Transfer the supernatants into clean tubes and discard the pellets. The concentration of MDA and the activity of SOD, GSH-Px, CAT, and T-AOC were normalized to the concentration of protein in the respective tissue lysate. The controls of assay validity in mammary cells or tissue were same as that in plasma.

Statistical analysis

In Experiment 1, the data of ROS level of plasma in different dry-off treatments were analyzed using the PROC MIXED procedure of SAS 9.4 software (SAS Institute, Inc., Cary, NC, USA) including experimental time (time), dry-off treatment (treat) and their interaction (time × treat) as the fixed effects. One-way analysis of variance (ANOVA) were used to analyze the differences of indicators in oxidative status and nutrition metabolism in plasma (IBM SPSS Statistics, IBM Corp., Armonk, N.Y., USA). In Experiment 2, one-way ANOVA with multiple comparisons (Tukey test) were used to analyze the differences of indicators in oxidative status and biochemical in plasma and mammary cells (IBM SPSS Statistics). The effect of different time was included as a repeated measure. GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA) was used to visualize the data, and the data of each group were expressed as mean ± standard error of the mean. $P < 0.05$ indicates that there is a significant difference between the two groups.

Results

Dynamic changes of ROS concentration in plasma

As shown in Figure 1, there was no significant changes of plasma ROS from −8 to −5 wk, then a transient increase at −4 wk and a decrease from −2 to −1 wk, then an increase from 0 to 1 wk. It is worth noting that plasma ROS concentrations were significantly elevated at −4 wk, while they were lower at −1, 0, and +1 wk. However, there were no significant differences in ROS concentration across all time points between the two dry off treatments (dry off at −8 or −12 wk, $P_{treat} = 0.36$). Additionally, there were no observed interactions between the drying-off treatments and time points ($P_{time \times treat} = 1.00$).

Oxidative status and biochemical parameters in plasma

The results for the plasma oxidative status and biochemical parameters in Experiment 1 are presented in Figure 2. Significant changes,

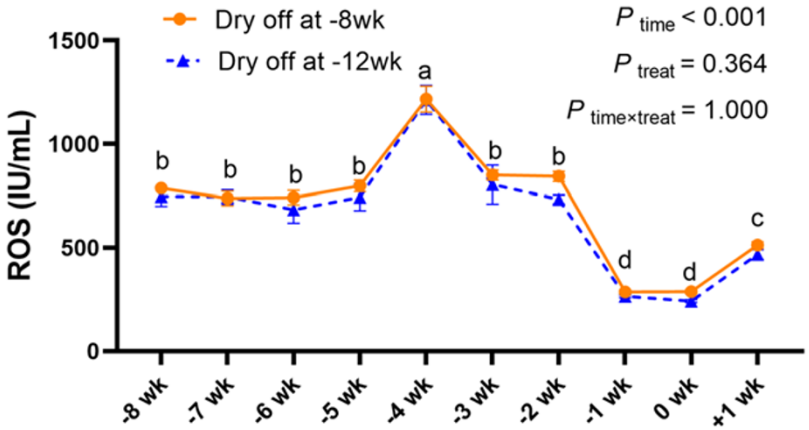

Figure 1. Mean plasma ROS concentration between goats dried off at 12 weeks (wk) before kidding (−12 wk) or −8 wk during regenerative involution. All goats had blood samples taken once a week at −8, −7, −6, −5, −4, −3, −2, −1 wk, on the day for kidding (0 wk) and the first week after kidding (+1 wk). The sample size was 14 and 6 in the −8 and −12 wk group, respectively. Bars indicate the SEM. The data in different dry-off treatments were analyzed using the PROC MIXED procedure of SAS including experimental time (time), dry-off treatment (treat), and their interaction (time × treat) as the fixed effects. Means with different letters are different in two time points in goats dried off at −8 wk group ($P < 0.05$).

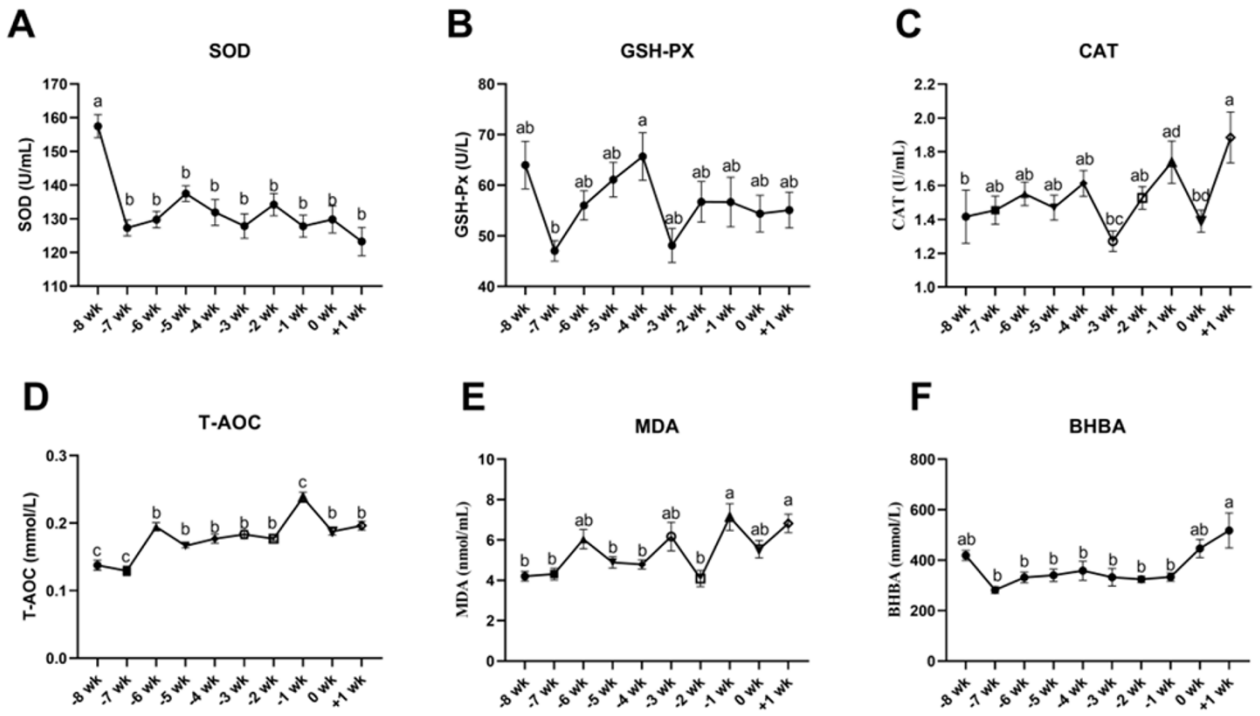

Figure 2. Mean plasma indicators of oxidative status and biochemistry in dairy goat dried-off at −8 wk during the regenerative involution in Experiment 1. (A) Activity of total SOD. (B) Activity of GSH-Px. (C) activity of CAT. (D) Activity of T-AOC. (E) Concentration of MDA. (E) Concentration of BHBA. All goats had blood samples taken once a week at 8 weeks (wk) before kidding (−8 wk), −7 wk, −6, −5, −4, −3, −2, −1 wk, on the day for kidding (0 wk) and the first week after kidding (+1 wk). The data are expressed as the mean ± SEM, $n = 14$ goats. Means with different letters are different in two time points ($P < 0.05$).

over time, were observed in SOD ($P < 0.01$, Figure 2A), GSH-Px ($P < 0.01$, Figure 2B), CAT ($P < 0.01$, Figure 2C), T-AOC ($P < 0.01$, Figure 2D), and MDA ($P < 0.01$, Figure 2E) from dry-off at −8 wk (starting to dry off) to early stage of the subsequent lactation (+1 wk). No significant changes in SOD were found from −7 to +1 wk (Figure 2A). The activity of the GSH-Px increased from the −7 wk to −4 wk and then showed a decreasing tendency until +1 wk (Figure 2B). The activity of CAT had minimal changes from −8 wk to −4 wk while it increased significantly from −3 wk to −1 wk ($P = 0.04$) and from 0 to +1 wk ($P = 0.02$, Figure 2C). The T-AOC activity had a lower level at −8 wk ($P < 0.01$) and −7 wk ($P < 0.01$) but increased significantly at −1 wk ($P < 0.01$) compared with −6, −5, −4, −3, −2, 0 and +1 wk (Figure 2D). No significant changes were observed in MDA from −8 to −2 wk, but there was a significant increase at −1 wk ($P < 0.01$) and +1 wk ($P < 0.01$) compared with −8 wk (Figure 2E). The BHBA gradually increased after kidding until +1 wk ($P < 0.01$) compared with that at −1 wk (Figure 2F).

Oxidative status parameters in the four key transition stages

The dynamic changes in ROS, oxidative and biochemical parameters during the transition phase of drying off were observed at several time points: −8 wk (starting to dry off), −4 wk (highest level of ROS) and −1 wk (higher level of T-AOC activity) and +1 wk (higher level of MDA and BHBA) in Experiment 1. To further support this observation, an additional seven goats were selected for plasma collection at these time points at −8, −4, −1, and +1 wk in Experiment 2. The results regarding plasma oxidative status are presented in Figure 3. Among the four time points, the highest level of ROS was found at −4 wk ($P< 0.01$, Figure 3A). SOD levels were lower at −4 wk ($P < 0.01$), −1 wk ($P < 0.01$) and +1 wk ($P < 0.01$) compared with −8 wk (Figure 3B). CAT activity showed a decreasing trend ($P = 0.01$), while no significant changes were observed for GSH-Px ($P = 0.34$). T-AOC activity ($P < 0.01$) and MAD ($P = 0.01$) gradually increased from −8 to +1 wk.

The plasma biochemical parameters including NEFA, BHBA, and triglyceride were measured and are shown in Table 1 for the four key transition stages. A significant and gradual increase in BHBA ($P < 0.01$) was observed from −8 to +1 wk. Triglyceride ($P = 0.01$) had an increased peak at −1 wk from −4 wk and followed a decrease at +1 wk. There were no significant changes for NEFA ($P = 0.16$) among these four time points.

Oxidative status indicators in mammary gland

The fluorescence intensity of ROS in the isolated mammary cells was significantly higher at −4 wk compared with other periods ($P < 0.01$, Figure 4A). The activities of SOD ($P < 0.01$) and GSH-Px ($P = 0.02$) were significantly increased at −1 and +1 wk (Figure 4B and C) compared with −8 and −4 wk. The GSH-Px activity was significantly elevated at −1 wk ($P = 0.05$) and +1 wk ($P = 0.04$) compared with −8 wk. No significant differences in T-AOC ($P = 0.13$) and CAT ($P = 0.98$) were observed among the four time points (Figure 4D and E). A dramatic increase in MDA was found at +1 wk compared with −8 wk ($P = 0.01$, Figure 4F).

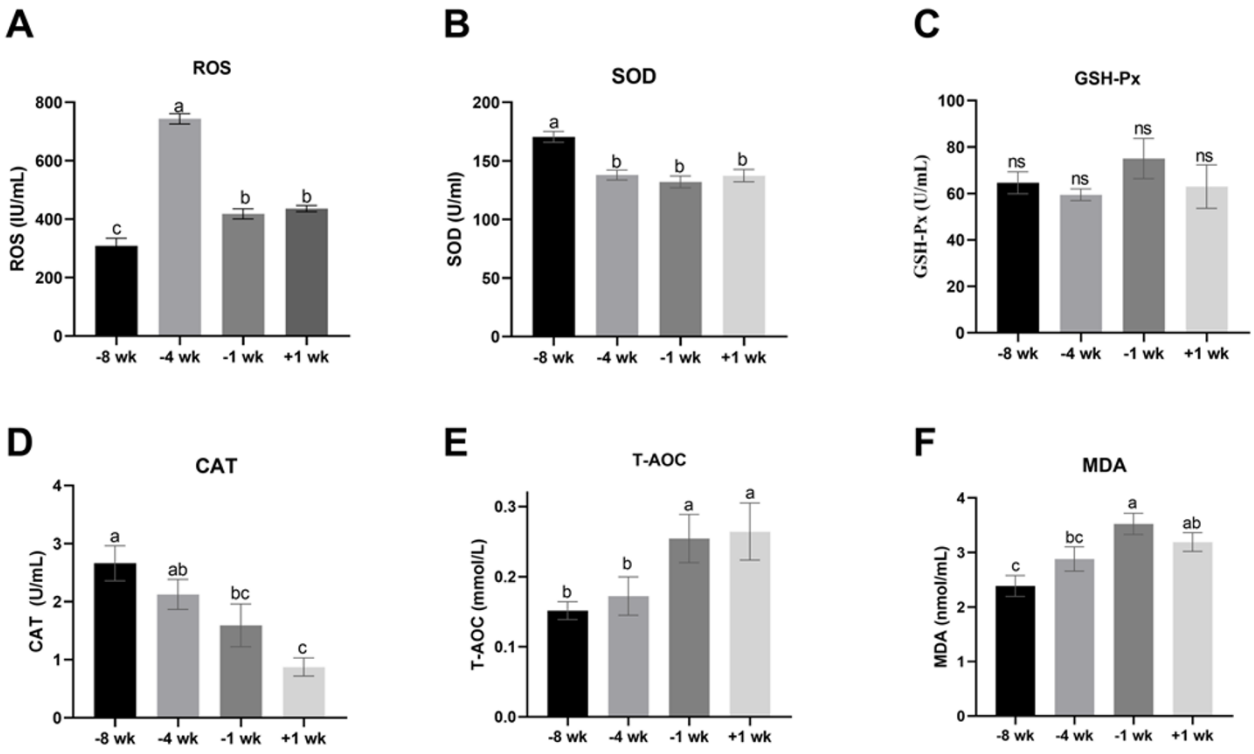

Figure 3. Mean plasma indicators of oxidative status in dairy goat at four key transition stages during the regenerative involution in Experiment 2. (A) Concentration of ROS. (B) Activity of total SOD. (C) Activity of GSH-Px. (D) Activity of CAT. (E) Activity of T-AOC. (F) Concentration of MDA. All goats had blood samples taken once a week at 8 weeks (wk) before kidding (−8 wk), −4, −1 wk, and the first week after kidding (+1 wk). The data are expressed as the mean ± SEM, $n = 7$ goats. Means with different letters are different in two time points ($P < 0.05$).

Table 1. Changes of metabolites in plasma during the regenerative involution in dairy goats

	Weeks relative to kidding					
Indicator	−8 wk	−4 wk	−1 wk	+1 wk	SEM	*P*-value
NEFA, μmol/L	494	574	752	750	48.5	0.16
BHBA, mmol/L	135[c]	197[bc]	269[ab]	353[a]	21.4	<0.01
TG, mmol/L	0.417[b]	0.380[b]	0.535[a]	0.420[b]	0.019	0.01

NEFA = nonesterified fatty acid; BHBA = β-hydroxybutyrate; TG = triglyceride.
[a-c]Means within a row with different superscripts differ ($P < 0.05$).
One-way ANOVA with multiple comparisons (Tukey test) were used to analyze the differences of metabolites. The data of each group were expressed as mean ± standard error of the mean. The sample size was 7 in each time point.

Discussion

During the dry period in dairy livestock, the mammary gland undergoes involution and overlaps with the late gestation period. This process allows for the restoration of mammary function in preparation for the subsequent lactation (Capuco and Choudhary 2020; De Vries et al. 2010). The balance of oxidative status is considered to be a critical signaling mechanism for cell renewal and tissue development (Sies et al. 2022; Sies and Jones 2020). However, the specific temporal profiles of indicators related to oxidative status during regenerative involution are not yet well understood. In this study, we found that indicators of oxidative status dynamically changed throughout the regenerative involution and early lactation stages, particularly at −4, −1, and +1 wk relative to kidding.

The ROS are continuously produced as metabolic byproducts, while endogenous antioxidant defense systems maintain ROS at a certain level by properly scavenging them (Sies et al. 2022; Sies and Jones 2020). Oxidative stress refers to high levels of ROS, which activate inflammatory responses, leading to cell growth arrest and even death (Niki 2009; Sies 2017). In this study, we observed a specific increase in ROS and only minor changes in antioxidant enzymes in plasma at −4 wk, indicating a condition of oxidative stress. The no changes in ROS from −8 to −5 wk in plasma are not consistent with previous data in dairy cows (Putman et al. 2018), in which ROS levels were low during the transition into the early dry period. The observation of highest level of ROS at −4 wk in the current study agrees with another study in dairy cows that reactive oxygen metabolites in the blood of dry dairy cows maintained at a high level from 30 days before parturition to 25 days before parturition (Bernabucci et al. 2005). Although the specific reason for the increase in ROS at this stage is unclear, the metabolic and endocrine adjustments related to the fetus and mammary metabolism may be responsible for some variations in oxidative status. These findings suggest that the transition period before parturition in dairy ruminants may be extended to −4 wk instead of the traditional −3 wk.

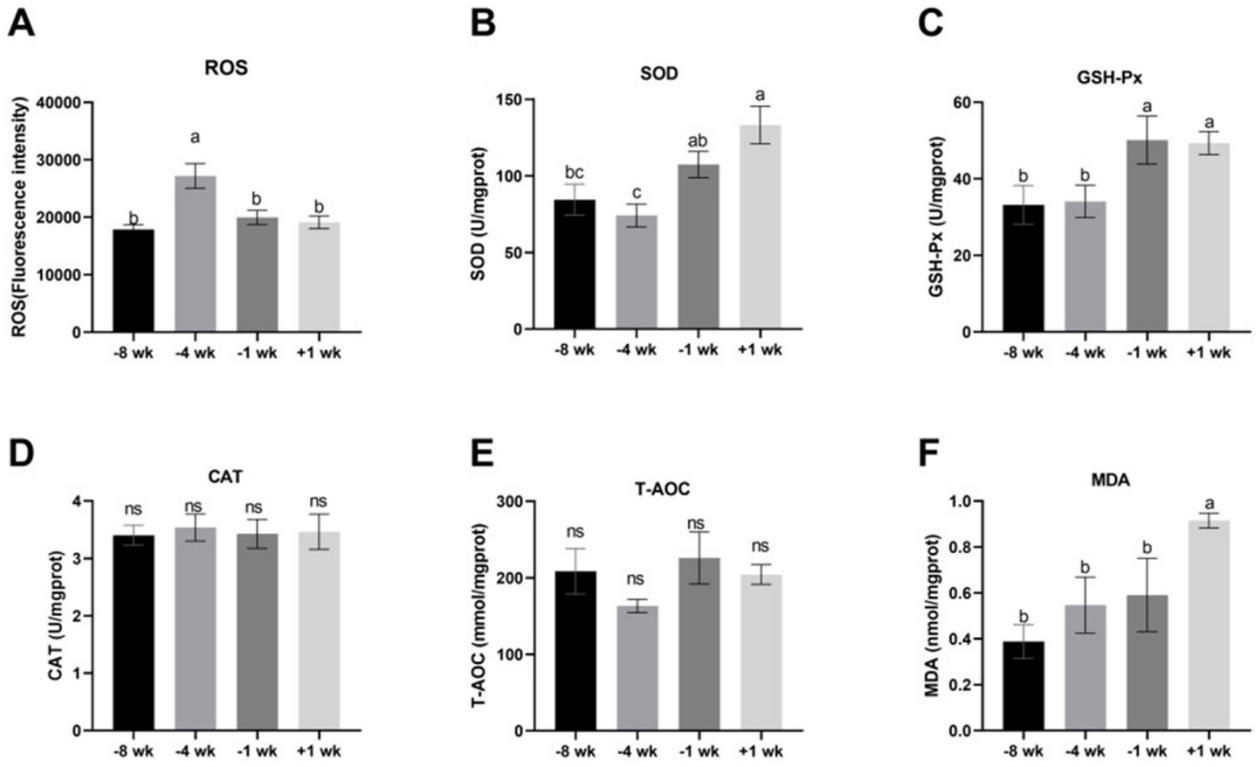

Figure 4. Indicators of oxidative status in isolated goat mammary cells at four key transition stages during the regenerative involution in Experiment 2. (A) Fluorescent intensity of ROS. (B) Activity of total SOD. (C) Activity of GSH-Px. (D) Activity of CAT. (E) Activity of T-AOC. (F) Concentration of MDA. All mammary cells are isolated from mammary tissue at 8 weeks (wk) before kidding (−8 wk), −4, −1 wk and the first week after kidding (+1 wk). The concentration of MDA and the activity of SOD, GSH-Px, CAT, and T-AOC were normalized to the concentration of protein in the respective tissue lysate (Per mg protein). The data are expressed as the mean ± SEM, $n = 7$ goats. Means with different letters are different in two time points ($P < 0.05$).

It is known that SOD and GSH-Px are the main enzymatic systems that prevent oxidative stress. Under physiological stress situations, the activation of these enzymatic antioxidants should decrease the production of ROS. In the current study, the significant increase in GSH-Px at −4 wk suggests that it is the main enzymatic system responding to the increasing ROS during the dry period. The low levels of plasma SOD, CAT, and T-AOC before kidding in the current study indicate a balanced oxidative status during the dry period of dairy goats. The increase in ROS might contribute to physiological conditions (Schieber and Chandel 2014).

Altered nutrient metabolism is close-linked to oxidative stress. The MDA, a byproduct of polyunsaturated fatty acid peroxidation in cells, is widely recognized as a marker of oxidative stress and antioxidant status (Cordiano et al. 2023). The lower level of plasma MDA at −4 wk compared with +1 wk indicate that these goats experienced less stress due to lipid peroxidation. The increase in MDA at −1 wk and +1 wk coincides with the rising levels of BHBA in plasma, which is a result of the negative energy balance during the transition into lactation. Minor changes in plasma NEFA may suggest that dairy goats have a lower degree of negative energy balance compared to dairy cows (Abuelo et al. 2019; Bernabucci et al. 2005; Ghavipanje et al. 2021). Using antioxidant compounds could be a means to maintain proper redox balance. Consistent with the increase in MDA at −1 wk, a higher activity of T-AOC was observed at −1 wk, indicating a balanced redox biology.

The regenerative involution leads to shrinking and collapse of the alveolar structure, resulting in a smaller lumen size at around −4 wk (Jiang et al. 2022). The restoration of mammary function is characterized by the reconstruction of the alveolar structure and milk secretion during parturition. The low rate of apoptosis in bovine mammary epithelial cells during early involution and increased cell proliferation during regenerative involution suggest a crucial role in gland renewal for function restoration (Sorensen et al. 2006). Despite no significant changes in antioxidants in plasma and mammary cells, the increase in ROS at −4 wk reveals that ROS might participate in the regenerative involution of mammary gland. This finding is consistent with the fact that balanced oxidative status could involve a small increase in ROS levels initiating biological processes (Niki 2016; Schieber and Chandel 2014). High levels of ROS in inner luminal cells are involved in lumen formation and the activity of luminal progenitor cells (Kannan et al. 2014; Schafer et al. 2009). The increase in ROS in mammary cells at −4 wk suggests a potential role of ROS in mammary gland regeneration. The ROS induce apoptosis in ROS-sensitive cells of the mammary gland while ROS-insensitive cells are maintained and subsequently differentiated (Baratta et al. 2018). To some extents, the process of mammary regenerative involution is similar to wound healing (Zaragozá et al. 2015). Tissue injury is associated with the production of ROS, especially in the early stages of trauma and regeneration, where ROS plays an essential role in the effective wound healing response (Diwanji and Bergmann 2018). Thus, the increase in ROS at −4 wk might have opposite effects at the same time point. Further studies on the biological activities of ROS in the mammary gland may provide answers to these questions.

The increase in ROS at −4 wk may be linked to the hormonal changes that occur during pregnancy. During regenerative involution, the renewal of the mammary gland is influenced by several reproductive hormones, including progesterone. This hormone promotes ROS production and inhibits the activity of antioxidant enzymes such as SOD and GSH-Px (Wassmann et al. 2005; Yuan et al. 2016). This agrees with our data that SOD and GSH-Px had a low level around −4 wk in the mammary gland. These differences in response to ROS may be due to alternative antioxidant mechanisms present in different luminal cells. For instance, rodent mammary luminal progenitor cells exhibit high ROS concentrations and rely on a robust antioxidant mechanism independent of glutathione, while basal cells have lower ROS levels and depend on less efficient antioxidant mechanisms that rely on glutathione (Kannan et al. 2014). Additionally, the minimal changes observed in CAT and T-AOC in the goat mammary gland indicate that they do not respond significantly to the increased oxidative stress during regenerative involution. The increase in SOD and GSH-Px around kidding might be a result of homeostatic control in the mammary gland. It is acknowledged that comprehensively assessing the oxidative status of an individual or tissue poses a significant challenge. Because these indicators of oxidative status are highly sensitive to these factors including diets, reproductive status, and environment. This may explain the differences observed in the ROS concentrations and BHBA between the two experiments in the current study. To addition surgical factors during the sample collection, including fixation of animal, drug use and pain, may also impact these indicators, which represents a limitation of this study. Overall, the fluctuations observed in all indicators of oxidant status throughout the sampling period suggest the involvement of redox biology in regenerative involution.

Conclusions

Our findings reveal a specific increase in ROS levels at −4 wk in both plasma and the mammary gland. However, the changes observed in antioxidant and nutrient metabolism indicators were not always proportional in terms of magnitude and duration during this period. The data obtained from this study provide insight into alterations of oxidative status indicators during regenerative involution. It is evident that all indicators of oxidative status varied over the sampling period, suggesting the involvement of redox biology in regenerative involution. Nonetheless, it remains unclear whether the temporal profiles of ROS merely play a physiological role in mammary gland development during this phase. Consequently, additional studies are needed to clarify the potential effects of ROS on the restoration of mammary gland function in ruminants.

Data availability statement. All data generated or analyzed during this study are included in the published article.

Author contributions. CW, TL, YYL, YL, and NJ performed the experiments. CW, KZ, and HS analyzed the data and wrote the original manuscript. HS obtained funding and revised the manuscript. All authors read and approved the final manuscript.

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Conflict(s) of interest. The authors declare that they have no competing interests.

Ethical standards. This study was conducted in accordance with the Chinese guidelines for animal welfare and the experimental protocols for animal care approved by the Animal Care Committee of Zhejiang University (Hangzhou, Zhejiang, China) and was in accordance with the university's guidelines for animal research.

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