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Expression profiling of α-SMA and Oct-4 with antioxidant activity in the testis during pre and post natal ontogenesis in non-descript goat (*Capra hircus*) in hot climatic conditions of Odisha

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Abstract

Background A thorough understanding of ontogenesis of parts of male reproductive system in non-descriptive goat, a prolific small ruminant is essential for achievement of better reproductive physiology and breeding practice. Oct-4, a stem cell marker, attributes to the maintenance of spermatogonial pleuripotency and self-renewal property in testis. α-SMA expression contributes to the maintenance of contractile action in the seminiferous tubules of testis, highly essential for sperm transport. Additionally, a favourable antioxidant status highly influences the appropriate expression pattern of Oct-4 and α-SMA in the male gonad. The intensity of immune-staining was measured by calculating the optical density (OD) value of Oct-4 and α-SMA. The anti-oxidant value in testis tissue was measured by using CUPRAC, FRAP, TBARS and GST assays.

Results Localization of Oct-4 confirmed the presence of spermatogonial stem cell (SSC) niches in prenatal and early postnatal testes, indicating regenerative potential. Alpha-SMA expression in the testi reflected increasing contractile capacity essential for sperm transport, with optical density and percentage of immunopositive cells rising in postnatal stages. CUPRAC and FRAP assays revealed a gradual increase in antioxidant capacity from prenatal to pubertal stages, suggesting enhanced defense mechanisms during spermatogenic activation. Conversely, TBARS levels, indicative of lipid peroxidation, were higher in

prenatal stages especially in late gestation reflecting oxidative stress during rapid cellular development. GST activity, indicative of detoxification capacity, increased significantly in postnatal stages, highlighting its role in maintaining redox balance and supporting testicular function.

Conclusions Collectively, these results highlighted the complex interplay of structural, biochemical, and functional changes that underpin the development and maturation of the male reproductive tract in non-descript goats, offering critical insights into reproductive physiology and age-related fertility assessment in domestic animals.

Keywords Oct-4, α-SMA, testis, anti-oxidant, pre natal, post natal, goat

Background

The continuity of any species is fundamentally reliant on its reproductive capacity. Reproduction is a multifaceted biological process encompassing sequential events such as gametogenesis, fertilization, gestation, reproductive behaviours, and parturition (lambing/kidding). Among domestic ruminants, sheep and goats are regarded as the most prolific breeders. Achieving a high level of reproductive efficiency is essential not only for species survival but also for the sustained production of meat, milk, fiber, and skin, as well as for the regular replacement of breeding animals (Abebe, 2001).

The reproductive capacity of goats is integral to their continued success as a livestock species. Increasing the percentage of kidding within a stipulated period is a goal for many goat farmers, as it directly impacts the productivity and profitability of goat farming operations. The male genitalia, particularly the testis, play a crucial role in determining reproductive efficiency. The testis is responsible for production of spermatozoa and the secretion of male sex hormone, testosterone, which is essential for the regulation of spermatogenesis and the manifestation of male secondary sexual characteristics (Li et al. 2024), a ductal network for sperm transport, accessory sex glands, and the penis. Presently, the epididymis is recognized not simply as a passive conduit for sperm from the testes to the exterior, but as a vital organ involved in the secretion of essential seminal components and in the maturation and functional preparation of spermatozoa for fertilization (James et al. 2020). The vas deferens also plays a vital role in conducting the mature spermatozoa from epididymis to the urethra. The glandular secretion from the ampulla of vas deferens also

impacts the quality and quantity of semen in the goats. For implementing appropriate reproductive management, understanding of the basic anatomy and reproductive physiology of goat testis, epididymis and vas deferens is a must.

Immunohistochemistry (IHC) is an essential tool for understanding the complex physiological processes within the male reproductive system, particularly in the testis, epididymis, and vas deferens of goats. IHC studies using specific protein allow for the assessment of key molecular markers involved in the localization of spermatogonial stem cell (SSC) niches and contractile myofibroblast cells in the seminiferous tubules of testis. These specific protein markers also play a key role in the development of epididymis and vas deferens in animals. Oct-4 is a transcription factor crucial for maintaining pluripotency of spermatogonial cells and regulating the process of spermatogenesis. This is vital for understanding the renewal of sperm-producing cells and ensuring continuous sperm production (Sahu et al. 2021). The expression of Oct-4 confirms the presence and localization of spermatogonial stem cell (SSC) niches, indicating their regenerative potential in the testis. Meanwhile, α-SMA expression in the testis, epididymis, and vas deferens reflects the contractile capacity of these tissues, essential for the transport of sperm during different developmental stages in goats. These antibodies, when incubated overnight at 4˚C, allow for detailed visualization of their localization in the cellular structures within the reproductive system, which could be correlated with their function, helping researchers understand the intricate mechanisms involved in male fertility (Hassan et al. 2009).

A comprehensive understanding of the reproductive anatomy of male goats, particularly the testis, epididymis, and vas deferens, is crucial for improving the fertility and reproductive efficiency of goats. By conducting comprehensive studies on immunohistochemistry reinforced by antioxidant assay of these organs, researchers can gain valuable insights into male goat reproduction and develop strategies to enhance fertility. This study will not only improve the productivity of goat farming, but also contribute to the sustainable development of the goat industry in the face of changing environmental conditions and increasing demand for animal products.

Materials and Methods

Ethical statement

The present research work was conducted under IAEC Approval No. 433/GO/Re/S/01/CCSEA, dated 29.07.2024 of the CVSc. & A.H., OUAT, Bhubaneswar.

Sample collection

The samples of testis, epididymis, and vas deferens of healthy non-descript goats (*Capra hircus*) were collected from slaughter house in and around Bhubaneswar across prenatal and postnatal stages. Prenatal samples were categorized into early (up to 50 days), mid (51–100 days), and late (101–150 days) trimesters, with fetal age estimated using the formula Y = 2.74X + 30.15, where 'Y' is the age of the embryos in days and 'X' is the crown-rump length (X) in centimetres (Gall et al. 1994). The postnatal samples were classified into neonatal (within 1 month), pre-pubertal (1–18 months), and pubertal (above 18 months) stages, with 10 samples collected per group as per the data collected from the records of the slaughter house. For immunohistochemical study, the tissue samples after collection were fixed in 10% Neutral Buffered Formalin. For antioxidant study fresh samples were collected and used.

Sample processing

The samples were routinely processed for immunohistochemicalstudy (Luna 1968). Four to five micrometer thick paraffin sections were cut. Following deparaffinization and rehydration, antigen retrieval was conducted by using heat-induced method with 10 times diluting concentrated (10×) EZ-Antigen Retrieval buffer (Invitrogen by Thermo Fisher Scientific). The sections were then immersed in 3% (v/v) $H_2O_2$for endogenous peroxidase activity blocking followed by washing (in 0.1 M phosphate buffered saline at pH 7.4). Then the sections were blocked by 2.5%normal horse serum in order to prevent non-specificbinding of antibodies.Incubation of the sections in a humid chamber at 4°C overnight was done with primary antibodies Oct-4 (Invitrogen by Thermo Fisher Scientific) and α-SMA (Invitrogen by Thermo Fisher Scientific). This step was followed by washing in 0.1 M phosphate buffered saline (at pH 7.4) and incubation with secondary antibody (ImmPRESS HRP Universal Antibody-Anti-Mouse IgG/Anti-Rabbit IgG, Peroxidase Polymer Detection Kit). As chromogen, 3, 3′-diaminobenzidinetetrahydrochloride(DAB) (ImmPACT DAB Substrate, Peroxidase-HRP by Vector Laboratories) and for counterstaining, Gill's III haematoxylin was used.Then the sections were subjected to running tap water, dehydrated,cleared and mounted with DPX. Observations and photomicrographs of

immunohistochemical stained slides were taken using microscope with a photomicrographic attachment (Make: Leica, Model: DM2500, Germany). Interpretation was done by using ImageJsoftware's cell counting plugin (version 1.49d), clicked images were executed and counted for immuno-positive cells (Schneider et al. 2012). Optical density (OD) value of IHC photographswere measured to assess the intensity of immunoreactions by using Fiji ImageJ's colour deconvolution plug-in 1.7 (Cizkova et al. 2021).For each region of testis, epididymis and vas deferensboth from prenatal and post-natal period,about 10-14 numbers of photomicrographs was opened in Fiji imageJ software. For 'H DAB' stain, colour deconvolution was done for each IHC image. Three numbers of images were obtained by clicking on colour deconvolution. The 'Colour_2' image was the DAB image.Following this, the image was screened to get the OD value. By using the formula, OD = log (max intensity/mean intensity), where max intensity = 255 for 8-bitimages the intensity unit in the results window was converted to OD numbers. The significance difference between the mean OD value was derived by followingOne Way ANOVA using SPSS 27.0 Software to determine the differences between the various developmental stages under study.

The antioxidant capacity of testicular tissue homogenates was assessed using the CUPRAC (Cupric Ion Reducing Antioxidant Capacity) method, following the standardized procedure outlined in the EZAssay Antioxidant Activity Estimation Kit. This assay relies on the reduction of a copper (II)-chromogen oxidant complex to its copper (I) form by antioxidants present in the sample, resulting in a measurable color change. The absorbance of the resulting Copper (I)-chromogen complex at 450 nm was directly proportional to the antioxidant potential of the test sample. For the assay, a 96-well microplate was prepared with wells designated for standards, controls, test samples, and blanks, each run in triplicate. Initially, 100 µL of deionized water was added to each well. Subsequently, 10 µL of test sample, standard solution, or deionized water (for blanks) was dispensed into the appropriate wells. Following this, 100 µL of the chromogenic substrate was added to all wells. The plate was incubated at room temperature in the dark for 10 minutes to allow the color development reaction to proceed. After incubation, the absorbance was recorded at 450 nm using a microplate reader. The blank values were subtracted from the corresponding readings, and a standard curve was constructed to calculate the antioxidant activity of the test samples. One-way ANOVA was used to compare mean values across groups and assess statistical correlations, with analyses performed using SPSS software (Version 27.0).

The antioxidant activity in testicular tissue homogenates was evaluated using the Ferric Reducing Antioxidant Power (FRAP) method, as outlined in the EZAssay Antioxidant Activity Estimation Kit protocol. This method is based on the reduction of a ferric (Fe^{3+})-chromogen complex to its ferrous (Fe^{2+}) form at low pH in the presence of antioxidants, resulting in the formation of a blue-colored complex. This complex exhibits a maximum absorbance at 593 nm, though it was recorded spectrophotometrically at 560 nm in this study. The intensity of absorbance was directly proportional to the antioxidant potential of the sample. To begin, tissue samples were thoroughly rinsed with 1X phosphate-buffered saline (PBS) to eliminate residual blood. Approximately 20–25 mg of tissue was homogenized in 200–250 µL of 1X PBS using a sonicator set at 40V for 15–20 seconds on ice. The homogenates were then centrifuged at 3428 g for 5 minutes, and the resulting supernatant was used for analysis. A 96-well microplate was prepared with designated wells for standards, controls, samples, and blanks, each run in triplicate. Into each well, 100 µL of the respective solution (standard/control/sample/blank) was pipetted, followed by the addition of 100 µL of the chromogenic reagent. The plate was then incubated for 10 minutes at room temperature in the dark. Absorbance was recorded at 593 nm using a microplate reader. Statistical comparisons between groups were made using one-way ANOVA, and data were analyzed with SPSS software (Version 27.0).

The concentration of thiobarbituric acid reactive substances (TBARS) in testicular tissue homogenates was estimated using the standard protocol outlined in the EZAssay TBARS Kit, which is designed to assess lipid peroxidation. Malondialdehyde (MDA), a by-product formed during the oxidation of polyunsaturated fatty acids, was used as the primary indicator of lipid peroxidation. The lipid peroxides generated during this process were quantified by measuring the reaction of MDA with thiobarbituric acid (TBA), following the kit's guidelines. In brief, the reagents, sample homogenates, and MDA standards were dispensed into clean, labeled glass tubes as instructed in the accompanying protocol. Each tube was sealed with aluminum foil to minimize evaporation and incubated in a boiling water bath for 60 minutes. After heating, the tubes were immediately cooled in an ice bath for 10 minutes and subsequently centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatants were then brought to room temperature for 30 minutes. An aliquot of 150 µL from each colored supernatant was transferred into individual wells of a 96-well microplate. Absorbance was recorded at 532 nm using a microplate reader. Blank values were subtracted from sample

readings, and a standard curve was constructed using the MDA standards to determine the TBARS concentration in each sample. Statistical analysis of the data, including comparison of mean values and assessment of group differences, was conducted using one-way ANOVA via SPSS software (Version 27.0).

The activity of Glutathione S-transferase (GST) in testicular tissue homogenates was evaluated following the standard protocol provided by the EZAssay GST Kit, designed to measure GSH-ST activity. This assay relies on the GST-mediated conjugation of reduced glutathione with the substrate 1-chloro-2,4-dinitrobenzene (CDNB). The enzymatic reaction results in a measurable increase in absorbance at 340 nm, as CDNB interacts with a broad range of GST isoenzymes. Prior to the procedure, the assay buffer and substrate solution were brought to room temperature. To establish a baseline, 930 µl of assay buffer was added to a 1 ml quartz cuvette and used as a blank to calibrate the spectrophotometer at 340 nm. Subsequently, 10 µl of reduced glutathione and 10 µl of the substrate solution were added to the cuvette, which was then sealed with parafilm and gently inverted several times for mixing. Absorbance was recorded at one-minute intervals for 5–6 minutes to generate blank readings. For the positive control, 4–6 µl of the GST standard was introduced into the reaction mixture and mixed thoroughly. In the case of the test sample, 50 µl of the tissue homogenate was added to the reaction mixture, followed by similar mixing. Absorbance readings at 340 nm were taken every minute for 5–6 minutes to assess enzymatic activity. Data obtained from the experiment were statistically analyzed using one-way ANOVA in SPSS software (Version 27.0) to determine the significance of differences among groups and assess intergroup correlations.

Results

Immunoreactivity of α-SMA

Immunoreactivity and Optical Density (OD) values of α-SMA in the testis of non-descript goat at different pre-natal and postnatal stages were recorded and presented in Tables 1-3. Immunoreactivity for α-SMA was consistently detected in the walls of blood vessels and in the cytoplasm of peritubular cells within the lamina propria of both the tubuli contorti of seminiferous tubules and the rete testis across all age groups, albeit with significant variation in intensity.During the early prenatal stage, the expression of α-SMA in the peritubular cells

was observed to be minimal (Fig. 1). As the development progressed, the immunoreactivity for α-SMA increased markedly during the mid and late trimesters. The peritubular immunoreactivity in the lamina propria observed to remain fair during the neonatal stage (Fig. 2), but showed further enhancement during pre-pubertal and pubertal stages, culminating in strong α-SMA expression at puberty.

The rete testis showed a gradual enhancement of α-SMA expression from low in the early trimester to intense by puberty. The findings of the present study demonstrated that α-SMA immunoreactivity served as a reliable indicator of developmental progression in testicular architecture.

Table 1. Immunoreactivity for α-SMA in testis at different ages in prenatal and post-natal stages of non-descript goat.

Sl. No.	Parameters	Early Trimester	Mid Trimester	Late Trimester	Neonatal	Pre-pubertal	Pubertal
1	Wall of blood vessels	P	P	P	P	P	P
2	Peritubular cells of lamina propria of tubulicontorti	+	++	++	++	+++	++++
3	Lamina propria of rete testis	+	++	++	++	+++	++++

Immunoreactivity for α-SMA was: P (Present), A (Absent), Ng (Negligible), +(Low), ++ (Moderate), +++ (Fair), ++++ (Intense) based on visual examination

Table 2. Optical Density (OD) value of immunostaining intensity of α-SMA in the testis of non-descript goat in different age groups at pre-natal stages.

Sl. No.	Parameters	Optical density of Lamina propria of tubuli contorti Mean ± SE	Level of Significance
1	Early Trimester	0.09 ± 0.01	***
2	Mid Trimester	0.18 ± 0.01	
3	Late Trimester	0.20 ± 0.01	

*** $p < 0.001$ (Highly significant), ** $0.001 < p < 0.01$ (Strongly significant), * $0.01 < p < 0.05$ (Moderately significant) & NS ($p > 0.05$): (Non-significant)

Table 3. Optical Density (OD) value of immunostaining intensity of α-SMA in the testis of non-descript goat in different age groups at post-natal stages.

Sl. No.	Parameters	Optical density of Lamina propria of tubuli contorti Mean ± SE	Level of Significance
1	Neonatal	0.23 ± 0.02	***
2	Pre-pubertal	0.32 ± 0.05	
3	Pubertal	0.38 ± 0.03	

*** p< 0.001 (Highly significant), ** 0.001<p<0.01 (Strongly significant), * 0.01<p<0.05 (Moderately significant) &NS (p > 0.05): (Non-significant)



Fig. 1. Photomicrograph showing low expression of α-SMA in the lamina propria of tubuli contorti (yellow arrow) and tunics of blood vessels (red arrow) in the testis at early trimester stage of non-descript goat. HDAB, x100



Fig. 2. Photomicrograph showing fair expression of α-SMA in the lamina propria of tubuli contorti (yellow arrow) in the testis at neonatal stage of non-descript goat. HDAB, x100

Immunoreactivity of Oct-4

Immunoreactivity and Optical Density (OD) values of Oct-4 in the testis of non-descript goat at different pre-natal and postnatal stages were recorded and presented in Tables 4-7. Immunoreactivity for Oct-4 was primarily localized in the nuclei of primordial germ cells (PGCs), gonocytes, and spermatogonial cells**,** consistent with its role in maintaining the undifferentiated state of germ cells.In the early trimester**,** the Oct-4 expression observed in PGCs. As development progressed into the mid trimester**,** gonocytes displayed Oct-4 immunoreactivity (Fig. 3). The increase in Oct-4 expression from mid to late trimester was noted in spermatogonial cells (Fig. 4). The peak immunoreactivity recorded in the neonatal stage (Fig. 5)**,** both in terms of staining intensity and the average number of Oct-4 immunopositive cells (37.53 ± 0.94) (Table 8). A declining trend was recorded in Oct-4 expression in pre-pubertal and pubertal stages (Fig. 6 and Fig. 7)**,** both in terms of staining and cell number (22.87 ± 0.71 and 13.22 ± 0.90, respectively).

Table 4. Immunoreactivity for Oct-4 in testis at different ages in prenatal and post-natal stages of non-descript goat.

Sl. No.	Parameters	Early Trimester	Mid Trimester	Late Trimester	Neonatal	Pre-pubertal	Pubertal
1	Primordial germ cell	+			-	-	-
2	Gonocytes		++		-	-	-
3	Lamina propria of tubulicontorti	-	-	-	-	-	-
4	Lamina propria of rete testis	-	-	-	++++	++	+
5	Spermatogonial cells		++	+++	-	-	-
6	Primary spermatocyte				-	-	-
7	Early Spermatid				-	-	-

Immunoreactivity for OCT-4 was: - (Absent), Ng (Negligible), +(Low), ++ (Moderate), +++ (Fair), ++++ (Intense)based on visual examination

Table 5. Optical Density (OD) value of immunostaining intensity of Oct-4 in the testis of non-descript goat in different age groups at pre-natal stages.

Sl. No.	Parameters	Optical density of Lamina propria of tubuli contorti Mean ± SE	Level of Significance
1	Early Trimester	0.17 ± 0.02	***
2	Mid Trimester	0.26 ± 0.02	
3	Late Trimester	0.36 ± 0.03	

*** p< 0.001 (Highly significant), ** 0.001<p<0.01 (Strongly significant), * 0.01<p<0.05 (Moderately significant) & NS (p > 0.05): (Non-significant)

Table 6. Optical Density (OD) value of immunostaining intensity of Oct-4 in the testis of non-descript goat in different age groups at post-natal stages.

Sl. No.	Parameters	Optical density of Lamina propria of tubuli contorti Mean ± SE	Level of Significance
1	Neonatal	0.41 ± 0.02	***
2	Pre-pubertal	0.23 ± 0.03	
3	Pubertal	0.16 ± 0.02	

Table 7. Average proportion of immunopositive cells for Oct-4 in testis of non-descript goat at pre-natal stages.

Sl. No.	Parameters	No. of field of immunopositive cells in a 'HDAB' image of OCT-4 Mean ± SE	Level of Significance
1	Early Trimester	20.70 ± 0.62	***
2	Mid Trimester	26.60 ± 0.76	
3	Late Trimester	32.88 ± 0.77	

*** p< 0.001 (Highly significant), ** 0.001<p<0.01 (Strongly significant), * 0.01<p<0.05 (Moderately significant) & NS (p > 0.05): (Non-significant)

Table 8. Average proportion of immunopositive cells for Oct-4 in testis of non-descript goat at post-natal stages.

Sl. No.	Parameters	No. of field of immunopositive cells in a 'HDAB' image of OCT-4 Mean ± SE	Level of Significance
1	Neonatal	37.53 ± 0.94	***
2	Pre-pubertal	22.87 ± 0.71	
3	Pubertal	13.22 ± 0.90	

*** p< 0.001 (Highly significant), ** 0.001<p<0.01 (Strongly significant), * 0.01<p<0.05 (Moderately significant) & NS (p > 0.05): (Non-significant)

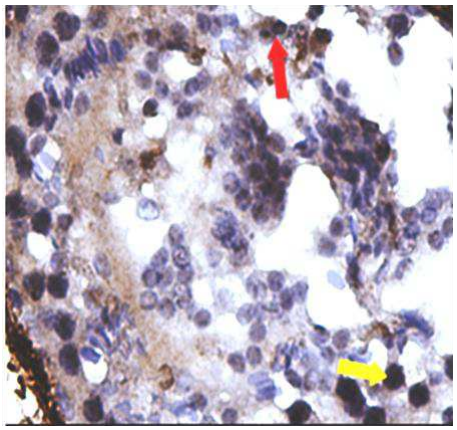


Fig. 3. Photomicrograph showing OCT-4 expression in gonocytes (yellow arrow) and spermatogonium (red arrows) in the stratified germinal epithelium of tubuli contorti of seminiferous tubules in the testis at mid trimester stage of non-descript goat.

HDAB, x400

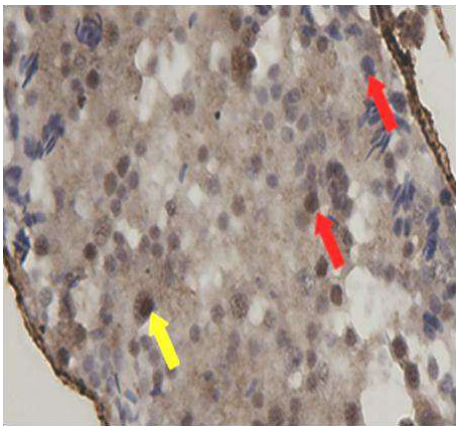


Fig. 4. Photomicrograph showing OCT-4 expression in the Dark type A spermatogonium (white arrow), Light type A spermatogonium (yellow arrow) and type B spermatogonium (red arrows) in the stratified germinal epithelium of tubuli contorti of seminiferous tubules in the testis at late trimester stage of non-descript goat.

HDAB, x400

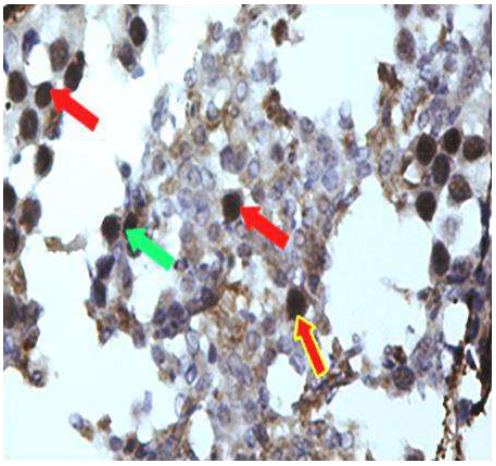


Fig. 5. Photomicrograph showing intense expression of OCT-4 in the type A spermatogonium (red arrows) and type B spermatogonium (green arrow) in stratified germinal epithelium of tubuli contorti of seminiferous tubules in the testis at neonatal stage in non-descript goat.

HDAB, x100

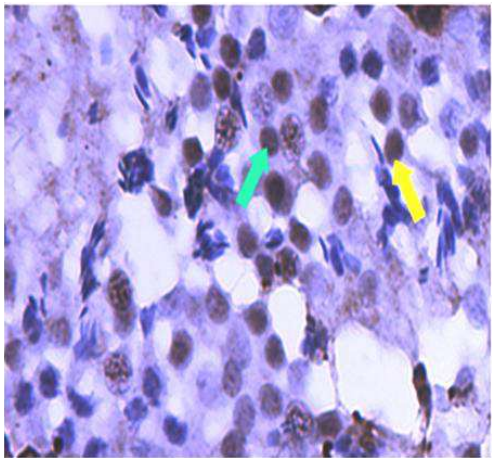


Fig. 6. Photomicrograph showing moderate expression of Oct-4 in the type-A spermatogonium (yellow arrows) and intense in type B spermatogonium (green arrow) in stratified germinal epithelium of tubuli contorti of seminiferous tubules in the testis at pre-pubertal stage in non-descript goat.

HDAB, x100

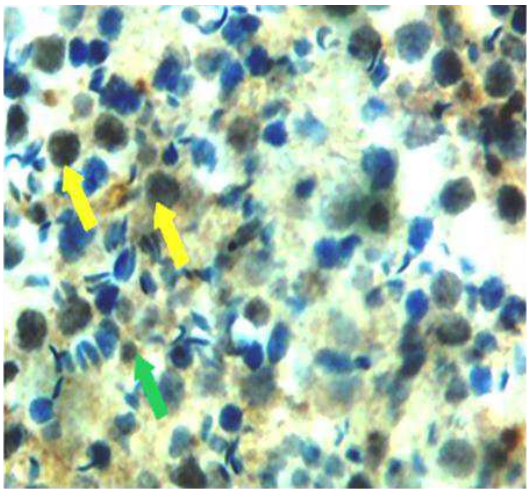


Fig. 7. Photomicrograph showing moderate expression of Oct-4 in the type-A spermatogonium (yellow arrows) and type B spermatogonium (green arrow) in stratified germinal epithelium of tubuli contorti of seminiferous tubules in the testis at pubertal stage in non-descript goat.

HDAB×100

Antioxidant assays on the testis

Different antioxidant assays were performed on the testis in non-descript goat at different pre-natal and postnatal stages to access the oxidative stress on the testicular environment.

Cupric Reduction Antioxidant Capacity (CUPRAC) of testis in non-descript goat at pre-natal and post-natal stages

The CUPRAC (Cupric Ion Reducing Antioxidant Capacity) assay was utilized to estimate the total antioxidant capacity in testicular tissues of non-descript goats across four developmental age groups, as presented in Table 9.

In the mid trimester stage**,** the mean optical density (OD) was 0.25 ± 0.01**,** corresponding to an antioxidant concentration of 484.27 ± 2.14 µM**.** In the late trimester stage, a higher OD of 0.30 ± 0.01 and antioxidant concentration of 565.86 ± 2.12 µM were recorded. In contrast, the pre-pubertal stage exhibited the lowest antioxidant concentration at 310.45 ± 1.00 µM (OD: 0.17 ± 0.01), which again increased to 543.39 ± 2.29 µM (OD: 0.29 ± 0.01) at the pubertal stage. The mean values of optical density and concentration of CUPRAC assay in the testis were statistically highly significant ($p<0.001$) among the groups of non-descript goat.

Table 9. Cupric reduction antioxidant capacity of testis in non-descript goat at pre-natal and post-natal stages.

Sl. No.	Age group	Mean ± SE Optical density	Mean ± SE Concentration (µM)	Level of Significance
1	Mid trimester stage	0.25 ± 0.01	484.27 ± 2.14	***
2	Late trimester stage	0.30 ± 0.01	565.86 ± 2.12	
3	Pre-pubertal stage	0.17 ± 0.01	310.45 ± 1.0	
4	Pubertal stage	0.29 ± 0.01	543.39 ± 2.29	

*** $p< 0.001$ (Highly significant), ** $0.001<p<0.01$ (Strongly significant), * $0.01<p<0.05$ (Moderately significant) & NS ($p > 0.05$): (Non-significant)

Ferric Reducing Antioxidant Potency (FRAP) values of testis in non-descript goat at pre-natal and post-natal stages

The Ferric Reducing Antioxidant Potency (FRAP) assay was conducted to assess the total antioxidant capacity in testicular tissues of non-descript goats across four age groups, as presented in Table 10. The antioxidant potential was measured in terms of micromolar (µM) concentration, derived from the ability of the samples to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions.

In the mid trimester stage, the mean optical density (OD) was 1.74 ± 0.01**,** corresponding to an antioxidant concentration of 348.70 ± 1.06 µM. In the late trimester stage, a higher OD of 1.98 ± 0.01 and antioxidant concentration of 392.54 ± 0.81 µM were recorded. In contrast, the pre-pubertal stage exhibited the lowest antioxidant concentration at 107.12 ± 0.62 µM (OD: 0.56 ± 0.01), which again increased to 183.81 ± 1.08 µM (OD: 0.93 ± 0.01) at the pubertal stage. The mean values of optical density and concentration of FRAP assay in the testis were statistically highly significant ($p<0.001$) among the groups of non-descript goat.

Table 10. Ferric Reducing Antioxidant Potency values of testis in non-descript goat at pre-natal and post-natal stages.

Sl. No.	Age group	Mean ± SE OD sample	Mean ± SE Concentration (µM)	Level of Significance
1	Mid trimester stage	1.74 ± 0.01	348.70 ± 1.06	***
2	Late trimester stage	1.98 ± 0.01	392.54 ± 0.81	
3	Pre-pubertal stage	0.56 ± 0.01	107 .12 ± 0.62	
4	Pubertal stage	0.93 ± 0.01	183.81 ± 1.08	

*** $p< 0.001$ (Highly significant), ** $0.001<p<0.01$ (Strongly significant), * $0.01<p<0.05$ (Moderately significant) & NS ($p > 0.05$): (Non-significant)

TBARS activity in testis in non-descript goat at pre-natal and post-natal stages

The TBARS (Thiobarbituric Acid Reactive Substances) assay was conducted to assess lipid peroxidation levels in the testicular tissue of non-descript goats across four developmental stages, as presented in Table 11. The results were expressed in nanomoles of malondialdehyde (MDA) per gram of tissue, which is indicative of the oxidative damage intensity.

In the mid trimester stage, the mean TBARS activity was 3.06 ± 0.14 nmol MDA/g tissue, indicating a higher degree of lipid peroxidation at this stage, which decreased to 1.24 ±

0.05 nmol MDA/g tissue at the late trimester stage. In contrast, the pre-pubertal stage demonstrated the highest TBARS level, recorded as 6.40 ± 0.27 nmol MDA/g tissue. The TBARS activity decreased to 5.38 ± 0.06 nmol MDA/g tissue at the pubertal stagesuggestive of less oxidative stress on the testicular environment as compared to the pre-pubertal stage. The mean values of TBARS activity in the testis were statistically highly significant ($p<0.001$) among the groups of non-descript goat.

Table 11. Concentration of malonaldehyde (MDA) in testis in non-descript goat at pre-natal and post-natal stages.

Sl. No.	Age group	Mean ± SE (TBARS Activity) MDA (µM)	Level of Significance
1	Mid trimester stage	3.06 ± 0.14	***
2	Late trimester stage	1.24 ± 0.05	
3	Pre-pubertal stage	6.4 ± 0.27	
4	Pubertal stage	5.38 ± 0.06	

*** p< 0.001 (Highly significant), ** 0.001<p<0.01 (Strongly significant), * 0.01<p<0.05 (Moderately significant) & NS (p > 0.05): (Non-significant)

Average GST activity values of testis in non-descript goat at pre-natal and post-natal stages

The activity of Glutathione S-transferase (GST), a key antioxidant enzyme, was evaluated in the testicular tissues of non-descript goats across four developmental age groups, as presented in Table 12. The enzyme activity was expressed in micromoles per milliliter per minute (µmol/ml/min).

In the mid trimester stage, the GST activity was 0.29 ± 0.01 µmol/ml/min. The GST activity decreased to 0.25 ± 0.01 µmol/ml/min at the late trimester stage. The GST activity again increased to 0.28 ± 0.01 µmol/ml/min in the pre-pubertal stage. The GST activity was comparatively less in the pubertal stage, which was recorded as 0.17 ± 0.01 µmol/ml/min. The mean values of GST activity in the testis were statistically highly significant ($p<0.001$) among the groups of non-descript goat.

Table 12. Average GST activity values of testis in non-descript goat at pre-natal and post-natal stages.

Sl. No.	Age group	Mean ± SE (GST Activity) µMole/ml/min	Level of Significance

1	Mid trimester stage	0.29 ± 0.01	***
2	Late trimester stage	0.25 ± 0.01	
3	Pre-pubertal stage	0.28 ± 0.01	
4	Pubertal stage	0.17 ± 0.01	

*** p< 0.001 (Highly significant), ** 0.001<p<0.01 (Strongly significant), * 0.01<p<0.05 (Moderately significant) & NS (p > 0.05): (Non-significant)

Discussion

The progressive and region-specific expression of α-smooth muscle actin (α-SMA) in the testis of non-descript goats, as observed in the present study, reflected the developmental maturation of testicular contractile elements essential for tubular integrity, spermatogenesis, and fluid transport. Minimal expression of α-SMA in the peritubular cells during the early prenatal stage as observed in the present study, partially consistent with the findings of Abd-Elmaksoud (2005) in embryonic bovine testes, where α-SMA immunolocalization was limited to the blood vessels, with no detectable expression in the tunica albuginea or peritubular areas.The low expression in the early trimester likely reflected the structural immaturity of the peritubularmyoid cells, which began to acquire contractile characteristics later in gestation. The immunoreactivity for α-SMA was intensified significantly along with developmental progress of the goat foetus towards mid and late trimesters. Devkota et al. (2006) also observed a pattern in cattle i.e. the α-SMA expression in peritubularmyoid cells emerged postnatally and intensified with age, correlating with the onset of spermatogonial division and structural maturation of the seminiferous epithelium.The statistically significant increase in α-SMA optical density from prenatal to postnatal stages observed in the current study supported this association with the testicular functional development. Moderate peri-tubular immune-reactivity in the lamina propria of testis during pre-pubertal and strong during neonatal and pubertal stage as noted in the present study aligned with the results of Acharya and Richburg. (2025), who described a well-developed α-SMA-positive peritubular sheath in adult llamas and vicunas, reflecting the high contractile and supportive demands of fully functional testes. A gradual increase in the α-SMA reactivity in the rete testis towards puberty in goat corresponded with the findings in adult bovine and caprine species, where α-SMA-positive cells contributed to the contractile regulation of fluid movement in the excurrent ducts (Devkota et al. 2006; Aire and Ozegbe 2007). The increasing immunoreactivity in both the tubuli contorti and rete testis highlighted the expanding role of myoid and stromal cells in ensuring efficient sperm transport and testicular homeostasis. The age-dependent increase in peritubular and rete testis regions in non-descript goats underlined

its essential role in supporting the structural and functional maturation of the male reproductive system.

In the early trimester, the Oct-4 expression observed in PGCs corroborated with earlier findings by Pesce and Scholer (1998), who reported Oct-4 localization in germ line cells of early mouse embryos and noted that its expression served as a marker of germ cell pluripotency prior to meiotic entry. Towards mid trimester, gonocytes displayed Oct-4 immunoreactivity, supporting the view of Mahla et al. (2012) and Mitchell et al. (2008) that Oct-4 was actively expressed in undifferentiated germ cells during the fetal development in both buffalo and primates. The spermatogonial cells reflected increased Oct-4 expression from mid to late trimester,might reflect the continued role of Oct-4 in early spermatogonial commitment, before its gradual down regulation upon meiotic differentiation. This observation aligned with the findings of Albert et al. (2010) in marmoset testis and Carlo (2018) in buffalo testis, who reported that Oct-4 expression peaks during the fetal and neonatal periods that subsequently declined postnatally. Strong Oct-4immunoreactivity as recorded in the current study highlighted the neonatal testis as a rich source of undifferentiated spermatogonial stem cells (SSCs). This was consistent with the conclusions drawn by Bafarani et al. (2014) and Carlo (2018), who emphasized that neonatal testicular tissue provided an ideal source for isolating SSCs for regenerative and reproductive biotechnologies and its use in regenerative medicine. The immunopositive cell count increased significantly ($p < 0.001$) from early (20.70 ± 0.62) to late trimester (32.88 ± 0.77) stages, indicating a progressive accumulation of Oct-4–positive undifferentiated cells during the fetal development.Oct-4 expression declined in the testis of non-descript goat during pre-pubertal and pubertal stages which is in agreement with the findings of Bhartiya et al. (2010) and He et al. (2010), who observed that Oct-4 expression diminished as spermatogonia underwent differentiation into meiotic and post-meiotic germ cells. The absence or reduced Oct-4 immunoreactivity in more differentiated cells like spermatocytes and spermatids, as reported in human and buffalo studies (He et al. 2010; Mahla et al. 2012), was in alignment with the present findings in the non-descript goats. Collectively, the present study confirmed that Oct-4 served as a reliable marker of undifferentiated germ cells in goats and supported its use in the identification and potential isolation of SSCs for germline preservation, genetic modification, or regenerative applications in caprine species.

The evaluation of total antioxidant capacity using the CUPRAC (Cupric Ion Reducing Antioxidant Capacity) assay revealed significant age-related variations in the testicular tissues of non-descript goats, reflecting dynamic changes in oxidative stress and antioxidant defense during development. In the prenatal age groups, a comparatively lower CUPRAC value was recorded in the mid trimester stage (484.27 ± 2.14 µM), indicating greater oxidative stress within the testicular environment. This period was characterized by the active cellular proliferation, testicular morphogenesis, and vascular remodeling, processes that were often associated with elevated production of reactive oxygen species (ROS). However, a marked increase in CUPRAC value was observed in the late trimester stage (565.86 ± 2.12 µM), signifying enhanced antioxidant defense mechanisms in response to the intensified metabolic activity and advanced testicular differentiation. These findings were in agreement with Apak et al. (2004), who demonstrated that the CUPRAC assay was a reliable method for detecting the changes in antioxidant status in biologically active tissues.

In the postnatal stages, a sharp decline in antioxidant concentration was evident in the pre-pubertal goats (310.45 ± 1.00 µM), indicating heightened oxidative stress during early postnatal development. This suppression of antioxidant capacity could be attributed to the delayed maturation of enzymatic antioxidant systems such as superoxide dismutase, catalase, and glutathione peroxidase. Similar observations were reported by Cecchini and Fazio (2021), who linked the reduced antioxidant status in neonatal and juvenile goats to increased vulnerability to oxidative damage during tissue growth and immune adaptation. Conversely, a notable restoration of antioxidant activity was recorded in the pubertal stage (543.39 ± 2.29 µM), suggesting the activation of fully mature antioxidant defense mechanisms that supported the metabolic and physiological demands of spermatogenesis. The increase in CUPRAC activity at pubertal stage was indicative of the testis reaching the functional maturity, which included the full development of oxidative stress mitigation pathways. This pattern aligned with the findings of Rubio et al. (2016), who validated the CUPRAC assay's sensitivity in detecting the developmental changes in antioxidant defenses in animal tissues.

The Ferric Reducing Antioxidant Potency (FRAP) assay revealed significant age-dependent variations in the total antioxidant capacity of testicular tissue in non-descript goats. During prenatal development, the antioxidant concentration increased from 348.70 ± 1.06 µM in the mid trimester to 392.54 ± 0.81 µM in the late trimester. This rise likely reflected enhanced metabolic and structural activity in the developing testis, including increased

vascularization and cellular proliferation, which demanded elevated antioxidant protection to mitigate the oxidative stress. As oxidative stress is closely associated with reactive oxygen species (ROS) production during tissue differentiation, this prenatal increase in FRAP values indicated an adaptive up regulation of the antioxidant defense system (Valko et al. 2007; Poljsak et al. 2025). The FRAP assay, as established by Benzie and Strain (1996), reliably measures the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions as an index of total antioxidant power in biological tissues.

Postnatally, a sharp decline in antioxidant capacity was observed in the pre-pubertal stage, with the FRAP value dropping to 107.12 ± 0.62 µM the lowest across all age groups. This reduction might be attributed to the underdeveloped state of the testicular antioxidant system in young goats, where enzymatic defenses such as catalase, superoxide dismutase, and glutathione peroxidase have not yet fully matured (Cecchini and Fazio 2021). However, a subsequent increase to 183.81 ± 1.08 µM in the pubertal stage suggested the re-establishment of the antioxidant mechanisms, likely associated with the onset of spermatogenic activity and increased mitochondrial biogenesis (Poljsak et al. 2025). These findings highlighted the dynamic nature of testicular redox status, where antioxidant capacity followed a biphasic trend, i.e. elevated during the late fetal development, suppressed in early postnatal life, and reactivated during the puberty.

The statistically significant differences ($p<0.001$) observed among the age groups emphasized the importance of developmental stage in influencing the testicular antioxidant status. This aligned with the previous observations that oxidative balance was tightly regulated across physiological transitions to maintain the reproductive function (Valko et al. 2007). Thus, the FRAP assay results in this study underscored the importance of antioxidant monitoring in goat reproduction and the potential need for redox support during the early postnatal development to ensure optimal testicular health and function.

The TBARS (Thiobarbituric Acid Reactive Substances) assay provided insights into the lipid peroxidation status and oxidative stress across developmental stages in the testicular tissue of non-descript goats. The mid trimester of prenatal development showed elevated TBARS activity (3.06 ± 0.14 nmol MDA/g tissue), suggesting high oxidative stress associated with increased metabolic activity during the organogenesis. This aligned with the notion that early fetal development demanded intense cellular proliferation and

differentiation, processes that were often accompanied by elevated reactive oxygen species (ROS) production and oxidative damage (Apak et al. 2004).

A notable decline in TBARS levels was observed in the late trimester (1.24 ± 0.05 nmol MDA/g tissue), indicating reduced oxidative stress. This decline might reflect the establishment of more mature antioxidant defenses and structural stabilization of the testis as development progressed. In the postnatal groups, a sharp rise in TBARS activity was recorded in the pre-pubertal stage (6.40 ± 0.27 nmol MDA/g tissue), marking the highest oxidative stress level across the age groups. This surge was likely due to the heightened metabolic and hormonal activity associated with the onset of testicular maturation, which, if not adequately counterbalanced by antioxidant systems, might impair the testicular development and compromise sperm quality (Cecchini and Fazio 2021).

At the pubertal stage, TBARS levels decreased to 5.38 ± 0.06 nmol MDA/g tissue, indicating a relative reduction in lipid peroxidation, possibly due to the improved efficacy of endogenous antioxidant mechanisms such as glutathione and catalase systems (Rubio et al. 2016). The statistically significant differences ($p<0.001$) observed across the developmental stages underscored the dynamic interplay between the oxidative stress and testicular maturation. The TBARS profile in this study highlighted the critical windows of vulnerability, especially in the pre-pubertal stage, where heightened oxidative stress might necessitate the targeted antioxidant interventions to safeguard the reproductive potential in goats.

Glutathione S-transferase (GST), an essential phase II antioxidant enzyme, played a crucial role in detoxifying reactive oxygen species (ROS) and protecting cellular structures against oxidative stress. In the present study, GST activity in the testicular tissue of non-descript goats showed dynamic changes across developmental stages, reflecting varying levels of oxidative stress. During the mid trimester of prenatal development, GST activity was highest (0.29 ± 0.01 µmol/ml/min), suggesting elevated metabolic activity and oxidative stress, potentially due to the heightened demand for cellular differentiation and organogenesis (Hayes et al. 2005; Valko et al. 2007). The subsequent decrease in GST activity observed during the late trimester (0.25 ± 0.01µmol/ml/min) might indicate a stabilization of cellular metabolism and enhanced intrinsic antioxidant capacity as the fetal testis matured.

In the postnatal groups, the pre-pubertal stage exhibited resurgence in GST activity (0.28 ± 0.01 μmol/ml/min), consistent with the increased metabolic turnover and testicular remodeling associated with the approach of puberty in the goats. Such elevated oxidative burden during this critical stage might compromise the germ cell integrity and impair future sperm quality, if not effectively neutralized (Sikka 2001; Aitken and Roman 2008). Interestingly, GST activity significantly declined in the pubertal stage (0.17 ± 0.01 μmol/ml/min), potentially reflecting a shift towards more efficient oxidative defense mechanisms and hormonal regulation that were well-established in the mature testis.

The statistically significant differences in GST activity among the age groups ($p<0.001$) underscored the enzyme's role as a key biomarker of testicular oxidative status during the development. These findings were in agreement with the earlier studies indicating the pivotal function of GST and other antioxidant systems in protecting testicular tissue from oxidative damage, particularly during periods of high cellular activity and transformation (Hayes et al. 2005; Valko et al. 2007). Monitoring such enzymatic activity during the early life stages might offer important insights for managing reproductive health and improving fertility outcomes in non-descript goats.

Conclusions

Quantitative analysis of optical density of Alpha-SMA and Oct-4 provided insights into the maturity and physiological functionality of testis with advancement of age in the non-descript goats. The Alpha-SMA, a marker of contractile function, was localized in the tunica albuginea and peritubular myoid cells of seminiferous tubules in testis. Its expression increased with age, corresponding with the development of contractile capacity in the testis, essential for the propulsion of sperm through the male reproductive tract. The expression of Oct-4, a pluripotency marker, was evident in the early prenatal germ cells (primordial germ cells and gonocytes) and persisted through mid-gestation, late gestation, neonatal stage, pre-pubertal stage and pubertal stage in the spermatogonial cells, confirming the presence and localization of spermatogonial stem cell (SSC) niches at various stages of postnatal development in non-descript goat, the regenerative potential crucial for initiating and maintaining spermatogenesis in the animal. However, the expression of Oct-4 was highest in the neonatal stage, which gradually decreased in the pre-pubertal and pubertal stages of the animal. Hence, the present study confirmed that Oct-4 served as a reliable marker of

551 undifferentiated germ cells in goats. The antioxidant profiling of testicular tissue across
552 developmental stages collectively illustrated the dynamic interplay between the oxidative
553 stress and antioxidant defense mechanisms. These biochemical markers provided critical
554 insights into the redox environment of the developing testis, which directly influenced the
555 sperm quality and reproductive competence in the non-descript goat.

556 **Supplementary information**

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572 **Declarations**

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576 **Consent for publication**

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578 **Competing interests**

579 The authors declare no conflicts of interest.

580 **References**

581 Abd-Elmaksoud A. Morphological, glycohistochemical and immunohistochemical studies
582 on the embryonic and adult bovine testis. Ph. D. thesis, Munich, Germany. 2005.
583
584 Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. Oxid Med
585 Cell Longev. 2008;1(1):15-24.
586
587 Albert S, Wistuba J, Ehmcke J, Schlatt S. Germ cell dynamics in the testis of the common
588 marmoset monkey (*Callithrix jacchus*). Biol Reprod. 2010;140(5):733-740.
589
590 Apak R, Guclu K, Ozyurek M, Karademir SE. Novel total antioxidant capacity index for
591 dietary polyphenols and vitamins C and E, using their cupric ion reducing capability
592 in the presence of neocuproine: CUPRAC method. J Agric Food Chem.
593 2004;52(26):7970-7981.
594
595 Bafarani AH, Mirhoseini Z, Heidari F. Achievement to the goat's spermatogonial stem
596 cells from embryos. J Anim Poult Sci. 2014;3(1):8-18.
597
598 Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of
599 antioxidant power: The FRAP assay. Anal Biochem. 1996;239(1):70-76.
600
601 Carlo G. Immunohistochemical localization of spermatogonial stem cells and their niche
602 in prenatal and postnatal testes in Indian buffalo. Ind J Vet Anat. 2018;30(2):54-60.
603
604 Cecchini S, Fazio F. Physiological responses and oxidative stress in goat kids during
605 growth: A review. Anim Sci J. 2021;11(3):849.
606

607 Devkota B, Sasaki M, Matsui M, Montoya CA, Miyake YI. Alterations in the
608 immunohistochemical localization patterns of α-smooth muscle actin (SMA) and
609 vimentin in the postnatally developing bovine cryptorchid testis. J Reprod Dev.
610 2006;52(3):329-34.
611 Gall CF, Stier CH, Fraham K. Age estimation of goat fetus. Small Rumin Res.
612 1994;14:91-94.
613 Li L, Lin W, Wang Z, Huang R, Xia H, Li Z, Deng J, Ye T, Huang Y, Yang Y. Hormone
614 regulation in testicular development and function. Int J Mol Sci. 2024;25(11):5805.
615
616 Hayes JD, Flanagan JU, Jowsey, IR. Glutathione transferases. Annu Rev Pharmacol
617 Toxicol. 2005;45:51-88.
618
619 He Z, Kokkinaki M, Jiang J, Dobrinski I, Dym M. Isolation, characterization, and culture
620 of human spermatogonia. Biol Reprod. 2010;82(2):363-372.
621
622 James ER, Carrell DT, Aston KI, Jenkins TG, Yeste M, Salas-Huetos A. The Role of the
623 Epididymis and the Contribution of Epididymosomes to Mammalian Reproduction.
624 Int J Mol Sci. 2020;21(15):5377.
625
626 Cizkova, K, Foltynkova T, Gachechiladze M, Tauber Z. Comparative Analysis of
627 Immunohistochemical Staining Intensity determined by Light microscopy, Image J
628 and QuPath in Placental Hofbauer Cells. Acta Histochem Cytochem. 2021;54(1):21-
629 29.
630
631 Mahla RS, Reddy N, Goel S. Spermatogonial stem cells (SSCs) in buffalo (*Bubalus*
632 *bubalis*) testis. PLoS One. 2012;7(4):36-40.
633
634 Mitchell RT, Cowan G, Morris KD, Anderson RA, Fraser HM, Mckenzie KJ, Wallace
635 WH, Kelnar CJ, Saunders PT, and Sharpe RM. Germ cell differentiation in the
636 marmoset (*Callithrix jacchus*) during fetal and neonatal life closely parallels that in
637 the human. Hum Reprod. 2008;23(12):2755-2765.
638

639 Aire TA and Ozebge PC. The testicular capsule and peritubular tissues of birds:
640 Morphometry, histology, ultrastructure and immunohistochemistry. J Anat.
641 2007;210(6):731-740.
642
643 Pesce M, Scholer HR. Oct-4 expression in early mouse embryos: an overview. Mech
644 Dev. 1998;71(12):165–171.
645
646 Acharya N, Richburg JH. Peritubular myoid cells of the testis produce monocyte
647 chemotactic protein 1 upon direct exposure to Mono-(2-Ethylhexyl) phthalate through
648 the IL-1 signaling pathway. Toxicology. 2025;13 (514):154118.
649
650 Rubio CP, Hernandez-Ruiz J, Martinez-Subiela S, Tvarijonaviciute A, Ceron JJ, Bernal
651 LJ. Spectrophotometric assays for total antioxidant capacity (TAC) in dog serum: an
652 update. BMC Vet Res. 2016;12: 166.
653
654 Poljsak B, Jamnik P, Milisav I. The importance of multifaceted approach for accurate and
655 comprehensive evaluation of oxidative stress status in biological system.
656 Antioxidants. 2025;14(9):1083.
657
658 Sahu SK, Mishra UK, Sathapathy S. Scanning Electron Microscopic studies on the
659 ventricular architecture of pre-natal non-descript sheep. Ind J Anim Res. 2021;10:
660 18805.
661
662 Schneider CA, Rasband WS, Eliceiri KW. NIH Image to Image J: 25 years of image
663 analysis. Nat Methods. 2012;9(7):671–675.
664
665 Sikka SC. Relative impact of oxidative stress on male reproductive function. Curr Med
666 Chem. 2001;8(7):851–862.
667
668 Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and
669 antioxidants in normal physiological functions and human disease. Int J Biochem Cell
670 Biol. 2007;39(1):44–84.