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Abstract

The objective of this study was to examine the influence of age on redox, immune, and transcriptional responses following surgical castration in calves. Twelve Wagyu calves were allocated to two age groups: 2 months (n = 6) and 4 months (n = 6). Blood was collected before castration and at 6, 24, and 72 hours after castration. Nitric oxide and total antioxidant capacity were measured. Gene expression (IFN- γ , TNF- α , and IL-10) was quantified using quantitative real-time PCR. T-lymphocyte subsets (CD4⁺, CD8⁺, and $\gamma\delta$ T cells) were determined by flow cytometry. RNA sequencing of peripheral blood mononuclear cells collected at 6 hours after castration was performed, followed by differential gene expression and pathway enrichment analyses. Nitric oxide concentrations were higher in 4-month-old calves than in 2-month-old calves ($p < 0.05$), whereas total antioxidant capacity decreased progressively after castration regardless of age ($p < 0.05$). IFN- γ and TNF- α expression increased significantly at 6 hours after castration, regardless of age. IL-10 expression exhibited an age $\times$ time interaction, with 2-month-old calves showing an enhanced anti-inflammatory response at 24-72 hours after castration ($p < 0.05$). Percentages of CD4⁺, CD8⁺, and $\gamma\delta$ T cells changed significantly over time following castration ($p < 0.05$), and CD8⁺ T-cell percentages were additionally influenced by age ($p < 0.05$), with 2-month-old calves showing a greater overall magnitude of response. Transcriptomic analysis identified 266 differentially expressed genes between age groups (164 upregulated, 102 downregulated in 4-month calves), with enrichment of immune- and inflammation-related pathways, including NF- κ B, MAPK, TNF, IL-17, Toll-like receptor, and NOD-like receptor signaling pathways. Upregulated genes in 4-month calves included CCL14, CSF3, PTX3, ALOX15, and IL17REL, which are associated with inflammatory signaling and leukocyte activation. These findings demonstrate that castration induces coordinated immune and redox responses in calves, with both age and time influencing the magnitude and dynamics of these changes. Older calves exhibited stronger inflammatory and redox-associated responses, whereas younger calves demonstrated distinct regulatory cytokine responses and differences in CD8⁺ T-cell proportions. Integration of physiological, cellular, and transcriptomic analyses suggests age-associated differences in postoperative inflammatory and redox regulation following castration.

Keywords (3 to 6): calves; castration; immune response; redox response; RNA sequencing; transcriptomics

Introduction

Meat is an important source of protein for human health, providing all essential amino acids. It is also rich in various fatty acids and micronutrients that contribute to nutritional balance [1]. In many regions of the world, particularly in developing countries, the demand for meat remains high [2]. In recent years, global meat consumption has increased significantly, largely driven by population growth and rising per capita income [1]. Therefore, improving beef production efficiency while ensuring optimal animal welfare through evidence-based management practices has become a key objective in modern livestock production systems [3].

Castration is a widely used practice in beef production systems to improve meat quality, reduce aggressive behavior, and facilitate herd management [4]. However, castration constitutes a major physiological stressor that triggers a cascade of molecular and cellular responses [5]. At the tissue level, surgical trauma activates damage-associated molecular patterns (DAMPs) that bind to pattern recognition receptors on innate immune cells, initiating transcription factor activation (particularly nuclear factor kappa B (NF- κ B) and activator protein-1 (AP-1)) and subsequent pro-inflammatory cytokine gene expression [6]. Meanwhile, the acute stress response activates the hypothalamic-pituitary-adrenal axis, leading to cortisol release that modulates immune cell function and metabolic processes [7]. These neuroendocrine and immune responses are accompanied by oxidative stress, characterized by increased production of reactive oxygen species (ROS) through NADPH oxidase activation in recruited neutrophils and macrophages, alongside superoxide generation via mitochondrial electron transport chain disruption, and nitric oxide via inducible nitric oxide synthase (iNOS) in inflammatory cells [8].

Previous studies have established several key aspects of castration-induced stress responses. At the endocrine level, cortisol elevation following castration has been well-documented, with peak concentrations occurring within 30 minutes to 9 hours post-procedure, though the magnitude varies with age and castration method [9,10]. At the cellular level, acute-phase protein responses, particularly haptoglobin elevation, have been characterized, indicating hepatic acute-phase response activation [11,12]. At the behavioral level, pain-associated behaviors and altered activity patterns have been quantified using a visual behavioral ethogram and electroencephalography [10,13]. At the immune level, leukocyte trafficking patterns have been described, showing initial neutrophilia followed by lymphocyte mobilization [14]. Additionally, recent work has demonstrated that surgical castration induces expression of pro-inflammatory cytokine genes, including interleukin-1-alpha (IL-1 α),

IL-1-beta (IL-1 β), IL-1 receptor antagonist (IL-1RN), and IL-6 in leukocytes, with peak mRNA expression occurring within the first 6-24 hours [15].

Despite these advances, critical mechanistic gaps prevent translation of phenotypic observations into optimized management strategies. First, while age-dependent differences in cortisol responses have been documented [10,14], the molecular mechanisms underlying differential immune-redox regulation between younger and older calves remain unexplored. Specifically, it is unknown whether age-related differences in inflammatory cytokine production reflect transcriptional regulation (differential gene expression) or feedback regulatory mechanisms. Second, the relationship between oxidative stress markers and inflammatory gene expression has not been mechanistically characterized. Third, the temporal coordination between redox signaling and immune activation is poorly understood. The existing literature provides snapshots of individual biomarkers at isolated time points but lacks an integrated analysis of how oxidative stress, antioxidant depletion, and inflammatory gene expression are temporally coordinated during the acute stress response [16,17]. Furthermore, the potential for transcriptional compensation mechanisms has not been explored. Recent work in other species suggests that immune system maturation involves not only increased inflammatory capacity but also enhanced regulatory mechanisms [18,19], including induction of negative feedback regulators (suppressor of cytokine signaling (SOCS) proteins, A20/tumor necrosis factor alpha-induced protein 3 (TNFAIP3), I κ B α) [20-22] and anti-inflammatory pathways (IL-10, transforming growth factor-beta (TGF- β) signaling) [23]. Whether such developmental differences in regulatory gene expression contribute to age-dependent castration responses in cattle remains unknown.

To address these knowledge gaps, the present study employed an integrated approach combining targeted biochemical measurements with whole-transcriptome sequencing. Our objectives were: (1) to quantify temporal changes in redox biomarkers (nitric oxide, total antioxidant capacity), inflammatory cytokines [Interferon-gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), IL-10], and adaptive immune cell populations (CD4⁺, CD8⁺, $\gamma\delta$ T cells) over 72 hours following surgical castration in 2- versus 4-month-old calves; (2) to perform RNA-sequencing on peripheral blood mononuclear cells (PBMCs) to identify age-associated transcriptional signatures and differentially expressed genes; (3) to conduct pathway enrichment analysis to determine which biological processes and signaling networks are differentially activated between age groups; and (4) to integrate transcriptomic findings with biochemical measurements to elucidate the molecular mechanisms underlying observed age-dependent physiological differences. Understanding these mechanisms is essential, as age at castration remains a modifiable management factor, yet current recommendations lack molecular evidence to support optimal timing decisions.

To our knowledge, this represents the first study to integrate whole-transcriptome profiling with temporal physiological measurements to elucidate molecular mechanisms of age-dependent castration responses. This integrative strategy moves beyond descriptive phenotyping toward mechanistic understanding, directly addressing the need for evidence-based optimization of castration timing to minimize physiological burden while maintaining production efficiency.

Materials and Methods

Ethical approval for all experimental procedures was granted by the Institutional Animal Care and Use Committee of Mahasarakham University (IACUC-MSU-090-082/2025).

1. Animal management and experimental procedure

Twelve male Wagyu calves were enrolled in the study. Animals were assigned to two age groups: 2 months old (2M; 57 ± 7 kg; $n = 6$) and 4 months old (4M; 130 ± 15 kg; $n = 6$). All calves originated from the same commercial herd located in Kalasin Province, northeastern Thailand, and were maintained under identical management conditions throughout the experiment.

Animals were housed on concrete flooring with straw bedding and had *ad libitum* access to drinking water. The feeding regimen differed according to age. Calves in the 2M group received milk replacer (4 L per calf per day), concentrate feed (CP 973®, CPF Thailand Public Company Limited, Saraburi Province, Thailand), and pangola grass. Calves in the 4M group were provided concentrate feed (CP 972®, CPF Thailand) together with pangola grass. All calves were clinically healthy as determined by rectal temperature ($<39.5^{\circ}\text{C}$), absence of respiratory signs, and normal appetite during a 7-day acclimation period before castration. Surgical castration was performed on day 0 between 10:30 and 12:30 h using a standardized surgical technique [5]. Briefly, the scrotum was cleaned with an antiseptic solution; a vertical incision was made through the scrotal skin and tunica vaginalis; the testicles were exteriorized; the spermatic cord was ligated and transected; and the wound was left to heal by second intention. The procedure duration was approximately 10-15 minutes per calf. During the procedure, calves were gently restrained using a halter to minimize unnecessary stress.

2. Experiment 1: physiological, biochemical, and immune responses

2.1 Experimental design and sampling procedure

The experiment employed a two-factor repeated-measures design to evaluate the effects of age at castration and time after surgery. The fixed factors were age at castration (2 and 4 months) and sampling time after castration (0, 6, 24, and 72 hours), with repeated measurements collected from the same calf across all time points. Sample size determination for each age group followed the method described by Ilyas et al. [24], resulting in six animals per group. This design enabled evaluation of age-related effects, temporal responses after castration, and their interaction.

2.2 Blood sample collection

Blood samples were collected from the jugular vein at 0, 6, 24, and 72 hours after castration. Approximately 8 mL of whole blood was collected into K3-EDTA tubes (ethylenediaminetetraacetic acid) to isolate peripheral blood mononuclear cells (PBMCs). Additional blood samples (2 mL) were collected in serum clot activator tubes for the determination of biochemical markers, including nitric oxide and total antioxidant capacity. Samples were centrifuged at $3000 \times g$ for 15 min to obtain the serum supernatant, which was then stored at -20°C until analysis.

2.3 Peripheral blood mononuclear cell isolation

PBMCs were isolated from whole blood as previously described [5]. Briefly, blood samples were diluted with two volumes of phosphate-buffered saline (PBS). Ficoll-Paque Plus (Cytiva, MA, USA) was placed at the bottom of centrifuge tubes, followed by careful layering of diluted blood. Samples were centrifuged at $800 \times g$ for 30 min. The supernatant was removed, and the interphase containing PBMCs was collected and resuspended in 10 mL PBS, followed by centrifugation at $1400 \times g$ for 10 min. The resulting cell pellet was treated with 3 mL red blood cell lysis buffer (Thermo Fisher Scientific Inc., USA) and incubated for 5 min at room temperature with gentle mixing. PBS was then added to stop the reaction, and samples were centrifuged again at $1400 \times g$ for 10 min. Cell pellets were washed twice with 10 mL PBS and subsequently resuspended in 1 mL RNAlater solution (Invitrogen, USA) for downstream molecular analysis.

2.4 Measurement of biochemical markers

Nitric oxide

Nitric oxide concentration was determined using the Griess colorimetric method [25]. Briefly, 100 μL of serum was incubated with 100 μL Griess reagent consisting of 1% sulfanilamide (DC Fine Chemicals, Terrassa, Spain) and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (PanReac AppliChem, Darmstadt, Germany) in

2.5% phosphoric acid (QR $\text{\textcircled{R}}$ C $^{\text{TM}}$, Auckland, New Zealand) at room temperature for 15 min. Absorbance was measured at 540 nm, and nitric oxide concentrations were calculated using a sodium nitrite (NaNO $_2$) (KemAus, Australia) standard curve.

Total antioxidant capacity analysis

The total antioxidant capacity of serum samples was evaluated using the ferric reducing antioxidant power (FRAP) assay. Fresh FRAP reagent was prepared by mixing 10 mL of 300 mM sodium acetate buffer (Ajax Finechem Pty. Ltd.), 1 mL of 10 mM tris(2-pyridyl)-s-triazine (Sigma-Aldrich, USA) in 40 mM HCl, and 1 mL of 20 mM ferric chloride (PanReac AppliChem, Darmstadt, Germany). For analysis, 20 μ L of serum was mixed with 180 μ L of FRAP reagent in microplate wells and incubated for 5 min at room temperature. Absorbance was measured at 595 nm using a Tecan Infinite $^{\text{\textcircled{R}}}$ 200 microplate reader (Tecan Group Ltd., Männedorf, Switzerland). Antioxidant capacity was calculated using FeSO $_4$ ·7H $_2$ O standards ranging from 100 to 1000 μ M.

2.5 cDNA synthesis and reverse transcription quantitative real-time PCR

Complementary DNA (cDNA) was synthesized from purified RNA using ReverTra Ace qPCR RT Master Mix (TOYOBO, Japan) according to the manufacturer's instructions. Gene expression levels were quantified using Maxima SYBR Green qPCR Mastermix (Thermo Fisher Scientific, USA) on a QuantStudio 3 Real-Time PCR system (Applied Biosystems). Relative gene expression was calculated using the $2^{-\Delta\Delta\text{CT}}$ method, with GAPDH used as the internal reference gene. Primer sequences used for amplification are listed in Table 1.

2.6 T-lymphocyte subset analysis by flow cytometry

Whole blood samples were treated with red blood cell lysis buffer (Thermo Fisher Scientific Inc., USA) and incubated at room temperature. After 10 min, PBS was added to stop the reaction, followed by centrifugation at 500 $\times$ g for 5 min. The supernatant was removed, and the remaining cell pellet was resuspended in 100 μ L PBS containing 2% fetal bovine serum. To prevent non-specific binding, polyclonal rat IgG (Sigma-Aldrich, USA) was added to all tubes and incubated at 4 $^{\circ}$ C for 15 min. Cells were then stained with fluorescently labeled antibodies, including CD3-Alexa Fluor 647, WC1-FITC, CD4-Pacific Blue, and CD8-RPE (Bio-Rad Laboratories Inc., UK). Samples were incubated at 4 $^{\circ}$ C for 30 min in the dark.

After staining, cells were washed with 1 mL PBS containing 2% fetal bovine serum and centrifuged at 400 $\times$ g for 5 min. The cells were suspended in PBS containing 2% fetal bovine serum. Samples were stored at 4 $^{\circ}$ C in the dark until flow cytometric analysis. Data acquisition was performed using a FACSCelesta flow cytometer (BD Biosciences, USA), and results were analyzed using FlowJo software (BD Life Sciences). Peripheral blood

lymphocytes were initially identified based on forward- and side-scatter characteristics (FSC-A vs. SSC-A), followed by singlet gating before analysis of T-cell subsets. No viability dye was included in the staining panel. Single-stained compensation controls were used to establish fluorescence compensation for each fluorochrome. Unstained cells were used to assess the negative fluorescence distribution. Fluorescence-minus-one (FMO) controls were not included in the original experimental staining panel. CD3⁺CD4⁺, CD3⁺CD8⁺, and CD3⁺WC1⁺ populations were identified using the established fluorescence distributions and the same gating hierarchy across all samples. The percentages of CD4⁺, CD8⁺, and WC1⁺ cells were calculated within the CD3⁺ T-cell population. The same photomultiplier tube voltage settings and fluorescence compensation matrix were applied consistently to all samples. A total of 100,000 events were acquired for each sample. The flow cytometric gating strategy is illustrated in Figure 1.

2.7 Statistical analysis for experiment 1

All statistical analyses were performed using R software (R Core Team, Vienna, Austria). Data were analyzed using linear mixed-effects models fitted with the lme4 package, with age at castration (2 and 4 months), time after castration (0, 6, 24, and 72 hours), and their interaction included as fixed effects. An individual calf was included as a random effect to account for repeated measurements over time. The significance of fixed effects was evaluated using Type III analysis of variance with Satterthwaite's approximation.

Model assumptions were assessed using a combination of graphical and statistical approaches. Residual normality was evaluated using the Shapiro–Wilk test and diagnostic plots generated from the DHARMA and performance packages. Variables that did not satisfy model assumptions were log-transformed before analysis. For CD8⁺ and $\gamma\delta$ T-cell percentages, statistical analyses were performed on log-transformed data. Estimated marginal means were subsequently back-transformed to the original percentage scale for presentation. In contrast, CD4⁺ T-cell percentages, nitric oxide, FRAP, and cytokine Δ Ct values were analyzed using raw data.

For cytokine gene expression analyses (IFN- γ , TNF- α , and IL-10), statistical analyses were performed using Δ Ct values. Fold change relative to baseline (Time 0 before castration) was subsequently calculated using the $2^{-\Delta\Delta\text{Ct}}$ method and used only for graphical and tabular presentation to facilitate biological interpretation.

Post hoc comparisons were performed using estimated marginal means with Tukey's adjustment for multiple comparisons. For variables analyzed after log transformation, results are presented as back-transformed geometric means with 95% confidence intervals. Statistical significance was declared at $p < 0.05$.

3. Experiment 2: Transcriptomic analysis

3.1 Sample selection, RNA extraction, and quality assessment

For transcriptomic analysis, PBMC samples collected 6 h after castration were selected because this time point represented the early phase of the inflammatory response, characterized by the initial upregulation of pro-inflammatory mediators. This time point was chosen to capture early transcriptional changes that may precede subsequent alterations in immune cell populations and redox-related parameters observed at later time points. Four calves were randomly selected from the six animals in each age group for RNA-seq analysis. No samples were excluded because of RNA quality or technical considerations. Total RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Germany). RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). RNA purity was assessed by evaluating the 260/280 and 260/230 absorbance ratios using the same instrument. RNA quality was further examined using 1% agarose gel electrophoresis to confirm the presence of clear 18S and 28S ribosomal RNA bands. RNA integrity number (RIN) was determined using the LabChip GX system (PerkinElmer LabChip GX Touch HT). Purified RNA samples were immediately frozen and stored at -20°C until further processing for cDNA synthesis and RNA sequencing.

3.2 RNA sequencing

Libraries were prepared using the Hieff NGS Ultima Dual-mode mRNA Library Prep Kit for Illumina (Yeasen) following the manufacturer's protocol. Sequencing was performed on the Illumina NovaSeq X Plus platform using the NovaSeq™ X Series 25B reagent kit with 150 bp paired-end reads. Sequencing generated an average of 46.2 million raw reads per sample (range: 43.2-53.5 million). Clean reads were mapped to the bovine reference genome (ARS-UCD1.2) using STAR aligner, achieving uniquely mapped read rates ranging from 88.43% to 92.58% (mean = 91.31%), indicating high-quality RNA sequencing data.

3.3 Differential expression and functional enrichment analysis

Differential gene expression analysis between 2- and 4-month-old calves was performed using the DESeq2 package. Differentially expressed genes (DEGs) were defined using the criteria false discovery rate (FDR) < 0.01 and $|\log_2(\text{fold change})| \geq 2$. Multiple testing correction was performed using the Benjamini–Hochberg method.

Principal component analysis (PCA) was conducted to evaluate clustering among samples. Functional enrichment analyses included Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. GO enrichment of DEGs was performed using the Goseq R package based on the Wallenius non-central hypergeometric distribution, and enrichment significance was defined as $q\text{-value} < 0.05$.

Results

1. Biochemical parameters

Nitric oxide concentrations were significantly lower in 2-month-old calves than in 4-month-old calves ($p = 0.01$). Across sampling times, nitric oxide concentrations decreased at 6 hours post-castration compared with pre-castration ($p = 0.01$). In contrast, concentrations at 24 and 72 hours did not differ significantly from either pre-castration or 6 hours (Table 2).

FRAP values were significantly lower at 72 hours post-castration than pre-castration ($p = 0.02$). However, FRAP values at 6 and 24 hours post-castration did not differ significantly from pre-castration or from those at 72 hours (Table 2).

2. Cytokine and immune responses

2.1 Cytokine responses

IFN- γ expression was affected by sampling time ($p = 0.03$), whereas age and the age $\times$ time interaction were not significant (Table 3). IFN- γ expression tended to increase at 6 hours post-castration; however, post hoc analysis detected no significant pairwise differences among sampling times.

TNF- α expression was influenced by sampling time ($p < 0.001$). In both age groups, TNF- α expression at 6 hours post-castration was significantly higher than pre-castration. In 4-month-old calves, TNF- α expression at 6 hours was also significantly higher than at 24 and 72 hours post-castration. In contrast, in 2-month-old calves, expression at 24 and 72 hours did not differ significantly from either 6 hours or pre-castration (Table 3).

IL-10 expression was significantly influenced by age ($p = 0.003$), sampling time ($p < 0.001$), and the age $\times$ time interaction ($p = 0.008$). Overall, 2-month-old calves exhibited higher IL-10 expression than 4-month-old calves, particularly at 24 and 72 hours post-castration (Figure 2; Table 3). In 2-month-old calves, IL-10 expression increased after castration and remained elevated through 72 hours compared with pre-castration. In 4-month-old calves, IL-10 expression peaked at 6 hours post-castration and subsequently declined at 24 and 72 hours.

2.2 T-lymphocyte subsets

The percentage of CD4⁺ T cells was significantly affected by sampling time ($p < 0.001$) (Table 4). In both age groups, the percentage of CD4 T cells at 72 hours post-castration was significantly higher than at 0, 6, and 24 hours. Percentages at 6 hours were significantly higher than at 24 hours, whereas the 6-hour value did not differ

significantly from pre-castration. Percentages at 0 and 24 hours did not differ significantly between sampling times (Figure 3A).

The percentage of CD8⁺ T cells was significantly influenced by age ($p = 0.007$) and sampling time ($p < 0.001$) (Table 4). Overall, 2-month-old calves exhibited significantly higher percentages of CD8⁺ T cells than 4-month-old calves across all sampling times, consistent with the non-significant age $\times$ time interaction. In both age groups, the percentage of CD8⁺ T cells at 6, 24, and 72 hours post-castration was significantly higher than pre-castration ($p < 0.05$). In 2-month-old calves, percentages at 6 hours did not differ significantly from those at 24 or 72 hours post-castration. In 4-month-old calves, the percentage at 24 hours was significantly higher than at 6 hours, while 72 hours did not differ significantly from either 6 or 24 hours. Percentages at 24 and 72 hours did not differ significantly between sampling times (Figure 3B).

The percentage of $\gamma\delta$ T cells was significantly affected by sampling time ($p < 0.001$) (Table 4). In both age groups, percentages at 24 and 72 hours post-castration were significantly higher than those pre-castration and at 6 hours post-castration. Percentages at 0 and 6 hours did not differ significantly between sampling times, and percentages at 24 hours did not differ significantly from 72 hours post-castration. (Figure 3C).

3. Transcriptomic analysis

3.1 Differentially expressed genes (DEGs)

PBMC samples collected 6 hours post-castration were subjected to RNA sequencing (RNA-seq) analysis. Sequencing yielded an average of 46.2 million paired-end reads per sample. Quality-filtered reads were aligned to the bovine reference genome (ARS-UCD1.2), achieving a mean mapping rate of 91.3% (range: 88.4%-92.6%), confirming high data quality. Principal component analysis (PCA) revealed a clear separation between the 2-month-old and 4-month-old groups (Figure 4A). In total, 266 genes were differentially expressed between calves castrated at 2 and 4 months of age ($FDR < 0.01$, $|\log_2FC| \geq 2$). Among these, 164 genes were upregulated, and 102 genes were downregulated in 4-month-old calves relative to the 2-month-old group (Figure 4B). The volcano plot illustrates the distribution of genes that are significantly upregulated or downregulated (Figure 4C). The top ten upregulated genes in the 4-month-old group included LOC112445693, CCL14, CSF3, PTX3, ALOX15, LOC101902555, HPGD, SDC4, CDC42EP2, and IL17REL (Table 5). These genes are primarily associated with inflammatory signaling, chemokine-mediated immune responses, lipid metabolism, and cellular stress responses. In contrast, the top ten downregulated genes in the 4-month-old group included LOC112445692, ENPP1, LOC786796, PER3, GZMA,

LOC112445731, NCMAP, TLL2, SIGLEC1, and ZNF697 (Table 6). These genes were primarily involved in immune regulation, extracellular matrix remodeling, circadian rhythms, and transcriptional control.

3.2 Functional enrichment analysis

To further characterize the biological functions associated with differentially expressed genes between 2- and 4-month-old calves, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed. GO enrichment analysis was classified into three functional categories: biological process (BP), cellular component (CC), and molecular function (MF). Among the enriched biological processes, significant pathways included positive regulation of protein deubiquitination, interleukin-1-mediated signaling, and negative regulation of MAP kinase activity (Figure 5A). For CC categories, enriched terms included mitochondrial electron transfer flavoprotein complex, proton-transporting two-sector ATPase complex (catalytic domain), and microtubule-associated structures (Figure 5B).

Within the MF category, the most significantly enriched terms were granulocyte colony-stimulating factor receptor binding, platelet-activating factor receptor activity, and arachidonate 12(S)-lipoxygenase activity (Figure 5C). KEGG pathway enrichment analysis also revealed significant enrichment ($FDR < 0.05$) in several immune- and signaling-related pathways. The top 20 enriched pathways included the TNF signaling pathway, IL-17 signaling pathway, NF- κ B signaling pathway, Toll-like receptor signaling pathway, NOD-like receptor signaling pathway, and mitogen-activated protein kinase (MAPK) signaling pathway (Figure 5D).

Discussion

Surgical castration causes acute tissue injury, which triggers complex changes in inflammatory responses and the regulation of redox homeostasis [17, 26]. Understanding how these responses are modulated by age is critically important, as immune status and redox regulation in calves undergo continuous development and adaptation [16, 19]. The present study found that both age and time play important roles in shaping post-castration biochemical and immune responses, although their effects differ across biological systems. Age-related differences were particularly evident in redox-associated parameters, whereas immune responses were largely determined by the time elapsed following surgical injury. The changes in biochemical and immune parameters observed in calves of different ages and at sampling time points after surgical injury are described as follows.

Nitric oxide concentrations were higher in 4-month-old calves than in 2-month-old calves, indicating an age-related difference in the magnitude of redox responses. Because no age $\times$ time interaction was detected, this difference remained consistent across all sampling time points. Castration induces acute tissue injury, which activates inflammatory cascades and stimulates iNOS, leading to increased nitric oxide production as part of host defense and wound-healing processes [27, 28]. Nitric oxide plays a dual role during inflammation, functioning both as a signaling molecule and as a mediator of oxidative stress when produced in excess [8, 29]. Therefore, tissue injury is associated with an elevation in nitric oxide levels [27]. Previous studies have shown that castration can trigger both neurohormonal and inflammatory stress responses in calves, with age influencing the magnitude of these physiological reactions [9-11]. The higher nitric oxide concentrations observed in older calves in the present study may reflect enhanced inflammatory signaling associated with age-related developmental differences and increased leukocyte responsiveness [16]. Age-related changes in immune function are well known to modulate sensitivity to redox and inflammatory reactions during early life [30], which may contribute to the increased nitric oxide production observed in 4-month-old calves following surgical injury. This pattern suggests that maturation of immune signaling pathways may promote nitric oxide-mediated inflammatory responses in older calves [31]. In contrast, total antioxidant capacity (FRAP) was influenced solely by time, showing a progressive decline after castration regardless of age. This pattern suggests that antioxidant depletion represents a general systemic response to surgical stress rather than an age-dependent mechanism [32]. Surgical trauma and acute inflammation are well known to disrupt redox homeostasis through increased production of reactive oxygen and nitrogen species [17, 33]. The concurrent increase in nitric oxide (particularly in older calves) and the time-dependent reduction in total antioxidant capacity indicate a transient redox imbalance during the early postoperative period, consistent with the dynamics of acute inflammatory wound healing [34].

In general, castration induces time-dependent changes in cytokine expression, reflecting the activation of both pro-inflammatory and regulatory immune pathways following surgical tissue injury [5]. In the present study, the pro-inflammatory cytokines IFN- γ and TNF- α increased over time during the early period after castration, with peak levels observed at 6 hours post-castration. These cytokines are key mediators of early innate immune activation and are rapidly induced following tissue damage through activation of intracellular signaling pathways such as NF- κ B and MAPK [22, 27]. IFN- γ plays an important role in macrophage activation and host defense, whereas TNF- α acts as a central amplifier of inflammation by promoting leukocyte recruitment and endothelial activation [35]. Similar transient increases in pro-inflammatory cytokines have also been reported in calves following castration,

reflecting acute immune activation associated with tissue injury and the early stages of wound healing [15, 27]. In contrast, IL-10 is a cytokine associated with immune regulation and anti-inflammatory processes [34]. In the present study, IL-10 expression exhibited an age $\times$ time interaction, with increased gene expression observed at later time points only in calves castrated at 2 months of age. IL-10 is a key immunoregulatory cytokine that limits excessive inflammation and promotes resolution of the immune response [35]. The delayed increase in IL-10 in younger calves may therefore reflect a compensatory regulatory mechanism aimed at restoring immune homeostasis following the early inflammatory response [4, 18].

Alterations in T-lymphocyte subsets following castration further demonstrate the dynamic nature of postoperative cellular immune responses. In the present study, percentages of CD4⁺ T cells, CD8⁺ T cells, and $\gamma\delta$ T cells were significantly influenced by sampling time, whereas age-related effects varied among individual T-cell populations. These findings indicate marked temporal changes in T-cell dynamics following surgical injury [36]. CD4⁺ T cells are key regulators of adaptive immunity and coordinate inflammatory and tissue-repair responses through cytokine production and immune-cell activation [36]. In the present study, CD4⁺ T-cell percentages increased at 6 hours, decreased at 24 hours, and reached their highest values at 72 hours post-castration in both age groups. This early decline is consistent with our previous findings in surgically castrated calves, where peripheral lymphocyte recovery began around 24 hours post-castration following an initial trauma-induced decrease in circulating lymphocytes within the first few hours [5], likely reflecting transient lymphocyte redistribution following acute surgical injury. The subsequent marked increase at 72 hours may reflect changes in the relative representation of CD4⁺ T cells during the transition from acute inflammation toward tissue repair [27]. The proportion of CD8⁺ T cells also changed significantly over time after castration. CD8⁺ T cells are major mediators of cytotoxic immune responses and participate in the elimination of damaged or stressed cells during inflammation [36]. In both age groups, CD8⁺ T-cell percentages reached their highest values at 24 hours post-castration, consistent with early cellular immune activation following tissue injury; this peak was statistically distinct from earlier and later time points in 4-month-old calves, whereas in 2-month-old calves, percentages at 6, 24, and 72 hours did not differ significantly from one another. Comparable transient increases in CD8⁺ T-cell populations have been reported during postoperative inflammatory responses [5]. Age additionally influenced overall CD8⁺ T-cell percentages, with 2-month-old calves showing higher values than 4-month-old calves across sampling times, suggesting an age-associated difference in the proportion of CD3⁺CD8⁺ cells following castration [37, 38]. Because FMO controls were not available, these findings should be interpreted as changes in the estimated CD8⁺ T-cell proportion rather

than direct evidence of altered cytotoxic T-cell function. $\gamma\delta$ T cells constitute a major T-cell population in cattle and are involved in innate-like immune surveillance, inflammatory regulation, and early responses to tissue injury [39]. In the present study, $\gamma\delta$ T-cell percentages increased at 24 and 72 hours post-castration in both age groups. In 4-month-old calves, $\gamma\delta$ T-cell percentages initially decreased at 6 hours post-castration before increasing at later time points, consistent with transient redistribution or early migration during acute inflammation [39, 40]. $\gamma\delta$ T cells rapidly respond to stress-associated molecules released from damaged tissues and contribute to cytokine production, leukocyte recruitment, and tissue repair [27, 39]. The temporal changes in the estimated $CD3^+WC1^+$ cell proportion are compatible with the dynamic redistribution of $\gamma\delta$ T cells during the post-castration period [39, 41]. However, because FMO controls were not included, these findings should be interpreted cautiously, and the present data do not establish functional activation of $\gamma\delta$ T cells. Collectively, these findings demonstrate coordinated alterations in T-lymphocyte subsets following surgical castration in calves, with distinct temporal patterns among $CD4^+$, $CD8^+$, and $\gamma\delta$ T-cell populations. The age-dependent differences observed, particularly in $CD8^+$ T-cell responses, suggest that developmental stage may contribute to postoperative cellular immune responses following surgical stress.

The transcriptomic analysis in this study revealed key mechanistic support for the observed immune responses. Specifically, the enrichment of signaling pathways related to NF- κ B signaling, MAPK activation, and cytokine-mediated signaling indicates coordinated transcriptional activation associated with inflammatory networks [42, 43]. Along with the enrichment of these pathways, multiple DEGs associated with immune activation and inflammatory signaling were identified between age groups [43, 44]. These DEGs were primarily involved in cytokine signaling, leukocyte activation, and cellular stress responses, suggesting coordinated transcriptional regulation of inflammatory pathways during the early post-castration period [45]. NF- κ B acts as a central integrator of inflammatory and redox-related signals and regulates the expression of numerous cytokines and immune mediators [22]. Similarly, MAPK pathways play important roles in regulating cytokine production, leukocyte activation, and cellular stress responses during inflammation [46]. Although RNA-seq was performed using total PBMCs rather than isolated lymphocyte subsets, the transcriptomic findings provide complementary evidence for the flow cytometric observations. The enrichment of inflammatory signaling pathways together with the differential expression of immune-related genes indicates early activation of immune regulatory networks before substantial changes in lymphocyte subset frequencies became apparent. This temporal pattern is consistent with the concept that transcriptional activation precedes phenotypic alterations in immune cell populations and may underlie the subsequent changes detected by flow cytometric analysis. Therefore, the transcriptomic and flow cytometric

findings should be regarded as complementary evidence describing different stages of the post-castration immune response rather than direct one-to-one relationships between specific genes and individual lymphocyte subsets. Because RNA-seq was conducted at 6 hours whereas the major changes in T-cell subset frequencies occurred predominantly at later time points, the transcriptomic findings are best interpreted as early molecular signatures associated with subsequent immune phenotypic changes rather than direct molecular explanations for individual T-cell populations. The consistency between cytokine dynamics and transcriptomic pathway enrichment suggests that the observed immune responses were driven by structured activation of inflammatory signaling pathways, rather than by isolated fluctuations in cytokine levels. Although several immunological and redox-related parameters exhibited more pronounced changes at later time points (24–72 h), transcriptomic analysis was intentionally performed at 6 h to characterize the early molecular events that may initiate these downstream responses. Thus, the transcriptomic findings should be interpreted as reflecting the early regulatory phase of the post-castration response rather than the peak of all physiological or immunological alterations.

Further mechanistic insight into the inflammatory responses observed after castration is provided by the differential expression of several immune-related genes identified through transcriptomic analysis [45]. Among the most strongly upregulated genes in 4-month-old calves were CCL14, CSF3, PTX3, ALOX15, and IL17REL, all of which are associated with innate immune activation and act as mediators in inflammatory processes. CCL14 functions as a chemokine that promotes leukocyte recruitment to sites of tissue injury [47], whereas CSF3 plays an essential role in neutrophil proliferation and mobilization during inflammatory responses [48]. PTX3 is an acute-phase protein involved in innate immune recognition and the regulation of inflammatory processes [49], while ALOX15 participates in the synthesis of lipid mediators that modulate inflammatory signaling pathways [50]. In addition, IL17REL is associated with IL-17 receptor signaling, a pathway known to promote the production of pro-inflammatory cytokines and the recruitment of neutrophils during tissue inflammation [51]. Therefore, the upregulation of these genes supports the activation of innate immune pathways following tissue injury induced by surgical castration.

Conversely, several genes associated with immune regulation, including GZMA, SIGLEC1, and ENPP1, were downregulated in the older calves. GZMA encodes granzyme A, a cytotoxic molecule produced by activated T cells and natural killer cells [52], while SIGLEC1 is involved in macrophage-mediated immune recognition and antigen presentation [53]. The various zinc finger transcription factors (ZNF697, LOC112445692, and LOC112445731) suggest altered transcriptional regulation and immune cell differentiation between age groups [54].

These transcriptional changes suggest age-dependent modulation of leukocyte activation and inflammatory signaling following castration, which is consistent with the enhanced nitric oxide production and inflammatory pathway enrichment observed in 4-month-old calves. Collectively, these transcriptional changes indicate that surgical castration triggers coordinated activation of innate immune pathways in PBMCs, particularly in older calves, thereby amplifying inflammatory signaling and the production of reactive oxygen and nitrogen mediators during the early postoperative period. These transcriptional patterns are consistent with the temporal increases in IFN- γ and TNF- α observed in the present study, suggesting that transcriptional activation of inflammatory pathways may contribute to the cytokine responses detected shortly after castration [46].

The observed differences between the two age groups are consistent with developmental changes that occur during early calf growth [19]. During the pre-weaning period, calves undergo continuous expansion and functional adaptation of both innate and adaptive immune systems, including $\gamma\delta$ T cells and conventional T-cell subsets [39, 40]. In ruminants, $\gamma\delta$ T cells play important roles in tissue surveillance, cytokine secretion, and wound repair [39]. As immune competence increases with age, leukocyte signaling capacity and transcriptional responsiveness to inflammatory stimuli become more robust [41], leading to enhanced activation of NF- κ B- and MAPK-dependent gene networks in older calves. At the same time, the maturation of immune regulatory mechanisms may influence the balance between pro-inflammatory activation and inflammation resolution [30].

The concordance between cytokine dynamics, redox markers, and transcriptional pathway enrichment indicates that inflammatory and redox responses following castration are not independent phenomena, but rather coordinated components of an integrated immune–redox regulatory network [26]. Taken together, the integration of redox biomarkers, cytokine profiles, and transcriptomic pathway enrichment supports a model in which castration triggers a coordinated redox–immune response. Tissue injury initiates innate immune activation and the production of redox mediators [55], which subsequently engage transcriptional programs that regulate cytokine signaling and leukocyte function. Age and developmental stage appear to modulate this cascade, influencing both the magnitude of inflammatory activation and the regulation of the immune response. These findings highlight the importance of developmental stage in shaping physiological responses to surgical stress and provide insight into age-associated differences in inflammatory and redox responses following castration in calves.

Although the present study focused on differences between calves castrated at two developmental ages, we acknowledge that the experimental groups also differed in body weight, developmental stage, and feeding regimen. Therefore, although the observed physiological and transcriptomic differences were associated with age, the

potential contribution of nutritional and growth-related factors cannot be excluded. Future studies designed to control these variables independently will be required to distinguish the specific effects of immune maturation from other developmental influences. Another limitation is that a viability dye was not used during flow cytometric analysis. Although lymphocyte gating was used to reduce the inclusion of debris and singlet gating was applied to exclude doublets, the absence of a viability dye means that non-viable cells could not be specifically excluded. In addition, the original experimental design did not include FMO controls. Although unstained and single-stained controls were used to assess negative fluorescence distributions and establish fluorescence compensation, respectively, FMO controls were not available to independently define the boundaries between negative and positive populations in the multicolor panel. Therefore, the precise discrimination and quantitative estimation of CD8⁺ and WC1⁺ populations may be subject to some uncertainty. The flow cytometry findings should consequently be interpreted as estimates of T-cell subset proportions and considered with appropriate caution. Future studies should incorporate viability dyes and FMO controls to improve the precision and reproducibility of positive-population identification.

Despite these limitations, the present findings suggest that age-associated differences in redox and inflammatory responses may have important practical implications for animal management. The higher nitric oxide levels and activation of inflammation-related gene networks observed in older calves suggest a potentially greater redox burden during the early phase of post-castration recovery. These findings may indicate that management practices or analgesic strategies according to developmental stage may help enhance recovery, reduce physiological stress, and ultimately improve animal welfare and the sustainability of beef production systems.

Conclusion

This study demonstrates that castration induces coordinated redox and immune responses in calves, with both age and time post-castration influencing changes in these biological parameters. Nitric oxide concentrations were higher in 4-month-old calves compared to 2-month-old calves, indicating a more pronounced inflammatory and redox response in older animals. Conversely, overall antioxidant capacity decreased after castration regardless of age, suggesting a disruption of redox homeostasis during the early post-castration period. Cytokine responses exhibited clear time-dependent dynamics following castration. Pro-inflammatory cytokines, including IFN- γ and TNF- α , increased rapidly at 6 hours post-castration, reflecting activation of early inflammatory pathways, whereas

IL-10 expression was influenced by both age and time, indicating the involvement of regulatory mechanisms in modulating the inflammatory response. Changes in T lymphocyte subsets, including increases in CD4⁺, CD8⁺, and $\gamma\delta$ T cells, were observed during the post-castration period, providing additional evidence of changes in peripheral T-cell composition. Furthermore, transcriptomic analysis revealed enrichment of immune- and inflammation-related pathways. These findings indicate that surgical castration triggers coordinated immune and redox responses, with the observed differences reflecting age-associated developmental variation.

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Table 1. Primer sequences for quantitative real-time PCR

Gene	Forward primer	Reverse primer	Reference
IFNG	5' - TTGAATGGCAGCTCTGAGAAAC	5' - TCTCTTCCGCTTTCTGAGGTTAGA	[5]
TNFA	5' - TGACGGGCTTTACCTCATCT	5' - TGATGGCAGACAGGATGTTG	[5]
IL-10	5' - AAGGTGAAGAGAGTCTTCAGTGAGC	5' - TGCATCTTCGTTGTCATGTAGG	[5]
GAPDH	5' - GGC GTG AAC CAC GAG AAG TAT AA	5' - CCC TCC ACG ATG CCA AAG T	[5]

Table 2. Changes in nitric oxide concentration and FRAP in 2- and 4-month-old calves before castration and at 6, 24, and 72 hours post-castration.

Parameter	Age	Time				<i>p</i> value		
		0 h	6 h	24 h	72 h	Age	Time	Age x Time
Nitric oxide (μ M)	2 M	0.64 $\pm$ 0.06	0.44 $\pm$ 0.06	0.59 $\pm$ 0.06	0.55 $\pm$ 0.06	0.01*	0.01*	0.70
	4 M	0.83 $\pm$ 0.06	0.63 $\pm$ 0.06	0.75 $\pm$ 0.06	0.61 $\pm$ 0.06			
FRAP (mM Fe ²⁺ /g)	2 M	584 $\pm$ 45.60 b	455 $\pm$ 45.60 ab	458 $\pm$ 45.60 ab	400 $\pm$ 45.60 a	0.12	0.02*	0.57
	4 M	582 $\pm$ 45.60	538 $\pm$ 45.60	558 $\pm$ 45.60	494 $\pm$ 45.60			

Values are presented as estimated marginal means $\pm$ standard error. Different superscript letters within the same row indicate significant differences across sampling times within each age group (Tukey's HSD, $p < 0.05$). Statistical analysis was performed using linear mixed-effects models with calf included as a random effect. * indicates a statistical difference ($p < 0.05$).

Table 3. Relative expression of inflammatory cytokines in 2- and 4-month-old calves before castration and at 6, 24, and 72 hours post-castration.

Parameter	Age	Time				p value		
		0 h	6 h	24 h	72 h	Age	Time	Age x Time
IFN- γ	2 M	1.00 (0.46- 2.19)	2.51 (1.14- 5.50)	1.07 (0.49- 2.35)	1.02 (0.47- 2.25)	0.22	0.03*	0.52
	4 M	1.00 (0.46- 2.19)	1.14 (0.52- 2.51)	0.50 (0.23- 1.10)	0.56 (0.25- 1.22)			
TNF- α	2 M	1.00 (0.49- 2.06) b	4.10 (1.99- 8.44) a	1.78 (0.86- 3.65) ab	2.89 (1.32- 6.30) a	0.38	<0.001**	0.08
	4 M	1.00 (0.49- 2.06) b	4.93 (2.40- 10.15) a	0.77 (0.37- 1.58) b	1.31 (0.64- 2.69) b			
IL-10	2 M	1.00 (0.48- 2.07) b	9.09 (4.39- 18.83) a	9.85 (4.76- 20.41) a	12.63 (6.10- 26.18) a	0.003*	<0.001**	0.008*
	4 M	1.00 (0.48- 2.07) c	15.34 (7.40- 31.78) a	3.06 (1.48- 6.33) b	4.14 (2.00- 8.58) b			

673

674 Values are presented as fold change relative to baseline (Time 0 before castration). Statistical analysis was

675 performed using Δ Ct values in a linear mixed-effects model with calf as a random effect. Different superscript

letters within each age group indicate significant differences among time points (Tukey's HSD, $p < 0.05$). * indicates a statistical difference ($p < 0.05$), and ** indicates a statistical difference ($p < 0.001$).

Table 4. Changes in peripheral blood T-lymphocyte subsets in 2- and 4-month-old calves before castration and at 6, 24, and 72 h after castration.

Parameter (%)	Age	Time				<i>p</i> value		
		0 h	6 h	24 h	72 h	Age	Time	Age x Time
CD4 ⁺ T cells	2 M	25.08 ± 2.9 ab	34.83 ± 2.9 b	17.65 ± 2.9 a	62.84 ± 3.2 c	0.16	<0.001**	0.74
	4 M	30.4 ± 2.9	39.75 ± 2.9	20.37 ± 2.9	62.02 ± 3.2			

		ab	b	a	c			
CD8 ⁺ T cells	2 M	0.66 ±	11.39 ±	24.24 ±	14.06 ±	0.007*	<0.001**	0.34
		0.17	2.87	6.11	3.92			
	4 M	a	b	b	b			
		0.37 ±	4.11 ±	15.97 ±	13.29 ±			
γδ T cells	2 M	0.09	1.04	4.03	3.7	0.09	<0.001**	0.06
		a	b	c	bc			
	4 M	0.86 ±	1.07 ±	25.09 ±	22.67 ±			
		0.2	0.25	5.86	5.8			
		a	a	b	b			
		0.5 ±	0.38 ±	25.72 ±	23.23 ±			
		0.12	0.09	6.0	5.95			
		a	a	b	b			

Values are presented as estimated marginal means ± standard error. CD8⁺ and γδ T-cell data were analyzed after log transformation to satisfy model assumptions and are presented as back-transformed geometric means with 95% confidence intervals. Statistical analysis was performed using a linear mixed-effects model with calf as a random effect. Different superscript letters within each age group indicate significant differences among time points (Tukey's HSD, $p < 0.05$). * indicates a statistical difference ($p < 0.05$), and ** indicates a statistical difference ($p < 0.001$). The p -values represent the main effects of age, sampling time, and the age × time interaction from the linear mixed-effects model.

Table 5 The top 10 up-regulated DEGs in PBMCs in the 4-month-old group.

Gene Symbol	Gene Description	Log ₂ FC	FDR
LOC112445693	Multidrug resistance-associated protein 4 isoform X1	5.705	0.004
LOC100297044	C-C motif chemokine 14	4.628	< 0.01
CSF3	Colony-stimulating factor 3	4.617	0.002

PTX3	Pentraxin 3	3.753	0.005
ALOX15	Arachidonate 15-lipoxygenase	3.716	< 0.001
LOC101902555	Multidrug resistance-associated protein 4-like isoform X2	3.228	< 0.001
HPGD	15-hydroxyprostaglandin dehydrogenase	3.204	< 0.001
SDC4	Syndecan-4	3.174	< 0.001
CDC42EP2	Cdc42 effector protein 2	3.131	0.001
IL17REL	Interleukin-17 receptor E-like	2.845	0.001

FC: fold change; FDR: false discovery rate.

Table 6 The top 10 down-regulated DEGs in PBMCs in the 4-month-old group.

Gene Symbol	Gene Description	Log ₂ FC	FDR
LOC112445692	Zinc finger protein OZF-like	-3.271	< 0.001
ENPP1	Ectonucleotide pyrophosphatase/ phosphodiesterase family member 1	-2.906	< 0.001
LOC786796	Antigen WC1.1	-2.844	< 0.001
PER3	Period circadian protein homolog 3	-2.609	0.001
GZMA	Granzyme A	-2.587	0.002
LOC112445731	Zinc finger protein OZF-like	-2.337	0.009
NCMAP	Noncompact myelin-associated protein	-2.232	< 0.001
TLL2	Tolloid-like protein 2	-2.164	0.006

SIGLEC1	Sialic acid binding Ig like lectin 1	-2.147	< 0.001
ZNF697	Zinc finger protein 697	-2.123	0.006

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Figure Legends

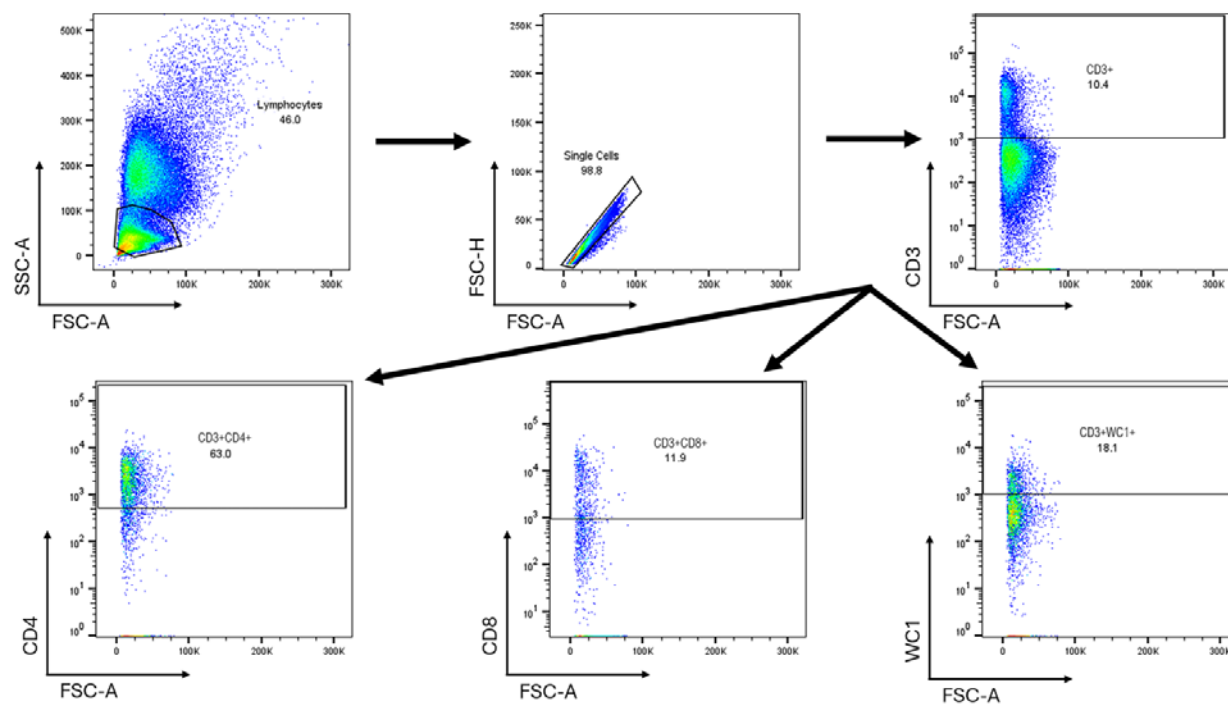


Figure 1 Representative flow cytometry plots showing the sequential gating strategy used to estimate the proportions of CD4⁺, CD8⁺, and WC1⁺ cells within the CD3⁺ T-cell population.

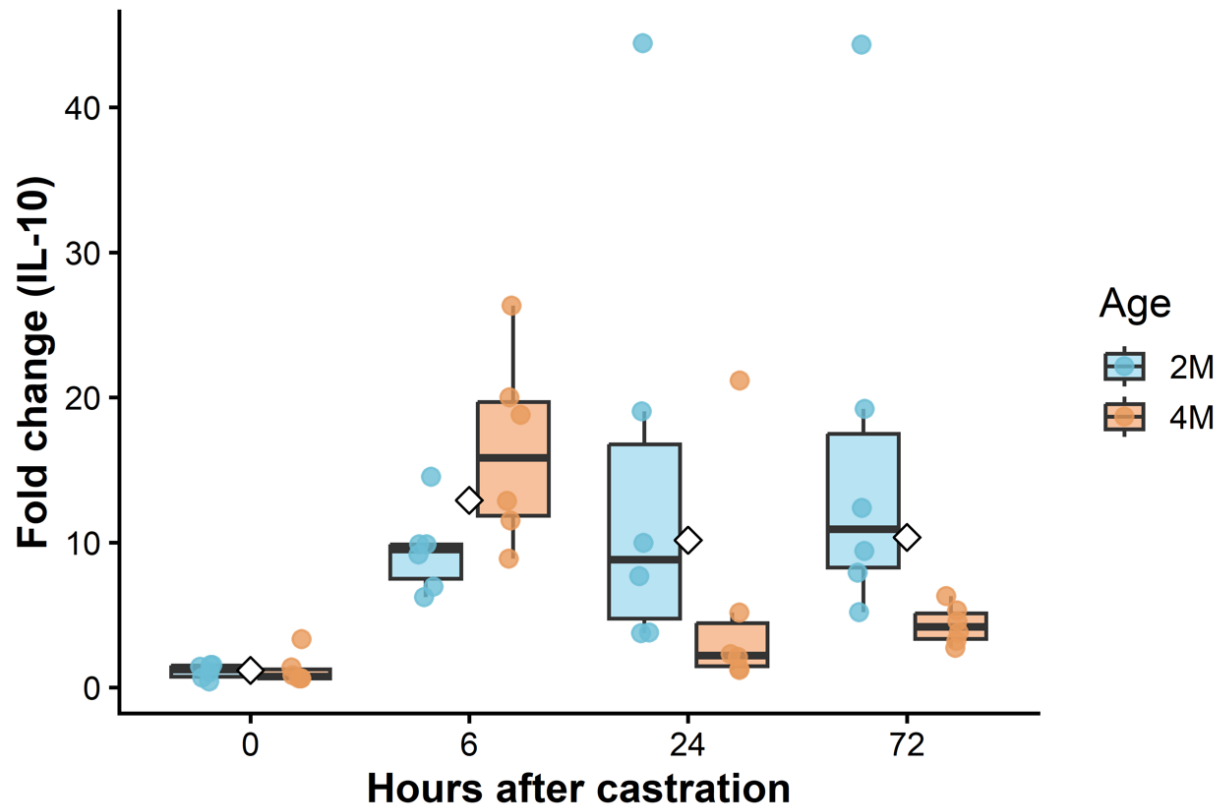
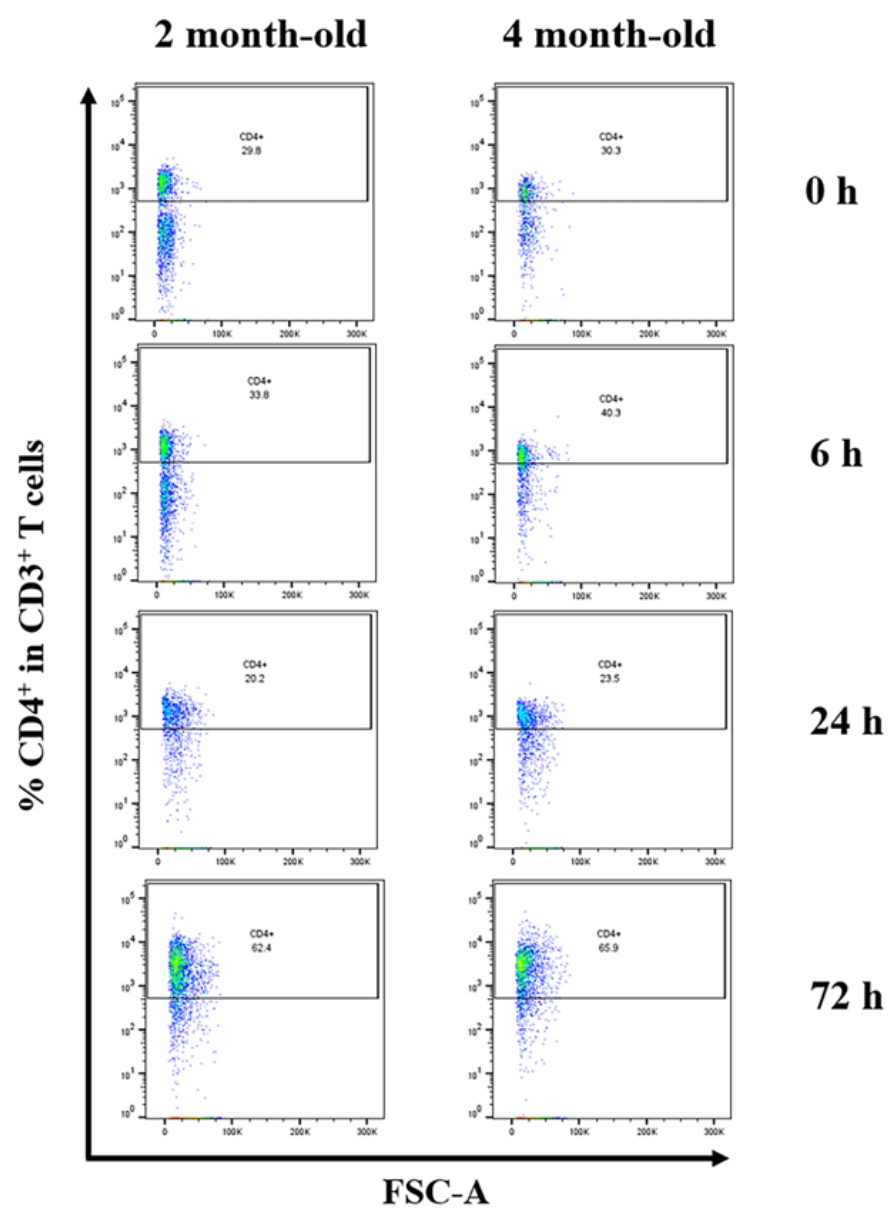


Figure 2. IL-10 gene expression in the peripheral blood of 2- and 4-month-old calves before and after castration. Data are expressed as fold change relative to the pre-castration value (0 h) and presented as box plots showing the median (horizontal line), interquartile range (box), and minimum–maximum values (whiskers), with individual data points overlaid. The diamond symbol indicates the group means. Blue and orange represent 2-month-old (2M) and 4-month-old (4M) calves, respectively.

738 (A)

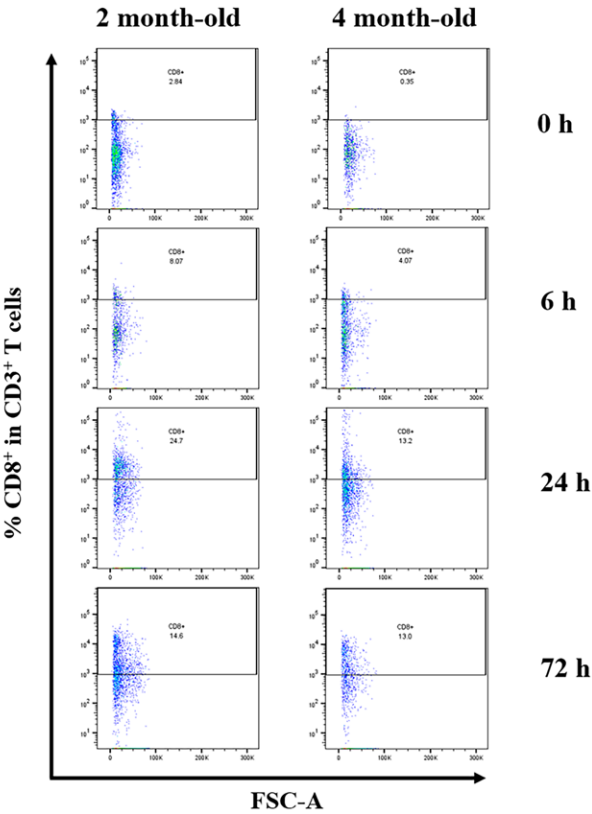


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(B)



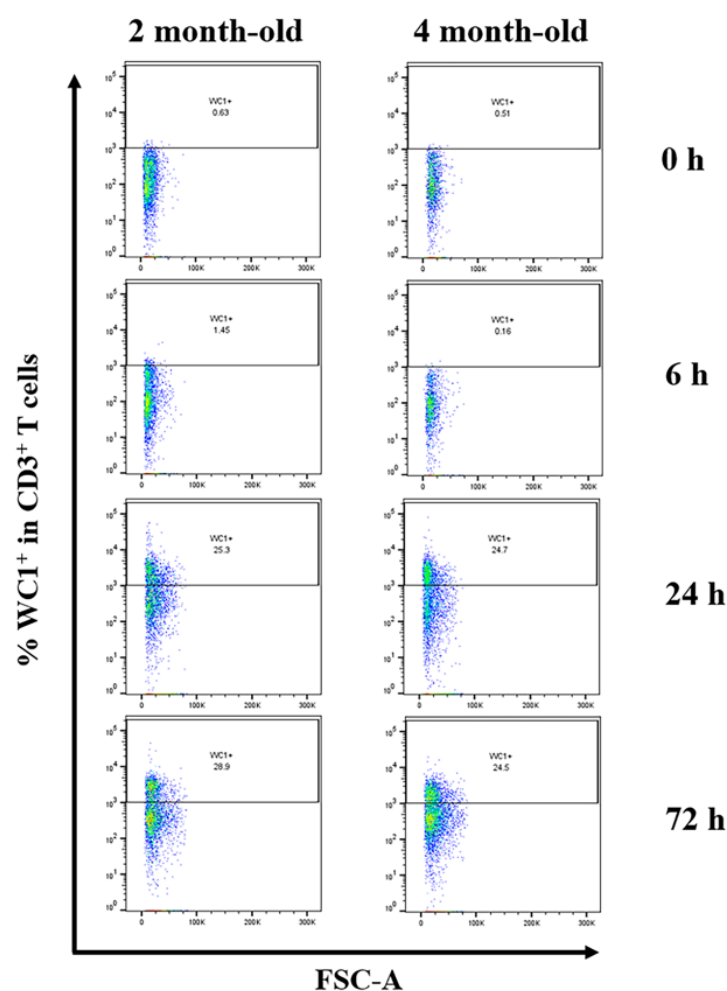
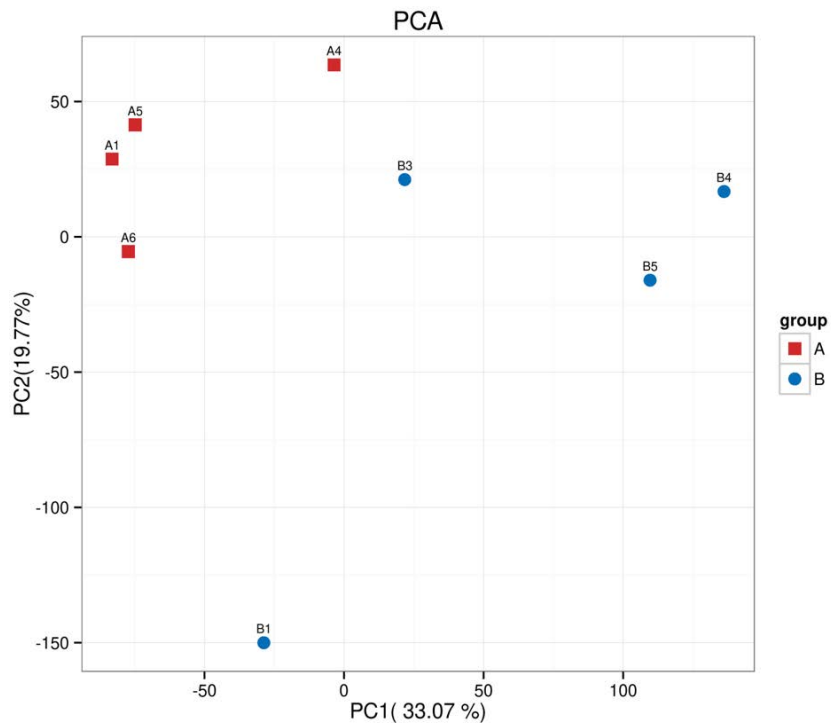
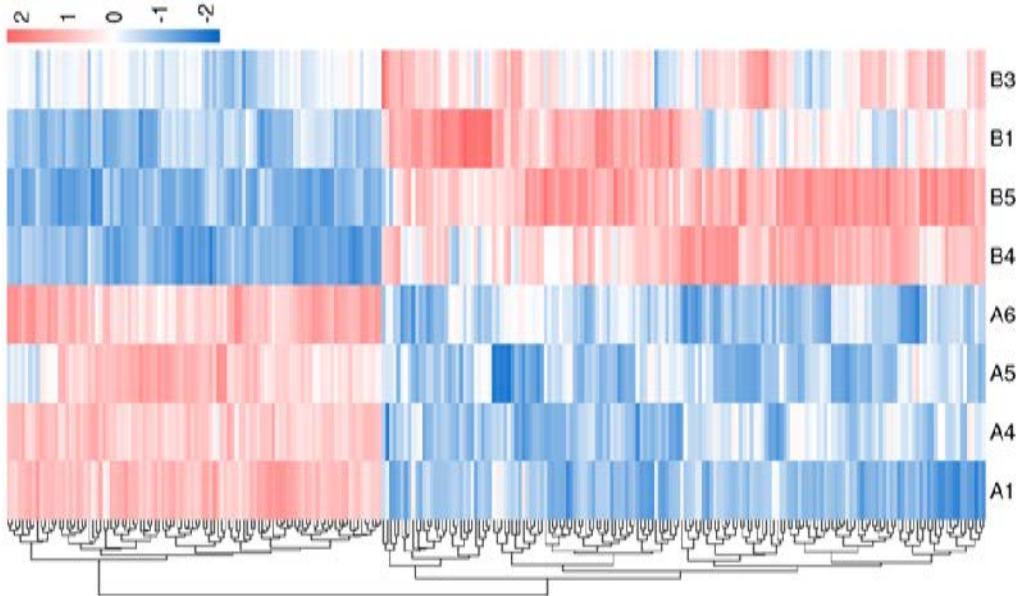


Figure 3. Changes in peripheral blood T-lymphocyte subsets in 2- and 4-month-old calves before and after castration. (A) CD4⁺ T cells, (B) CD8⁺ T cells, and (C) $\gamma\delta$ T cells. Percentages of CD4⁺, CD8⁺, and $\gamma\delta$ T cells were calculated within the CD3⁺ T-cell population. Flow cytometric analysis was performed using a consistent lymphocyte $\rightarrow$ singlet $\rightarrow$ CD3⁺ gating hierarchy, with identical gate boundaries, PMT voltage settings, and fluorescence compensation applied across all samples and sampling time points.

(A)



(B)



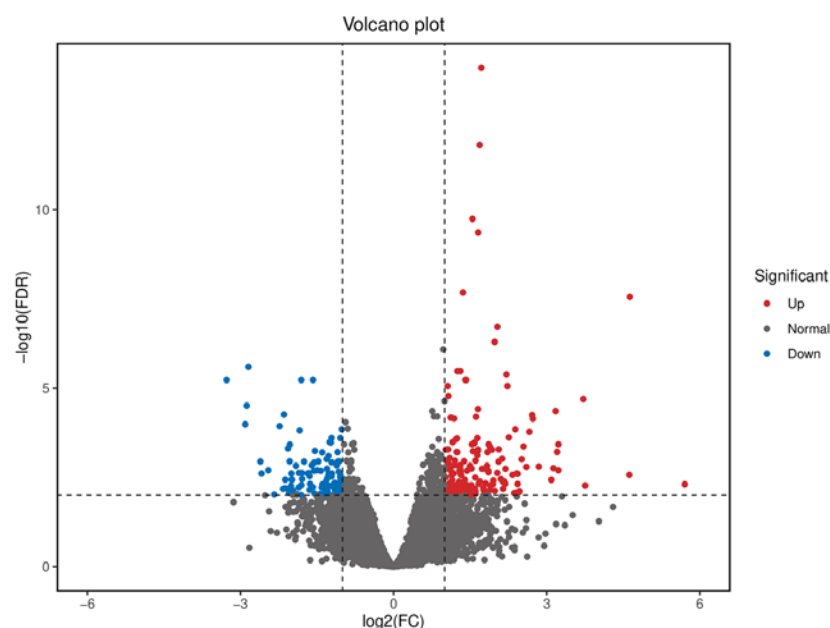


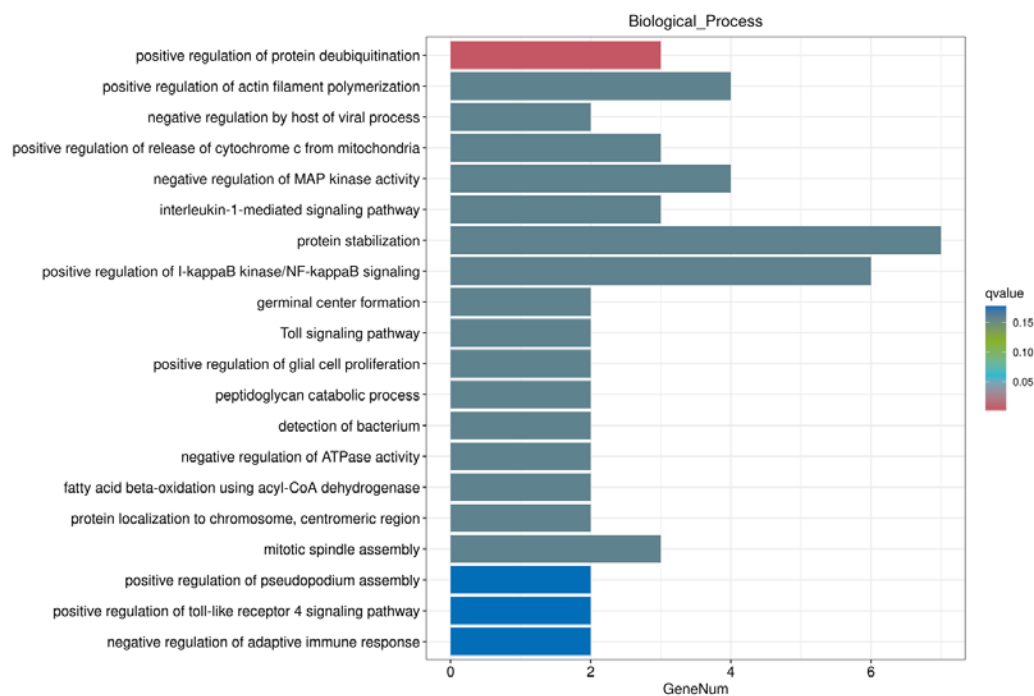
Figure 4 PCA plot, heatmap, and volcano plot of differentially expressed genes (DEGs) in PBMCs collected 6 h after castration.

(A) Principal component analysis (PCA) plot showing clear separation between 2-month-old calves (Group A; A1, A4, A5, A6) and 4-month-old calves (Group B; B1, B3, B4, B5). Different colors represent biological replicates from each group.

(B) Heatmap showing expression profiles of differentially expressed genes between the two age groups. Columns represent individual samples, and rows represent genes. Expression levels are represented by color intensity, with red indicating upregulated genes and blue indicating downregulated genes.

(C) Volcano plot of DEGs between the two age groups. The x-axis represents $\log_2$ fold change in gene expression, and the y-axis represents $-\log_{10}(\text{FDR})$ or $-\log_{10}(p\text{-value})$. Red dots indicate significantly upregulated genes, green dots indicate significantly downregulated genes, and black dots indicate genes that are not significantly differentially expressed in 4-month-old calves compared with 2-month-old calves.

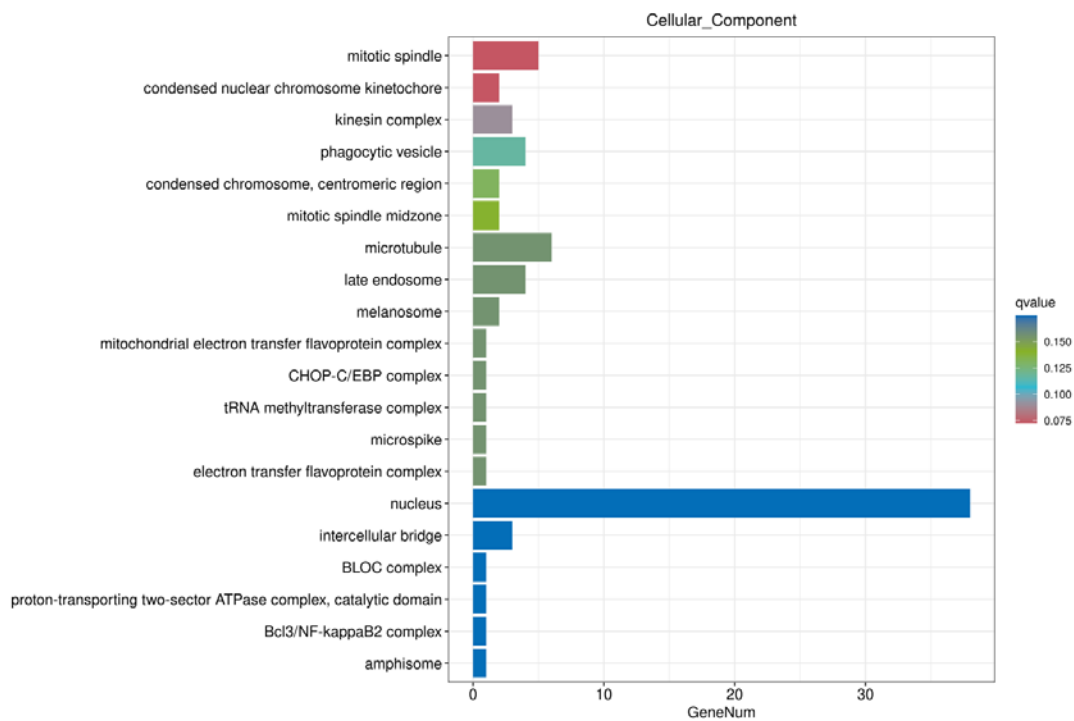
778 (A)



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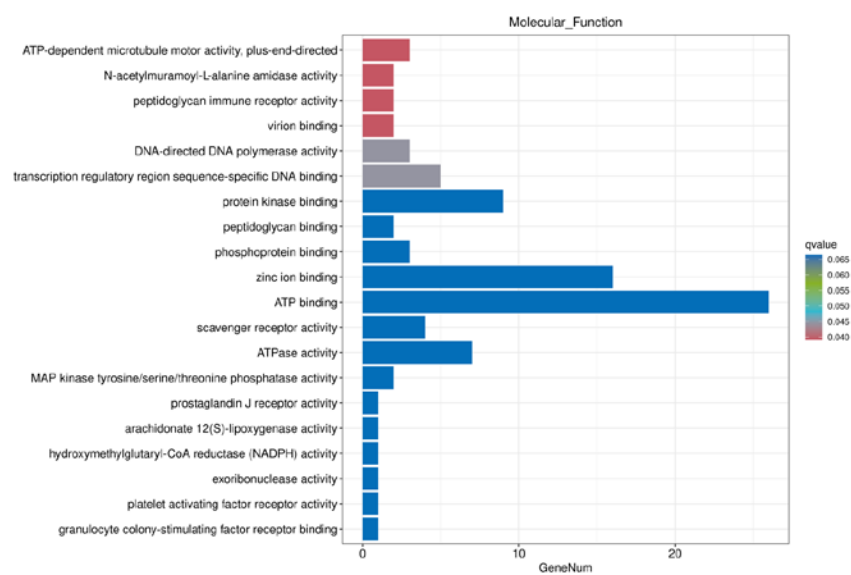
781 (B)



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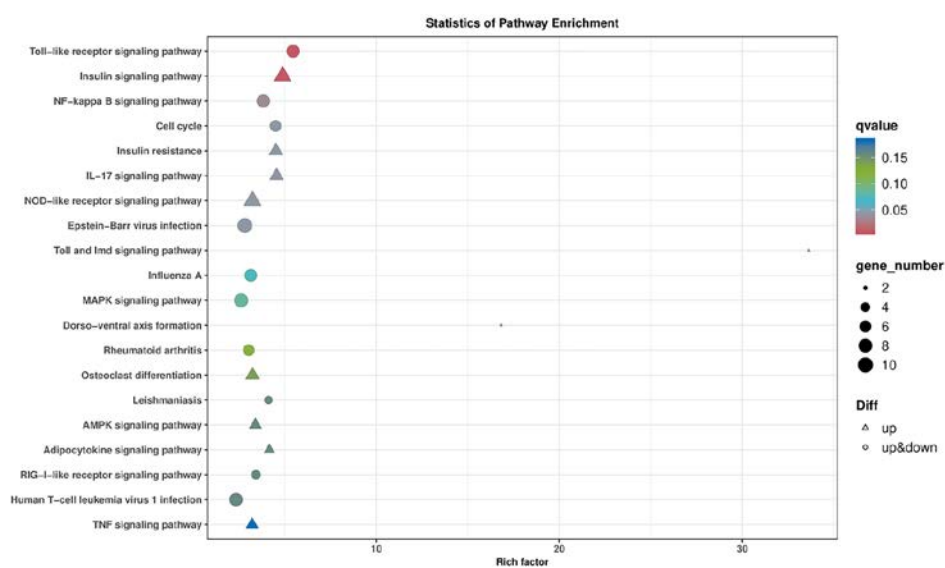
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784 (C)



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786 (D)



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788 **Figure 5** Functional enrichment analysis of differentially expressed genes (DEGs) in PBMCs of 2-month calves

789 compared to 4-month calves. Gene Ontology (GO) enrichment analysis is divided into (a) biological process, (b)

790 cellular component, and (c) molecular function. The bar length and color represent the number of enriched genes

791 and statistical significance (*q*-value), respectively. (d) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway

792 enrichment analysis of DEGs. Each dot represents a KEGG pathway, with the x-axis displaying the Rich factor

793 (Ratio of DEGs annotated to the term over all DEGs)/ (Ratio of genes annotated to the term over all genes), and the

794 y-axis displays the enrichment pathways. The dot color refers to the *q*-value, and the size refers to the number of

795 DEGs enriched in this pathway.