RESEARCH ARTICLE

Site-Specific Enrichment of FOXP3-Expressing Regulatory T-Cells in the Bovine Oviduct and Utero-Tubal Junction

Sofi Imran ul Umar, Vinay Kharb, Arif A. Pandit*

Abstract

The bovine reproductive tract must accept foreign cells while protecting against pathogens. Regulatory T cells expressing FOXP3 are central to this balance. This study investigated whether FOXP3 expression differs among anatomical regions. We analysed tissues from four sites, uterus, utero-tubal junction (UTJ), oviduct ampulla, and ovary, from eight clinically healthy cows at slaughter, restricted to animals displaying follicular-phase ovarian morphology. FOXP3 mRNA was quantified using q-PCR; protein localization was determined using immunofluorescence. Both the oviduct ampulla and UTJ exhibited approximately 4.5-fold higher FOXP3 expression than the uterus. The ovaries displayed levels equivalent to the uterine baseline. This enrichment demonstrated considerable anatomical specificity. FOXP3 is concentrated in the sperm reservoir, where spermatozoa reside awaiting ovulation. This suggests increased FOXP3 expression may represent targeted immunoregulatory investment in zones with prolonged allogeneic contact. These baseline values enable meaningful comparisons in subfertile animals. In the present study, all animals were selected based on follicular-phase ovarian morphology, whereas as FOXP3 expression patterns during the luteal phase remain to be characterized

Keywords: *Bos taurus,* Gene expression, Immune tolerance, Sperm reservoir, T-Lymphocytes, Regulatory.

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Introduction

The bovine female reproductive tract faces an unusual immunological challenge: it must tolerate spermatozoa carrying paternal antigens and, subsequently, a semi-allogeneic embryo, yet still defend mucosal surfaces against infection (Pollard and Chong, 2022). The oviduct and uterotubal junction are central to this challenge. Spermatozoa bind the oviductal epithelium via fucose-containing glycoproteins and wait in the isthmus until ovulation (Lefebvre *et al.,* 1997; Suarez, 2015), undergoing capacitation during this period (Mostek *et al.,* 2021). Because spermatozoa express MHC antigens foreign to the female immune system, the local immune response must be actively suppressed rather than simply absent (Rapacz-Leonard *et al.,* 2014; Sereshki *et al.,* 2021). FOXP3-expressing regulatory T cells (Tregs) provide this suppression. Their necessity is well documented: mice without functional Tregs cannot sustain pregnancies, and women with unexplained infertility consistently show lower endometrial FOXP3 expression than fertile controls (Jasper *et al.,* 2006; Moldenhauer *et al.,* 2024; Pandit *et al.,* 2025a,b).

Where exactly FOXP3-expressing cells are distributed within the bovine reproductive tract is not yet known. Our earlier work showed reduced FOXP3 expression in peripheral blood mononuclear cells of repeat-breeder cows, together with diminished IL-10, altered CCR7, and decreased WC1 (Pandit *et al.,* 2025a), but that study did not examine reproductive tissues. Before systemic deficits in subfertile animals can be interpreted, baseline tissue-level data from healthy cattle are needed. The present study mapped FOXP3 expression across the uterus, uterotubal junction, oviduct, and ovary to provide those reference values, and tested the hypothesis whether enrichment would be greatest in regions carrying the highest immunological load, the sperm reservoir and fertilization site.

Mountain Livestock Research Institute, Sher E Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar-193504, J&K, India

Corresponding Author: Dr. Arif Pandit, Assistant Professor, Mountain Livestock Research Institute, Sher E Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar-193504, J & K, India. e-mail: arif.pandit@skuastkashmir.ac.in

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Materials and Methods

For this study, tissues were collected from local slaughterhouses. The samples were by-products of routine slaughter; no animals were killed for this study, hence ethics committee approval was not required under local regulations. All specimens were handled according to the standard biosafety protocols.

Sample Collection and Processing

The reproductive tracts from 8 apparently healthy cows were collected at local slaughter-houses. All animals were non-

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lactating (dry) at the time of slaughter, but their metadata were not available. To minimize hormonal variability, only tracts displaying features consistent with the follicular phase were included: a dominant follicle larger than 10 mm in diameter and, crucially, no corpus luteum. This was essential for our data to represent only one hormonal context.

Immediately after removal, each reproductive tract was rinsed with sterile phosphate-buffered saline (PBS) to remove surface contaminants and blood from the lumen. The samples were then stored on ice in an insulated cooler and transported to the laboratory within 2 h of collection to ensure tissue integrity. Each reproductive tract was carefully dissected into four anatomical regions: uterine horn (uterus), uterotubal junction, oviduct (ampulla), and ovary. We specifically selected the ampulla region as the ampulla is the primary site of fertilization in cattle and it exhibits the most pronounced epithelial folding and secretory activity, which may indicate increased immunoregulatory demand. Moreover, practical considerations also played a role; the larger diameter of the ampulla allowed for more consistent tissue sampling compared to the narrower isthmus.

The tissue samples were divided into two parts for further analysis. One part was immediately snap-frozen in liquid nitrogen and stored at -80°C for RNA extraction. The other part was fixed in 10% neutral buffered formalin solution for 24-48 h and subsequently used for histopathological and immunofluorescence analyses.

Histopathological Examination

Fixed tissue samples were processed using standard histological methods (Luna, 1968). The H&E stained sections were examined under a light microscope (Leica DM 2000) to assess tissue health and rule out pathological lesions, inflammatory infiltrates and abnormal morphology. Only samples with a normal histological architecture were included in the subsequent analyses.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from approximately 30 mg of frozen tissue using TRIzol reagent (Invitrogen, Thermo Fisher Scientific) following the manufacturer's protocol. Concentration and purity (A260/A280: 1.8-2.0) were assessed on a NanoDrop spectrophotometer (Agilent BioTek, Epoch 2) and integrity confirmed by agarose gel electrophoresis. cDNA was synthesised from 100 ng of total RNA using the Verso cDNA Synthesis Kit (Thermo Scientific, Cat. No. AB-1453/A) as per the manufacturer's instructions.

Quantitative Real-Time PCR (q-RT-PCR)

We performed gene expression analyses using quantitative real-time PCR on a Bio-Rad CFX Opus 96 Real-Time PCR system. Primer pairs for bovine FOXP3 and the reference gene ACTB (β-actin) were designed using Primer3 software, and their specificity was validated using BLAST analysis against the bovine genome. The primer sequences and their characteristics are listed in Table 1.

Table 1: Primer sequences and characteristics for qRT-PCR analysis

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
FOXP3	TGGTGCAATCTCTGGAGCAA	GTCAGATGATGCCGCAGATG
ACTB	TGGGCATGGAATCCTG	GGCGCGATGATCTTGAT

For tissue sampling in qPCR experiments, representative samples from each anatomical region were collected. In the case of the uterus, tissue was taken from the middle section of the uterine horn, approximately 5 cm from the uterine body, irrespective of ovarian functional side.

Each qRT-PCR reaction was performed in triplicate in a total volume of 20 µL and contained 10 µL of GoTaq® qPCR Master Mix (Promega, catalog number A6001), 1 µL each of forward and reverse primers (final concentration 0.5 µM), 4 µL of diluted cDNA template, and 4 µL of nuclease-free water. Negative controls without DNA templates (NTC) were included in each run to detect potential contamination. The thermal cycling conditions were: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15s, annealing at 60 °C for 30s, and extension at 72 °C for 30s. Following amplification, a melting curve analysis (65-95 °C, 0.5 °C/5 s increments) was performed to verify product specificity and the absence of primer dimers. Primer amplification efficiency was assessed using five-point serial dilutions (1:2 through 1:32) of pooled cDNA. Efficiencies were 91.3% ($R^2 = 0.992$) for FOXP3 and 94.6% ($R^2 = 0.996$) for ACTB, confirming that both primer pairs performed within the accepted range of 90-110%. Representative melting curves for FOXP3 and ACTB are shown in Figure 1.

Gene expression data were analyzed using the 2^(-ΔΔCt) method (Livak and Schmittgen, 2001) Ct (threshold cycle) values were determined using Bio-Rad CFX Maestro software with automatic baseline and threshold settings.

Immunofluorescence Staining and Analysis

Paraffin sections (4-5 µm) were deparaffinised, rehydrated, and processed for heat-induced antigen retrieval in citrate buffer (10 mM, pH 6.0), then fixed and permeabilized with the Foxp3 Fixation/Permeabilization Kit (Thermo Fisher Scientific, Cat. No. 00-5523-00) as per the manufacturer's protocol. The anti-FOXP3 monoclonal antibody (clone FJK-16s, Alexa Fluor™ 561, eBioscience™, Cat. No. 505-5773-82; 1:50), a clone with documented bovine cross-reactivity was applied, and nuclei counterstained with DAPI (1 µg/mL, Sigma-Aldrich, Cat. No. D9542). Ten non-overlapping fields per region were imaged on a Leica DMi8 microscope (200× and 400×); FOXP3-positive cells were counted in ImageJ (v1.53k, NIH) by two blinded observers (ICC = 0.87).

Statistical Analysis

All statistical analyses were performed using R (version 4.3.1). Data are reported as the mean ± SEM. Regional differences were assessed using one-way ANOVA with Tukey's post-hoc comparisons. The significance threshold was set at $p < 0.05$. The correlation between qRT-PCR and immunofluorescence data was tested using Pearson's coefficient.

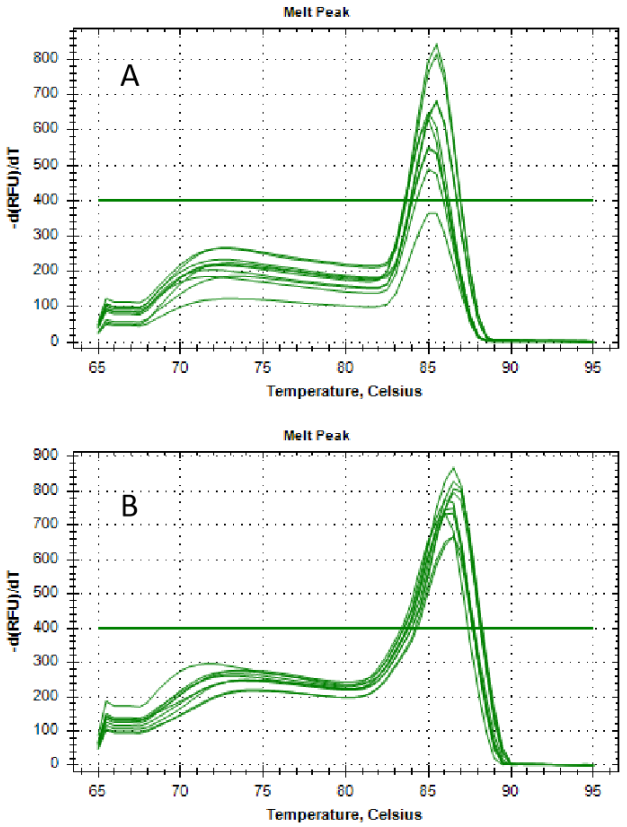


Fig. 1: Representative melting curve analysis for qRT-PCR primers. (A) FOXP3: a single dominant melting peak at approximately 85°C (B) ACTB: a single sharp peak at approximately 87°C across all samples

Results and Discussion

Histopathological Characterization of Tissue Samples

H&E staining confirmed normal morphological architecture in all four compartments, consistent with descriptions of healthy bovine reproductive tissues (Dejarnette and Nebel, 2013). No pathological lesions, inflammatory infiltrates, necrosis, or cellular atypia were detected in any region (Fig. 2A-D): the endometrium showed intact glandular architecture with columnar epithelium (Fig. 2D); the utero-tubal junction displayed characteristic folded mucosa with prominent ciliated cells (Fig. 2C); the oviduct had well-preserved mucosal folds (plica) lined by ciliated epithelium (Fig. 2B); and the ovarian cortex contained follicular structures at various developmental stages (Fig. 2A). The absence of inflammatory infiltrates matters beyond routine quality control. Subclinical inflammation reshapes local leukocyte composition including T cell subsets and can elevate FOXP3 expression independently of physiological Treg enrichment (Marey *et al.,* 2016). Its exclusion here means the regional FOXP3 differences reported below are unlikely to reflect inflammatory artefact. The normal tissue architecture confirmed here makes pathology-driven expression unlikely. All samples were thus confirmed suitable for subsequent immunological and molecular analysis.

Regional Expression of FOXP3 mRNA in the Reproductive Tract

Using the uterus as a reference (1.0-fold), FOXP3 mRNA was approximately 5-fold higher in both the oviduct ampulla (4.69 ± 0.37-fold; $p < 0.01$) and the uterotubal junction (4.42 ± 0.56-fold; $p < 0.01$) (Fig. 3A). Ovarian expression was only marginally above uterine levels (1.44 ± 0.26-fold; $p > 0.05$). Post-hoc comparisons confirmed that oviduct and UTJ differed significantly from both uterus and ovary ($p < 0.01$ each) but not from each other ($p > 0.05$).

The distribution makes biological sense. The UTJ and isthmus are the primary sperm reservoir in cattle, where spermatozoa bind oviductal epithelium via fucose-containing glycoproteins and remain viable for 24-48 h until ovulation (Lefebvre *et al.,* 1997; Suarez, 2015). Spermatozoa carry paternal MHC antigens, so their extended dwell in host tissue requires active immune suppression (Marey *et al.,* 2016). High FOXP3 expression in the UTJ is consistent with a resident Treg population programmed to maintain this tolerogenic microenvironment through IL-10 and TGF-β secretion, as we have previously shown to be compromised in repeat-breeder cattle (Pandit *et al.,* 2025a). The near-identical elevation in the oviduct ampulla, the fertilization site was, frankly, greater than anticipated. This likely reflects an extension of tolerogenic demand beyond sperm storage: the newly formed zygote expresses paternal antigens from its earliest stages and must transit the oviduct under immune protection (Rapacz-Leonard *et al.,* 2014; Maillo *et al.,* 2016). The low and non-significant ovarian FOXP3 expression is also biologically plausible. Follicular rupture, ovulation, and luteal formation are inflammatory events requiring pro-inflammatory immune activity; broad immunosuppression at this site could interfere with these processes (Poole and Pate, 2012; Chen and Chu, 2025). Sampling was restricted to follicular-phase ovaries without corpora lutea; whether progesterone-dominant conditions alter the FOXP3 profile remains to be tested (Brenner and West, 1975). The mRNA data point to a reproductive tract in which FOXP3 is concentrated where it is needed most, not spread evenly across compartments.

Immunofluorescence Localization and Quantification

The protein data estimated mirrored the mRNA findings (Fig. 4). FOXP3-positive cells (red signal, Alexa Fluor 561) were readily apparent in the oviduct ampulla and UTJ sections (Fig. 4B and 4C). They were concentrated in the lamina propria and stromal compartments, exactly where tissue-infiltrating lymphocytes would be expected (Fig. 4A-D). The nuclear staining patterns were consistent with FOXP3's role as a transcription factor. Interestingly, the UTJ had the highest absolute cell count. Relative to the uterus (2.1 ± 0.4 cells per field), the UTJ reached 8.3 ± 1.2 cells per field, approximately a fourfold increase. The oviduct was close behind at 6.7 ± 0.9 cells per field followed by ovary with just 1.8 ± 0.3 cells per field (statistically not significant) (Fig. 5).

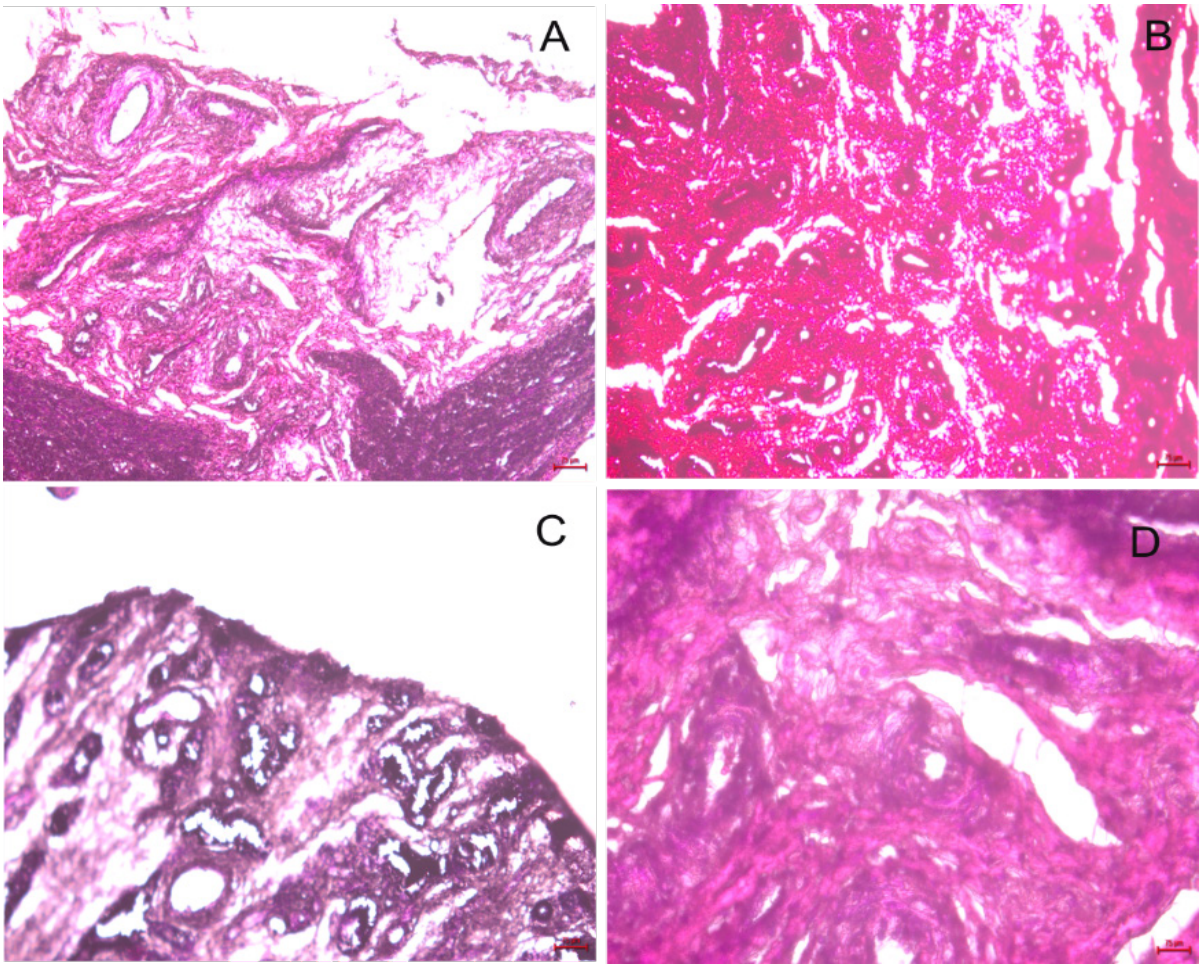


Fig. 2: Histopathological characterization of bovine reproductive tract tissues. Representative hematoxylin and eosin (H&E) stained sections showing normal morphological architecture. (A) Ovary: ovarian cortex with follicular structures at various developmental stages. (B) Oviduct: ampulla region displaying mucosal folds (plicae) lined by ciliated columnar epithelium. (C) Utero-tubal junction (UTJ): transitional region with folded mucosa and prominent ciliated cells. (D) Uterus: endometrium showing normal glandular architecture with columnar epithelium and intact stroma. All samples exhibited normal histology without pathological lesions, inflammatory infiltrates, or cellular atypia. Scale bars: 75 µm.

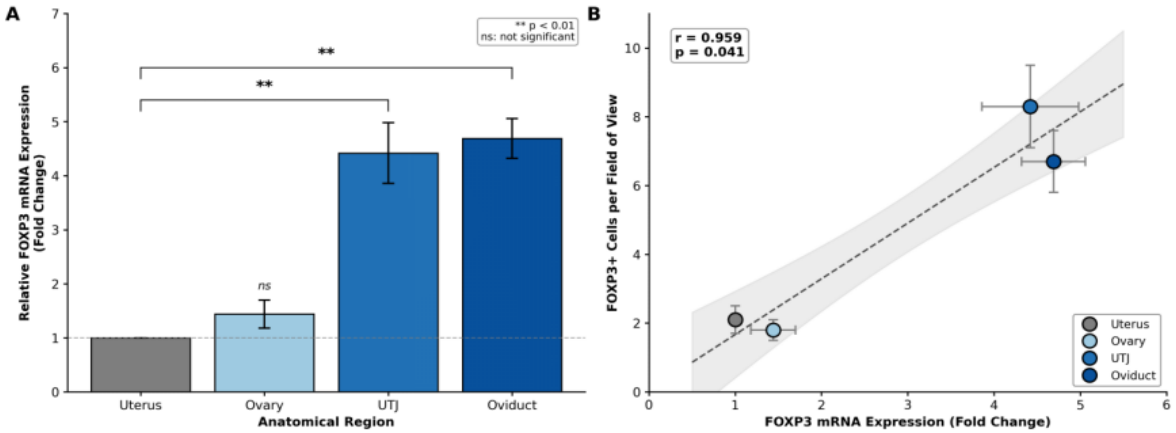


Fig. 3: Regional FOXP3 mRNA expression and correlation with protein levels in the bovine reproductive tract. (A) Quantitative real-time PCR analysis of FOXP3 mRNA expression across four anatomical regions. Data are expressed as fold change relative to uterus (reference = 1.0) using the 2^−ΔΔCt method with ACTB as the reference gene. (B) Correlation between FOXP3 mRNA expression (qRT-PCR) and FOXP3+ cell density (immunofluorescence). Pearson correlation analysis revealed a strong positive correlation (r = 0.959, p = 0.041). Dashed line indicates linear regression; shaded area represents 95% confidence interval. Data are presented as mean ± SEM (n = 8). **p < 0.01; ns, not significant.

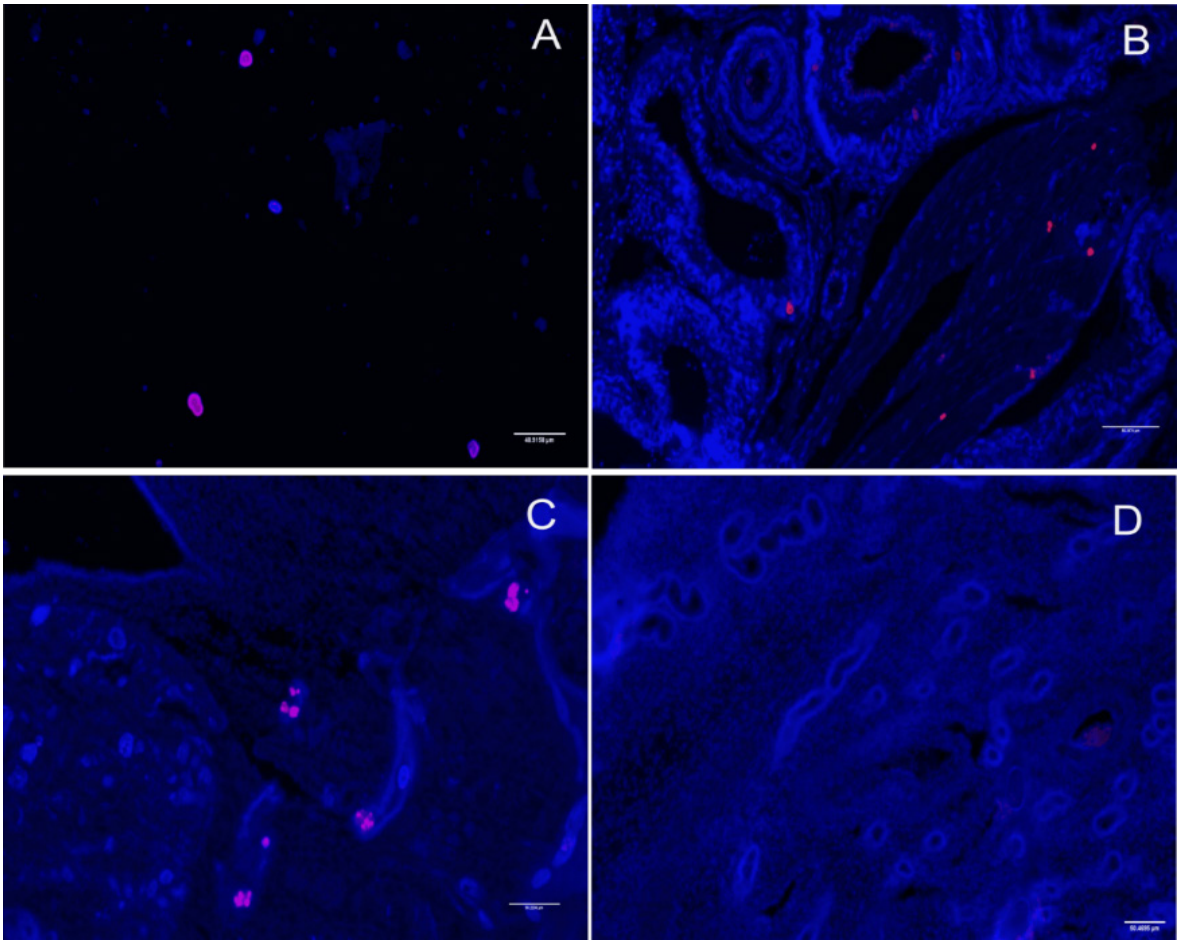


Fig. 4: Immunofluorescence localization of FOXP3-expressing cells in the bovine reproductive tract. Representative fluorescence micrographs showing FOXP3+ cells (red, Alexa Fluor 561) with DAPI nuclear counterstain (blue). (A) Ovary: sparse FOXP3+ cells in the ovarian cortex. (B) Oviduct (ampulla): abundant FOXP3+ cells concentrated in the lamina propria and stromal compartments. (C) Utero-tubal junction (UTJ): highest density of FOXP3+ cells observed among all regions, localized primarily in subepithelial stromal tissue. (D) Uterus: scattered FOXP3+ cells in the endometrial stroma. Scale bars as indicated.

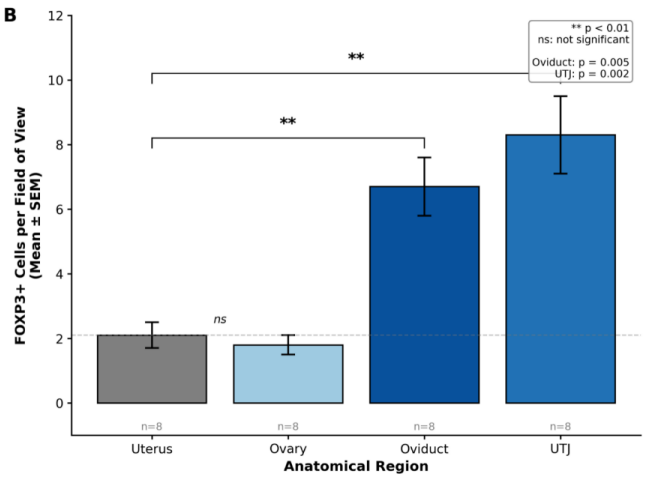


Fig. 5: Quantification of FOXP3+ cells by immunofluorescence. Cell counts per field of view across anatomical regions. UTJ (8.3 ± 1.2) and oviduct (6.7 ± 0.9) showed significantly higher FOXP3+ cell density than uterus (2.1 ± 0.4) and ovary (1.8 ± 0.3). Mean ± SEM, n = 8. **p < 0.01; ns, not significant.

Both qRT-PCR and immunofluorescence pointed to the same sites adds credibility to the pattern observed, especially given the sample size of eight animals (r =0.959, p=0.041; Fig. 3B). The UTJ and oviduct ampulla are the functional sperm reservoir and fertilization site in cattle (Coy *et al.,* 2012). Spermatozoa bind oviductal epithelial glycoproteins at these sites and dwell for 24-48 h awaiting ovulation, carrying paternal MHC antigens foreign to the female immune system throughout that period (Lefebvre *et al.,* 1997; Suarez, 2015). The high density of FOXP3-positive cells in the subjacent lamina propria and stroma is consistent with a resident Treg population that suppresses effector T cell activation and maintains local tolerance through IL-10 and TGF-β secretion, a mechanism we previously showed to be disrupted in repeat-breeder cattle (Pandit *et al.,* 2025a). The near-equivalent enrichment in the ampulla was greater than we had expected. One interpretation is that immune tolerance requirements extend beyond sperm storage into fertilization itself, given that the newly formed zygote expresses paternal antigens

from its earliest stages and must transit the oviduct under immune protection (Rapacz-Leonard *et al.,* 2014; Maillo *et al.,* 2016). A caveat here deserves mention that FOXP3 expression does not, by itself, confirm suppressive function. Activated conventional T cells can transiently upregulate FOXP3 without acquiring regulatory activity (Gavin *et al.,* 2006), and stable Treg identity also requires CD25 and CTLA-4 co-expression (Sojka *et al.,* 2009) and demonstrable suppressive capacity. These data identify where FOXP3-expressing cells are concentrated; whether they are functionally suppressive at these sites requires direct testing.

ANOVA confirmed significant regional variation ($p=0.012$). The effect size was substantial ($\eta^2 = 0.35$), indicating that the tissue region accounted for approximately 35% of the variance in FOXP3+ cell counts. Tukey's test identified the protein levels in the UTJ ($p=0.002$) and oviduct ($p=0.005$) as significantly elevated relative to the uterus, but not in the ovary.

These baseline values gain additional meaning when placed against our earlier data in repeat-breeder cattle, where systemic FOXP3 expression in peripheral blood mononuclear cells was 0.68-fold that of fertile controls (Pandit *et al.,* (Pandit *et al*., 2025a). A 32% systemic reduction would hit the oviduct and UTJ harder than the uterus precisely because their normal expression is 4-5 times higher. Whether the sperm reservoir can sustain a tolerogenic micro-environment under that kind of deficit is an open question, but one the current baseline makes testable. The low ovarian FOXP3 is not surprising. Follicular development, ovulation, and luteal formation all require acute inflammation; Tregs suppressing those responses would be counterproductive (Poole and Pate, 2012; Chen and Chu, 2025). All ovaries here were also follicular phase, so the luteal context remains uncharacterised. What drives the enrichment in the oviduct and UTJ is less clear. The oviductal epithelium expresses estrogen receptors and shifts its secretory activity across the cycle (Hunter, 2012). Oviductal fluid contains TGF-β and CSF2, both known to promote Treg differentiation locally (de Moraes *et al.,* 1999; Loureiro *et al.,* 2009). Seminal plasma expands Tregs in rodents and humans, in part through extracellular vesicles that push T cells toward a regulatory phenotype (Ahmadi *et al.,* 2022; Zhang *et al.,* 2024). Whether the same signals operate at the bovine sperm reservoir has not been tested directly.

Taken together, these findings map FOXP3-expressing cells to the precise anatomical zones where the bovine immune system faces its greatest reproductive challenge, providing a reference framework against which expression in subfertile animals can be compared.

Conclusions

This study provides the first fundamental characterization of FOXP3 expression across the bovine female reproductive tract. FOXP3 expression was markedly elevated 4- to 5-fold in the oviduct and uterotubal junction compared to that in the uterus and ovary. Two independent methods of identification converged to this result. The anatomical pattern was functionally coherent. FOXP3 is concentrated precisely where the immune system faces its greatest reproductive challenge: tolerating allogeneic spermatozoa in the sperm reservoir and permitting fertilization in the ampulla. To our knowledge, this is the first systematic mapping of FOXP3 across these anatomical regions in cattle. The data presented are preliminary, and several questions remain; however, a solid foundation has been established. Subsequent research can utilize these baseline values to further examine the role of FOXP3 dysregulation in bovine infertility and potentially inform the development of interventions aimed at restoring reproductive immune tolerance in animals with subfertility.

Acknowledgments

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