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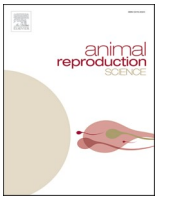

Eosinophils from jennies form extracellular traps (EETs) *in vitro* in response to seminal plasma: A new perspective on uterine immune defense in donkeys

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ABSTRACT

Jennies exhibit a post-insemination uterine inflammatory response characterized by the infiltration of polymorphonuclear cells (PMNs), primarily neutrophils, followed by eosinophils and basophils. Neutrophils are known to release neutrophil extracelular traps (NETs) to capture spermatozoa. Recently, eosinophil extracellular traps (EETs) have been identified in humans, and eosinophil infiltration into the uterus has been reported in jennies during estrus and diestrus, increasing after semen exposure. This study aimed to develop an in vitro model to evaluate EET formation in response to semen components. PMNs were isolated from jenny peripheral blood, using a standardized protocol. Eosinophils were separated using spectral flow cytometry cell sorter. Semen from three fertile jackasses was used to prepare treatments, including two negative controls (RPMI and Kenney solution), a positive control (A23187), and four experimental groups: seminal plasma (SP), spermatozoa (SPZ)-Kenney (K), SPZ-SP, SPZ-K-SP. Samples were analyzed at 0 and 3 h of incubation. EET formation was assessed by measuring changes in eosinophil nuclear fluorescence, stained with SYTOX Green, using a spectral flow cytometer. Confocal microscopy confirmed EET structures. At time 3, most of the analyzed parameters differed significantly ($p < 0.05$) between the treatment with seminal plasma and the negative control (RPMI). Notably,

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cell size already showed significant differences at time 0 ($p < 0.05$). A similar but less pronounced response was observed in the SPZ-SP and SPZ-K-SP groups, likely due to dilution of seminal plasma. No significant effect was seen with spermatozoa alone. These findings suggest that eosinophils rapidly release extracellular traps in response to seminal plasma, potentially modulating the post-insemination uterine immune response.

1. Introduction

Previous studies have demonstrated the presence of eosinophils in the endometrium of jennies during both estrus and diestrus, suggesting that these cells are involved in the normal uterine physiology of the species. Following artificial insemination (AI), there is a marked increase in the number of eosinophils in the uterine environment of jennies, suggesting a uterine inflammatory response distinct from that observed in other species (Costa et al., 2023; Freitas et al., 2025; Miró and Papas, 2018; Miró et al., 2011; Radar-Chafirovitch et al., 2025; Sokkar et al., 2001; Vilés et al., 2013a, 2013b). This increase is even more pronounced when using frozen-thawed semen, as jennies tend to exhibit a prolonged inflammatory response characterized by a more pronounced eosinophilic infiltration compared to mares (Costa et al., 2023). These differences may partly explain the lower fertility rates observed in jennies inseminated with cryopreserved semen (Vidament et al., 2009).

Eosinophils contain granules rich in preformed cytotoxic proteins — such as major basic proteins (MBP-1 and MBP-2), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) — as well as cytokines, among other molecules, which may be released upon stimulation (Brosnahan, 2020a; Hogan et al., 2008). These mediators give eosinophils the ability to destroy microorganisms and participate in hypersensitivity reactions. However, due to their high cytotoxic potential, dysregulated activation may lead to tissue damage and dysfunction (Stockert, 2005). Traditionally, these cells have been associated with parasitic infections, autoimmune diseases, and allergic processes across several species (reviwed by Birkmann et al., 2024; Brosnahan, 2020b). Nevertheless, their role in physiological and localized inflammation remains poorly understood and appears to be strongly dependent on the microenvironment and specific stimuli.

The modulation of polymorphonuclear cells (PMNs) in the jenny's uterus following artificial insemination (AI) has been the focus of several studies. Vilés et al. (2013a, 2013b) initially explored the use of seminal plasma and ketoprofen as strategies to mitigate post-AI inflammation. The study reported stable neutrophil counts, whereas eosinophil numbers showed a decrease that returned to baseline levels; however, this change did not reach statistical significance, likely due to the limited sample size. More recently, Chapero et al. (2023) demonstrated that systemic administration of dexamethasone at the time of AI significantly reduced the number of endometrial eosinophils 24 h post-insemination, without altering neutrophil counts—suggesting a selective eosinophilic response to this anti-inflammatory treatment. In contrast, intrauterine infusion of platelet-rich plasma (PRP) was shown to increase eosinophil percentages in the uterus of jennies (Freitas et al., 2025).

An innate immune mechanism of growing interest is the formation of extracellular traps (ETs), first described in neutrophils (NETs), which release structures composed of DNA and cytotoxic proteins aimed at containing the spread of pathogens (Brinkmann et al., 2004). In jennies, Miró et al. (2020) demonstrated that neutrophils are activated in the presence of seminal plasma and form NETs in vitro. In this model, it was observed that neutrophils phagocytosed only a small fraction of spermatozoa, while the majority remained trapped in extracellular DNA networks, forming a halo around the cells. After approximately three hours, some spermatozoa managed to escape these traps, suggesting that this process contributes to sperm selection by favoring only the most viable gametes.

Studies in humans have shown that eosinophils are also capable of forming extracellular traps associated with their granules, a process called EETosis (Echevarría et al., 2017; Ueki et al., 2013). The occurrence of EETs reveals an additional dimension of the immune function of eosinophils, expanding the understanding of their role in inflammatory and immune processes. Although EETs have already been characterized in humans and experimental models (Ueki et al., 2013; Yousefi et al., 2008), there are currently no reports of EET formation in eosinophils from jennies or their possible induction by reproductive stimuli such as semen or its components.

In this context, the present study aimed to: (1) establish a method for obtaining an eosinophil-rich fraction from the peripheral blood of jennies, as repeated uterine lavages to collect them may negatively impact fertility; (2) assess the ability of these eosinophils to undergo EETosis in vitro after exposure to activators; and (3) determine whether isolated or combined spermatozoa and seminal plasma (i.e., semen) are capable of inducing EETosis.

2. Material and methods

2.1. Animals

Three Catalan jack donkeys (aged 5–11 years) and one 5-year-old Catalan jenny were used in this study. All animals were clinically healthy and had proven fertility. The males provided the semen samples used in the experiments, while the female served as the blood donor for eosinophil isolation. During the experimental period, all donkeys used in this study (male and female) were fed a diet of mixed hay and basic concentrate, had ad libitum access to water, and were housed at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain). This is a center approved by Generalitat de Catalunya (Regional Government of Catalonia), Spain, and the European Union (authorization number: ES09018), which operates under rigorous health

and animal welfare protocols. The health status was in accordance with the guidelines established by Regulation (EU) 2016/429 of the European Parliament and of the Council of 9 March 2016, and guaranteed that the jackasses were free from equine infectious anemia, contagious equine metritis and equine viral arteritis.

All animal procedures were conducted in accordance with the Spanish ethical and legal requirements (5/1995/Generalitat de Catalunya, Spanish Royal Decree 53/2013, 86/609/EEC, 91/628/EEC, 92/65/EEC, Directive 2010/63/EU) regarding the use of animals in research. Therefore, the protocol was reviewed and approved by the Ethics Committee on Animal and Human Experimentation (CEEAH 1424) of the Universitat Autònoma de Barcelona, and no permit number issued by the competent authority was required.

2.2. Semen collection and processing

Semen was collected using a Hannover model artificial vagina equipped with an in-line filter to retain the gel fraction. Immediately after collection, the ejaculate volume was recorded, and the semen was initially diluted at a 1:5 (v/v) ratio using a skim milk-based extender (modified Kenney extender, maintained at 37 °C). Sperm concentration was assessed using a Neubauer chamber after diluting an aliquot in buffered formalin saline and adjusted as needed. Viability was assessed after counting 200 cells in a smear stained with eosin-nigrosin staining using a light microscope at 100X. Sperm motility parameters were analyzed using a Computer-Assisted Sperm Analysis (CASA.; ISAS v. 1.0.; Proiser S.L.; Valencia, Spain) system to assess initial sperm kinetics.

The settings of CASA used were those recommended by the provider for equine sperm, i.e., frames/s: 25 images captured per second; connectivity: 6; particle area > 4 and < 75 μm2; minimum number of images to calculate the ALH: 10. Cut-off value for motile sperm was VAP ≥ 10 μm/s and for progressively motile sperm was STR ≥ 75 %.

Subsequently, the diluted semen was fractionated to obtain two distinct sperm presentations:

Sample A (Spermatozoa + Kenney extender) – An aliquot of semen was centrifuged at 600 x g for 15 min at 20 °C. The seminal plasma was discarded, and the sperm pellet was resuspended in Kenney extender pre-warmed to 20 °C, achieving a final concentration of 12 × 106 sperm/mL.

Sample B (Spermatozoa + seminal plasma) – Another semen aliquot was centrifuged under the same conditions (600 x g, 15 min, 20 °C), and the sperm pellet was resuspended exclusively in autologous seminal plasma maintained at 20 °C, also at a final concentration of 12 × 106 sperm/mL. The procedure for obtaining the autologous seminal plasma is described in Section 2.3.

2.3. Seminal plasma separation

An aliquot of raw semen was centrifuged at 3000 x g for 30 min at 4 °C (Medifriger BL-S; JP Selecta S.A., Barcelona, Spain). The supernatant (seminal plasma) was collected and subjected to repeated centrifugation under the same conditions until complete absence of spermatozoa was confirmed by phase-contrast microscopy (Olympus BH-2 Europa, Hamburg, Germany). Fresh seminal plasma aliquots were stored at 20 °C and used immediately in each experiment.

2.4. Eosinophil isolation from peripheral blood

2.4.1. Blood collection

Approximately 80 mL of whole blood was collected from the donor jenny via external jugular venipuncture and distributed into eight 10 mL Vacutainer® tubes containing EDTA (18 mg/tube) as an anticoagulant. Samples were kept at room temperature, protected from light, and processed within 10 min of collection to preserve cell integrity.

2.4.2. PMN enrichment

Initial processing aimed to obtain a PMN-rich fraction, following protocols adapted from Siemsen et al. (2014) and Yildiz et al. (2019). Each blood tube was incubated in a water bath at 37 °C for 30 min to allow spontaneous sedimentation of erythrocytes. Subsequently, approximately 5 mL of the leukocyte-rich plasma layer (located just above the erythrocyte sediment) was gently transferred to a sterile tube and mixed 1:1 with phosphate-buffered saline containing 0.02 % EDTA (PBS-EDTA).

This suspension was carefully layered over an equal volume of Ficoll-Paque® in 15 mL conical tubes and centrifuged at 500 x g for 30 min at 20 °C. After density gradient centrifugation, the upper layers (plasma and the mononuclear cell layer containing lymphocytes and monocytes) were removed, retaining the red pellet at the bottom, composed of residual erythrocytes and polymorphonuclear cells (PMNs). The pellet was washed with 25 mL of 1 x PBS and centrifuged at 500 x g for 10 min at 20 ºC to remove residual Ficoll. Subsequently, selective erythrocyte lysis was performed by adding 5 mL of red blood cell lysis buffer (RBC Lysis Buffer, ThermoFisher) and incubating for 15 min with gentle agitation, according to the manufacturer's instructions. The lysis reaction was stopped by adding 25 mL of 1 x PBS to restore normal osmolarity, followed by another centrifugation (500 x g, 10 min, 20 °C). The final PMN-enriched pellet was resuspended in 1 mL of RPMI-1640 medium supplemented with 1 % penicillin-streptomycin at room temperature.

To assess both the isolation efficiency and the purity of the collected samples, a hematology flow cytometer (Sysmex XN-1000™, Sysmex Corporation, Chuo-ku, Kobe, Japan) was used for analysis.

2.4.3. Eosinophil purification

The purified PMN fraction (containing primarly neutrophils and eosinophils) was then processed using a BD FACSDiscoverTM S8

cell sorter with Image CellView Technology (San Jose, CA, USA) for image analysis. A 70 μm sorting nozzle was used, and both the samples and the instrument were maintained at 20 °C throughout the procedure. Granulocytes were initially gated based on forward and side scatter (Light LossViolet-Area/SSC-imaging-Area) properties, excluding debris and big aggregates. Doublets were excluded using a two step gating strategy. First, events were gated based on SSC (Imaging)-Height vs. SSC (Imaging)-Width to discriminate aggregates. Subsequaently Light Loss (violet)-H vs. Light Loss (violet)-W was used to further exclude remaining doublets.

Eosinophils were distinguished from neutrophils based on their higher intrinsic autofluorescence. This autofluorescence was detected in the B2-(515)-A channel (excitation laser: 488 nm) in combination with the V2-(440)-A channel where eosinophils exhibited a stronger signal due to their flavoprotein-rich granules. Additionally, the cytometer's integrated imaging module enabled basic assessment of nuclear and granular morphology to aid identification. Based on these combined criteria, eosinophils were isolated by gating and separated from neutrophils through flow sorting using the purity mode.

The purified eosinophils were collected into tubes containing RPMI-1640 supplemented with 20 % FCS to preserve the cell viability. On average, approximately 3×10^6 viable eosinophils were recovered within 60 – 90 min of sorting.

3. Experimental design – *in vitro* treatments

The experimental design aimed to investigate: (i) whether the ionophore A23187 can induce EETosis in jenny eosinophils; (ii) whether the semen extender used is capable of triggering EETosis in these eosinophils; (iii) whether donkey seminal plasma alone (in the absence of spermatozoa) can induce EETosis; (iv) whether donkey spermatozoa alone (without seminal plasma) can also induce EETosis; and (v) whether whole semen (spermatozoa + seminal plasma) enhances EETosis compared to its individual components.

To this end, 1 mL aliquots of purified eosinophil suspension (3×10^5 cells/mL in RPMI supplemented with 2 % BSA) were distributed into the following experimental groups:

- Positive Control Group (CT-A23): 1 mL eosinophil suspension + 1 mL RPMI containing calcium ionophore A23187 (2 μM).
- Diluent Control Group (CT-K): 1 mL eosinophil suspension + 1 mL Kenney extender.
- Negative Control Group (CT-RPMI): 1 mL eosinophil suspension + 1 mL RPMI (2 % BSA).
- Seminal Plasma Group (Trat-SP): 1 mL eosinophil suspension + 1 mL homologous seminal plasma.
- Spermatozoa without Seminal Plasma Group (Trat-S+K): 1 mL eosinophil suspension + 0.5 mL of Sample A (12 ×106 sperm/mL, diluted in Kenney) + 0.5 mL additional Kenney.

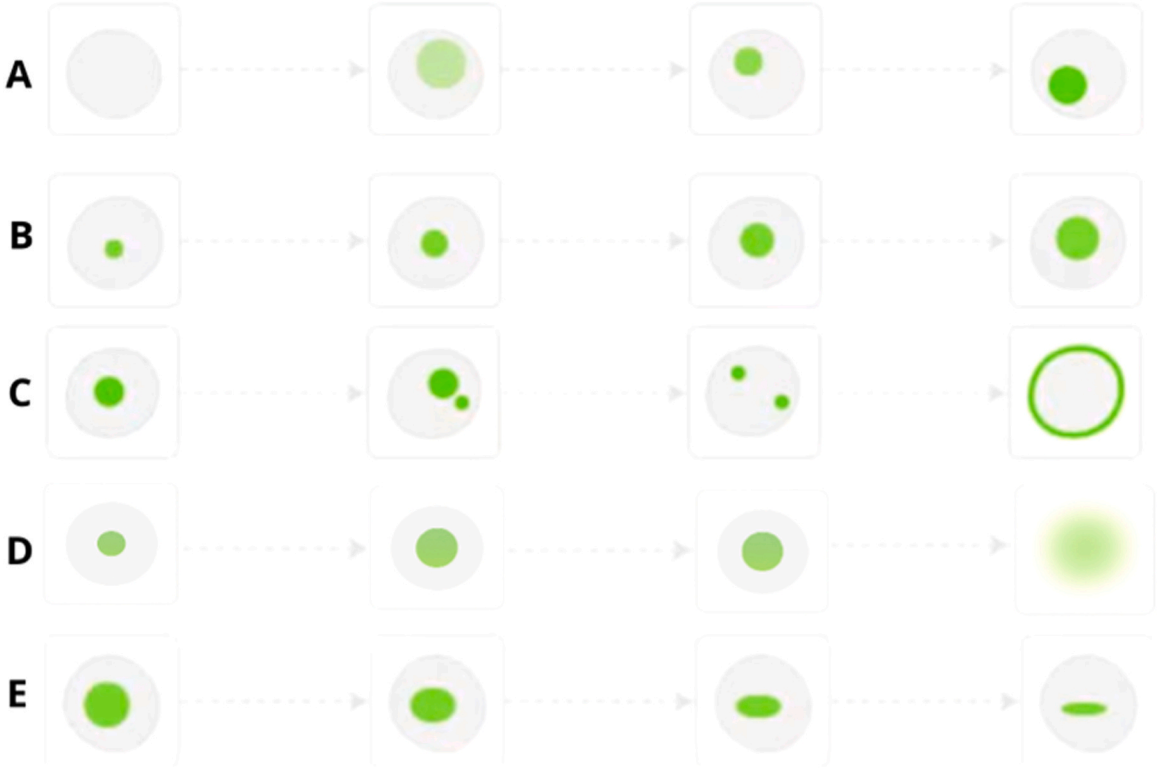

Fig. 1. Imaging-derived parameters measured using the spectral flow cytometer BD FACSDiscover™ S8 to assess cellular characteristics during EETosis. (A) Intensity, (B) Size (C) Radial Moment, (D) Diffusivity, (E) Eccentricity. Adapted from (BD Biosciences, n.d.).

- Spermatozoa with Seminal Plasma Group (Trat-S+SP): 1 mL eosinophil suspension + 0.5 mL of Sample B (12 ×106 sperm/mL, diluted in autologous seminal plasma) + 0.5 mL additional autologous seminal plasma.
- Whole Semen Group (Trat-S+SP+K): 1 mL eosinophil suspension + 0.5 mL of Sample A (12 ×106 sperm/mL, diluted in Kenney) + 0.5 mL homologous seminal plasma.

All media, eosinophil suspensions, and semen samples were maintained at 20 °C during treatment preparation. Each treatment was prepared in duplicate, with one milliliter immediately fixed using 200 μL of 4 % paraformaldehyde (PFA) for baseline at time zero (0 h, T0) analysis. The remaining 1 mL was incubated for 3 h (T3) at 37 °C in 5 % CO2 and fixed at the end of incubation.

EETosis was analyzed in samples from all groups at baseline (T0, 0 h) and after 3 h of incubation and fixation (T3, 3 h) using flow cytometry and confocal microscopy, as detailed below. A total of five independent experimental replicates were performed, each conducted approximately one week apart.

In treatment groups containing semen, the eosinophil-to-spermatozoa ratio was approximately 1:20. This ratio was established based on typical sperm concentrations observed in the uterus following artificial insemination and the average number of eosinophils reported in the uterine environment in previous studies (Miró et al., 2020; Radar-Chafirovitch et al., 2025).

4. EETosis analysis by flow cytometry

For the flow cytometric analysis of EETosis, SYTOX Green dye (ThermoFisher), used to identify extracellular DNA, was added to each previously fixed sample at a 1:2500 dilution, followed by a 15-minute incubation in the dark. EETosis detection was based on imaging-derived parameters (Fig. 1) acquired using the spectral flow cytometer BD FACSDiscover™ S8 equipped with Image Cell-View™ Technology for integrated image-based analysis. SYTOX Green was detected in the B2 channel associated with the 488 nm laser.

For each sample, SYTOX Green fluorescence distribution was analyzed at timepoints 0 (pre-incubation) and 3 h (post-incubation), aiming to evaluate both intragroup variations – differences between timepoints within the same treatment; and intergroup variations – differences among treatments at each timepoint.

Cell size was estimated based on image analysis, defined as the number of pixels in the image that are brighter than a user-defined intensity threshold set at 3245 arbitrary units. This approach allows for a more direct assessment of cell dimensions, as cells with expanded nuclei or dispersed DNA leads to a less compact fluorescente signal associated with the cell body. Consequently, the measured cell size may decrease because the dispersed DNA occupies less dense area within the defined cellular region. Therefore, a reduction in measured size can reflect DNA extrusion and cellular morphological changes.

Radial dispersion (Radial Moment) and diffusivity (Diffusivity) of SYTOX Green fluorescence were analyzed to assess nuclear morphological changes. Radial Moment represents the average distance of fluorescent pixels from the centroid, while Diffusivity measures how broadly these pixels are spatially distributed. Both parameters indicate the degree of DNA dispersion, a hallmark of extracellular trap formation.

Mean fluorescence intensity was measured in the B2 Green channel of the cytometer, which detects the SYTOX Green signal. As this dye binds specifically to DNA, cells with intact DNA content exhibit stronger fluorescence in this channel, providing an additional parameter for DNA quantification. Thus, the reduction of the B2 Green signal may indicate loss or degradation of DNA during the EETosis process.

Eccentricity was also evaluated and it is defined as the ratio of the shortest to the longest axis of the identified particle within the region of analysis. Lower eccentricity values indicate more elongated or irregular structures, which may reflect nuclear deformation or chromatin extrusion associated with EETosis.

5. EETosis analysis by confocal microscopy

An aliquot from each treatment group was processed immediately following the addition of 4 % paraformaldehyde (PFA) at both timepoints (T0 and T3). A volume of 100 μL from each sample was transferred into individual wells of an 8-well Millicell EZ SLIDE glass chamber slide (Merck Millipore, Carrigtwohill, Ireland). Samples were fixed for 20 min at room temperature. After fixation, the slides were carefully washed twice with phosphate-buffered saline (PBS 1 ×). Following the second wash, 100 μL of PBS was added to each well, and the slides were stored under refrigeration (4°C) until analysis.

Slides were stained with SYTOX Green (ThermoFisher Scientific) diluted 1:2500 (v/v) in 200 μL of PBS, and incubated for 15 min. After incubation, the staining solution was removed and the wells were washed twice with 200 μL of PBS. For imaging, slides were mounted using Fluoroshield Mounting Medium with DAPI (Abcam, Cambridge, UK) and analyzed using a Nikon A1R confocal microscope (Nikon Corp., Tokyo, Japan). Six random fields per treatment were imaged using a 20 × objective.

Eosinophils were assessed based on nuclear morphology: cells were classified as non-reactive if they exhibited an intact, lobulated, and condensed nucleus, or as reactive if they displayed decondensed chromatin and an expanded or elongated nucleus—features indicative of extracellular DNA filament release. Confocal microscopy was performed to qualitatively assess nuclear morphology and verify that the fluorescence-based changes observed by flow cytometry corresponded to the formation of extracellular traps.

6. Statistical analysis

Data were analyzed using the R statistical package (version 4.0.3, R Core Team; Vienna, Austria) and graphs were created using

GraphPad Prism software (version 8.4.0, GraphPad Software LLC; San Diego, CA, USA). Data normality was checked using the Shapiro–Wilk test and homoscedasticity of variances was checked using Levene's test. The arcsine function $\sqrt{x}$ was used to transform the data and obtain a normal distribution only when necessary. Flow cytometry analyses of EETosis (Size, Radial Moment, Diffusivity, Eccentricity, Intensity) were compared using a generalized linear mixed model with repeated measures over time. The effect of different EETosis experimental groups in donkey ejaculates (CT-A23, CT-K, CT-RPMI, TRAT-PS, TRAT-S+K, TRAT-S+PS, TRAT-S+PS+K) was the fixed effect factor, the 0 h (preincubation) and 3 h (postincubation) time points was the within-subject factor, and donkey was the random effect factor. In all analyses, the Bonferroni post-hoc test was used for pairwise comparisons, with a minimum level of statistical significance of $p \leq 0.05$. Results are expressed as mean ± standard error of the mean (SEM).

7. Results

7.1. Semen analyses

The semen samples showed an average sperm viability of 83.0 ± 7.6 %, with an initial concentration of $468.2 \times 10^6 \pm 78.5 \times 10^6$ spermatozoa/mL. Motility descriptors assessed using a Computer-Assisted Sperm Analysis (CASA) system indicated a total motility (TM) of 83.9 ± 3.8 %. The corresponding kinematic parameters were: curvilinear velocity (VCL), 85.6 ± 4.0 mm/s; straight-line velocity (VSL), 39.5 ± 0.7 mm/s; and average path velocity (VAP), 58.4 ± 1.9 mm/s. Linearity (LIN) and straightness (STR) reached 46.4 ± 2.1 % and 67.7 ± 1.1 %, respectively, while wobble (WOB) averaged 68.5 ± 3.1 %. The amplitude of lateral head displacement (ALH) was 3.2 ± 0.2 mm, and the beat cross frequency (BCF) was 9.7 ± 0.8 Hz. Data are expressed as mean ± standard error of the mean (SEM) from five independent replicates. For the in vitro assays, sperm suspensions were always prepared from the ejaculate of a single male; ejaculates from different donkeys were never pooled.

7.2. PMNs enrichment

The methods used for processing and isolating PMN cells from whole blood, as well as for eosinophil separation from the purified PMN fraction, were effective in obtaining eosinophils with a high degree of purity and viability at the end of the procedure.

Polymorphonuclear (PMN) proportions and total leukocyte counts obtained from the purified samples using the Sysmex XN-1000™ revealed that neutrophils represented the predominant population, accounting for 76.1 ± 1.6 % ($56.3 \pm 2.1 \times 10^3$/μL). Eosinophils were the second most abundant subtype (11.7 ± 0.8 %; $8.7 \pm 0.7 \times 10^3$/μL), followed by lymphocytes (10.1 ± 1.6 %; $7.4 \pm 1.1 \times 10^3$/μL) and monocytes (1.9 ± 0.2 %; $1.4 \pm 0.1 \times 10^3$/μL). Basophils were scarcely detected, representing only 0.3 ± 0.1 % ($0.2 \pm 0.1 \times 10^3$/μL). Values are expressed as mean ± standard error of the mean (SEM) from five independent replicates.

According to the hematological analysis performed with the Sysmex system, polymorphonuclear cells (neutrophils and eosinphils) represented, on average, 88.1 % of the total leukocyte population. Within this subset, eosinophils accounted, on average, for 11.7 %, indicating a moderate enrichment of this granulocyte subtype in the purified sample. Considering that the eosinophil percentage of total leukocytes in peripheral blood of donkeys averages around 4.0 %, with a range of 0.9–9.1 %, this represents a highly efficient isolation of eosinophils (Burden et al., 2016).

7.3. Eosinophil purification

The separation of the main granulocyte populations from isolated PMNs, as achieved by cell sorting using the BD FACSDiscover™ S8 spectral cell sorter, is illustrated in Fig. 2.

A mean eosinophil purity of 97.7 ± 1.2 % was obtained after cell sorting (Fig. 3), with an average viability of 93.9 ± 7.3 %, as assessed by Propidium Iodide staining through flow cytometry.

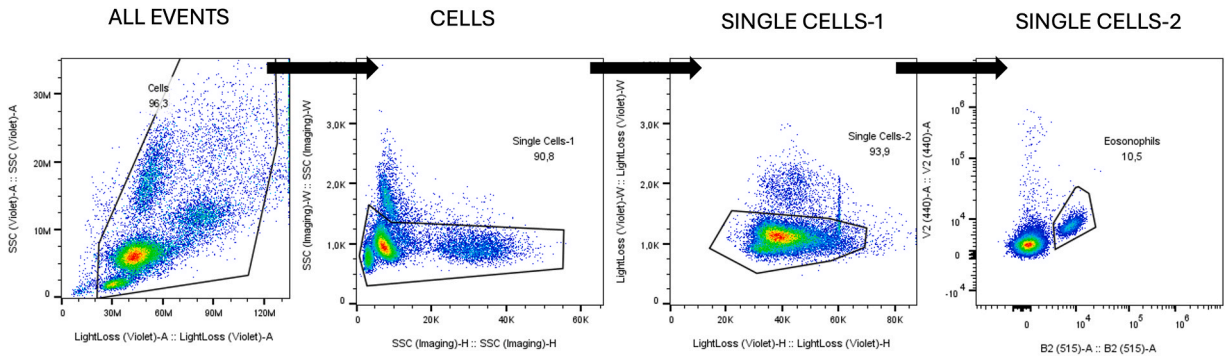

Fig. 2. Cell sorting was performed using the spectral cell sorter BD FACSDiscover™ S8. Leukocyte population initially gated based on forward and side scatter parameters (Light Loss Violet-Area / SSC Imaging-Area). Doublets were removed by sequential gating on SSC (Imaging)-Height vs. SSC (Imaging)-Width, followed by Light Loss (Violet)-Height vs. Light Loss (Violet)-Width.

Representative smear images of the purified leukocyte populations are shown in Fig. 4, illustrating the morphological characteristics of PMNs (A) and isolated eosinophils (B). The typical bilobed nucleus with highly condensed chromatin, along with a cytoplasm densely filled with granules, is clearly observable, confirming the identity and purity of the sorted eosinophils.

7.4. EETosis analysis by flow cytometry

Overall, differences between treatment groups at time 0 h were observed in only one parameter. Most eosinophils exhibited similar size and internal complexity, as well as intact nuclei, across all groups (controls and treated samples prior to incubation). However, after 3 h of incubation, marked differences were observed between groups, reflecting varying degrees of EETosis induction depending on the stimulus (Figs. 5 and 6).

Cell size increased significantly ($p < 0.05$) at T3 compared to T0 in both the positive control group (CT-A23) and the diluent control group using Kenney (CT-K). Additionally, at time 0, a significant reduction ($p < 0.05$) in cell size was already observed in the SP group compared to RPMI and the TRAT-S+K, which lacked seminal plasma. At T3, the treated group TRAT-PS exhibited significantly lower values ($p < 0.05$) than the three control groups (CT-A23, CT-K, CT-RPMI), as well as the group lacking seminal plasma (TRAT-S+K).

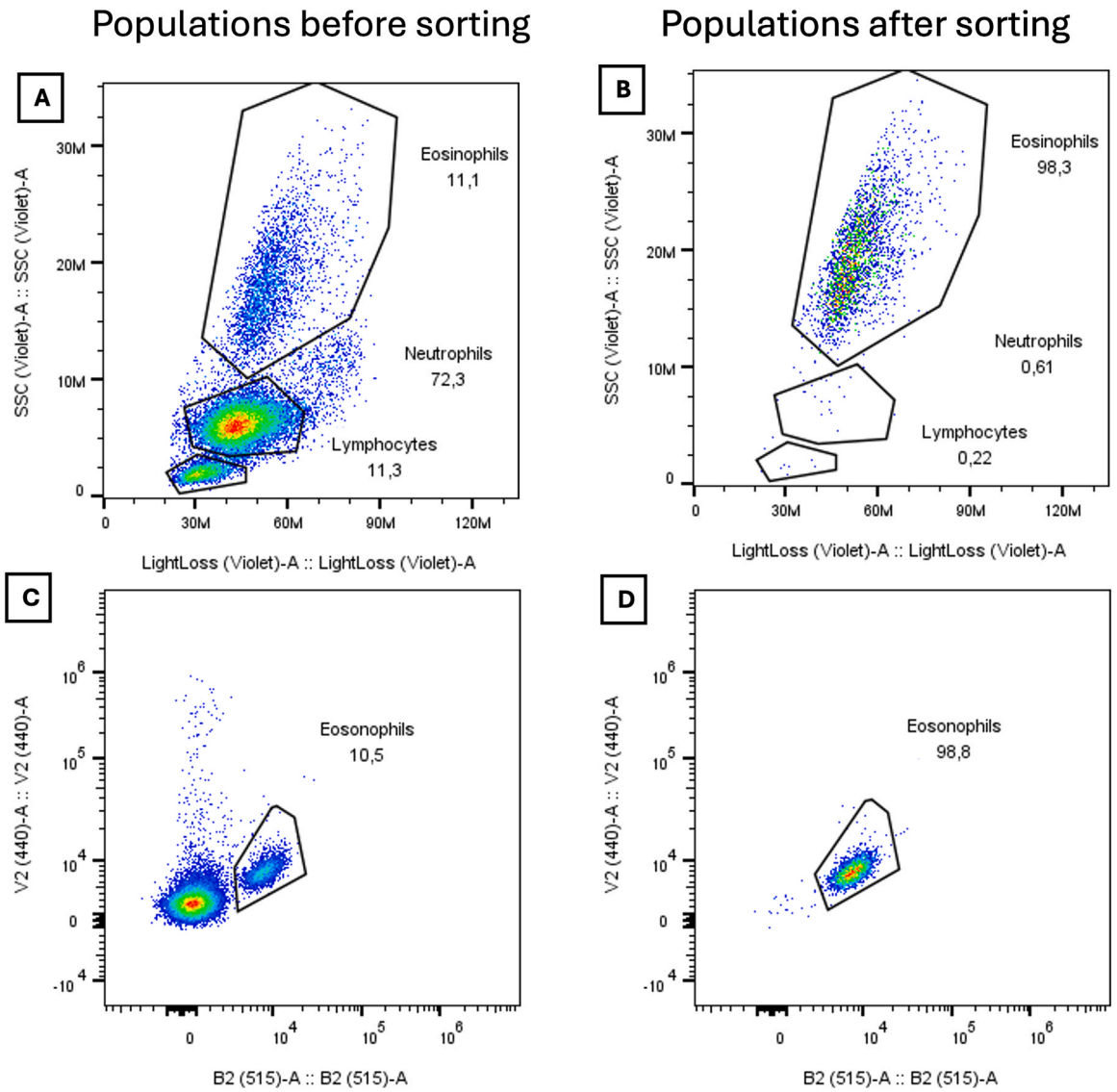

Fig. 3. Eosinophil purification using the BD FACSDiscover™ S8 spectral cell sorter. Representative plots of leukocyte populations before (left) and after (right) cell sorting. (A–B) Initial gating strategy based on forward and side scatter parameters to identify different leukocyte populations. (C–D) Eosinophils were distinguished based on their intrinsic autofluorescence properties. After sorting, eosinophil purity increased from approximately 10–11 % to over 98 %.

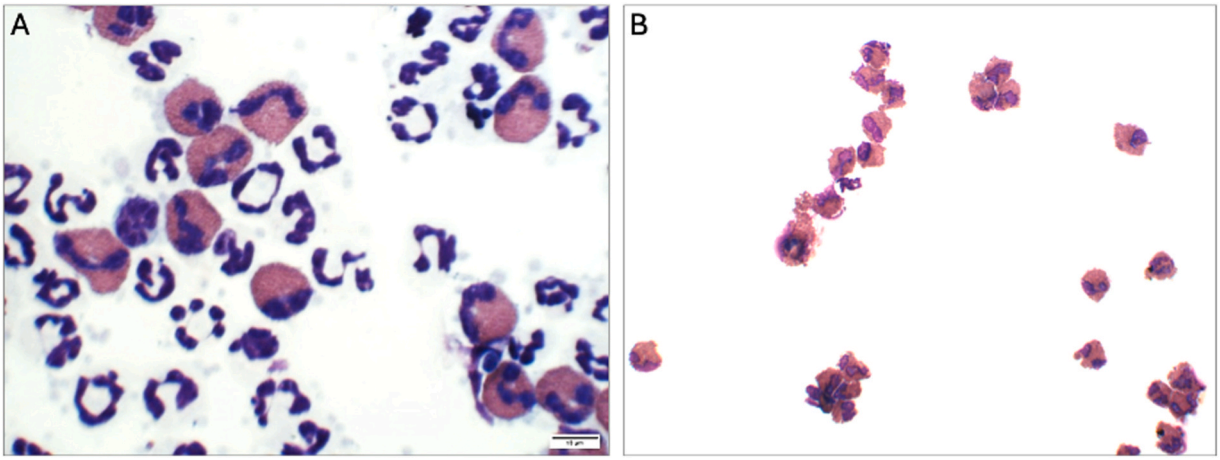

Fig. 4. Giemsa-stained leukocyte smears analyzed by bright-field light microscopy. (A) Smear of the polymorphonuclear cell suspension (neutrophils and eosinophils) following the PMN enrichment on protocol from jenny whole blood (100x magnification); (B) Smear of the suspension containing eosinophils isolated by cell sorting from the PMN-enriched sample (40x magnification).

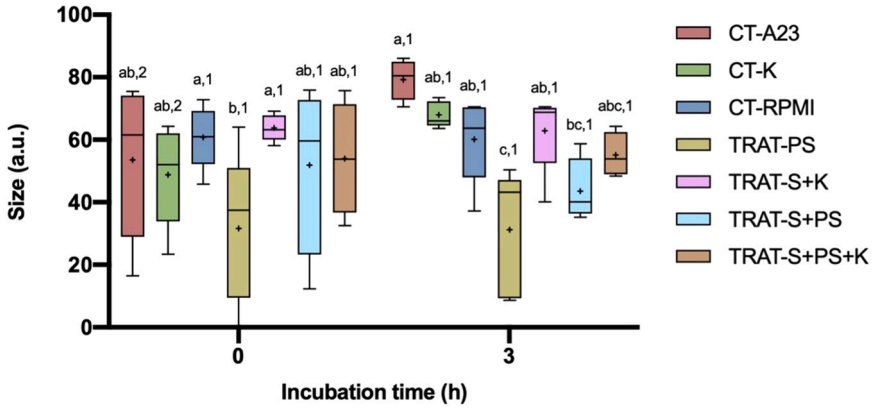

Fig. 5. Apparent cell size assessed by flow cytometry with integrated image analysis at 0 and 3 h across all treatment groups. Data are presented as mean ± standard error of the mean (SEM) from five independent replicates. **CT-A23 = Positive Control (eosinophils + calcium ionophore); CT-K = Extender Control (eosinophils + Kenney extender); CT-RPMI = Negative Control (eosinophils + RPMI medium); TRAT-PS = Seminal Plasma Treatment (eosinophils + homologous seminal plasma); TRAT-S+K = Spermatozoa without Seminal Plasma (eosinophils + spermatozoa + Kenney extender); TRAT-S+PS = Spermatozoa with Seminal Plasma (eosinophils + spermatozoa + homologous seminal plasma); TRAT-S+PS+K = Whole Semen Treatment (eosinophils + spermatozoa + homologous seminal plasma + Kenney extender). **Different letters indicate statistically significant differences between treatments at the same timepoint. Different numbers indicate significant differences between timepoints (T0 vs. T3) within the same treatment.*

This decrease was followed by the TRAT-S+PS group, and subsequently by TRAT-S+K+PS group. TRAT-S+PS group also showed lower values than CT-A23 (Fig. 5).

Parameters related to nuclear content dispersion, including Radial Moment and Diffusivity, are shown in Fig. 6, respectively. No significant differences in radial fluorescence dispersion were observed between T0 and T3 for any group (Fig. 6). In contrast, an increase in fluorescence diffusivity was detected in the TRAT-S+PS group at T3 compared to T0 (Fig. 6).

In addition, at time 0, both parameters already showed a clear tendency to increase in response to the seminal plasma treatment. At T3, the TRAT-PS and TRAT-S+PS groups exhibited significantly greater radial fluorescence dispersion ($p < 0.05$) than the control groups CT-A23 and CT-RPMI (Fig. 6), while only the TRAT-PS group showed higher fluorescence diffusivity compared to the negative control CT-RPMI (Fig. 6).

Regarding the fluorescence intensity parameter (B2 Green channel) corresponding to SYTOX Green bound to DNA, no significant differences were observed between timepoints (T0 and T3) for any of the treatment groups. However, a visual trend toward decreased B2 Green fluorescence intensity was observed in the group treated with seminal plasma at both timepoints. Similarly, at T3, the TRAT-S+PS group showed lower intensity values, followed by TRAT-S+PS+K, in which the effect appears to be diluted. Both TRAT-SP and TRAT-S+PS groups differed significantly ($p < 0.05$) from the A23 control group (Fig. 6).

Eccentricity showed a significant decrease ($p < 0.05$) in the seminal plasma-treated group (TRAT-SP) at time point T3, compared to the other groups not treated with seminal plasma (CT-A23, CT-K, and TRAT-S+K) (Fig. 6).

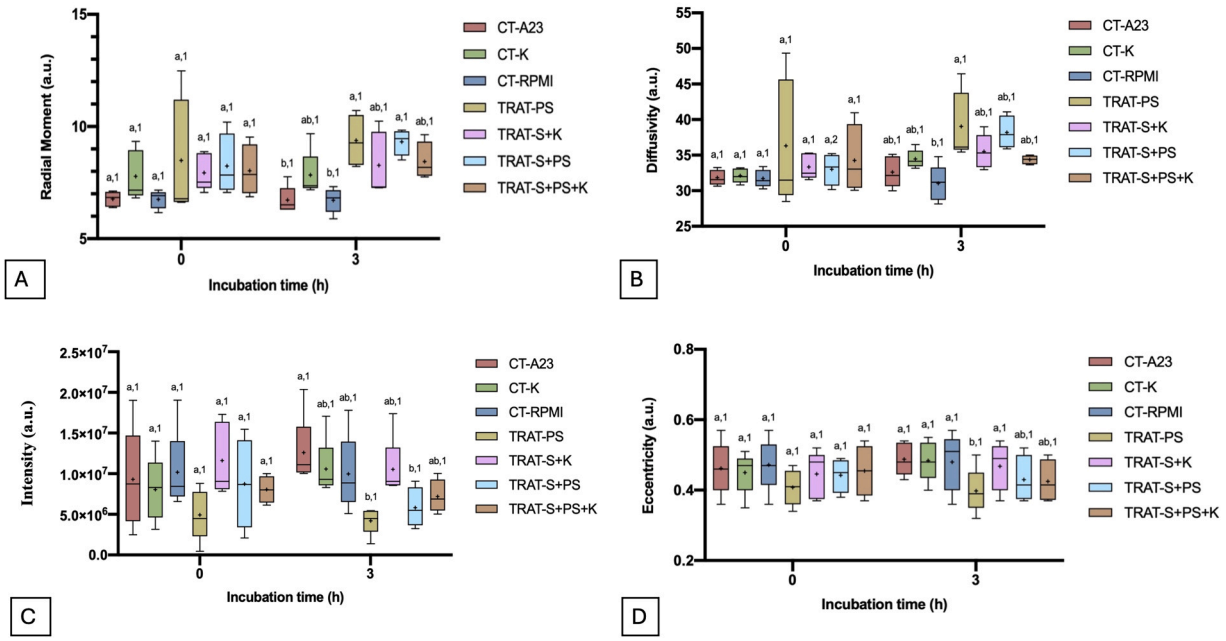

Fig. 6. Fluorescence and nuclear morphometric parameters of eosinophils assessed by flow cytometry with integrated image analysis at 0 and 3 h across all treatment groups. (A) Radial dispersion of SYTOX Green fluorescence, (B) Fluorescence diffusivity, (C) B2 Green channel fluorescence intensity and (D) Nuclear eccentricity. Data are presented as mean ± standard error of the mean (SEM) from five independent replicates. **CT-A23 = Positive Control (eosinophils + calcium ionophore); CT-K = Extender Control (eosinophils + Kenney extender); CT-RPMI = Negative Control (eosinophils + RPMI medium); TRAT-PS = Seminal Plasma Treatment (eosinophils + homologous seminal plasma); TRAT-S+K = Spermatozoa without Seminal Plasma (eosinophils + spermatozoa + Kenney extender); TRAT-S+PS = Spermatozoa with Seminal Plasma (eosinophils + spermatozoa + homologous seminal plasma); TRAT-S+PS+K = Whole Semen Treatment (eosinophils + spermatozoa + homologous seminal plasma + Kenney extender). **Different letters indicate statistically significant differences between treatments at the same timepoint. Different numbers indicate significant differences between timepoints (T0 vs. T3) within the same treatment.*

As a complementary observation, the real-time visualization of cells through CellView™ Technology of the cytometer enabled the visualization of dynamic changes in the nuclear fluorescence intensity of SYTOX Green-stained eosinophils over time and in response to treatment. Initially, a well-defined nucleus was visible at the center of the cells. When the EETosis process began, the nuclei appeared more rounded. A marked loss of nuclear fluorescence was detected in eosinophils treated with seminal plasma (Fig. 7).

Additionally, in samples containing spermatozoa, eosinophils with reduced nuclear fluorescence were frequently seen in association with sperm cells.

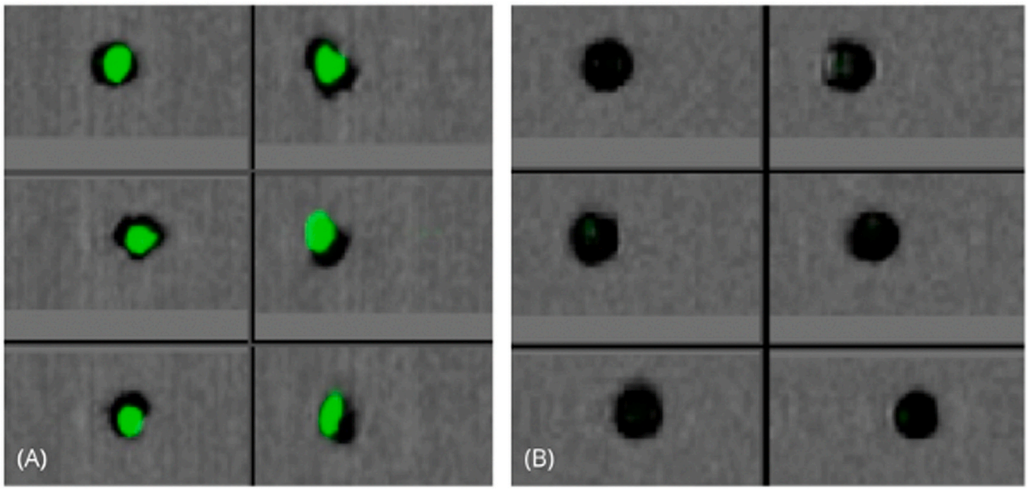

Fig. 7. Loss of nuclear fluorescence intensity of SYTOX Green-stained eosinophils. Eosinophil in the RPMI control (A) and eosinophils with seminal plasma (B) are shown.

7.5. EETosis analysis by confocal microscopy

Qualitative analysis of EETosis by confocal microscopy confirmed the presence of this process in several treatment groups, as evidenced by the visualization of chromatin decondensation and DNA expansion (Fig. 8B). In some groups, extrusion of DNA interacting with spermatozoa was also observed.

8. Discussion

To the best of the authors' knowledge, this is the first study to provide a detailed description of eosinophil extracellular trap (EET) formation in jennies, revealing a novel aspect of the post-insemination uterine immune response in this species.

A key strength of this study was the use of the BD FACSDiscover™ S8 Cell Sorter with BD CellView™ technology, which enabled advanced cellular characterization and sorting and also represents the first report describing the isolation of eosinophils by flow cytometry in domestic animals with high purity. This technology combined spectral cytometry with real-time morphological analysis via innovative camera-less imaging, enabling precise identification of eosinophil subpopulations beyond the capabilities of conventional cytometers.

There is no doubt that the events observed by flow cytometry correspond to extracellular trap formation, as confirmed by confocal microscopy. Key morphological features include the loss of nuclear lobulation and the decondensation of nuclear chromatin, evidenced by the appearance of more rounded nuclei (Shen et al., 2023). These changes culminate in the rupture of both the nuclear envelope and plasma membrane, resulting in the release of large, unfragmented, and decondensed chromatin fibers that form web-like extracellular DNA traps (Ueki et al., 2013). This process represents a form of cell death distinct from apoptosis, which is characterized by chromatin that becomes highly condensed against the nuclear envelope, cellular shrinkage and preservation of plasma membrane integrity (Fukuchi et al., 2021; Ueki et al., 2013).

Eosinophils exposed to seminal plasma alone exhibited higher EETosis rates compared to those incubated with whole semen, where spermatozoa are diluted in seminal plasma and Kenney extender. In contrast, minimal activation occurred when eosinophils were exposed to isolated spermatozoa in the absence of seminal plasma. These findings suggest that specific bioactive components within seminal plasma – possibly cytokines, chemokines, complement fragments, or specific seminal fluid metabolites – are recognized by eosinophils and initiate the cascade leading to DNA and cytotoxic granule extrusion.

Most of the current konwledge about extracelular traps (ETs) and their mechanism of release is still based on neutrophils. These findings are consistent with previous studies in donkey neutrophils, where seminal plasma—but not spermatozoa—was shown to induce NETosis (neutrophil extracellular trap formation) in a time- and concentration-dependent manner (Mateo-Otero et al., 2022; Miró et al., 2020). Miró et al. (2020) reported that incubation of equine neutrophils with seminal plasma led to significant NET release, whereas spermatozoa alone did not provoke such a response. The present study expands this understanding by demonstrating that eosinophils respond similarly, forming extracellular traps in response to seminal plasma stimulation. This represents an important novel finding, as eosinophil extracellular trap formation has previously been documented primarily in humans and in the context of allergic diseases, asthma, and parasitic infections (Echevarría et al., 2017; Ueki et al., 2013), but not within a reproductive setting.

Comparative analyses of the structural organization of extracellular traps formed by neutrophils and eosinophils have demonstrated that eosinophil-derived DNA fibers are both thicker and more resistant to degradation than those from neutrophils. This enhanced stability is attributed to the lower protease activity found in eosinophil granules, suggesting that EETs may persist longer in tissues and exert prolonged effects, even after the eosinophils are no longer morphologically identifiable (Fukuchi et al., 2021; Thompson-Souza et al., 2022). Scanning electron microscopy further supports this, showing that neutrophil extracellular traps are composed of 15–17 nm smooth stretches (Brinkmann et al., 2004), whereas eosinophil traps primarily consist of continuous fibers measuring between 25 and 35 nm in diameter (Ueki et al., 2013).

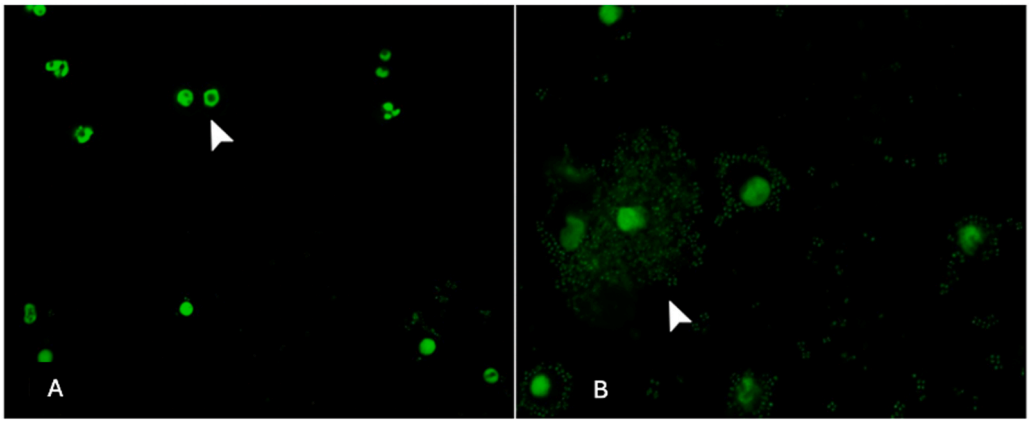

Fig. 8. Confocal microscopy image of eosinophils stained with SYTOX Green showing the morphology of a non-activated donkey eosinophil (white arrow) (A), and an eosinophil undergoing EETosis with nuclear expansion and visible extracellular DNA extrusion (white arrow) (B).

Given that eosinophil traps are more stable than those of neutrophils, they are also expected to exert sustained microbicidal effects through passive mechanisms, including hydrophobic interactions (Ueki et al., 2016). Moreover, the DNA backbone of these traps serves as a framework for antimicrobial mediators, such as granule-derived proteins from imune cells (Wu et al., 2024). Consequently, eosinophils may play a complementary role in immune defense by contributing to antimicrobial activity, further supporting uterine defense mechanisms.

It has been observed that, similar to NETs, EETs can be degraded by DNase I (Shen et al., 2023). In the context of NETs, DNase I present in seminal plasma has been shown to release trapped spermatozoa, thereby improving sperm motility and fertility outcomes (Alghamdi and Foster, 2005). A similar mechanism is hypothesized for EETs, supported by the observations from both flow cytometry and confocal microscopy, where some spermatozoa appear potentially trapped within the extracellular structures. It is plausible that this dual mechanism, neutrophil NETs combined with eosinophil EETs, represents an evolutionary adaptation in jennies to optimize reproductive success, ensuring that only high-quality spermatozoa reach the oocyte, while simultaneously neutralizing potential pathogens. The interaction between these traps and spermatozoa warrants further investigation, particularly regarding their potential influence on sperm viability and function.

The reaction appeared to be remarkably rapid. The presence of diverse receptors on the surface of eosinophils enables these cells to detect and quickly react to changes in their surrounding microenvironment (Fukuchi et al., 2021). Differences in cell size were already significant ($p < 0.05$) at time 0 between the seminal plasma group and the RPMI control. Although other parameters showed a trend toward activation in the seminal plasma group, statistically significant differences ($p < 0.05$) did not emerge until the 3-hour time point. These observations indicate a rapid but progressively amplifying response, likely reflecting early morphological changes associated with EETosis that become more pronounced over time. It has been observed that seminal plasma induces higher rates of EET formation, reaching more advanced stages within the same incubation time compared to its A23 control. This is consistent with reports in the literature, which indicate that in vitro experimental factors such as the type of stimulus applied, exposure duration, and specific assessment methods can significantly influence eosinophil extracellular trap formation from viable cells (Mukherjee et al., 2018). This suggests the possibility of a species-specific response, where donkeys may exhibit a lower sensitivity to this particular inducer.

The rapid response of eosinophils suggest a potential immunomodulatory role influencing other immune cell populations. Eosinophils have recently been recognized as multifunctional leukocytes involved not only in inflammatory responses but also as key modulators of both innate and adaptive immunity (Rothenberg and Hogan, 2006).

Based on the various functions described for eosinophils, we hypothesize that eosinophils may serve as primary triggers for neutrophil activation, thereby facilitating sperm capture and potentially enhancing fertility. First, major basic protein 1 (MBP-1), has been shown to activate neutrophils through the stimulation of tyrosine kinase and calmodulin-dependent signaling pathways (Acharya and Ackerman, 2014; Akuthota et al., 2008; Aoki et al., 2021). Second, cellular debris released during EETosis may accumulate in tissues and act as a chemotactic stimulus that attracts neutrophils to phagocytose this material. This can initiate and sustain neutrophil extracellular trap (NETosis) formation (Echevarría et al., 2017). Third, eosinophils produce the neutrophil-attracting chemokine CXCL8/IL-8 (Braun et al., 1993; Brinkmann et al., 2004), indicating their potential involvement in recruiting neutrophils to inflammatory sites (Kushwah and Hu, 2011).

It is hypothesized that another potential immune role of eosinophils is their involvement in embryo recognition, which may be crucial to the establishment of maternal-fetal tolerance (Araujo et al., 2018; Rothenberg and Hogan, 2006). In this context, eosinophils may contribute to the modulation of immune responses by releasing regulatory mediators such as galectin-10 and transforming growth factor beta (TGF-β), which support the expansion of regulatory T cells (Tregs) and suppress effector T cell activity (Kheshtchin et al., 2024; Lingblom et al., 2021, 2017). Galectin-10 is one of the most abundant cytoplasmatic proteins in eosinopils, comprising aproximately 7–10 % of their total protein content (Lingblom et al., 2017). Interestingly, EETosis is closely associated with the formation of Charot-Leyden crytals – bipyramidal structures primarly composed of crytallized galectin-10 (Ueki et al., 2018), highlighting the association between eosinophil cell death and immunoregulatory processes. In addition, eosinophil cationic protein (ECP) exerts immunomodulatory effects beyond its cytotoxic activity, such as suppressing T cell proliferation and inhibiting immunoglobulin production by B lymphocytes (Santos et al., 2024). Moreover, eosinophil granule protein eosinophil-derived neurotoxin (EDN) promotes the maturation and activation of Dendritic cells (Yang et al., 2003), a antigen-presenting cells that help to initiate and regulate the establishment of immune tolerance and either supporting Treg function (Kushwah and Hu, 2011; Tagliani and Erlebacher, 2011). All of these functions are important for maintaining immune tolerance during pregnancy and preventing fetal rejection.

The consistent presence of eosinophils in the uterus of jennies suggests a potential homeostatic role, as well as involvement in tissue repair preventing cumulative damage (Acharya and Ackerman, 2014; Santos et al., 2024). Notably, jennies maintain reproductive fertility at more advanced ages than mares (Canisso et al., 2019). In this context, Miró et al. (2020b) suggested that chronic eosinophil depletion may predispose to endometrial fibrosis via the IL-33 pathway, impairing uterine function. Eosinophils may also contribute to endometrial repair by releasing interleukin-4 (IL-4), a known activator of fibro/adipogenic progenitors (FAPs) involved in muscle tissue regeneration (Aoki et al., 2021). Therefore, controlled eosinophilic activity, including EET formation during immune challenges such as insemination, may benefit uterine homeostasis in jennies.

The role of seminal plasma in modulating the uterine inflammatory response remains a key area of investigation in reproductive medicine. As our understanding of the immune mechanisms triggered after insemination deepens, it becomes increasingly clear that seminal plasma is not merely a vehicle for spermatozoa, but a bioactive component with complex immunoregulatory functions. In jennies, its ability to induce both NETosis and EETosis suggests that it acts as a potent modulator of the uterine microenvironment, promoting a balanced immune response that supports pathogen clearance, sperm selection, and potentially endometrial regeneration. However, identifying which specific component of seminal plasma is responsible for triggering these effects remains challenging (Morelli et al., 2021), since its composition varies between species, among individuals, between ejaculates of the same individual, and

even between fractions of a single ejaculate—particularly in species like donkeys that ejaculate in jets (Rodríguez-Martínez et al., 2011).

Considering that the reduced fertility observed using frozen semen appears to be of maternal origin, and that the main cytological difference between jennies and mares lies in the higher number and activity of eosinophils in the former, the absence of seminal plasma in frozen semen and the resulting lack of EET formation, may contribute to this decreased reproductive efficiency.

Although the results are clear and promising, it is possible that more evidence of EET presence may have been missed due to the incubation time used. Some in vitro studies have defined optimal EET formation within a time frame of 30 min to 2 h (Ueki et al., 2016, 2013), whereas in our study, samples were incubated for 3 h. This extended duration may have led to more advanced stages of EET evolution, in which the extracellular DNA structures become more dispersed and, consequently, less detectable by SYTOX Green staining. Other factors that can be influenced and even downgrade the EETosis rate is that the use of FCS in the culture medium may affect the observation of ETs because induce dissociation of aggregated EETs because of a heat-stable nuclease capable of degrading DNA (Fukuchi et al., 2021; Ueki et al., 2016).

9. Conclusion

In conclusion, this study highlights that flow cytometry with cell sorting is an effective method for isolating eosinophils, and demonstrates that these cells are capable of forming EETs. Furthermore, the findings underscore the pivotal role of seminal plasma in modulating eosinophil responses, suggesting that EETosis may play a beneficial role in the reproductive immunophysiology of jennies. Future studies should aim to identify the specific components within seminal plasma that trigger EET formation and to elucidate the broader functional implications of EETs in fertility, pathogen defense, and endometrial health.

CRediT authorship contribution statement

Manuela Costa: Validation, Methodology, Investigation, Formal analysis. **Jesús Martínez-Hernández:** Investigation, Formal analysis. **Iván Yánez-Ortiz:** Validation, Software, Data curation. **Yame Fabres Robaina Sancler-Silva:** Writing – original draft, Investigation, Formal analysis, Data curation. **Miro Jordi:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Sebastià Company:** Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Jaime Catalán:** Writing – review & editing, Validation, Supervision, Project administration, Investigation, Data curation.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported herein.

Data Availability

The datasets supporting the conclusions of this article are available from the corresponding author on reasonable request.

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