

# In vitro exposure of porcine sperm to functionalized superparamagnetic nanoparticles

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## Funding information

Fundación Séneca; Ministry of Science and Innovation

## Abstract

Nanotechnology and its applications have advanced significantly in recent decades, contributing to various fields, including reproduction. This study introduces a novel method to label porcine oocytes with nanoparticles (NPs) bound to oviductin (OVGP1, Ov) for use in Assisted Reproductive Technologies (ARTs). Despite promising developments, concerns about NP toxicity in gametes necessitate thorough investigation. This research aims to assess the impact of functionalized NPs (NPOv) on sperm functionality. Boar sperm were co-incubated with NPOv for 0, 0.5 and 1 h in two media: BTS (semen dilution and conservation) and TALP (sperm capacitation and in vitro fertilization-IVF). Sperm quality parameters (viability, motility and kinematics) showed no significant differences in TALP medium ( $p > .05$ ). In BTS, although some kinetic parameters were altered, motility, progressive motility and viability remained unaffected ( $p > .05$ ). Additionally, NPs presence on the zona pellucida (ZP) of oocytes did not affect sperm attachment ( $p > .05$ ). In conclusion, in vitro exposure of boar sperm to OVGP1-functionalized NPs in IVF medium or attached to the ZP surface of matured oocytes does not impair sperm functionality, including their binding ability to the ZP.

## KEYWORDS

boar, nanomaterial, nanotechnology, paramagnetic, spermatozoon

## 1 | INTRODUCTION

Nanotechnology, a relatively new field focused on the study of nanoparticles (NPs), is rapidly expanding and finding widespread applications across various domains (Durfey et al., 2019; Hill & Li, 2017). The numberless applications have garnered interest in nearly all aspects of life, including the field of reproduction (Angelakeris, 2017; Mehta, 2017; Mohammed et al., 2017) where

the use of NPs has seen a considerable increase (Gil et al., 2013; Zhang et al., 2017). Yet, numerous toxic effects of NPs on reproductive cells have been reported, especially on sperm cells (Durfey et al., 2017; Feugang, 2017; Moretti et al., 2013; Taylor et al., 2014; Wiwanitkit et al., 2009; Zakhidov et al., 2010, 2013). Consequently, ensuring the safe application of any novel nanomaterials in Assisted Reproductive Technologies (ARTs) requires demonstrating their lack of negative effect on gametes.

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It has been well documented that the properties of NPs such as size, surface charge, core material or surface coating, are closely related to their potential effects on sperm cell function (Falchi et al., 2018; Feugang, 2017; Habas et al., 2021; Singh et al., 2010). Additionally, the components and chemical properties of the NPs dilution media (pH, ionic compositions, or ionic strength) can alter NP properties, such as NP aggregation, surface charge and chemical forms. These alterations can increase the potential toxicity of NPs for cells (Djurišić et al., 2015; Li et al., 2011; Yue et al., 2015). Furthermore, the incubation medium can affect the interaction between the cells and NPs, which could lead to negative effects of NPs on cells (Djurišić et al., 2015).

Likewise, *in vitro* dose-dependent cytotoxicity caused by NPs on spermatogonia and DNA integrity of sperm cells has been reported (Braydich-Stolle et al., 2005; Moradi et al., 2021). Additionally, the core material and surface coating of NPs were highly correlated with the detrimental effects observed in reproduction (Feugang, 2017; Singh et al., 2010). In this sense, while gold NPs showed nanotoxicity in human, mouse and bovine sperm (Moretti et al., 2013; Taylor et al., 2014; Wiwanitkit et al., 2009; Zakhidov et al., 2010, 2013), iron oxide NPs showed no harmful effect on sperm after *in vitro* incubation (Makhluף et al., 2006; Taylor et al., 2014; Zakhidov et al., 2013).

Furthermore, one of the remarkable properties of NPs is their exceptional versatility, allowing them to be functionalized with a broad range of agents such as proteins, DNA, cell internalization agents, drugs and many others (Angelakeris, 2017; Mehta, 2017; Mohammed et al., 2017). Given these considerations, it is essential to consider not only the inherent harmful effects of NPs but also the effects after their functionalization on sperm cells (Truong et al., 2012, 2013). These concerns highlight the need for comprehensive safety studies to fully assess the risks associated with NPs use in ARTs.

In a recent study conducted by our group, we reported a novel method where magnetic NPs are linked to the zona pellucida (ZP) of mature oocytes through their functionalization with a recombinant protein, oviductin (OVGP1), which has the intrinsic and specific ability to bind to the ZP (García-Vázquez et al., 2024). This new technology may become an auspicious tool for *in vitro* fertilization (IVF) laboratories since it could simplify the manipulation of oocytes and embryos by allowing them to be moved or retained using an external magnetic force.

Adapting this technique for oocytes intended for IVF involves addressing several questions to ensure the safety of this system for gametes. The mere presence of these NPs in the media represents a potential risk for sperm cells. Furthermore, the presence of these NPs around the ZP surface of mature oocytes for IVF may block the binding sites for sperm. The main objective of this study is to assess sperm functionality in systems that include oviductin (Ov)-functionalized nanoparticles (NPs). The first experiment evaluates the influence of medium composition and dosage on sperm quality when sperm are co-incubated with NPs (NPs or NOPv). In the second experiment, sperm are co-incubated with oocyte-NPOv or oocyte-NPs in an IVF system to further examine the ability of sperm to bind to the ZP.

## 2 | MATERIALS AND METHODS

### 2.1 | NP functionalization

Carboxyl-modified superparamagnetic NPs (Estapor®) with a diameter of  $\sim 0.365 \mu\text{m}$  were functionalized as previously described by García-Vázquez et al. (2024). Recombinant truncated porcine (pOVGP1) was expressed and purified as described by Algarra et al. (2016). For conjugation,  $10 \mu\text{L}$  (concentration/solid content [%]=0.1) of NPs were incubated with pOVGP1 ( $6 \mu\text{g}$ ;  $\sim 75 \text{kDa}$ ) in a coupling buffer at room temperature for 2 h. Also, as a control group,  $10 \mu\text{L}$  of NPs were incubated for the same time and under the same condition, but pOVGP1 was replaced for the same volume of coupling buffer during conjugation.

### 2.2 | Assessment of sperm quality following co-incubation with NPs

The sperm-rich fraction of semen from five mature and fertility-tested boars was collected by the gloved hand method and immediately transported to the laboratory. A minimal criterion was established to use the ejaculates:  $>85\%$  spermatozoa total motility and  $<20\%$  abnormal spermatozoa morphology. A total of five different experimental groups: (1) NPOv10: sperm +  $10 \mu\text{L}$  NPOv, (2) NPOv20: sperm +  $20 \mu\text{L}$  NPOv, (3) NP10: sperm +  $10 \mu\text{L}$  NPs, (4) NP20: sperm +  $20 \mu\text{L}$  NPs and (5) Control: sperm without NPs. Groups of NPs without pOVGP1 were tested to establish if there was any inner toxicity of the NPs after the conjugation protocol without the protein. Sperm were incubated in two different media: Beltsville Thawing Solution extender (BTS, Zoitech), commonly used for the handling and storage of porcine sperm, and Tyrode's albumin-lactate-pyruvate (TALP), the usual medium employed in porcine IVF systems and for sperm capacitation, to analyse the effects of NPOv on sperm when they were under an IVF protocol. A final concentration of  $30 \times 10^6$  sperm/mL was defined for each group. The TALP medium was supplemented with 1.5% BSA to reduce sperm agglomerations (Caldeira et al., 2017). During the co-incubation period, samples in BTS were kept at  $15^\circ\text{C}$  and samples in TALP were kept at  $38.5^\circ\text{C}$  with  $5\% \text{CO}_2$ .

The sperm analysis (motility, kinematic parameters and viability) was performed at different periods of incubation: 0 (when the groups were formed), 0.5 and 1 h. For motility analysis, the Computer Assisted Semen Analysis (CASA) was used (ISAS® software, PROiSER R+D S.L., Valencia, Spain) coupled to a phase-contrast microscope (negative-pH  $10\times$  objective; Leica DMR, Wetzlar, Germany) and a digital camera (Basler Vision, Ahrensburg, Germany). A  $4 \mu\text{L}$  drop of the sample was placed in a prewarmed ( $38^\circ\text{C}$ ) chamber (20-micron Spermtrack® chamber, Proiser R+D, SL; Paterna, Spain) and evaluated by a negative phase-contrast microscope ( $10\times$  objective; Leica DMR, Wetzlar, Germany). Parameters provided by CASA were total motility-MOT (%), progressive motility-PMOT (%), mean velocity-VAP ( $\mu\text{m/s}$ ), linear velocity-VSL ( $\mu\text{m/s}$ ), curvilinear velocity-VCL

( $\mu\text{m/s}$ ), mean lateral head amplitude-ALH ( $\mu\text{m}$ ), frequency of head displacement-BCF (Hz), straightness coefficient-STR (%), linearity coefficient-LIN (%). At least three different fields were analysed per sample. A spermatozoon was considered motile when there was a VAP  $>10\mu\text{m/s}$ . PMOT was considered to exist when there was a STR  $>45\%$ . Motility determinations were made at 25 frames per second for 1 s (25 images).

Sperm viability was assessed by eosin-nigrosin staining, according to a method previously described by Wells and Awa (Wells & Awa, 1970). A bright-field microscope was used for the evaluation (40 $\times$  objective; Nikon® Model YS100, Tokyo, Japan). Spermatozoa were classified as follows: (1) intact membrane, indicated by colourless spermatozoa and (2) damage membrane, indicated by pink-coloured spermatozoa. Sperm viability was expressed as the percentage of cells with an intact membrane. At least 200 spermatozoa per sample were evaluated.

### 2.3 | Sperm-ZP binding analysis

Porcine oocytes were obtained from ovaries of prepubertal females collected at local slaughterhouses. The oocytes were in vitro matured and decumulated by soft pipetting. The resulting denuded oocytes were then co-incubated with NPOv (20  $\mu\text{L}$ ), NPs (20  $\mu\text{L}$ ) or without NPs (control) in 500  $\mu\text{L}$  of DPBS-BSA in a Nunc® four-well dish for at least 20 min at 38.5°C, as described by García-Vázquez et al. (2024). These oocytes then underwent an IVF protocol to test the ability of sperm to bind to the ZP of oocytes. For the IVF process, semen, previously selected by a discontinuous Percoll® (Pharmacia, Uppsala, Sweden) gradient (45%–90%), was added to each well of Nunc® four-well dish containing 40 oocytes in TALP, achieving a final concentration of  $1.5 \times 10^6$  spermatozoa per well in a final volume of 500  $\mu\text{L}$ . Finally, at 18–20 hpi, the oocytes were fixed for 15 min in 10% glutaraldehyde in PBS, washed in PBS and mounted

on glass slides for subsequent evaluation under a negative phase-contrast microscope (Leica DMR, Wetzlar, Germany). The number of sperm bound to the ZP per oocyte was determined.

### 2.4 | Statistical analysis

Statistical analysis for sperm parameters was performed using the free statistical software SAS University Edition (SAS, 2016). All the motion parameters (MOT, PMOT, VCL, VAP, VSL, LIN, STR, WOB, BCF and ALH) and viability were compared with the mixed model of SAS. The model included the experimental groups (NP10 and NP20, NPOv10 and NPOv20, control) for both types of media (BTS or TALP), the time related to experimental groups (0, 0.5 and 1 h) and their interaction as the main effects, with spermatozoa as a random effect. A first-order autoregressive covariance structure was used to adjust the difference in data according to the differences over time.

For sperm bound to ZP per oocyte, statistical analysis was performed using IBM SPSS v.23 (SPSS Inc. Chicago, IL, USA). Since the homogeneity of variance was probed by a Levene's test an ANOVA test was used for media comparison. In any case, differences were considered statistically significant at  $p < .05$ . Data are expressed as mean  $\pm$  standard error of the mean (SEM).

## 3 | RESULTS

### 3.1 | Sperm quality after exposure to NPs

The evaluation of sperm parameters after co-incubation with NPs using BTS as the incubation medium indicated that both MOT and PMOT of the spermatozoa did not differ between the control and experimental groups ( $p > .05$ ; Table 1). Additionally, no interactions between time and groups were observed ( $p > .05$ ). Yet, the analysis

**TABLE 1** Sperm quality parameters analysed after co-incubation with NPs or NPOv in two volumes (10 and 20  $\mu\text{L}$ ) using BTS medium (analysed at 0, 0.5 and 1 h of incubation) at 15°C.

Parameters	Treatments					p-value	SEM
	CONTROL (BTS)	NP10	NP20	NPOv10	NPOv20		
MOT (%)	85.89	83.39	81.83	85.06	83.17	.07	4.30
PMOT (%)	59.89	58.67	58.72	61.89	60.56	.19	3.08
VCL ( $\mu\text{m/s}$ )	84.43 <sup>a</sup>	74.51 <sup>b</sup>	71.30 <sup>b</sup>	74.11 <sup>b</sup>	68.23 <sup>b</sup>	.001	8.71
VSL ( $\mu\text{m/s}$ )	27.13 <sup>a</sup>	25.74 <sup>b,c</sup>	25.27 <sup>c</sup>	26.60 <sup>a,b</sup>	25.84 <sup>b,c</sup>	.02	1.65
VAP ( $\mu\text{m/s}$ )	45.10 <sup>a</sup>	41.10 <sup>b,c</sup>	39.03 <sup>c</sup>	42.16 <sup>a,b</sup>	39.27 <sup>b,c</sup>	.001	3.96
LIN (%)	33.67 <sup>b</sup>	36.62 <sup>a,b</sup>	37.26 <sup>a</sup>	36.87 <sup>a,b</sup>	39.19 <sup>a</sup>	.04	2.58
STR (%)	61.44 <sup>b</sup>	64.50 <sup>a,b</sup>	66.21 <sup>a</sup>	63.87 <sup>a,b</sup>	67.07 <sup>a</sup>	.02	2.78
WOB (%)	54.41	56.25	55.90	57.41	58.24	.05	1.76
BCF (Hz)	8.01	7.82	7.66	7.73	7.61	.06	0.29
ALH ( $\mu\text{m}$ )	3.01 <sup>a</sup>	2.74 <sup>b</sup>	2.70 <sup>b</sup>	2.70 <sup>b</sup>	2.55 <sup>b</sup>	.01	0.18
Viability (%)	90.33	88.44	89.00	89.22	88.77	.84	1.20

Note: Statistical analysis was carried out through a study of repeated measures over time. Superscripts (a, b, c) indicate significant differences ( $p < .05$ ) ( $n = 5$  replicates).

identified some differences in sperm kinetic parameters (Table 1). Specifically, VCL and ALH were significantly greater in the control group compared to the experimental groups ( $p < .001$  and  $p < .007$ , respectively). VSL and VAP were also higher in the control group than in the NP10, NP20 and NPOv20 groups ( $p < .02$  and  $p < .001$ , respectively), although these parameters did not differ significantly from NPOv10. Additionally, VSL and VAP for NP20 were significantly different from those for NPOv10 ( $p < .03$  and  $p < .05$ , respectively). Furthermore, LIN and STR were lower in the control group than in both NP20 and NPOv20 groups ( $p < .001$  and  $p < .007$ , respectively).

Conversely, when sperm quality was assessed in TALP medium, there were no significant differences in the analysed parameters between the control and experimental groups ( $p > .05$ ), and no interactions were observed between time and groups (Table 2). Likewise, there were no significant differences in sperm viability among the experimental groups, whether in BTS or TALP medium ( $p > .05$ ).

### 3.2 | Assessment of sperm binding to the ZP of NPOv

No significant differences were observed in the number of sperm adhering to the ZP per oocyte among the oocytes exposed to NPs, NPOv and control oocytes ( $p > .05$ ; Figure 1a,b). An average of  $20.70 \pm 6.50$  sperm per oocyte was recorded, indicating a consistent binding capacity across all experimental groups.

## 4 | DISCUSSION

The development of new biomedical applications for NPs is constantly expanding. Alongside this growth, concerns are also increasing about their potential harmful effects on cells (Hussain et al., 2009;

Schrand et al., 2010). Magnetic NPs, a subclass of nanomaterials, have become widely used in biomedical applications, including in the reproductive field (Ali et al., 2016; Barkalina et al., 2014), especially for enhancing ARTs. Recent research by our group (García-Vázquez et al., 2024) has demonstrated that oocytes and embryos can be indirectly manipulated through external magnetic fields by attaching magnetic NPs conjugated with a recombinant protein-ligand (pOVGP1) to the surface of the ZP. This promising technology has the potential to be integrated into routine oocyte and embryo manipulation procedures in fertilization laboratories to simplify and automate tasks. Nevertheless, before its full implementation in ARTs can be realized, a comprehensive evaluation of the potentially harmful effects of these NPs on sperm cell functions is essential. Accordingly, this study provides relevant insights into the effects of NPs on porcine sperm functionality.

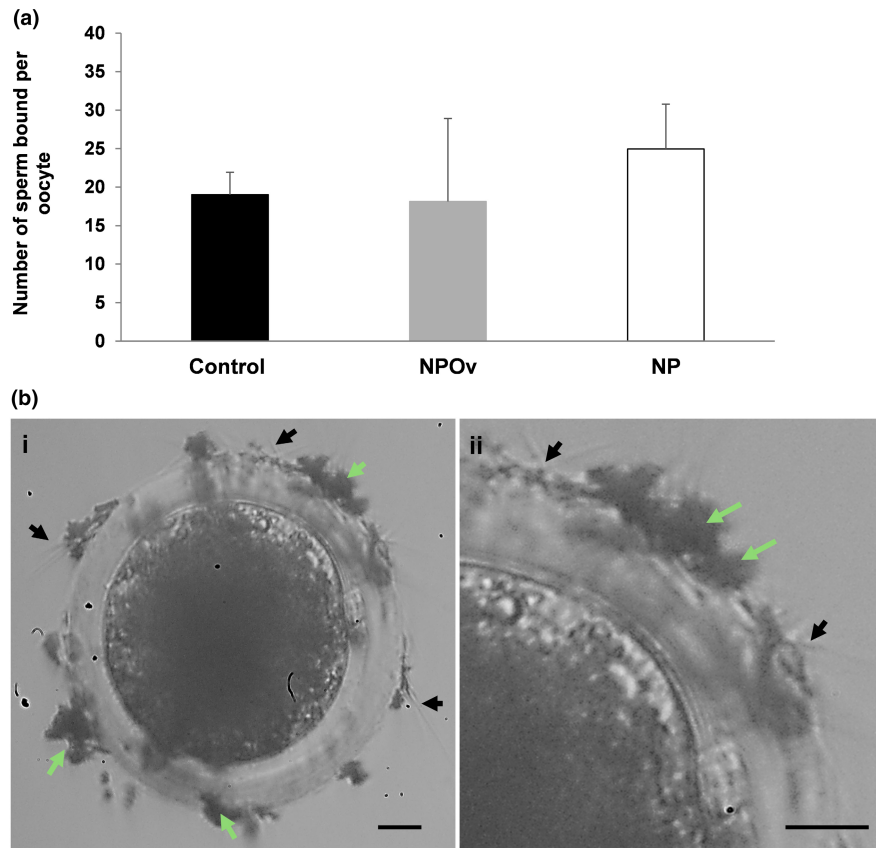
In the first experiment, we tested the sperm functionality in terms of sperm quality after the co-incubation of sperm with NPs (with or without OVGP1). Furthermore, the incubation media could be a determining factor for the interaction between sperm and NPs, so we tested two different media: BTS and TALP. In this study, no alterations in sperm viability, PMOT or MOT were found when spermatozoa were incubated with NPs or NPOv in either of the media used for up to 1 h. In the same sense, other authors have observed similar results with no variations in MOT or PMOT when NPs coated with polyvinyl alcohol (Makhluf et al., 2006) or MNP-DMSA (Caldeira et al., 2017) were incubated with bovine sperm cells for up to 4 h (Caldeira et al., 2017).

It has been established that the impaired effects of NPs on sperm cell function are linked to both the dosage and duration of exposure (Hou & Zhu, 2017; Moretti et al., 2013). In light of this, the absence of adverse effects observed in our study may be attributed to the relatively low doses of NPs/NPOv used. Additionally, the short incubation period stipulated for this study may have contributed to the lack of detrimental effects. Yet, in the second experiment of this

Treatments							
Parameters	CONTROL (TALP)	NP10	NP20	NPOv10	NPOv20	p-value	SEM
MOT (%)	88.44	88.17	87.28	88.22	88.28	.82	0.01
PMOT (%)	68.50	65.72	64.44	67.06	69.28	.24	0.02
VCL ( $\mu\text{m/s}$ )	66.56	65.94	66.33	67.72	68.77	.85	3.25
VSL ( $\mu\text{m/s}$ )	48.06	45.61	44.94	47.56	47.44	.81	3.14
VAP ( $\mu\text{m/s}$ )	54.33	52.56	52.39	54.33	54.61	.83	2.90
LIN (%)	71.83	68.56	67.28	69.61	68.78	.60	3.09
STR (%)	87.56	85.56	85.33	86.39	86.17	.89	2.24
WOB (%)	80.56	78.28	77.44	79.67	79.00	.06	1.71
BCF (Hz)	7.61	7.33	7.39	7.67	7.50	.31	0.16
ALH ( $\mu\text{m}$ )	1.67	1.67	2.00	1.72	1.83	.09	0.13
Viability (%)	76.89	79.28	78.56	77.78	77.00	.45	3.94

TABLE 2 Sperm quality parameters analysed after co-incubation with NPs or NPOv in two volumes (10 and 20  $\mu\text{L}$ ) using TALP medium (analysed at 0, 0.5 and 1 h of incubation) at 38.5°C with 5%  $\text{CO}_2$ .

Note: Statistical analysis was carried out through a study of repeated measures over time ( $n = 5$  replicates).



**FIGURE 1** Sperm binding to ZP of oocytes covered by NPs. (a) The bar chart illustrates the average number of sperm bound to the ZP per oocyte in each analysed group (Control, NPOv, NP). Data are presented as mean  $\pm$  SEM ( $n=3$  replicates). (b) Bright-field microscopy images illustrate an oocyte with NPOv attached around the ZP surface with sperm attached to the ZP. Black arrows highlight the sperm, while green arrows indicate aggregates of NPOv. Scale bar, 25  $\mu$ m.

research, the incubation period for the IVF process was extended to 18–20h, yet non-detrimental effects on sperm were observed.

Furthermore, among the NP materials, superparamagnetic iron oxide NPs (SPIONs) are preferred for biological applications since they are generally well tolerated by cells, as this type of iron is endogenous to the body. Furthermore, SPION coatings are mainly made of biocompatible materials and can be easily functionalized with various molecules (Neuberger et al., 2005). Despite these advantages, it has been proposed that functionalized particles could present some effects on cells that should be carefully considered (Truong et al., 2012, 2013). The NPs selected for our technology development belonged to this category and was functionalized with a recombinant protein, pOVGP1. To study the cause of any possible negative effects, we evaluated the impact of NPs, considering the conjugation media and the inclusion of OVGP1. According to our results, neither the NPs nor the recombinant protein used for their functionalization, nor the buffers used in the conjugation process, presented negative effects on the sperm after their co-incubation. Similarly, no effects on sperm functionality were documented after exposure to magnetic NPs functionalized with a variety of molecules (Feugang et al., 2015; Odhiambo et al., 2014; Yousef et al., 2020).

Differences in certain sperm kinetic parameters were observed between the control and NP groups when the BTS medium was

used. The affected parameters included those related to velocity (VSL, VCL and VAP). It is possible to speculate that the reduction in these parameters in the NP groups may be due to sperm agglutination around the NPs, which acts as impediments to the free movement of sperm cells (Ibănescu et al., 2016; Pérez-Duran et al., 2020). These aggregates could also explain the observed decrease in the ALH in the NP groups, as the presence of aggregates likely impedes sperm head movement. In addition, the LIN parameter, which is calculated as  $VSL/VAP \times 100$ , was affected due to its dependence on the previously mentioned velocity parameters affected by increased agglutination. These differences were detected when using BTS, but no such differences were observed with TALP, a more complex medium. To counteract NPs and sperm aggregation, an additional 1.5% BSA was incorporated into TALP (Caldeira et al., 2017). This modification resulted in no detectable differences in speed parameters between the control and experimental groups with NPs, as agglutination was comparably reduced in all groups.

It is well known that initial sperm-egg recognition on the surface of the ZP is an essential step for fertilization (Rankin et al., 2001). In this context, the possible masking of sperm binding sites on ZP due to the presence of NPs attached to ZP was a concern. Analysing the number of sperm bound to the ZP in oocytes with NPs showed no differences between the number of sperm

bound to the ZP when oocytes were exposed to NPs, NPOv or left untreated. This indicates that there was no blockage of the binding sites. Previously, we showed that the maximum surface coverage of the ZP with NPOv was 40%, ensuring that the binding of the sperm is not affected. Consequently, we observed the same binding values as in techniques where sperm have been pre-selected (Canovas et al., 2017).

To conclude, our findings reveal that the in vitro exposure of boar sperm cells to functionalized superparamagnetic NPs, either through their presence in the IVF medium or attached to the ZP surface of oocytes during the IVF protocol, did not compromise sperm functionality or their ability to bind to the ZP. This suggests that the application of this novel method for oocyte manipulation during ARTs is possible without negative effects on sperm cells that could compromise fertility outputs.

#### AUTHOR CONTRIBUTIONS

MJM and FAGV contributed to the study conception and design. Methods and data analysis were performed by GG and CML. MJM and FAGV provided the resources. GG drafted the manuscript and all authors read and approved the final version of the manuscript.

#### FUNDING INFORMATION

This publication was funded by Fundación Seneca-Agencia de Ciencia y Tecnología de la Región de Murcia 'Ayudas a la realización de proyectos para el desarrollo de investigación científica y técnica por grupos competitivos' (20887/PI/18, M.J.M.; 22001/PI/22, F.A.G.V.) and 'Fundación Séneca-Prueba de concepto' (21637/PDC/21, F.A.G.V. and M.J.M.) Spain, also by projects from Spanish Ministry of Science and Innovation PID 2020-114109GB-I00 (M.J.M.) and PID2019-106380RB-I00 (F.A.G.V.) funded both by MCIN/AEI/10.13039/501100011033.

#### CONFLICT OF INTEREST STATEMENT


None of the authors have any conflict of interest to declare.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**How to cite this article:** Garrappa, G., Martínez-López, C., Jiménez-Movilla, M., & García-Vázquez, F. A. (2024). In vitro exposure of porcine sperm to functionalized superparamagnetic nanoparticles. *Reproduction in Domestic Animals*, 59(Suppl. 3), e14654. <https://doi.org/10.1111/rda.14654>