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Acquisition of fertilization competence in guinea pig spermatozoa under different capacitation protocols

Karina Cañón-Beltrán [a, b, *], Yulia N. Cajas [a, c], Encina González [d], Raúl Fernández-González [a], Natacha Fierro [e], Pedro L. Lorenzo [f], María Arias-Álvarez [g], Rosa M. García-García [f], Alfonso Gutiérrez-Adán [a], Dimitrios Rizos [a]

[a] Department of Animal Reproduction, National Institute for Agriculture and Food Research and Technology (INIA-CSIC), Madrid, Spain
[b] Programa de Medicina Veterinaria y Zootecnia, Grupo Kyron, Corporación Universitaria del Huila (CORHUILA), Huila, Colombia
[c] Laboratorio de Biotecnología de la Reproducción Animal, Facultad de Ciencias Agropecuarias, Universidad de Cuenca, Cuenca, Ecuador
[d] Department of Anatomy and Embryology, Veterinary Faculty, Complutense University of Madrid, Madrid, Spain
[e] Escuela de Ingeniería Agropecuaria, Departamento de Ciencias Biológicas, Universidad Técnica Particular de Loja, Loja, Ecuador
[f] Department of Physiology, Veterinary Faculty, Complutense University of Madrid, Madrid, Spain
[g] Department of Animal Production, Veterinary Faculty, Complutense University of Madrid, Madrid, Spain

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ABSTRACT

Guinea pig *in vitro* fertilization (IVF) are poorly developed due to the limited accessibility to oocytes and the lack of an efficient method of sperm capacitation. Thus, we aimed to evaluate different capacitation protocols that we validated through sperm analysis and using heterologous (He) IVF with zona-intact bovine oocytes. Spermatozoa of guinea pigs were collected and processed separately by 4 different protocols: A) Spermatozoa were obtained by flushing the lumen of one cauda epididymis and incubated in a minimal culture medium (MCM); B) One epididymis was placed in a prewarmed of M2 medium and gently minced with fine scissors. Spermatozoa were incubated in a modified human tubal fluid medium (HTF). In both protocols, the spermatozoa were capacitated at 37 °C under an atmosphere of 5% CO2 for 2 h. In the protocols C and D, the spermatozoa were collected by flushing the lumen of the cauda epididymis and selected by commercial density gradient Bovipure® (Nidacon Laboratories AB, Göthenborg, Sweden), according to the manufacturer's instructions. Then for Protocol C) spermatozoa were incubated in MCM medium supplemented with 10 mg/mL heparin (MCM-Hep); while for Protocol D) spermatozoa were incubated in FERT medium supplemented 10 mg/mL heparin (FERT-Hep). Incubation of C and D protocols were performed at 38.5 °C under an atmosphere of 5% CO_2 for 2 h. Capacitation protocols C and D showed a higher percentage of viability, total and hyperactive-like motility, and acrosome reaction compared to protocols A and B. For this reason, protocols C and D were used for further He-IVF analysis. Guinea pig sperm and matured zona-intact bovine oocytes were co-incubated at 5% CO_2 and 38.5 °C. Sperm-oocyte interaction was assessed at 2.5 h post-insemination (hpi) and pronuclear formation (PrF) were evaluated at 18, 20, 22, 24 and 26 hpi, while the cleavage rate was evaluated at 48 hpi. In protocol D, PrF was significantly higher than in protocol C ($P \leq 0.05$) at every time point evaluated. Also, the cleavage rate at 48 hpi was higher ($P \leq 0.05$) in He-IVF protocol D (69.8 ± 1.7%) compared to He-IVF protocol C (49.1 ± 1.1%). In conclusion, we determined the most adequate sperm capacitation conditions for guinea pig that allow zona-intact bovine oocyte penetration and lead to hybrid embryo formation, suggesting that these conditions could be optimal to develop IVF in guinea pigs.

1. Introduction

The guinea pig is an important meat-producing livestock species in south American countries, capable of efficient feed conversion in meat but depends upon the selection of animals with superior production

* Corresponding Author. Department of Animal Reproduction, National Institute for Agriculture and Food Research and Technology (INIA-CSIC), Ctra de la Coruña KM 5, Madrid, Spain.
E-mail address: kecanon@gmail.com (K. Cañón-Beltrán).

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traits and reproductive performance [1,2]. *However, genetic improvement is limited such as the use of reproductively untested males is common due to the difficulty of semen collection and quality evaluation. Furthermore, assisted reproductive technologies, like in vitro fertilization (IVF), are of great importance for increasing the genetic diversity in species like guinea pig (Cavia porcellus) that have a low ovulation rate* [3,4], *where conventional superovulation methods are not effective* [5], *and sperm capacitation is insufficient* [6,7]. *Although, nowadays the guinea pig is used to study cellular and molecular mechanisms of human reproduction including sperm technologies* [6,7] *and human infectious diseases such as this specie is more physiologically and immunologically similar to humans than other small animal models* [8].

Till today it has not been possible to establish an adequate protocol for guinea pig semen capacitation to identify the best fertile males, which could be used to develop reproductive biotechnologies such as artificial insemination and IVF. Many factors may affect the success of IVF, principally sperm motility-hyperactivation and capacitation-acrosome reaction. Previous studies have reported that *in vitro* guinea pig capacitation can occur in defined media [6] supplemented with certain components such as Ca^{2+}, HCO_3^- and BSA [7]. It has been demonstrated that heparin (a glycosaminoglycan), commonly used for bull sperm capacitation *in vitro* [8], can bind to sperm to facilitate the entry of ions, especially calcium and induce changes in the intracellular environment [9–11]. In fact, Sánchez-Vásquez et al. [9], showed that decondensation of guinea pig sperm nuclei was also time- and heparin-concentration-dependent. Furthermore, Huang et al. [12] showed that guinea pig sperm capacitation required Ca^{2+} and bicarbonate to support *in vitro* hyperactivation, but despite this, there is no standard guinea pig sperm capacitation protocol conventionally used to fertilize a mature egg *in vitro*.

One of the best systems to analyze the conditions and media necessary for sperm capacitation is the evaluation of fertilizing capacity [13]. This parameter encompasses several physiological processes, such as sperm-oocyte interaction, penetration, and pronuclear formation. Ideally, IVF procedures should use homologous (Ho) oocytes. Nonetheless, obtaining guinea pig oocytes in sufficient quantities for IVF can be very challenging due to the low ovulation rate [3,4] and the inefficiency of superovulation method [5]. Thus, heterologous (He) IVF may represent an effective approach to evaluate the fertilizing capacity of guinea pig sperm. Besides, He-IVF has further advantages over Ho-IVF, such as the accessibility to oocytes and the possibility of using well-developed *in vitro* maturation systems. He-IVF has been successfully performed to evaluate the ability of dolphin, horse and ram sperm to fertilize matured oocytes from mice [14,15], hamster [16,17] or cattle [18–20]. In the guinea pig, some authors have carried out studies using ZP-free oocytes [21]. However, IVF assays using ZP-free oocytes might be considered incomplete for assessing fertility, since sperm fertility can be described as the ability of the spermatozoa to bind and cross the ZP, to perform the fusion of its membrane with the oocyte's oolema, and to achieve the formation of the male pronucleus [22]. In addition, the cumulus oophorus cells are removed with the ZP, which can alter oocyte homeostasis and influence parameters such as pronuclei formation, and embryo cleavage [23,24]. Despite the apparent advantages, He-IVF using zona-intact oocytes and guinea pig sperm has never been conducted. Therefore, this study aimed to evaluate the influence of four different *in vitro* capacitation protocols of guinea pig spermatozoa analyzing both, sperm capacitation parameters and sperm ability to fertilize bovine matured oocyte.

2. Materials and methods

All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institute for Agriculture and Food Research and Technology (INIA-CSIC) and approved by the Ethics Committee for Animal experimentation of the Universidad Técnica Particular de Loja (Constitución de la República del Ecuador. 2008; Art 83. No.12) and the Spanish law for animal welfare and experimentation (RD 53/2013).

All the chemicals used in this study were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA) unless otherwise stated.

2.1. Sperm isolation and incubation of guinea pig spermatozoa

Male guinea pigs (n = 6) were fed *ad libitum* with a standard diet and maintained in a temperature and light-controlled room (23 °C; 12 h light, 12 h dark). Spermatozoa were collected from the cauda epididymis and processed individually using the following protocols: Protocol A) Guinea pig spermatozoa were obtained by flushing the lumen of one cauda epididymis with 0.154 M NaCl (2 mL per vas) at 37 °C (n = 3 replicates) as previously described by Mújica and Ruíz [25] with minor modifications. Spermatozoa were washed twice with 0.154 M NaCl (2 mL each time) by centrifugation at 600×*g* for 4 min. Before the second centrifugation, an aliquot was withdrawn to determine sperm concentration of 35×10^6 cells/mL and was incubated in minimal culture medium (MCM) containing: 102.30 mM NaCl, 1.71 mM $CaCl_2$, 25.07 mM $NaHCO_3$, 0.25 mM Na-pyruvate, 20.00 mM Na-lactate and 5.56 mM glucose. Protocol B) one epididymis (n = 3 replicates) was placed in a prewarmed 500-μL drop of M2 medium. Adipose tissue and blood vessels were carefully dissected away, and the epididymis gently minced with fine scissors and spermatozoa were incubated in 500 μL of modified human tubal fluid medium (HTF; 2.04 mM $CaCl_2 \times 2H_2O$, 101.6 mM NaCl, 4.69 mM KCl, 0.37 mM KH_2PO_4, 0.2 mM $MgSO_4 \times 7H_2O$, 21.4 mM Na-lactate, 0.33 mM Na-pyruvate, 2.78 mM glucose, 25 mM $NaHCO_3$, 100 U/mL penicillin, 50 μg/mL streptomycin SO_4 and 0.001% (w/v) phenol red) supplemented with 1% BSA. This protocol is commonly used for mice sperm capacitation [26]. For capacitation, incubation of A and B protocols was performed at 37 °C under an atmosphere of 5% CO_2 for 2 h.

For the following two protocols (C and D), the spermatozoa were collected by flushing the lumen of the cauda epididymis with 2 mL 0.154 M NaCl at 37 °C and were processed separately. Sperm were centrifuged for 10 min at 250×*g* through a gradient of 1 mL of 40% and 1 mL of 80% Bovipure® (Nidacon Laboratories AB, Göthenborg, Sweden), according to the manufacturer's instructions. The sperm pellet was isolated and washed in 3 mL of Boviwash® (Nidacon Laboratories AB, Göthenborg, Sweden) by centrifugation at 250×*g* for 5 min. The pellet was re-suspended in the remaining 300 μL of Boviwash®. Then for Protocol C) spermatozoa were incubated in 500 μL of MCM medium supplemented with 10 mg/mL heparin sodium salt (Calbiochem) (MCM-Hep) at concentration of 35×10^6 spermatozoa/mL (n = 4 replicates); while for Protocol D) spermatozoa were incubated in 500 μL of fertilization FERT-TALP medium (Merck) supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/mL fatty acid-free BSA and 10 mg/mL heparin sodium salt (Calbiochem) (FERT-Hep) at concentration of 35×10^6 spermatozoa/mL (n = 4 replicates). This protocol is commonly used for bull sperm capacitation in our lab [27–30]. For capacitation, incubation of C and D protocols were

performed at 38.5 °C under an atmosphere of 5% CO_2 for 2 h.

The evaluation of the quantitative and qualitative parameters of guinea pig sperm was performed at 0, 60 and 120 min of incubation for the 4 protocols used.

2.2. Sperm viability

Viability was evaluated by eosin-nigrosin staining. A 5 μL drop containing approximately 25,000 spermatozoa was placed on a glass slide warmed at 37 °C, mixed with 5 μL of stain solution (0.67% eosin and 10% nigrosin), incubated by 30 s and smeared, and then the slide was allowed to air dry at 37 °C. A total of 200 spermatozoa per replicate/protocol/time were counted individually using bright-field microscopy (400X). Normal live sperm exclude the eosin stain and appear white, whereas "dead" sperm (i.e. those with loss of membrane integrity) take up eosin and appear pinkish (Fig. 1A, B, C)

2.3. Sperm morphology

Semen smears were prepared, air-dried, fixed, and stained with Spermac® according to the manufacturer's instructions (Minitube). For morphological assessment, a minimum of 200 spermatozoa per replicate/protocol/time were assessed individually using bright-field microscopy (400X). Spermatozoa exhibiting normal morphology were categorized as 'normal' (Fig. 1D and E), and those exhibiting morphological abnormalities in the head were categorized as 'major anomalies' (Fig. 1F) while in the mid-piece or flagellum were categorized as 'minor anomalies' (Fig. 1G, H, I).

2.4. Acrosome reaction (AR)

For this procedure, a 20 μL sample droplet (approximately 25,000 spermatozoa) was placed on a glass slide and air-dried at 37 °C. It was then immersed for 30 s in 100% methanol at −20 °C, air-dried inside an extraction chamber, and stored in a dry atmosphere at room temperature for later processing. The slide was washed twice for 5 min in PBS, the excess PBS was removed, and 20 μL of 15 μg/mL FITC-PNA prediluted in 5 μg/mL Hoechst were added. This was incubated for 30 min in a wet chamber under darkness, washed for 10 min in double distilled water, dried at 37 °C, and mounted with FluoroMount™ (F4680). AR was quantified based on the percentage of sperm without an acrosome. Two slides were counted (200 cells/replicate/protocol/time) [31].

2.5. Motility and kinetics

Ten microliters of sperm suspension were placed in a Mackler chamber on the heated stage (37 °C) of a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) fitted with a digital camera (Basler acA1300-200uc, Basler AG, Ahrensburg, Germany). Five to eight 1.5s movies were recorded at 60 frames/s using the Pylon Viewer software provided by Basler, capturing at least 100 moving spermatozoa/replicate/protocol/time. The motility and sperm kinetics were analyzed using the free software ImageJ 1.x [32] with the plugin CASA_bmg following instructions for analyzing guinea pig spermatozoa [33,34]. The parameters analyzed were as described by Mortimer [35]: straight-line velocity (VSL; μm/s), curvilinear velocity (VCL; μm/s), average path velocity (VAP; μm/s), linearity (LIN) (defined as (VSL/VCL) × 100), straightness (STR) (defined as (VSL/VAP) × 100), wobble (WOB) (defined as (VAP/VCL) × 100), amplitude of lateral head (ALH) displacement (μm), and beat-cross frequency (BCF; Hz). In addition, we examined the percentage of spermatozoa showing more signs of hyperactivation (HYP) by determining out of all the analyzed spermatozoa, the lower VCL and

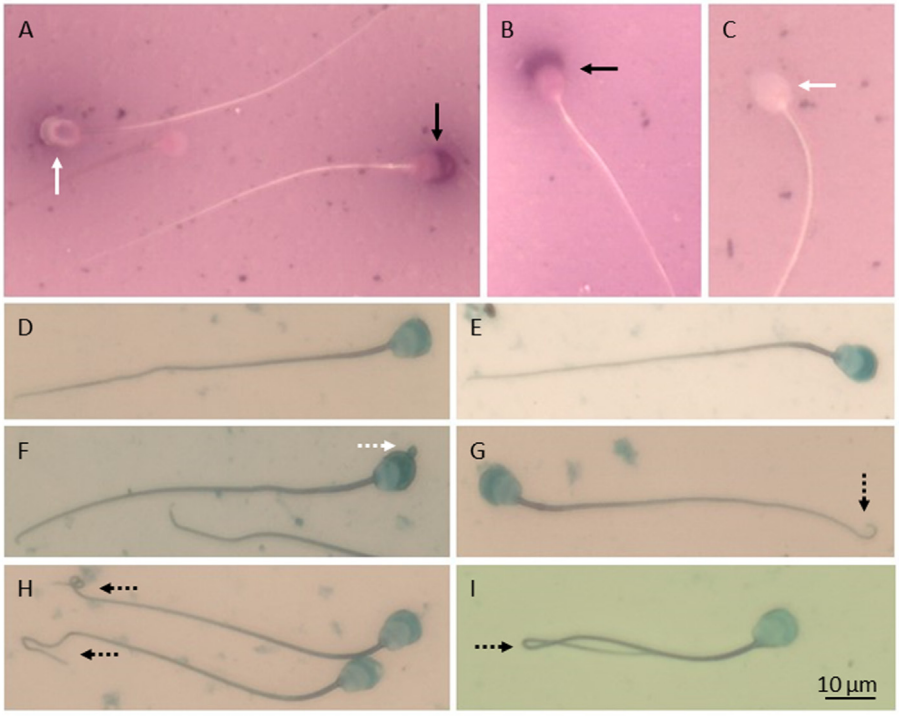

Fig. 1. Evaluation of sperm viability and morphology. (A, B, C) Representative images of eosin-nigrosin staining for the determination of guinea pig sperm viability. Normal "live" sperm exclude the eosin stain and appear white (white arrow), whereas "dead" sperm (i.e. those with loss of membrane integrity) take up eosin and appear pinkish (black arrow); (D, E) representative images of spermatozoa exhibiting normal morphology; (F) representative image of spermatozoa exhibiting morphological abnormalities in the head (major anomalies – white dotted arrow); (G, H, I) representative images of spermatozoa exhibiting morphological abnormalities in the flagellum (minor anomalies – black dotted arrow) (n = 200 per replicate/protocol/time). Images were captured on 40X objective. Scale bar 10 μm.

ALH values of the 10% of spermatozoa with the highest VCL and ALH. These values were: VCL = 250 μm/s and ALH = 9.0 μm. Thus, we defined spermatozoa showing hyperactive-like motility as those showing VCL >250 μm/s and ALH >9.0 μm [33,34].

2.6. Heterologous fertilization

2.6.1. Zona-intact bovine oocyte/guinea pig fertilization sperm assay

The guinea pig sperm samples used in this experiment were those capacitated with protocols C and D, described in section 2.1.

For bovine homologous (Ho) fertilization, frozen-thawed (in a water bath at 37 °C for 50 s) bovine sperm from a single Asturian Valley bull previously tested in IVF was used (Asturgen, Gijon, Spain). Motile sperm were selected by density gradient centrifugation (BoviPure®, Nidacon International, Sweden) and were diluted in a 30 μL droplet of FERT medium (FERT-TALP media supplemented with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/mL fatty acid-free BSA and 10 mg/mL heparin).

Four experimental groups were used: a negative control group (parthenotes: in which no sperm was added for coincubation (n = 75)), a Ho IVF group (n = 263), and two He IVF groups which included guinea pig spermatozoa obtained with C and D protocols: HeC group (n = 705) and HeD group (n = 707), respectively. For *in vitro* fertilization a total of four replicates were performed.

Immature cumulus oocyte complexes (COCs) were obtained by aspirating follicles (2–8 mm diameter) from the ovaries of mature heifers (i.e., at least one corpus luteum or remained scars from previous ovulations in one or both ovaries) collected at local slaughterhouses and matured in 30 μL droplets (~30 COCs per droplet) of maturation medium (TCM-199) supplemented with 10 ng/mL EGF and 10% (v:v) fetal calf serum (FCS) for 24 h, at 38.5 °C under an atmosphere of 5% CO_2 in air, with maximum humidity [36]. After 24 h, *in vitro* matured oocytes were washed twice in FERT-Hep medium and transferred to 30 μL droplet of MCM plus 100 U/mL penicillin, 50 μg/mL streptomycin and 10 mg/mL heparin (~30 COCs) for the HeC group (MCM-Hep) or into a 30 μL droplets of FERT-Hep (~30 COCs) for the HeD, Ho IVF and parthenogenetic groups. COCs were co-incubated for 18 h in the presence of 1×10^6 guinea pig or bovine spermatozoa/mL according to the experimental design.

After coincubation, presumptive zygotes or COCs were denuded of cumulus cells (CCs) by vortexing for 3 min and then cultured in groups of ~25 in 25 μL droplets of synthetic oviductal fluid (SOF); with 4.2 mM sodium lactate, 0.73 mM sodium pyruvate, 30 μL/mL BME amino acids, 10 μL/mL minimum essential medium (MEM) amino acids and 1 μg/mL phenol red supplemented with 5% FCS under mineral oil at 38.5 °C under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 with maximum humidity to complete a total of 48 h of incubation.

Sperm-egg interaction was assessed by sperm-zona pellucida binding assay at 2.5 hpi. For that purpose, COCs were vortexed for 3 min, fixed and stained with Hoechst 33,342 to count the number of sperm that remained bound to the ZP by widefield fluorescence microscope with structured illumination (ApoTome, Zeiss) UV-2E/C, excitation: 340e380 nm, emission: 435e485 nm). In addition, pronuclei formation was evaluated at 18, 20, 22, 24 and 26 hpi for He IVF and 18 hpi for Ho IVF. For this evaluation, presumptive zygotes were treated and examined using procedures as explained above for sperm-ZP binding. The cleavage rate was evaluated at 48 hpi in all groups. Times used in this study for hybrid embryo analysis were similar to those used in co-incubation studies in other species [15,19,37].

2.7. Experimental design

The effects of the different protocols for collection and capacitation of guinea pig sperm through sperm analysis and using heterologous (He) IVF with zona-intact bovine oocytes were examined. Spermatozoa were collected from the cauda epididymis and processed individually using the following protocols: Protocol A) Spermatozoa were obtained by flushing the lumen of one cauda epididymis and incubated in a minimal culture medium (MCM). Protocol B) One epididymis was placed in a prewarmed of M2 medium and gently minced with fine scissors. Spermatozoa were incubated in a modified human tubal fluid medium (HTF). This protocol is commonly used for mice sperm capacitation. In both protocols, the spermatozoa were capacitated at 37 °C under an atmosphere of 5% CO_2 for 2 h. For the following two protocols (C and D), the spermatozoa were collected by flushing the lumen of the cauda epididymis and selected by commercial density gradient Bovipure® and isolated and washed with Boviwash (Nidacon Laboratories AB, Göthenborg, Sweden), according to the manufacturer's instructions. Then for Protocol C) spermatozoa were incubated in MCM medium supplemented with 10 mg/mL heparin (MCM-Hep); while for Protocol D) spermatozoa were incubated in FERT medium supplemented 10 μg/mL heparin (FERT-Hep). This protocol is commonly used for bull sperm capacitation in our lab. Incubation of C and D protocols were performed at 38.5 °C under an atmosphere of 5% CO_2 for 2 h. In all protocols, sperm parameters were assessed at 0, 60 and 120 min (Fig. 2).

The fertilizing capacity of guinea pig sperm capacitated with protocol C and D (selected based on the optimal sperm quality parameters measured in the previous analysis), was tested by He-IVF with zona-intact bovine oocytes. Homologous fertilization and parthenogenesis were used as control. A total of 1750 COCs obtained from ovaries from heifers and cows at slaughter, in 4 individual replicates were used for heterologous [HeC (n = 705) or HeD (n = 707)] and homologous IVF (n = 263), or parthenogenesis (non-fertilized oocytes, n = 75). Sperm zona binding (2.5 hpi), and pronuclei formation (18, 20, 22, 24 and 26 hpi) and cleavage rate (48 hpi) were evaluated in all groups (Fig. 2).

2.8. Statistical analysis

Statistical analysis was carried out using the SigmaStat software package (Jandel Scientific, San Rafael, CA). Results are expressed as means ± standard error of the mean (SEM). Means were compared and analyzed using a repeated measures one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Significance was set at $P < 0.05$.

3. Results

3.1. The viability, morphology, and kinetics of guinea pig sperm improves by different capacitation protocols

To determine the effect of Protocol A, Protocol B, Protocol C, and Protocol D on sperm quality, we conducted a comparative analysis of viability, morphology, and sperm kinetics over 120 min of incubation. At the onset of incubation and, during the first 60 min, no significant differences in sperm viability and morphology were found between the protocols studied. However, at 120 min of incubation, protocols C and D showed better percentages of viability compared to protocols A and B ($P < 0.05$) (Table 1).

Total motility of the sperm incubated in all protocols tended to increase ($P < 0.05$) up to 60 min, after which it remained stable. When we compared the total motility between protocols for each time point we found that at 60 and 120 min increased for protocols

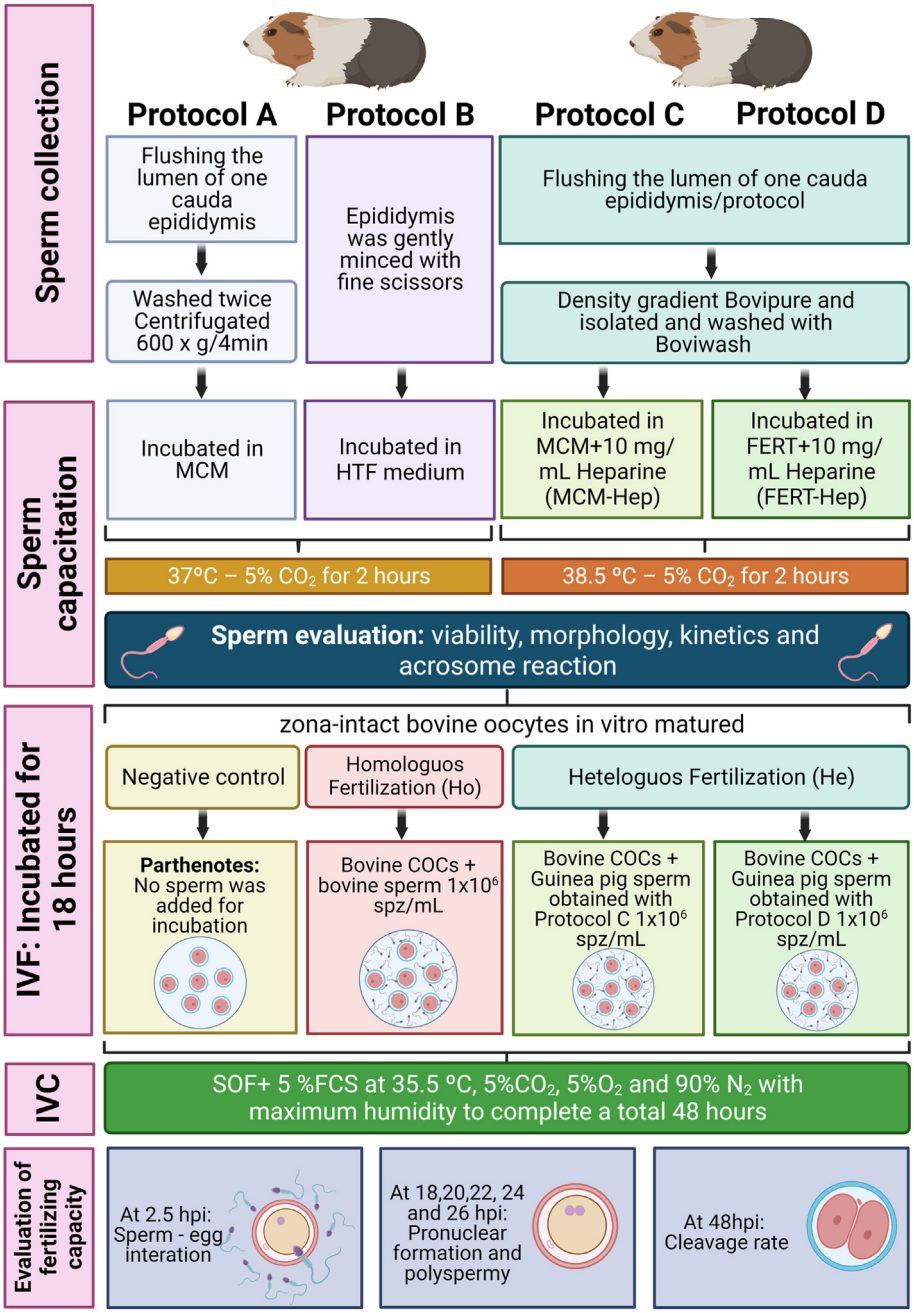

Fig. 2. Experimental design: Effects of the different protocols for collection and capacitation of guinea pig sperm through sperm analysis and using heterologous (He) IVF with zona-intact bovine oocytes. MCM: minimal culture medium. HTF: human tubal fluid medium. MCM-Hep: minimal culture medium supplemented with 10 mg/mL heparin. FERT-Hep: FERT medium supplemented 10 μg/mL heparin. Ho: Homologous fertilization. He: Heterologous fertilization. COCs: cumulus oocyte complexes. SOF: synthetic oviductal fluid. IVF: In vitro fertilization. IVC: In vitro culture.

C and D compared to protocols A and B. The progressive motility of sperm remained unchanged during all the time of incubation in the protocols A and B, and in the protocols C and D increase since to 60 min, after which it remained stable (Supplementary Table 1).

At the onset of incubation, no significant differences in sperm kinetics were found. At 120 min of incubation VCL of swimming spermatozoa were higher in Protocol D that A and B and similar that Protocol C (Supplementary Table 1). Thus, in Protocol D we detected a higher fraction of spermatozoa showing motility classified as indicating hyperactivation, hereafter referred to as hyperactive-like motility) ($P < 0.05$, Fig. 3). In addition, sperm kinetics in Protocol B returned to the initial values in VCL and ALH after 120 min, while the other parameters did not significantly vary during incubation. In A, C and D protocols sperm gradually acquired a less progressive motility type (LIN and STR reduced after 60 min), as well as a decrease in WOB and beat cross frequency (Supplementary Table 1).

These motion changes produced during incubation in Protocol A, C and D were recorded as a significant increase in the percentage of spermatozoa showing hyperactive-like motility after 60 and 120 min of incubation, recording in a higher percentage in protocol D ($P < 0.05$, Fig. 3). However, in Protocol B was recorded as a

Table 1
Percentages of viability and spermatozoa exhibiting normal morphology, morphological abnormalities in the head (major anomalies), and in flagellum (minor anomalies).

Parameter	Protocol	0 min	60 min	120 min
Viability (%)	**A**	87.7 ± 0.4	86.7 ± 0.4	84.3 ± 0.9^{X}
	B	88.5 ± 0.8	87.5 ± 0.9	86.2 ± 0.4^{X}
	C	88.9 ± 0.8	88.3 ± 0.9	89.3 ± 0.3^{Y}
	D	89.1 ± 0.4	87.9 ± 0.8	88.9 ± 0.5^{Y}
Normal morphology (%)	**A**	80.8 ± 1.6	80.5 ± 1.0	81.0 ± 1.2
	B	78.8 ± 0.7	79.3 ± 1.0	79.2 ± 0.7
	C	79.4 ± 2.0	78.5 ± 1.9	77.9 ± 1.9
	D	80.8 ± 3.2	78.5 ± 1.8	77.8 ± 7.2
Major anomalies (%)	**A**	7.0 ± 0.6	7.5 ± 0.3	8.0 ± 0.5
	B	7.5 ± 0.3	6.0 ± 0.6	7.0 ± 0.3
	C	7.6 ± 0.9	6.4 ± 1.0	7.5 ± 1.2
	D	6.9 ± 1.3	9.5 ± 0.8	9.8 ± 1.6
Minor anomalies (%)	**A**	12.2 ± 1.2	12.0 ± 1.3	11.0 ± 1.6
	B	13.7 ± 1.0	14.7 ± 0.9	13.8 ± 0.7
	C	13.0 ± 1.3	15.1 ± 1.0	14.6 ± 0.7
	D	12.3 ± 1.9	12.0 ± 2.2	12.5 ± 2.5

Results are expressed as mean ± SEM ($P < 0.05$, n = 3). Different capital letters (X,Y) indicate significant differences between protocols for each time point.

significant increase in the percentage of spermatozoa showing hyperactive-like motility after 60 min, but after 120 min, hyperactive-like motility returned to the levels observed at the start of incubation ($P < 0.05$).

3.2. Spontaneous acrosome reaction is induced by different protocols in guinea pig spermatozoa

At the onset of incubation and after 60 min of incubation no significant differences were found between the protocols. After 120 min incubation, the percentage of spontaneous acrosome reaction in the protocol D (28.4 ± 2.1) was significantly higher than in A (12.3 ± 1.0), B (18.8 ± 0.7) and C (20.4 ± 2.0) protocols ($P < 0.05$), respectively. In addition, within the times for each protocol, the percentage of spontaneous acrosome reaction increased after 60 min, but between 60 and 120 min only significant differences were observed for protocol D (Fig. 4).

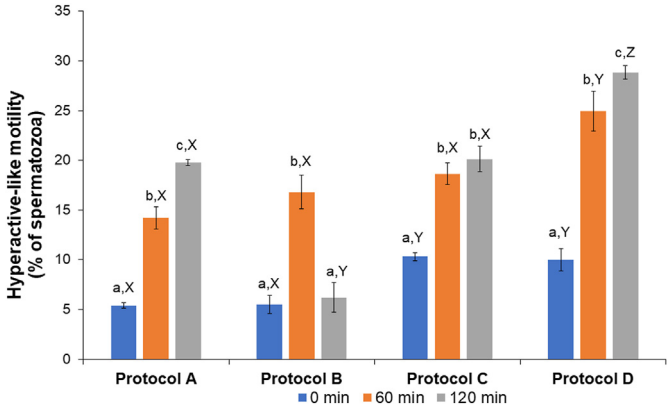

Fig. 3. Percentage of spermatozoa showing hyperactive-like motility. Kinetics were examined at the time 0, 60 and 120 min of incubation in A, B, C and D protocols. Spermatozoa showing hyperactive-like motility were defined as those showing VCL >250 μm/s and ALH >9.0 μm. Different lowercase letters indicate significant differences between time points for each protocol ($P < 0.05$, ANOVA). Different capital letters (X, Y, Z) indicate significant differences between protocols for each time point ($P < 0.05$).

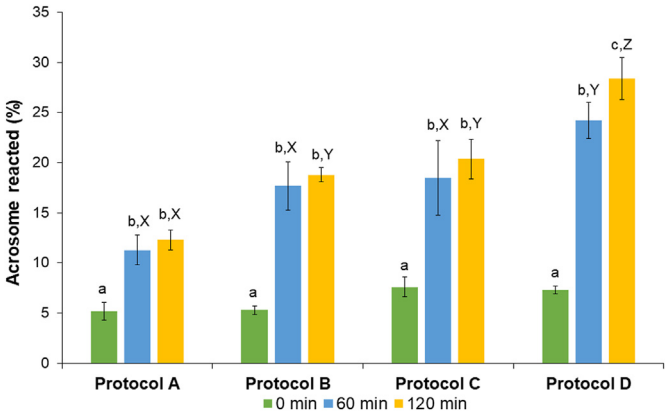

Fig. 4. Percentage of spermatozoa showing spontaneous acrosome reaction (AR). AR was examined at the time 0, 60 and 120 min of incubation in A, B, C and D protocols. Different lowercase letters indicate significant differences between time points for each protocol ($P < 0.05$, ANOVA). Different capital letters (X, Y, Z) indicate significant differences between protocols for each time point ($P < 0.05$).

3.3. Guinea pig spermatozoa capacitated with protocols C and D can fertilize bovine oocytes

Only the experimental groups (Protocol C and Protocol D) that showed better sperm viability, kinetics, and spontaneous acrosome reaction in the previous experiments (Section 3.1 and 3.2) were used to evaluate the fertilizing capacity of the guinea pig sperm.

Results of He-IVF are depicted in Fig. 5 and values are provided in Table 2. The number of bound sperm were similar (Ho = 0.5 ± 0.1; HeC = 0.4 ± 0.1; HeD = 0.4 ± 0.1). Heterologous IVF showed that guinea pig sperm obtained with protocol C or D, were equally capable of penetrating bovine oocytes, leading to pronuclear formation and hybrid embryo cleavage. Significant differences were seen in pronuclei formation (18, 20, 22, 24 and 26 hpi) or cleavage rate (48 hpi) between C and D protocols, with higher rates for protocol D ($P < 0.05$). Similar to the previous experiment, Ho-IVF was associated with higher percentages of pronuclei formation at 18 hpi compared to He-IVF. As expected, the cleavage rate was higher ($P < 0.05$) in Ho-IVF than He-IVF at 48 hpi. There was a spontaneous parthenogenetic activation rate of 4.2 ± 2.5% in mature unfertilized bovine oocytes at 48 hpi.

4. Discussion

The results of this study showed that using a density gradient in guinea pig sperm selection protocol and capacitation with heparin-supplemented FERT medium (Protocol D) preserves sperm motility, acrosome and morphological integrity, and promotes hypermotility better than when using the protocols, A, B and C. Likewise, the present work revealed the ability of guinea pig spermatozoa to penetrate zona-intact bovine oocytes and generate hybrid embryos.

All protocols used did not have a negative effect on the viability and morphology of guinea pig sperm. However, incubation conditions in Protocols A, C and D led to a time-dependent effect on sperm kinetics whereby hyperactive-type motility was acquired by a fraction of the spermatozoa. This effect was not observed when spermatozoa were incubated in HTF medium (Protocol B). Further, although all media significantly increased AR levels after 120 min of incubation, higher levels were attained with Protocol D. The AR results were similar to those reported by Delgado-Buenrostro et al. [38], whose study after 2 h of incubation in MCM of guinea pig sperm showed a spontaneous acrosome-reacted cells, that is, spermatozoa without acrosome and with hyperactivated motility,

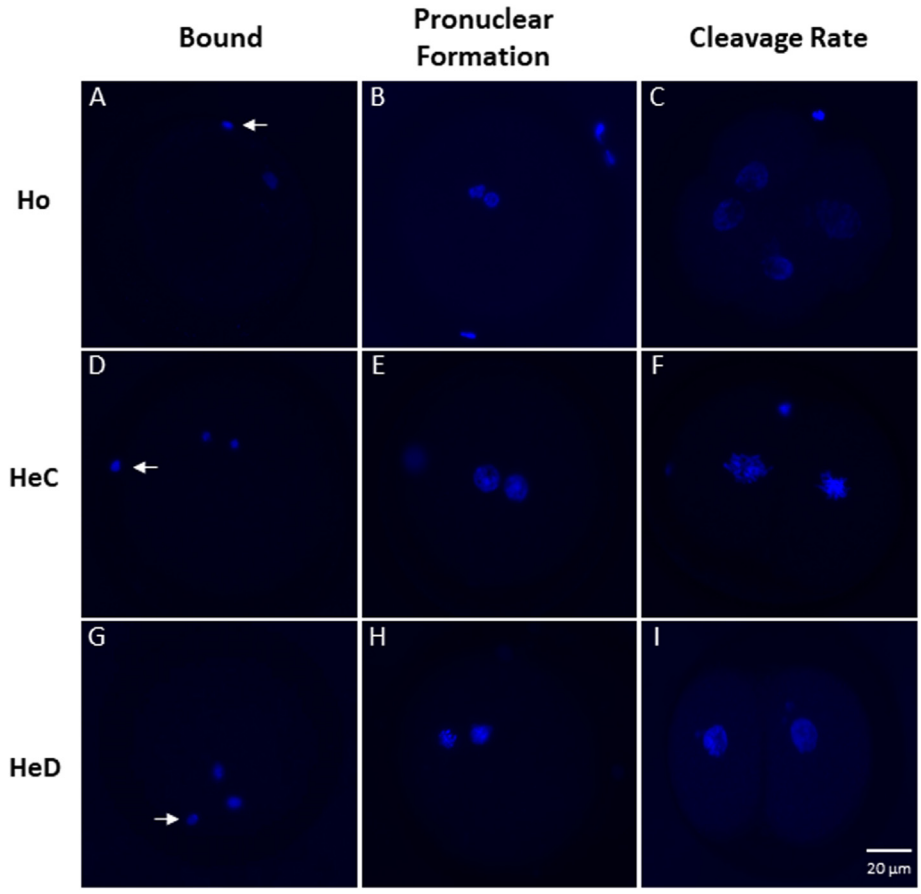

Fig. 5. Evaluation of sperm-oocyte binding, pronuclear formation, and cleavage after homologous (Ho) (bovine sperm) and heterologous (He) (guinea pig sperm obtained with protocol C (HeC) and D (HeD)) IVF. Gametes were stained with Hoechst 33,342. Ho: Bound bovine sperm after 2.5 h of coincubation with zona-intact bovine oocytes (A); pronuclei formation after 6 hpi (B); and embryo cleavage after 48 hpi (C). HeC and HeD: Bound guinea pig sperm after 2.5 h of gametes coincubation (D, G); pronuclei formation after 18 hpi (E, H); and hybrid-embryo cleavage after 48 hpi (F, I), respectively. Arrow indicates sperm head chromatin. Images were captured on 63X objective. Scale bar 20 µm.

while that were no observed acrosome-reacted spermatozoa in Tyrodes-capacitated sperm at 45 min of incubation. In this sense, the differences between both studies could be explained by the use of different supplements in the media (protocol D contain heparin and not glucose), or the different times analyzed (45 min vs 60 and 120 min) or selection sperm method.

In our study, the media differ to a greater extent in the amount of glucose and pyruvate that they contain. For example, MCM, MCM-Hep, and HTF media have ~3 to 4 times less concentration of pyruvate than FERT-Hep medium. In effect, pyruvate is one of the main sources of energy utilized by guinea pig spermatozoa [39–42]. Hereng et al. [43] demonstrated that exogenous pyruvate enhances ATP production because of its ability to regenerate NAD + following its conversion to lactate and, therefore, promotes capacitation in human spermatozoa. In addition, it has been demonstrated that a higher concentration of an energy source that can be utilized for the mitochondria activity, enhancing reactive oxygen species production and its associated hyperactive-like motility [44,45]. Hence, in Protocol D a greater mitochondrial activity could explain the higher VCL and hyperactive-like motility

Table 2
Rates of pronucleus formation and cleavage after homologous (bovine sperm) and heterologous (guinea pig sperm obtained with protocol C (HeC) and D (HeD)) coincubation with bovine oocytes at different hours post-insemination (hpi). "N" refer to the total number of oocytes fertilized per treatment or no-fertilized for parthenotic; "n" refers to the total number of fertilized oocytes/presumptive zygotes or no-fertilized (partenotic) evaluated at each time point to assess Bound sperm, Pronuclear formation and Cleavage rate.

Semen Groups N	Bound sperm	Pronuclear formation (%)					Cleavage rate (%)
	2.5 hpi n	18 hpi n	20 hpi n	22 hpi n	24hpi n	26 hpi n	48 hpi n
263 Ho	54 (0.5 ± 0.1)	98 (77.6 ± 2.0)^{a}					111 (83.5 ± 0.7)^{a}
705 HeC	52 (0.4 ± 0.1)	102 (45.3 ± 2.5)^{c}	106 (46.2 ± 1.1)^{b}	104 (45.4 ± 4.2)^{b}	102 (47.2 ± 4.2)^{b}	103 (45.3 ± 5.7)^{b}	136 (49.1 ± 1.1)^{c}
707 HeD	50 (0.4 ± 0.1)	104 (58.8 ± 1.9)^{b}	101 (58.2 ± 1.5)^{a}	106 (61.5 ± 4.2)^{a}	108 (64.4 ± 4.9)^{a}	107 (64.9 ± 2.1)^{a}	131 (69.8 ± 1.7)^{b}
75 Parthenotic							75 (4.2 ± 2.5)^{d}

Ho = homologous *in vitro* fertilization with bovine sperm; HeC = heterologous *in vitro* fertilization with guinea pig sperm using protocol C; HeD = heterologous *in vitro* fertilization with guinea pig sperm using protocol D; Parthenogenic = non-fertilized oocytes.
Bound sperm was expressed as the average number of spermatozoa that remained bound to the ZP. Pronuclear formation was expressed as the percentage of oocytes with two pronuclei at each time point over the number (n) of oocytes evaluated. Cleavage rate was expressed as the percentage of ≥2 cell embryos over the number (n) of fertilized or no-fertilized (parthenotic) oocytes. Different superscripts (a, b, c, d) in the same column indicate differences ($P < 0.05$; n = 4 replicates). Values are expressed as mean ± SEM.

observed from the onset of incubation until 120 min. Lactate is also an energy source used for sperm capacitation, including the guinea pig. In our study, all the media contained lactate and in a slightly higher amount in the FERT-Hep medium. It is also important to point out that lactate is the most abundant source of energy within the oviduct where capacitation, and thus hyperactivation, is triggered *in vivo* [46].

Conversely, FERT-Hep medium does not have glucose, which has been shown to have an inhibiting effect on sperm capacitation in some species such as bovine [10], and canine [47]. However, guinea pig studies are controversial. While the first interpretation of Rogers et al. [48] was that glucose retards sperm capacitation, the results of Mújica and Ruíz [25], did not support it by demonstrated that glucose did not retard sperm capacitation, because no significant difference in the percentage of acrosome-reacted spermatozoa was observed between the sperm that pre-incubated in medium with glucose and then transferred to MCM and their corresponding MCM incubated controls. When sperm were capacitated in a glucose-free medium, sperm activation was inhibited to various degrees, thereby suggesting a key role of this hexose for capacitation, motility hyperactivation, and oocyte-fusion in mammalian spermatozoa including humans [43,49–51]. In the sperm cells, an important regulatory point of glycolysis, besides the control of sugar uptake, is the hexose phosphorylation, since monosaccharides have to be converted into glucose 6-phosphate, before being introduced into the general sperm metabolic system [52,53]. This way, the kinetics and specificity of hexose kinases are able to modulate glucose phosphorylation in sperm cells. Taking these results together, we can suggest that the guinea pig may metabolize different types of energy through the various metabolic pathways for sperm capacitation.

In our study, heparin is another compound found exclusively in Protocol C and D. Heparin can bind to sperm, removes decapacitating factors from the sperm plasmalemma, or acts in the membrane, to facilitate the entry of ions, especially calcium, inducing changes in the intracellular environment [8,11]. Because we used epididymal sperm, there are no decapacitating factors from seminal plasm, for this reason, the effect of the hearing should be related to membrane alterations and Ca^{2+} intake that leads to an increase in the intracellular pH and consequently hyperactivation [54,55]. This response was observed to be the best in guinea pig sperm capacitated in Protocol D than in Protocol C. Thus, in guinea pig sperm the heparin can have a capacitating effect that may be dimmed by the interaction of glucose, but more studies are needed to address this hypothesis.

Another different parameter between protocols (A and B vs C and D) were the incubation temperature and the sperm selection method. In our results, there was no clear indication that the 1.5 °C variation in incubation temperature influenced capacitation. On the other hand, in Procotol C and D sperm selection method by density-gradient centrifugation (BoviPure®) were used. This method could contribute to the improvement of the parameters evaluated how has been demonstrated in bovine by Samardzija et al. [56] when BoviPure® was used by sperm selection resulted in spermatozoa showing a greater progressive motility and viability, as well as a higher blastocyst yield. Then, Arias et al. [57] reported that BoviPure® density gradient is a good alternative for bovine sperm separation, since important sperm quality parameters, such as DNA integrity, plasma and acrosomal membrane integrity and gene expression, are not affected. In canine, the use of BoviPure® enhanced the motilities, velocities, movement trajectory, and integrity of plasma and acrosome membranes of fresh, chilled, and frozen-thawed dog epididymal sperm Galarza et al. [58].

Once the two protocols that produce the best sperm capacitation results were selected, protocol C (including MCM medium previously used in guinea pig) and protocol D (FERT-Hep medium commonly used in bovine IVF), we performed a He-IVF test to determine which of the two produces the best fertilizing results. Heterologous fertilization was evident by the presence of 2 pronuclei and polar bodies in bovine oocytes, indicating that guinea pig sperm can activate the oocyte, in a process that includes the completion of meiosis and pronuclei formation. We observed that guinea pig spermatozoa were able to bind bovine oocytes after 2.5 h of co-incubation in both capacitation media used. These results agree with previous He-IVF studies realized in ZP-free hamster eggs. In the first Sánchez-Gutiérrez et al. [21], demonstrated that ZP-free hamster eggs were fertilized after being co-incubated for 4 h with procaine-treated guinea pig spermatozoa. In the second study, Chen et al. [39] used complete or Cl− -deficient MCM for guinea pig spermatozoa capacitation and they found that spermatozoa penetration to oocytes (zona-free hamster eggs) and fertilizing was reduced with decreased Cl− concentrations, indicating that Cl− is required to capacitation and fertilization [39,59]. In the third, Bhattacharyya [60] determined that guinea pig spermatozoa are able of having an acrosomal reaction and fertilize the zona-free egg, in the absence of albumin or other synthetic macromolecules. However, our study is the first to use an oocyte with an intact ZP to analyze guinea pig spermatozoa He-IVF, so the sperm must bind and cross the ZP before binding to the plasma membrane of the oocyte.

The high pronuclei formation at 24 and 26 h (64.6%) obtained when guinea pig spermatozoa were capacitated with Protocol D is probably due to the composition of the medium, basically the combination of FERT medium and heparin. Thus, our results suggested that the physiological and biochemical changes produced by heparin could be important to the formation of pronuclei. Sánchez-Vázquez et al. [9] observed that in guinea pig sperm that heparin and reduced glutathione have the ability to decondense the nuclei and this decondensation of sperm nuclei is a prerequisite for the removal of sperm-specific chromatin-associated proteins (protamines and/or histones), this process then is followed by immediate replacement with egg-derived histones before the final restructuring of the male pronucleus. Furthermore, as suggested in some studies of He-IVF, the relationship between chromatin stability, protamination, and fertility would provide essential information on chromatin status, which is expected to have a strong prognostic value on sperm function [15,19,61]. Taking this into account, we can suggest that heparin could affect early development by better capacitation and acrosome reaction without altering DNA fragmentation of guinea pig sperm, making it easier for pronuclei formation.

In conclusion, the results of the present study showed that using a density gradient in guinea pig sperm selection and the capacitation with different sources of energy preserves sperm motility, morphological integrity, promoting acrosome reaction, and hypermotility. Besides, when FERT medium is supplemented with heparin it improved all sperm capacitation parameters. This suggests that the guinea pig may metabolize different types of energy through the various metabolic pathways for sperm capacitation. Furthermore, our results revealed the ability of guinea pig spermatozoa to penetrate zona-intact bovine oocytes and generate hybrid embryos in *in vitro* conditions, supporting its potential use to assess the fertilizing ability of guinea pig spermatozoa obtained from the epididymis.

CRediT authorship contribution statement

Karina Cañón-Beltrán: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Yulia N. Cajas:** Methodology, Investigation, Writing – review & editing.

Encina González: Methodology. **Raúl Fernández-González:** Methodology. **Natacha Fierro:** Methodology. **Pedro L. Lorenzo:** Conceptualization. **María Arias-Álvarez:** Conceptualization, Methodology. **Rosa M. García-García:** Conceptualization, Methodology. **Alfonso Gutiérrez-Adán:** Conceptualization, Writing – review & editing. **Dimitrios Rizos:** Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2022.12.042.

References

[1] Producción de cuyes (Cavia porcellus) en los países andinos n.d. https://www.fao.org/3/V6200T/v6200T05.htm (accessed December 6, 2022).
[2] Avilés DF, Martínez AM, Landi V, Delgado JV. El cuy (Cavia porcellus): un recurso andino de interés agroalimentario the Guinea pig (Cavia porcellus): an Andean resource of interest as an agricultural food source. Anim Genet Resour Génétiques Anim Genéticos Anim 2014;55:87–91. https://doi.org/10.1017/S2078633614000368.
[3] Suzuki O, Koura M, Noguchi Y, Takano K, Yamamoto Y, Matsuda J. Optimization of superovulation induction by human menopausal gonadotropin in Guinea pigs based on follicular waves and FSH-receptor homologies. Mol Reprod Dev 2003;64:219–25. https://doi.org/10.1002/mrd.10242.
[4] Sadeu JC, Adriaens I, Cortvrindt R, Smitz J. Study of folliculogenesis in vivo in Guinea pig. Theriogenology 2007;68:1228–39. https://doi.org/10.1016/j.theriogenology.2007.08.028.
[5] Grégoire A, Allard A, Huamán E, León S, Silva RM, Buff S, et al. Control of the estrous cycle in Guinea-pig (Cavia porcellus). Theriogenology 2012;78:842–7. https://doi.org/10.1016/j.theriogenology.2012.03.034.
[6] Yanagimachi R. Fertility of mammalian spermatozoa: its development and relativity. Zygote Camb Engl 1994;2:371–2. https://doi.org/10.1017/s0967199400002240.
[7] Hiroshi H, Seishiro K. Factors regulating changes of head-to-head agglutinability in boar spermatozoa during epididymal transit and capacitation in vitro - review-. Asian-Australas J Anim Sci 2001;14:1196–202. https://doi.org/10.5713/ajas.2001.1196.
[8] Parrish JJ. Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin. Theriogenology 2014;81. https://doi.org/10.1016/j.theriogenology.2013.08.005. 10.1016/j.theriogenology.2013.08.005.
[9] Sánchez-Vázquez ML, Reyes R, Ramírez G, Merchant-Larios H, Rosado A, Delgado NM. DNA unpacking in Guinea pig sperm chromatin by heparin and reduced glutathione. Arch Androl 1998;40:15–28. https://doi.org/10.3109/01485019808987924.
[10] Parrish JJ, Susko-Parrish JL, First NL. Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. Biol Reprod 1989;41:683–99. https://doi.org/10.1095/biolreprod41.4.683.
[11] Li G-P, Seidel GE, Squires EL. Improved cleavage of bovine ICSI ova cultured in heparin-containing medium. Theriogenology 2004;61:1077–84. https://doi.org/10.1016/j.theriogenology.2003.05.005.
[12] Huang J, Wang G, Kong L. Effects of Ca2+ and HCO3- on capacitation, hyperactivation and protein tyrosine phosphorylation in Guinea pig spermatozoa. Asian-Australas J Anim Sci 2009;22:181–6.
[13] Brahmkshtri BP, Edwin MJ, John MC, Nainar AM, Krishnan AR. Relative efficacy of conventional sperm parameters and sperm penetration bioassay to assess bull fertility in vitro. Anim Reprod Sci 1999;54:159–68. https://doi.org/10.1016/s0378-4320(98)00108-0.
[14] Liang LF, Dean J. Conservation of mammalian secondary sperm receptor genes enables the promoter of the human gene to function in mouse oocytes. Dev Biol 1993;156:399–408. https://doi.org/10.1006/dbio.1993.1087.
[15] Sánchez-Calabuig MJ, de la Fuente J, Laguna-Barraza R, Beltrán-Breña P, Martínez-Nevado E, Johnston SD, et al. Heterologous murine and bovine IVF using bottlenose dolphin (Tursiops truncatus) spermatozoa. Theriogenology 2015;84:983–94. https://doi.org/10.1016/j.theriogenology.2015.06.001.
[16] Berger T, Parker K. Modification of the zona-free hamster ova bioassay of boar sperm fertility and correlation with in vivo fertility. Gamete Res 1989;22:385–97. https://doi.org/10.1002/mrd.1120220405.
[17] Matsukawa K, Takahashi M, Hanada A. Assay for penetrability of frozen-thawed stallion spermatozoa into zona-free hamster oocytes. J Reprod Dev 2002;48:539–44. https://doi.org/10.1262/jrd.48.539.
[18] Al-Essawe EM, Wallgren M, Wulf M, Aurich C, Macías-García B, Sjunnesson Y, et al. Seminal plasma influences the fertilizing potential of cryopreserved stallion sperm. Theriogenology 2018;115:99–107. https://doi.org/10.1016/j.theriogenology.2018.04.021.
[19] Galarza DA, Ladrón de Guevara M, Beltrán-Breña P, Sánchez-Calabuig MJ, Rizos D, López-Sebastián A, et al. Influence of sperm filtration and the addition of glycerol to UHT skimmed milk- and TEST-based extenders on the quality and fertilizing capacity of chilled ram sperm. Theriogenology 2019;133:29–37. https://doi.org/10.1016/j.theriogenology.2019.04.027.
[20] Consuegra C, Crespo F, Dorado J, Diaz-Jimenez M, Pereira B, Sánchez-Calabuig MJ, et al. Fertilizing capacity of vitrified stallion sperm assessed utilizing heterologous IVF after different semen warming procedures. Anim Reprod Sci 2020;223:106627. https://doi.org/10.1016/j.anireprosci.2020.106627.
[21] Sánchez-Gutiérrez M, Delgado-Buenrostro NL, Zárate-Grande M, Uribe S, Mújica A. In Guinea pig spermatozoa, the procaine-promoted synchronous acrosome reaction results in highly fertile cells exhibiting normal F-actin distribution. Reprod Toxicol Elmsford N 2006;21:208–15. https://doi.org/10.1016/j.reprotox.2005.08.007.
[22] Levron J, Willadsen S, Munné S, Cohen J. Formation of male pronuclei in partitioned human oocytes. Biol Reprod 1995;53:209–13. https://doi.org/10.1095/biolreprod53.1.209.
[23] Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol Reprod Dev 2002;61:414–24. https://doi.org/10.1002/mrd.10102.
[24] Tanihara F, Nakai M, Kaneko H, Noguchi J, Otoi T, Kikuchi K. Evaluation of zona pellucida function for sperm penetration during in vitro fertilization in pigs. J Reprod Dev 2013;59:385–92. https://doi.org/10.1262/jrd.2013-021.
[25] Mújica A, Ruíz MAV. On the role of glucose in capacitation and acrosomal reaction of Guinea pig sperm. Gamete Res 1983;8:335–44. https://doi.org/10.1002/mrd.1120080404.
[26] Hourcade JD, Pérez-Crespo M, Fernández-González R, Pintado B, Gutiérrez-Adán A. Selection against spermatozoa with fragmented DNA after post-ovulatory mating depends on the type of damage. Reprod Biol Endocrinol 2010;8:9. https://doi.org/10.1186/1477-7827-8-9.
[27] Lopera-Vasquez R, Hamdi M, Maillo V, Lloreda V, Coy P, Gutierrez-Adan A, et al. Effect of bovine oviductal fluid on development and quality of bovine embryos produced in vitro. Reprod Fertil Dev 2017;29:621–9. https://doi.org/10.1071/RD15238.
[28] Hamdi M, Lopera-Vasquez R, Maillo V, Sanchez-Calabuig MJ, Núnez C, Gutierrez-Adan A, et al. Bovine oviductal and uterine fluid support in vitro embryo development. Reprod Fertil Dev 2018;30:935–45. https://doi.org/10.1071/RD17286.
[29] Cañón-Beltrán K, Giraldo-Giraldo J, Cajas YN, Beltrán-Breña P, Hidalgo CO, Vásquez N, et al. Inhibiting diacylglycerol acyltransferase-1 reduces lipid biosynthesis in bovine blastocysts produced in vitro. Theriogenology 2020;158:267–76. https://doi.org/10.1016/j.theriogenology.2020.09.014.
[30] Sánchez-Calabuig MJ, García-Vázquez FA, Laguna-Barraza R, Barros-García C, García-Parraga D, Rizos D, et al. Bottlenose dolphin (Tursiops truncatus) spermatozoa: collection, cryopreservation, and heterologous in vitro fertilization. J Vis Exp JoVE 2017. https://doi.org/10.3791/55237.
[31] Ruiz-Díaz S, Oseguera-López I, De La Cuesta-Díaz D, García-López B, Serres C, Sanchez-Calabuig MJ, et al. The presence of D-penicillamine during the in vitro capacitation of stallion spermatozoa prolongs hyperactive-like motility and allows for sperm selection by thermotaxis. Animals 2020;10:1467. https://doi.org/10.3390/ani10091467.
[32] Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671–5.
[33] Schroer SC, Yudin AI, Myles DG, Overstreet JW. Acrosomal status and motility of Guinea pig spermatozoa during in vitro penetration of the cumulus oophorus. Zygote 2000;8:107–17. https://doi.org/10.1017/S0967199400000885.
[34] Cordero-Martínez J, Reyes-Miguel T, Rodríguez-Páez L, Garduño-Siciliano L, Maldonado-García D, Roa-Espitia AL, et al. TMEM16A inhibition impedes capacitation and acquisition of hyperactivated motility in Guinea pig sperm. J Cell Biochem 2018;119:5944–59. https://doi.org/10.1002/jcb.26789.
[35] Mortimer ST. CASA—practical aspects. J Androl 2000;21:515–24. https://doi.org/10.1002/j.1939-4640.2000.tb02116.x.
[36] Cajas YN, Cañón-Beltrán K, Ladrón de Guevara M, Millán de la Blanca MG, Ramos-Ibeas P, Gutiérrez-Adán A, et al. Antioxidant nobiletin enhances oocyte

maturation and subsequent embryo development and quality. Int J Mol Sci 2020;21:5340. https://doi.org/10.3390/ijms21155340.
[37] Sessions-Bresnahan DR, Graham JK, Carnevale EM. Validation of a heterologous fertilization assay and comparison of fertilization rates of equine oocytes using in vitro fertilization, perivitelline, and intracytoplasmic sperm injections. Theriogenology 2014;82:274–82. https://doi.org/10.1016/j.theriogenology.2014.04.002.
[38] Delgado-Buenrostro NL, Hernández-González EO, Segura-Nieto M, Mújica A. Actin polymerization in the equatorial and postacrosomal regions of Guinea pig spermatozoa during the acrosome reaction is regulated by G proteins. Mol Reprod Dev 2005;70:198–210. https://doi.org/10.1002/mrd.20192.
[39] Chen WY, Xu WM, Chen ZH, Ni Y, Yuan YY, Zhou SC, et al. Cl- is required for HCO3- entry necessary for sperm capacitation in Guinea pig: involvement of a Cl-/HCO3- exchanger (SLC26A3) and CFTR. Biol Reprod 2009;80:115–23. https://doi.org/10.1095/biolreprod.108.068528.
[40] Kim K-S, Foster JA, Kvasnicka KW, Gerton GL. Transitional states of acrosomal exocytosis and proteolytic processing of the acrosomal matrix in Guinea pig sperm. Mol Reprod Dev 2011;78:930–41. https://doi.org/10.1002/mrd.21387.
[41] Pastén K, Bastian Y, Roa-Espitia AL, Maldonado-García D, Mendoza-Hernández G, Ortiz-García CI, et al. ADAM15 participates in fertilization through a physical interaction with acrogranin. Reproduction 2014;148:623–34. https://doi.org/10.1530/REP-14-0179.
[42] Ramírez-Ramírez D, Salgado-Lucio ML, Roa-Espitia AL, Fierro R, González-Márquez H, Cordero-Martínez J, et al. Rac1 is necessary for capacitation and acrosome reaction in Guinea pig spermatozoa. J Cell Biochem 2020;121:2864–76. https://doi.org/10.1002/jcb.29521.
[43] Hereng TH, Elgstøen KBP, Cederkvist FH, Eide L, Jahnsen T, Skålhegg BS, et al. Exogenous pyruvate accelerates glycolysis and promotes capacitation in human spermatozoa. Hum Reprod Oxf Engl 2011;26:3249–63. https://doi.org/10.1093/humrep/der317.
[44] Darr CR, Varner DD, Teague S, Cortopassi GA, Datta S, Meyers SA. Lactate and pyruvate are major sources of energy for stallion sperm with dose effects on mitochondrial function, motility, and ROS production. Biol Reprod 2016;95:34. https://doi.org/10.1095/biolreprod.116.140707.
[45] Jane Rogers B, Yanagimachi R. Retardation of Guinea pig sperm acrosome reaction by glucose: the possible importance of pyruvate and lactate metabolism in capacitation and the acrosome reaction. Biol Reprod 1975;13:568–75. https://doi.org/10.1095/biolreprod13.5.568.
[46] Pérez-Cerezales S, Ramos-Ibeas P, Acuña OS, Avilés M, Coy P, Rizos D, et al. The oviduct: from sperm selection to the epigenetic landscape of the embryo. Biol Reprod 2018;98:262–76. https://doi.org/10.1093/biolre/iox173.
[47] Albarracín JL, Mogas T, Palomo MJ, Peña A, Rigau T, Rodríguez-Gil JE. In vitro capacitation and acrosome reaction of dog spermatozoa can be feasibly attained in a defined medium without glucose. Reprod Domest Anim Zuchthyg 2004;39:129–35. https://doi.org/10.1111/j.1439-0531.2004.00485.x.
[48] Rogers BJ, Chang L, Yanagimachi R. Glucose effect on respiration: possible mechanism for capacitation in Guinea pig spermatozoa. J Exp Zool 1979;207:107–12. https://doi.org/10.1002/jez.1402070111.
[49] Fraser LR, Herod JE. Expression of capacitation-dependent changes in chlortetracycline fluorescence patterns in mouse spermatozoa requires a suitable glycolysable substrate. J Reprod Fertil 1990;88:611–21. https://doi.org/10.1530/jrf.0.0880611.
[50] Mukai C, Okuno M. Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. Biol Reprod 2004;71:540–7. https://doi.org/10.1095/biolreprod.103.026054.
[51] Williams AC, Ford WC. The role of glucose in supporting motility and capacitation in human spermatozoa. J Androl 2001;22:680–95.
[52] Fernández-Novell JM, Ballester J, Medrano A, Otaegui PJ, Rigau T, Guinovart JJ, et al. The presence of a high-Km hexokinase activity in dog, but not in boar, sperm. FEBS Lett 2004;570:211–6. https://doi.org/10.1016/j.febslet.2004.06.015.
[53] Medrano A, García-Gil N, Ramió L, Montserrat Rivera M, Fernández-Novell JM, Ramírez A, et al. Hexose-specificity of hexokinase and ADP-dependence of pyruvate kinase play important roles in the control of monosaccharide utilization in freshly diluted boar spermatozoa. Mol Reprod Dev 2006;73:1179–94. https://doi.org/10.1002/mrd.20480.
[54] Parrish JJ. Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin. Theriogenology 2014;81:67–73. https://doi.org/10.1016/j.theriogenology.2013.08.005.
[55] Gangwar D, Atreja S. Signalling events and associated pathways related to the mammalian sperm capacitation. Reprod Domest Anim 2015;50:705–11. https://doi.org/10.1111/rda.12541.
[56] Samardzija M, Karadjole M, Getz I, Makek Z, Cergolj M, Dobranic T. Effects of bovine spermatozoa preparation on embryonic development in vitro. Reprod Biol Endocrinol 2006;4:58. https://doi.org/10.1186/1477-7827-4-58.
[57] Arias ME, Andara K, Briones E, Felmer R. Bovine sperm separation by Swim-up and density gradients (Percoll and BoviPure): effect on sperm quality, function and gene expression. Reprod Biol 2017;17:126–32. https://doi.org/10.1016/j.repbio.2017.03.002.
[58] Galarza DA, Jara DI, Paredes EB, Samaniego JX, Méndez MS, Soria ME, et al. BoviPure® density-gradient centrifugation procedure enhances the quality of fresh and cryopreserved dog epididymal spermatozoa. Anim Reprod Sci 2022;242:107003. https://doi.org/10.1016/j.anireprosci.2022.107003.
[59] Wertheimer EV, Salicioni AM, Liu W, Trevino CL, Chavez J, Hernández-González EO, et al. Chloride Is essential for capacitation and for the capacitation-associated increase in tyrosine phosphorylation. J Biol Chem 2008;283:35539–50. https://doi.org/10.1074/jbc.M804586200.
[60] Bhattacharyya A. Albumin is required for the Guinea pig sperm capacitation but is not essential for acrosome reaction and fusion with eggs. Arch Androl 1992;28:235–9. https://doi.org/10.3109/01485019208987702.
[61] García-Álvarez O, Maroto-Morales A, Martínez-Pastor F, Fernández-Santos MR, Esteso MC, Pérez-Guzmán MD, et al. Heterologous in vitro fertilization is a good procedure to assess the fertility of thawed ram spermatozoa. Theriogenology 2009;71:643–50. https://doi.org/10.1016/j.theriogenology.2008.09.036.