

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

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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, Gothenborg, 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% CO₂ 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% CO₂ 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 ($69.8 \pm 1.7\%$) compared to He-IVF protocol C ($49.1 \pm 1.1\%$). 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.

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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

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 $600\times g$ 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 $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 101.6 mM NaCl, 4.69 mM KCl, 0.37 mM KH_2PO_4 , 0.2 mM $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 21.4 mM Na-lactate, 0.33 mM Na-pyruvate, 2.78 mM glucose, 25 mM NaHCO_3 , 100 U/mL penicillin, 50 $\mu\text{g}/\text{mL}$ 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 $250\times g$ through a gradient of 1 mL of 40% and 1 mL of 80% Bovipure® (Nidacon Laboratories AB, Gothenborg, Sweden), according to the manufacturer's instructions. The sperm pellet was isolated and washed in 3 mL of Boviwash® (Nidacon Laboratories AB, Gothenborg, Sweden) by centrifugation at $250\times g$ 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% CO₂ 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

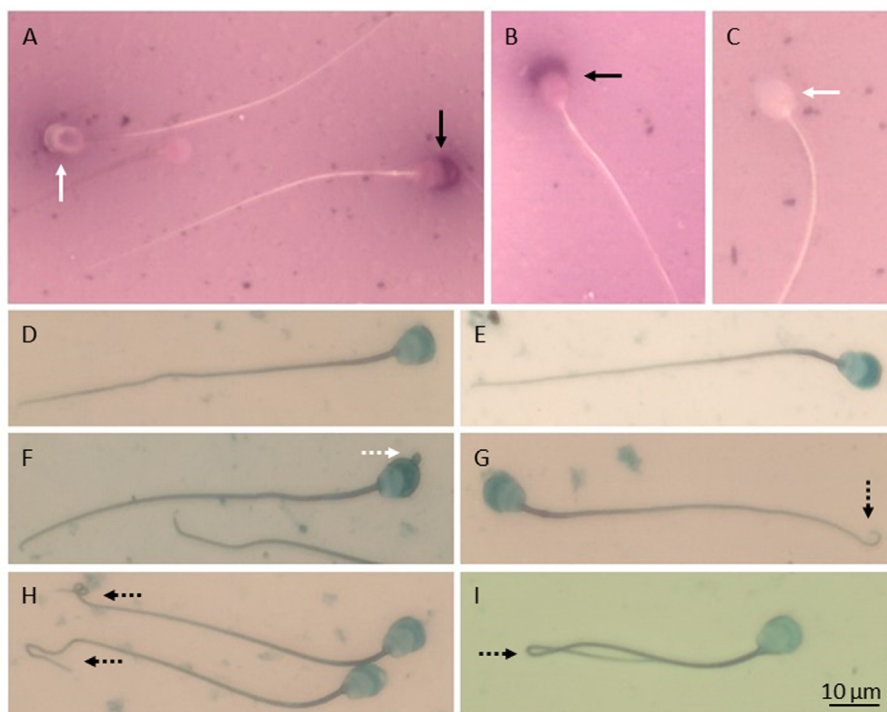


Fig. 1. Evaluation of sperm viability and morphology. (A, B, C) Representative images of eosin-nigrosin staining for the determination of guinea pig sperm viability. Normal "live" sperm exclude the eosin stain and appear white (white arrow), whereas "dead" sperm (i.e. those with loss of membrane integrity) 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 $\mu\text{m/s}$ and ALH = 9.0 μm . Thus, we defined spermatozoa showing hyperactive-like motility as those showing VCL >250 $\mu\text{m/s}$ 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, Gijón, 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 $\mu\text{g/mL}$ 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 $\mu\text{L/mL}$ BME amino acids, 10 $\mu\text{L/mL}$ minimum essential medium (MEM) amino acids and 1 $\mu\text{g/mL}$ 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, Gothenborg, 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 $\mu\text{g/mL}$ 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 $\pm$ 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

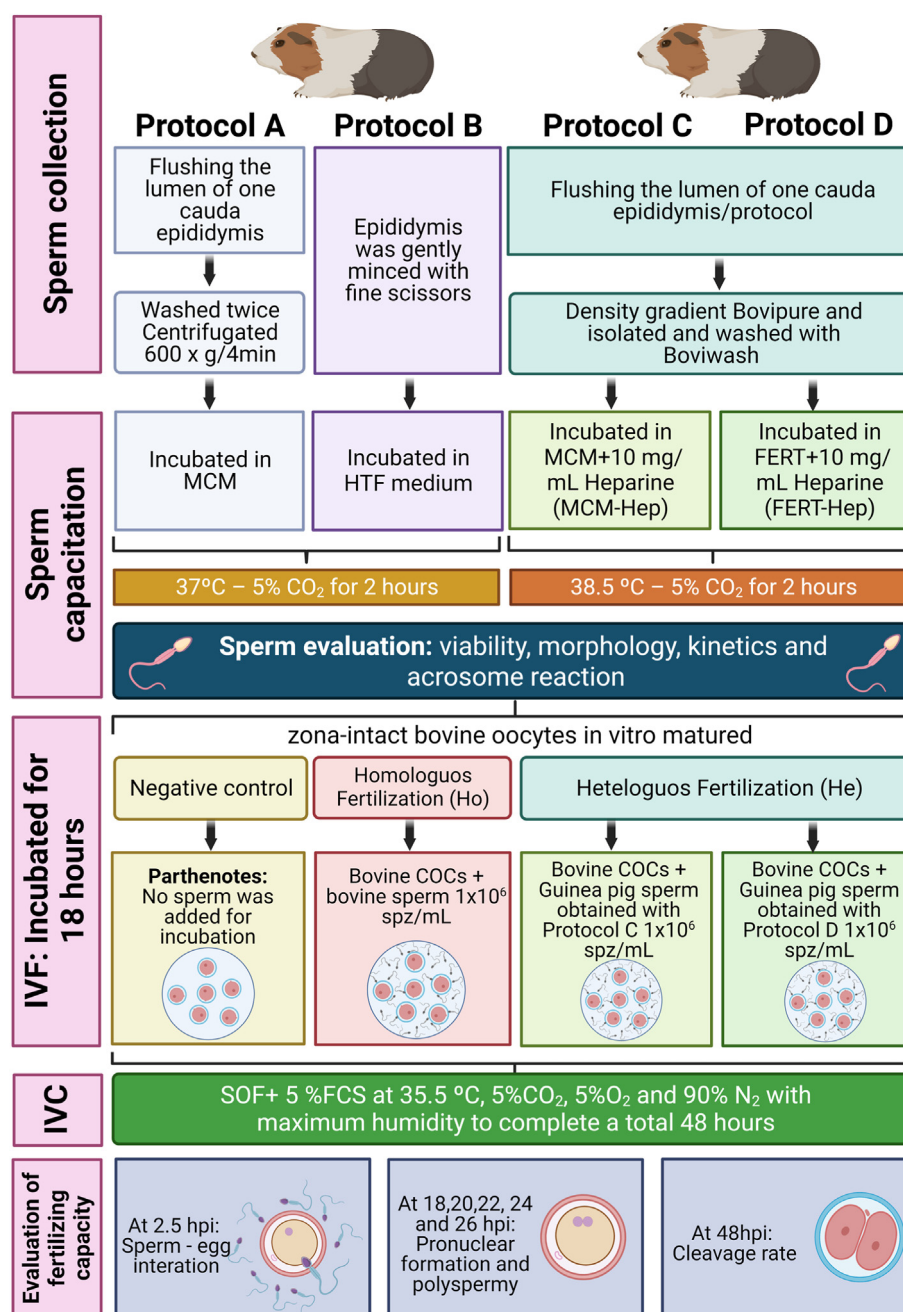


Fig. 2. Experimental design: Effects of the different protocols for collection and capacitation of guinea pig sperm through sperm analysis and using heterologous (He) IVF with zona-intact bovine oocytes. MCM: minimal culture medium. HTF: human tubal fluid medium. MCM-Hep: minimal culture medium supplemented 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 than 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).

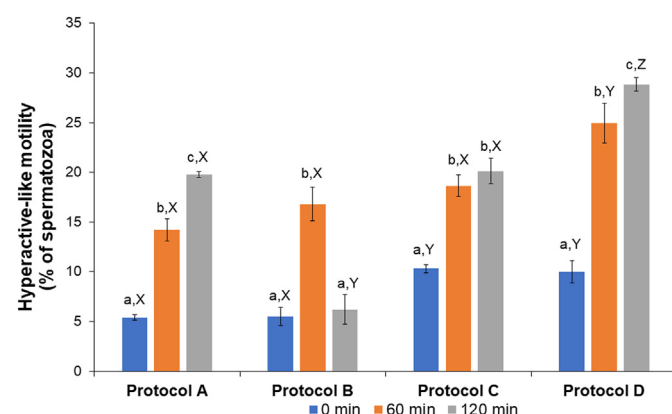


Fig. 3. Percentage of spermatozoa showing hyperactive-like motility. Kinetics were examined at the time 0, 60 and 120 min of incubation in A, B, C and D protocols. Spermatozoa showing hyperactive-like motility were defined as those showing VCL $> 250 \mu\text{m/s}$ and ALH $> 9.0 \mu\text{m}$. Different 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 ($P < 0.05$).

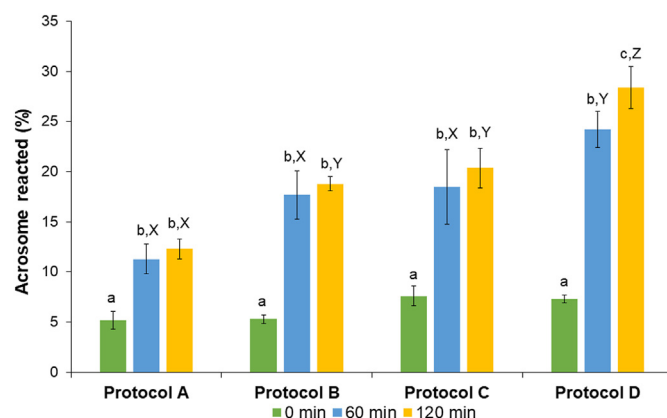


Fig. 4. Percentage of spermatozoa showing spontaneous acrosome reaction (AR). AR was examined at the time 0, 60 and 120 min of incubation in A, B, C and D protocols. Different lowercase letters indicate significant differences between time points for each protocol ($P < 0.05$, ANOVA). Different capital 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 ($H_o = 0.5 \pm 0.1$; $H_eC = 0.4 \pm 0.1$; $H_eD = 0.4 \pm 0.1$). 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 $4.2 \pm 2.5\%$ 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,

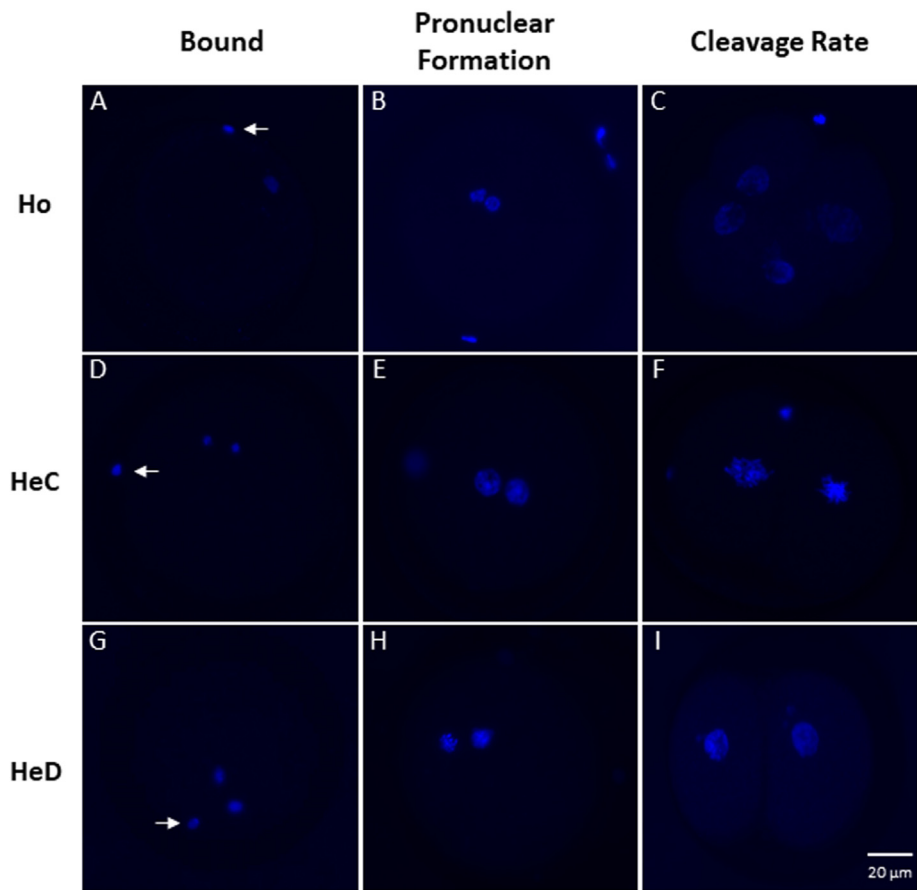


Fig. 5. Evaluation of sperm-oocyte binding, pronuclear formation, and cleavage after homologous (Ho) (bovine sperm) and heterologous (He) (guinea pig sperm obtained with protocol C (HeC) and D (HeD)) IVF. Gametes were stained with Hoechst 33,342. Ho: Bound bovine sperm after 2.5 h of coincubation with 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 μ m.

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 (parthenotic) 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 $\pm$ 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 demonstrating 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 plasma, for this reason, the effect of the heparin 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 Protocol 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>.

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