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ORIGINAL ARTICLE

Melatonin affects the motility and adhesiveness of in vitro capacitated boar spermatozoa via a mechanism that does not depend on intracellular ROS levels

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Summary Sentence
Melatonin activates sperm adhesiveness and affects motion patterns of boar spermatozoa subjected to in vitro capacitation. These effects were not apparently linked to a direct antioxidant action, although they were related to the maintenance of proper levels of intact disulphide bonds.
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SUMMARY

This work sought to address the effects of melatonin during in vitro capacitation (IVC) and progesterone-induced acrosome exocytosis (IVAE) in boar spermatozoa. With this purpose, two different experiments were set. In the first one, IVC and IVAE were induced in the absence or presence of melatonin, which was added either at the start of IVC or upon triggering the IVAE with progesterone. Different parameters were evaluated, including intracellular levels of peroxides and superoxides, free cysteine radicals and distribution of specific lectins. While melatonin neither affected most capacitation-associated parameters nor IVAE, it dramatically decreased sperm motility, with a maximal effect at 5 μM. This effect was accompanied by a significant increase in the percentage of agglutinated spermatozoa, which was independent from noticeable changes in the distribution of lectins. Levels of free cysteine radicals were significantly lower in melatonin treatments than in the control after 4 h of incubation in capacitating medium. The second experiment evaluated the effects of melatonin on in vitro fertilising ability of boar spermatozoa. Spermatozoa previously subjected to IVC in the presence of 1 μM melatonin and used for in vitro fertilisation exhibited less ability to bind the zona pellucida (ZP) and higher percentages of monospermy. In conclusion, melatonin affects sperm motility and the stability of nucleoprotein structure and also modulates the ability of in vitro capacitated boar spermatozoa to bind the oocyte ZP. However, such effects do not seem to be related to either its antioxidant properties or changes in the sperm glycocalix.

INTRODUCTION

Mounting evidence supports the existence of a functional machinery related to melatonin metabolism in the mammalian reproductive tract (Reiter *et al.*, 2009). While melatonin receptors MT1 and MT2 are present in the spermatozoa from humans, hamsters, pigs, dogs, cattle, deer and sheep (González-Arto *et al.*, 2016), they are absent from other species, such as horses (Da Silva *et al.*, 2011). These two receptors are detected in both seasonal and non-seasonal species, and their presence is concomitant with that of melatonin in the seminal plasma (concentration range: 0.5–5 μM; Luboshitzky *et al.*, 2002; Casao *et al.*, 2010; Pérez- Patiño *et al.*, 2016). All these data suggest the existence of an active melatonin pathway system in mammalian spermatozoa.

The main role of melatonin has usually been linked to the regulation of circadian rhythms, including those related to the reproductive function (Reiter *et al.*, 2009). However, while the presence of melatonin in seminal plasma and MT receptors in

spermatozoa has been clearly associated with circadian modulation in seasonal breeders, such as the sheep (Casao *et al.*, 2010), its relationship with the circadian rhythm in non-seasonal breeders, such as the pig, seems to be dismissed (González-Arto *et al.*, 2016).

Focusing on the effects upon sperm function, melatonin seems to have a vital regulatory role for sperm capacitation in the sheep and water buffalo (Casao *et al.*, 2009; Ashrafi *et al.*, 2013). Furthermore, melatonin has been shown to improve motility and other sperm functional parameters in human, ram, equine and boar spermatozoa (reviewed in Cebrián-Pérez *et al.*, 2014). The mechanism through which melatonin exerts these effects has been suggested to be linked with a reduction in oxidative stress by scavenging intracellular free radicals (Reiter *et al.*, 2000). In agreement with this hypothesis, in vitro treatment of spermatozoa with melatonin decreases intracellular levels of reactive oxygen (ROS) and nitrogen species (RNS; Rao & Gangadharan, 2008; Du Plessis *et al.*, 2010; Jang *et al.*, 2010; Najafi *et al.*, 2018), membrane lipid peroxidation (Gadella *et al.*, 2008; Du Plessis *et al.*, 2010; Da Silva *et al.*, 2011), apoptosis markers (Casao *et al.*, 2010; Da Silva *et al.*, 2011; Espino *et al.*, 2011; Najafi *et al.*, 2018) and DNA fragmentation (Sarabia *et al.*, 2009). In spite of all these data, little is known about whether melatonin could exert any effect on sperm function through a mechanism independent from its antioxidant properties. This is especially relevant for some species such as the pig, in which ROS levels produced by their spermatozoa in response to cryopreservation are marginal when compared to other species, such as the horse and cattle (Bilodeau *et al.*, 2000; Guthrie & Welch, 2006; Yeste *et al.*, 2013, 2015a,b). Thus, ROS production or accumulation seems to play a minor role to explain specific events, such as boar sperm cryodamage (Yeste *et al.*, 2013, 2015b). Likewise, changes in ROS levels are also low during the achievement of in vitro boar sperm capacitation (IVC), which again suggests they play a marginal role (Awda *et al.*, 2009). Taking all these data into account, we can hypothesise that melatonin could affect boar sperm function through mechanisms other than its ability to modulate intracellular ROS levels.

This study sought to determine the influence of melatonin on the achievement of IVC and subsequent progesterone-induced in vitro acrosome exocytosis (IVAE) of boar spermatozoa, as well as on their ability to adhere and further penetrate in vitro matured oocytes. With this purpose, two experiments were devised. In the first one, boar spermatozoa were subjected to IVC/IVAE in the presence of increasing concentrations of melatonin, added either before or after 4 h of incubation under capacitating conditions. Several parameters related to the achievement of IVC and IVAE were evaluated. In the second experiment, in vitro fertilisation (IVF) was conducted with spermatozoa previously capacitated with 1 μM melatonin. The sperm ability to bind the oocyte ZP and penetrate in vitro matured oocytes was assessed.

MATERIALS AND METHODS

Seminal samples

A total of 57 ejaculates collected from 32 healthy Pietrain boars aged between two and three years were used. These animals were housed in climate-controlled commercial farms (Servicios Genéticos Porcinos, S.L., Roda de Ter, Spain), fed with a commercial adjusted diet and provided with water ad libitum. Boar housing fulfilled with the welfare standards established by European regulations on livestock species, specifically, on pig farms. Furthermore, and despite not being required as we did not manipulate any boar and only worked with seminal doses provided by the commercial farm, the experimental protocol was approved by the Ethics Committee of our institution (Bioethics Commission, Autonomous University of Barcelona; Bellaterra, Cerdanyola del Vallès, Spain). In all the cases, samples came from sperm-rich fractions that were obtained through manual collection with the conventional hand-gloved method. Upon collection, samples were immediately diluted with a commercial extender (Androstar Plus®; Minitub Ibérica SL, Tarragona, Spain) to a final sperm concentration of 2×10^7 spermatozoa/mL and cooled down to 16 °C. Diluted semen was packaged in 90-mL commercial AI doses and transported in an insulated container at 16 °C for approximately 45 min, which was the time required to arrive to our laboratory.

In vitro capacitation and progesterone-induced acrosome exocytosis

As aforementioned, two experiments were set. The first experiment was subdivided into two parts. The first one aimed at testing how melatonin affected the achievement of IVC (i.e. addition at 0 h). The second part sought to determine the impact of melatonin upon triggering IVAE in fully in vitro capacitated boar spermatozoa (i.e. addition at 4 h). With this purpose, different concentrations of melatonin were added after 4 h of incubation in CM, as this time period has previously been reported to induce IVC in boar spermatozoa (Ramió *et al.*, 2008). In this case, the addition of melatonin was performed together with that of progesterone, which was used to induce the acrosome reaction (Jimenez *et al.*, 2003; Wu *et al.*, 2006). Regardless of when melatonin was added (i.e. either at 0 h or at 4 h), five different treatments were assayed: a positive control (C+), which consisted of spermatozoa incubated in CM containing bicarbonate and bovine serum albumin (BSA); three treatments with different concentrations of melatonin (0.5, 1, 5 μM) in CM; and a negative control (C−), which consisted of spermatozoa incubated in non-capacitating medium without bicarbonate or BSA (NCM). As aforementioned, the tested concentrations of melatonin were within the physiological range of the genital tract, as described in previous works (Luboshitzky *et al.*, 2002; Casao *et al.*, 2009, 2010; Ashrafi *et al.*, 2013; Cebrián-Pérez *et al.*, 2014; Pérez- Patiño *et al.*, 2016).

For both parts, 50 mL of a given semen sample was split into five aliquots of 10 mL each. Aliquots were centrifuged at 600 ***g*** and 16 °C for 10 min and resuspended either with NCM (C−), CM (C+) or melatonin treatments (i.e. CM supplemented with melatonin at final concentrations of 0.5, 1 or 5 μM). In all cases, final sperm concentration was adjusted to 2×10^7 sperm/mL. The composition of NCM was 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer (pH = 7.4), 112 mM NaCl, 3.1 mM KCl, 5 mM glucose, 21.7 mM sodium L-lactate, 1 mM sodium pyruvate, 0.3 mM $NaHPO_4$, 0.4 mM $MgSO_4$ and 4.5 mM $CaCl_2$ (osmolarity: 287 mOsm/kg ± 6 mOsm/kg). Capacitation medium (CM) consisted of NCM supplemented with 37.6 mM $NaHCO_3$ and 5 mg/mL BSA (pH adjusted to 7.4; osmolarity: 304 mOsm/kg ± 5 mOsm/kg).

Aliquots were incubated at 38.5 °C and 5% CO_2 in humidified air for 4 h either with or without melatonin, as described in

Ramió *et al.* (2008). Samples were taken at 0 h and 4 h of incubation for analysis of sperm parameters. After 4 h of incubation, spermatozoa were subjected to progesterone-induced in vitro acrosome exocytosis (IVAE) through adding 10 mg/mL progesterone. After thoroughly mixing, sperm samples were incubated at 38.5 °C and 5% CO_2 in humidified air for a further hour. Separate aliquots were taken at 1, 5 and 60 min after the addition of progesterone. In the case of the second part, melatonin was directly added to the corresponding tube at final concentrations of 0.5, 1 or 5 μM after 4 h of starting the experiment rather than at 0 h.

At each relevant time point (i.e. 0, 4 h, and after 1, 5 and 60 min of progesterone addition), the achievement of both IVC and IVAE was evaluated on the basis of the following parameters: sperm motility, agglutination, viability, acrosome exocytosis, membrane lipid disorder and tyrosine phosphorylation of P32 protein, as a specific capacitation marker of boar spermatozoa (Bravo *et al.*, 2005). Furthermore, intracellular ROS levels, free cysteine residues in sperm head and tail extracts and lectin distribution over sperm membrane were also evaluated as parameters that could be related to the effects of melatonin on IVC/IVAE.

Unless stated otherwise, all fluorochromes and lectins were purchased from Molecular Probes® (Invitrogen, ThermoFisher; Eugene, OR, USA) and diluted with dimethyl sulfoxide (DMSO; Sigma-Aldrich; Saint Louis, MO, USA).

Evaluation of sperm motility and agglutination

Sperm motility and agglutination were evaluated by utilising a commercial, computer-assisted sperm analysis (CASA) system (Integrated Sperm Analysis System V1.0; Proiser, Valencia, Spain). This system is based on the analysis of 25 consecutive digitalised photographic images obtained from a single field at a magnification of 100× (Olympus BX41 microscope; Olympus 10 × 0.30 PLAN objective lens, negative phase contrast; Olympus Europa GmbH, Hamburg, Germany). These 25 consecutive photographs were taken at a time lapse of 1 sec, which implies that an image was captured every 40 ms. Five to six separate fields were taken for each replicate, and five replicates were evaluated per sample and treatment. Prior to evaluation with CASA, a 5 μL drop was placed onto a warmed Makler chamber (Sefi Medical Instruments, Haifa, Israel). In the case of samples evaluated at 0 h, they were previously warmed at 38 °C for 15 min in a water bath. Recorded sperm motility and kinematic parameters were those described in Ramió *et al.* (2008). Settings for the CASA system were as follows: area of particles: 10–80 μm^2; curvilinear velocity (VCL): 1–500 μm/sec; mean velocity (VAP): 1–500 μm/sec; linearity coefficient (LIN): 10–98%; straightness coefficient (STR): 10–98%; mean amplitude of lateral head displacement (ALH): 0–100 μm; and beat cross-frequency (BCF): 0–100 Hz. A spermatozoon was considered to be motile when its VAP was higher than 10 μm/sec.

Furthermore, the number of spermatozoa included in agglutination complexes and the percentage of agglutinated spermatozoa that showed apparent tail movement were determined by evaluation of each consecutive photograph obtained from CASA analyses. Specifically, we determined the number of sperm heads agglutinated divided by the total number of sperm heads, and the number of beating tails observed in each agglutination complex divided by the total number of tails.

Flow cytometry analyses

Sperm viability, acrosome exocytosis, membrane lipid disorder and intracellular ROS levels were evaluated by flow cytometry. Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry (ISAC; Lee *et al.*, 2008). Prior to evaluation, sperm concentration was adjusted to 1×10^6 spermatozoa/mL in a volume of 0.5 mL. Thereafter, spermatozoa were stained with the appropriate combinations of fluorochromes, following the protocols described below.

Samples were evaluated through a Cell Laboratory QuantaSC cytometre (Beckman Coulter, Fullerton, CA, USA) and were excited using single-line visible light from an argon laser (wavelength: 488 nm; power: 22 mW). Sheath flow rate was set at 4.17 μL/min, and electronic volume (EV; equivalent to forward scatter) and side scatter (SS) were recorded for each event. Calibration of the equipment was periodically performed using 10-μm Flow-Check fluorospheres (Beckman Coulter). Three optical filters with the following characteristics were used: FL1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, band-pass filter: 525 nm, detection width 505–545 nm; FL2 (orange fluorescence): DRLP: 600 nm, BP filter: 575 nm, detection width: 560–590 nm; and FL3 (red fluorescence): long pass filter: 670 nm. Signals were logarithmically amplified, and photomultiplier (PMT) settings were adjusted to each particular staining method; compensation was used to minimise spillover of the fluorescence into a different channel. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter<7 μm) and cell aggregates (particle diameter>12 μm) and sperm-specific events were positively gated on the basis of EV/SS distributions. Three independent replicates were examined, and 10,000 events were evaluated per replicate. Information on the events was collected as list-mode data files (LMD), and data were analysed through Cell Lab QuantaSC MPL Analysis Software (version 1.0; Beckman Coulter).

In all assessments except SYBR14/PI, data obtained from flow cytometry experiments were corrected according to the procedure described in Petrunkina *et al.* (2010).

Evaluation of sperm viability

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR14/ PI) following the protocol set in Garner & Johnson (1995). With this purpose, sperm samples were incubated with SYBR14 (final concentration = 100 nM) at 38 °C for 10 min and then with propidium iodide (PI; final concentration = 10 μM) at the same temperature for 5 min. Fluorescence emitted by SYBR14 was measured through FL1, whereas that emitted by PI was detected through FL3. Three sperm populations were identified as follows: (i) viable green-stained spermatozoa ($SYBR14^+/PI^-$); (ii) non-viable, red-stained spermatozoa ($SYBR14^-/PI^+$); and (iii) non-viable spermatozoa that were stained both in green and in red ($SYBR14^+/PI^+$). Non-sperm particles (debris) were found in the $SYBR14^-/PI^-$ quadrant. Single-stained samples were used for setting PMT voltages of EV, FL1 and FL3, and for compensation of SYBR14 spillover into the FL3 channel (2.45%).

Evaluation of acrosome exocytosis

True acrosome exocytosis was determined through costaining of spermatozoa with *Arachis hypogaea* agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC-PNA) and ethidium homodimer (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide; EthD-1). This protocol was originally described by Cooper & Yeung (1998) and has been adapted to boar spermatozoa in our laboratory. In brief, samples were incubated with EthD-1 (final concentration: 2.5 μg/mL) at 38 °C for 5 min in the dark. Following this step, samples were centrifuged at 2000 ***g*** for 30 sec and then resuspended with PBS containing 4 mg/mL bovine serum albumin (BSA) to remove the free dye. Thereafter, samples were again centrifuged at the aforementioned conditions and then fixed and permeabilised by adding 100 μL of ice-cold methanol (100%) for 30 sec. Methanol was removed by centrifugation at 2000 ***g*** for 30 sec, and pellets were resuspended with 250 μL PBS. Following this step, 0.8 μL PNA-FITC (final concentration: 2.5 μM) was added, and samples were incubated at 15 °C in the dark for 15 min. Next, samples were washed twice with PBS at 2000 ***g*** for 30 sec and finally resuspended in PBS.

Following staining, samples were evaluated with the flow cytometry and the following four sperm populations were identified: (i) viable spermatozoa with an intact acrosome membrane (PNA-FITC$^+$/EthD-1$^-$); (ii) viable spermatozoa with a non-intact acrosome membrane (PNA-FITC$^-$/EthD-1$^-$); (iii) non-viable spermatozoa with an intact acrosome membrane (PNA-FITC$^+$/EthD-1$^+$); and (iv) non-viable spermatozoa with a non-intact acrosome membrane (PNA-FITC$^-$/EthD-1$^+$). Fluorescence of EthD-1 was detected through FL3, whereas that of PNA-FITC was detected through FL1.

Evaluation of sperm membrane lipid disorder

Lipid disorder of boar sperm membrane was evaluated by costaining with merocyanine-540 (M540) and YO-PRO-1, following a procedure modified from Rathi *et al.* (2001). Briefly, spermatozoa were stained with M540 (final concentration: 2.6 μM) and YO-PRO-1 (final concentration: 25 nM) and incubated at 38 °C for 10 min in the dark. Red fluorescence from M540 was collected through FL3, and green fluorescence from YO-PRO-1 was collected through FL1. The following four sperm populations were distinguished: (i) viable spermatozoa with low membrane lipid disorder (M540$^-$/YO-PRO-1$^-$); (ii) viable spermatozoa with high membrane lipid disorder (M540$^+$/YO-PRO-1$^-$); (iii) non-viable spermatozoa with low membrane lipid disorder (M540$^-$/YO-PRO-1$^+$); and (iv) non-viable spermatozoa with high membrane lipid disorder (M540$^+$/YO-PRO-1$^+$). In this test, data were not compensated.

Evaluation of intracellular levels of superoxides and peroxides

Intracellular superoxide ($O_2^{-\bullet}$) and peroxide (H_2O_2) levels were determined using two different oxidation-sensitive fluorescent probes: hydroethidine (HE) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). Following a procedure modified from Guthrie & Welch (2006), a simultaneous differentiation of viable from non-viable spermatozoa was performed using either YO-PRO-1 or PI.

In the case of superoxides, samples were stained with HE (final concentration: 4 μM) and YO-PRO®-1 (final concentration: 25 nM) and incubated at 15 °C for 40 min in the dark [17]. Hydroethidine is freely permeable to cells and is oxidised by $O_2^{-\bullet}$ to ethidium (E$^+$) and other products. Fluorescence of ethidium (E$^+$) was detected through FL3, and that of YO-PRO-1 was collected through FL1. Viable spermatozoa with high intracellular superoxide levels were positive for ethidium and negative for YO-PRO-1 (E$^+$/YO-PRO-1$^-$).

With regard to peroxides, spermatozoa were stained with H_2DCFDA at a final concentration of 200 μM and PI at a final concentration of 10 μM, and incubated at 15 °C for 60 min in the dark. H_2DCFDA is a cell-permeable, non-fluorescent probe that is intracellularly de-esterified and converted into highly fluorescent, 2′,7′-dichlorofluorescein (DCF$^+$) upon oxidation (Guthrie & Welch, 2006). This DCF$^+$ fluorescence was collected through FL1, whereas PI fluorescence was detected through FL3. Data were not compensated, and viable spermatozoa with high intracellular peroxide levels were positive for DCF and negative for PI (DCF$^+$/PI$^-$).

In both cases, unstained and single-stained samples were used for setting EV, FL1 and FL3 PMT voltages and data were not compensated.

Immunoblotting

Aliquots of 1 mL corresponding to each experimental point were centrifuged at 1000 ***g*** and 15 °C for 30 sec, and pellets were stored at −80 °C until the beginning of the assay. Pellets were resuspended and sonicated in 300 μL ice-cold lysis buffer containing 50 mM Tris-HCl, 1 mM EDTA, 10 mM EGTA, 25 mM dithiothreitol, 1.5% (v:v) Triton® X-100, 1 mM PMSF, 10 μg/mL leupeptin, 1 mM orthovanadate and 1 mM benzamidine (pH = 7.4). After 30 min on ice, the homogenised suspensions were centrifuged at 600 ***g*** and 4 °C for 20 min, and total protein content in supernatants was calculated through the Bradford method (Bradford, 1976) using a commercial kit (Bio-Rad Laboratories). Afterwards, samples were mixed with loading buffer (1 : 5; v:v) containing 250 mM Tris-HCl (pH = 6.8), 50 mM dithiothreitol, 10% (w:v) SDS, 0.5% (v:v) bromophenol blue and 50% (v:v) glycerol and stored at −20 °C until gel electrophoresis (SDS-PAGE; Laemmli, 1970).

Prepared samples were loaded onto 0.75-mm gels containing 10% acrylamide (w:v). After running the gels at constant voltage (180 V), proteins were transferred onto Immun-Blot® low-fluorescence polyvinylidene fluoride (PVDF) membranes (Bio-Rad) using the Trans-Blot® Turbo Transfer System with Trans-Blot® Turbo Midi Transfer Packs (Bio-Rad). Membranes were subsequently immersed for 60 min into blocking solution, consisting of Tris-buffered saline solution added with 5% (w:v) BSA and 0.1% (v:v) Tween-20. Thereafter, membranes were incubated with a mouse monoclonal PY20 antiphosphotyrosine antibody (ref. P4110; Sigma-Aldrich; dilution factor: 1 : 1000 (v:v) in blocking solution) at 4 °C for 8 h. Membranes were washed three times with blocking solution (5 min per wash) and then incubated at 15 °C for 1 h with a horseradish peroxidase (HRP)-conjugated, polyclonal rabbit anti-mouse antibody (Dako; Glostrup, Denmark) at a dilution of 1 : 5000 (v:v) in blocking solution. After washing membranes with blocking solution for six times (5 min per wash), membranes were incubated with chemiluminescent HRP substrate (ImmunoCruz Western Blotting Luminol Reagent; Santa Cruz Biotechnology®, Dallas, TX, USA) at 15 °C for 2 min, following manufacturer's instructions. Revealed images were analysed through IMAGEJ ver. 1.49 (National Institute of Health, Bethesda, MD, USA), and the intensity/densitometry

of each band was quantified. Following this, membranes were stripped and then incubated with a mouse monoclonal anti-β-tubulin (ref. T5201; Sigma-Aldrich; 1 : 1000 (v:v) in blocking solution) and the same secondary HRP antibody. Images were also analysed through ImageJ. A total of seven semen samples were used for Western blot assays.

Evaluation of free cysteine residues in spermatozoa and tail extracts

Determination of free cysteine radicals in sperm head and tail extracts as an indirect measure of disrupted disulphide bridges within proteins was carried out following the protocol described in Flores *et al.* (2011). Briefly, samples were centrifuged at 600 ***g*** and 16 °C for 10 min and then resuspended in an ice-cold lysis buffer made up as follows: 50 mM Tris buffer, 150 mM NaCl, 1% (v:v) Non-idet, 0.5% (w:v) sodium deoxycholate, 1 mM benzamidine, 10 μg/mL leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Na_2VO_4 (pH adjusted to 7.4). Samples were homogenised through sonication (12 pulses; Ikasonic U50 sonicator; Ika Labortechnick, Staufen, Germany), and obtained homogenates were centrifuged at 850 ***g*** and 4 °C for 20 min. After this centrifugation step, the supernatant mainly contained the sperm tails, whereas the pellet mainly contained the sperm heads. This was confirmed by separate evaluations through phase-contrast microscopy of both fractions, the pellets being previously resuspended with 300 μL Tris buffer. Indeed, percentages of tails in supernatants and heads in pellets were found to be above 90%, respectively (data not shown).

Levels of free cysteine radicals in both fractions (i.e. supernatants and Tris-solubilised pellets) were determined using the 2,2′-dithiodipyridine technique (2,2′-dipyridyl disulphide; Sigma-Aldrich) as described in Brocklehurst *et al.* (1979). Briefly, 10 μL of the supernatant or resuspended pellet was added with 990 mL of an aqueous solution containing 0.4 mM 2,2′-dithiodipyridine. Standard curves were generated with 10-μL aliquots containing different concentrations of cysteine (Sigma-Aldrich; from 0.1 to 5 mM), which were also added with 990 mL of 0.4 mM 2,2′-dithiodipyridine. Samples were incubated at 37 °C for 60 min, and levels of free cysteine radicals were subsequently determined through spectrophotometry at a wavelength of 340 nm. The results obtained were normalised through a parallel determination of the total protein content by the Bradford method (Bradford, 1976), using a commercial kit (Quick Start Bradford Protein Assay; Bio-Rad, Hercules, CA, USA). Five replicates per sample and treatment were evaluated, and the corresponding mean ± standard error of the mean (SEM) was calculated.

Distribution of lectins over sperm membrane

Four FITC-conjugated lectins were used as follows: *Triticum vulgaris* agglutinin (WGA), *Solanum lycopersicum* lectin (STL), *Pisum Sativum* agglutinin (PSA) and *Arachis Hypogaea* agglutinin (PNA). Semen samples were centrifuged at 1000 ***g*** and 15 °C for 30 sec, and the resultant pellets were resuspended with 400 μL PBS containing 4% (w:v) paraformaldehyde. Fixation was conducted at 4 °C in the dark for 2 h. Samples were subsequently spread onto poly-lysine (1% poly-lysine solution in water; Sigma-Aldrich)-coated microscope slides and then left to dry. Samples were permeabilised by incubation with 0.3% (v:v) Triton® X-100 in PBS (pH = 7.4) at 15 °C for 10 min. Next, slides were washed three times with PBS and then blocked through incubation with PBS containing 0.1% (v:v) Tween-20 and 5% (w:v) BSA at 15 °C for 60 min. After blocking, samples were incubated at 15 °C in a humid chamber for 1 h with the corresponding lectin at the following dilutions in PBS: 1 : 200 (w:v) for WGA and PSA, 1 : 300 (w:v) for PNA and 1 : 50 (w:v) for STL. Slides were further washed three times with PBS (5 min each wash) and then mounted with antifading medium Vectashield H-1000 (Vector Laboratories, Burlingame, CA, USA). After being covered by coverslips, slides were compressed to eliminate any excess of liquid. Coverslips were finally sealed with colourless nail polish, and slides were stored at 4 °C in the dark until observation. Negative control experiments were performed omitting the lectin.

Samples were observed using a confocal laser scanning microscope (Leica TCS 4D; Leica Lasertechnik, Heidelberg, Germany) at 63× magnification. The light source was an argon/krypton laser. Successive confocal slices of images (image thickness: 0.5 μm) were integrated to create three-dimensional images that were saved in TIFF format. Each lectin generated distinct staining patterns that were examined in non-capacitated, capacitated and acrosome-exocytosed spermatozoa.

In vitro oocyte–sperm co-incubation and evaluation of sperm adhesiveness and penetration ability

As previously mentioned, the current work was divided into two experiments. In the second experiment, and following the results obtained in the first one, one melatonin treatment (1 μM) was compared with the control. The sperm ability to bind the ZP and to penetrate in vitro matured oocytes was evaluated after previous incubation with 1 μM melatonin, following a modified protocol from Castillo-Martín *et al.* (2014).

Ovaries were obtained from a local slaughterhouse and were brought to the laboratory in a 0.9% (w:v) NaCl solution containing 100 μg kanamycin sulphate per mL previously warmed at 37 °C. Oocyte–cumulus cell complexes (COCs) were collected from follicles of 3–6 mm diameter and only those showing at least two layers of cumulus cells and a homogeneous cytoplasm were selected. COCs were washed twice with DPBS supplemented with 4 mg/mL polyvinyl alcohol (PVA) and then with maturation medium, previously equilibrated at 38.5 °C and 5% CO_2 in humidified air for at least 3 h. Groups of 50 oocytes were cultured in 500 μL maturation medium for 22 h at 38.5 °C and 5% CO_2 in humidified air. Thereafter, oocytes were transferred to fresh maturation medium without hormones or dibutyryl cAMP, and cultured for further 22 h. The maturation medium was NCSU-37 (Petters & Wells, 1993) supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 μg/mL insulin, 50 μM β-mercaptoethanol, 10 IU/mL equine chorionic gonadotrophin (Folligon, Intervet International BV, Boxmeer), 10 IU/mL human chorionic gonadotrophin (Veterin Corion, Divasa Farmavic, Barcelona, Spain) and 10% (v:v) pig follicular fluid.

After maturation, oocytes were mechanically stripped of cumulus cells by gentle aspiration with a pipette. Denuded oocytes were washed with TALP medium, and groups of 25 oocytes were then transferred to each well of four-well Nunc multidishes (Nunc; Roskilde, Denmark) containing 250 μL TALP medium, previously equilibrated at 38.5 °C under 5% CO_2 in humidified air. The composition of TALP medium was as follows: 114.06 mM NaCl, 3.2 mM KCl, 8 mM calcium lactate·$5H_2O$,

0.5 mM $MgCl_2 \cdot 6H_2O$, 0.35 mM NaH_2PO_4, 25.07 mM $NaHCO_3$, 10 mL/L sodium lactate, 5 mM glucose, 2 mM caffeine, 1 g/L PVA, and 0.17 mM kanamycin sulphate supplemented with 3 mg/mL fatty acid-free BSA (FAF-BSA) and 1.1 mM sodium pyruvate (Rath *et al.*, 1999).

Two hundred fifty microlitres of sperm suspensions from each treatment group was added to the fertilisation wells at a final concentration of 5×10^4 sperm/mL. Those spermatozoa had previously been incubated in CM at 38.5 °C and 5% CO_2 in humidified air for 4 h, either in the presence or absence of 1 μM melatonin. Specifically, three treatments were set as follows: (i) control, which consisted of oocytes incubated with spermatozoa previously incubated in CM without melatonin; (ii) experimental treatment 1, which consisted of oocytes co-incubated with spermatozoa previously in vitro capacitated with CM added with 1 μM melatonin at 38.5 °C and 5% CO_2 in humidified air for 4 h; and (iii) experimental treatment 2, which consisted of oocytes added with both spermatozoa (previously incubated in CM without melatonin) and melatonin to a final concentration of 1 μM. In all treatments, co-incubation of spermatozoa with in vitro matured oocytes was performed at 38.5 °C and 5% CO_2 in humidified air for 1 h. Free, non-attached spermatozoa were removed by washing oocyte–sperm complexes with TALP medium, and 500 μL fresh TALP medium was subsequently added. Oocyte–sperm complexes were incubated at 38.5 °C and 5% CO_2 in humidified air for further 7 h and subsequently prepared for nuclear staining. Following this, oocytes were gently aspirated with a pipette, washed with TALP medium and subsequently transferred to a new well containing 500 μL TALP. Oocyte–sperm complexes were maintained in this medium at 38.5 °C and 5% CO_2 in humidified air for further 7 h and then collected to perform the following nuclear staining protocol.

Oocyte–sperm complexes were washed with warmed PBS and then fixed with 4% (w:v) paraformaldehyde in PBS at 38.5 °C for 30 min. After fixation, complexes were washed twice with PBS and subsequently stained with 1% (v:v) Hoechst® 33342 in PBS at 15 °C for 25 min. Oocyte–sperm complexes were then washed two times with PBS, mounted on glass slides and examined under a TCS 4D laser confocal scanning microscope (Leica Lasertechnik) at 63× magnification. The following parameters were evaluated: (i) spermatozoa bound to the ZP: number of nuclear spermatozoa attached to the ZP; (ii) total penetration rate: number of oocytes that showed evident signs of sperm penetration divided by the total number of sperm–oocytes complexes; lack of penetration consisted of sperm–oocytes complexes that showed a unique nucleus with or without apparent polar bodies; (iii) percentage of monospermy: number of oocytes showing the presence of two pronuclei, or one sperm head inside the oocyte with or without signs of decondensation, divided by the total number of sperm–oocytes complexes; and (iv): percentage of polyspermy: number of oocytes showing more than two pronuclei, or more than one sperm head inside the oocyte with or without signs of decondensation, divided by the total number of sperm–oocytes complexes.

Statistical analyses

Statistical analyses were performed using a statistical package (IBM SPSS for Windows version 21.0, IBM Corp; Chicago, IL, USA). Data are presented as mean ± standard error of the mean (SEM), and the level of significance was set at $p \leq 0.05$.

In the case of experiment 1, data were first tested for normality and homogeneity of variances through Shapiro–Wilk and Levene tests, respectively. When required, data (x) were transformed through arcsine square root ($\arcsin\sqrt{x}$) before a general mixed model (i.e. with repeated measures) was run. In this model, the intersubject factor was the treatment (i.e. composition of capacitation media), and the intrasubject factor was the incubation time (i.e. 0 h, 4 h, 4 h + 1 min, 4 h + 5 min, 4 h + 60 min). In all cases, each sperm functional parameter was the dependent variable, and pairwise comparisons were made with Sidak post hoc test. When no transformation remedied the normality, nonparametric procedures were conducted with raw data. Friedman's test and the Wilcoxon matched-pairs test were performed as nonparametric alternatives to repeated measures ANOVA.

With regard to experiment 2, the number of spermatozoa attached to oocyte ZP was checked for normality and homogeneity of variances as previously described, and compared through one-way ANOVA followed by post hoc Sidak's test. For the analysis of monospermy/polyspermy, a chi-square test (χ^2) was used.

RESULTS

Effects of melatonin on viability, acrosome exocytosis and capacitation-like changes in sperm membrane

As shown in Figure S1A, incubation of boar spermatozoa in CM for 4 h reduced their viability, which went from 80.4% ± 3.7% at 0 h to 67.9% ± 2.8% after 4 h of incubation. This decline was maintained after the addition of progesterone. While the addition of melatonin to CM at 0 h or 4 h did not significantly modify the observed drop in sperm viability, the extent of that decrease was higher when spermatozoa were incubated in NCM (Figure S1A,B).

Percentages of true acrosome exocytosis (PNA-FITC$^-$/EthD-1$^-$) were very low in cells incubated in CM during 4 h. The addition of progesterone at 4 h induced an increase in this percentage, which reached maximal values after 60 min of that addition (67.4% ± 2.3%; Figure S2A,B). This increase was not observed when spermatozoa were incubated in NCM. The addition of melatonin either at 0 h or at 4 h did not modify the pattern observed in spermatozoa incubated in CM (Figure S2A,B).

Incubation of boar spermatozoa in CM significantly ($p < 0.05$) increased the percentage of viable spermatozoa with high membrane lipid disorder (from 9.4% ± 2.6% at 0 h to 45.7% ± 4.6% at 4 h; Figure S3A,B). The subsequent addition of progesterone was associated with a progressive decrease in this percentage, which reached values of 34.6% ± 3.0% after 60 min of progesterone addition. The addition of melatonin either at 0 h or at 4 h did not change the dynamics observed in spermatozoa incubated in CM (i.e. positive control; Figure S3A,B).

Effects of melatonin on P32 tyrosine phosphorylation levels

As expected, incubation of boar spermatozoa in CM for 4 h induced a noticeable increase in tyrosine phosphorylation (pTyr) levels of P32 protein (from 100.0 arbitrary units at 0 h of incubation to 231.7 ± 14.3 arbitrary units after 4 h), which was roughly maintained after progesterone addition (Figures S4 and S5). Addition of melatonin at 0 h did not significantly modify that pattern (Figure S4). The addition of progesterone after 4 h of incubation in CM did not have any prominent effect on

pTyr-P32 levels. Only the treatment containing melatonin at 5 μM showed a slight decrease in pTyr-P32 values when compared to incubation in CM (60 min after progesterone addition; melatonin at 5 μM: 203.5 ± 7.4 arbitrary units vs. CM: 228.2 ± 7.6 arbitrary units; see Figure S5). Finally, the addition of 5 μM melatonin at 4 h was found to decrease the intensity of tyrosine phosphorylation in P32 band after 5 min and 60 min of progesterone addition (60 min after progesterone addition; melatonin at 5 μM: 206.8 ± 6.5 arbitrary units vs. CM: 226.1 ± 6.9 arbitrary units; see Figure S5).

Effects of melatonin on sperm motility

Total motility of spermatozoa incubated in CM significantly ($p < 0.05$) decreased throughout the experiment, reaching minimal values of 27.0% ± 2.5% after 60 min of the addition of progesterone (Fig. 1). Incubation of spermatozoa in NCM led to even worse motility values, with complete immobilisation at the end of the experiment. The addition of melatonin at 0 h induced an immediate decrease in total motility, which was more apparent at the highest melatonin concentration (0 h: 47.2% ± 3.0% in melatonin at 5 μM vs. 64.0% ± 3.9% in CM; Fig. 1A). This adverse effect on sperm motility was observed throughout all the incubation period. Melatonin also decreased sperm motility when added together with progesterone at 4 h, the treatments containing melatonin at 1 and 5 μM showing values near to complete immobility after 60 min of progesterone addition (Fig. 1B).

Regarding kinetic parameters, spermatozoa incubated in CM for 4 h showed significant ($p < 0.05$) increases in several parameters, including VCL, VAP and ALH (as an example, VAP at 0 h of incubation in CM: 65.7 μm/sec ± 2.1 μm/sec vs. VAP after 4 h of incubation in CM: 72.9 μm/sec ± 2.9 μm/sec; Table 1). However, the addition of melatonin at 0 h significantly ($p < 0.05$) decreased VAP values (after 4 h of incubation; 57.9 μm/sec ± 1.7 μm/sec in the treatment containing 0.5 μM melatonin vs. 72.9 μm/sec ± 2.9 μm/sec in CM; Table 1). When melatonin was added together with progesterone at 4 h, there was an immediate decrease in VAP (1 min after progesterone addition: 40.9 μm/sec ± 2.0 μm/sec in the treatment containing 1 μM melatonin vs. 59.8 μm/sec ± 2.7 μm/sec in CM; Table 1), LIN (1 min after progesterone addition: 25.7% ± 1.6% in the treatment containing 1 μM melatonin vs. 39.9% ± 1.7% in CM; Table 2) and STR (1 min after progesterone addition: 58.1% ± 1.8% in the treatment containing 1 μM melatonin vs. 67.7% ± 2.8% in CM; Table 2). Melatonin-induced decreases of both VAP and STR were recovered after 5 min and 60 min of the addition of progesterone and melatonin at 0.5 and 1 μM (VAP after 60 min of the addition of progesterone and melatonin at 1 μM: 50.1 μm/sec ± 3.1 μm/sec vs. 56.8 μm/sec ± 3.3 μm/sec in CM; STR after 60 min of the addition of progesterone and melatonin at 1 μM: 77.1% ± 4.4% vs. 76.2% ± 3.3% in CM; Tables 1 and 2). In contrast, LIN was only recovered after 60 min of the addition of progesterone and melatonin at the same concentrations (LIN after 60 min of the addition of progesterone and melatonin at 1 μM: 32.7% ± 1.5% vs. 37.1% ± 2.0% in CM; Table 2).

Effects of melatonin on sperm agglutination

Incubation of spermatozoa in CM increased their degree agglutination, which was 60.9% ± 7.5% at 4 h (Fig. 2). Agglutinations were of medium size (Figure S6D,E), and about 45% of agglutinated spermatozoa showed appreciable tail beating at 4 h of incubation (Fig. 3). Although the percentage of agglutinated spermatozoa did not vary after the addition of progesterone (Fig. 2, Figure S6F), the percentage of agglutinated spermatozoa with appreciable tail beating showed a transient increase upon progesterone addition and then started to decrease, reaching a value of 17.3% ± 2.6% at the end of the experiment (Fig. 3). In contrast to the aforementioned, spermatozoa incubated in NCM did show low percentages of agglutinated spermatozoa (Fig. 2, Figure S6A–C). The addition of melatonin at 0 h induced an immediate and significant ($p < 0.05$) increase in the percentage of agglutinated spermatozoa (5 μM melatonin: 56.2% ± 6.4% vs. CM: 26.1% ± 3.8%; Fig. 2A and Figure S6G). This increase

Figure 1 Effects of melatonin on total motility of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis. (A): Melatonin added at 0 h. (B): Melatonin added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM medium (C−). Light grey bars: spermatozoa incubated in CM medium (C+). Medium grey bars: spermatozoa incubated in CM added with 0.5 μM melatonin. Dark green bars: spermatozoa incubated in CM added with 1 μM melatonin. Black bars: spermatozoa incubated in CM added with 5 μM melatonin. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.

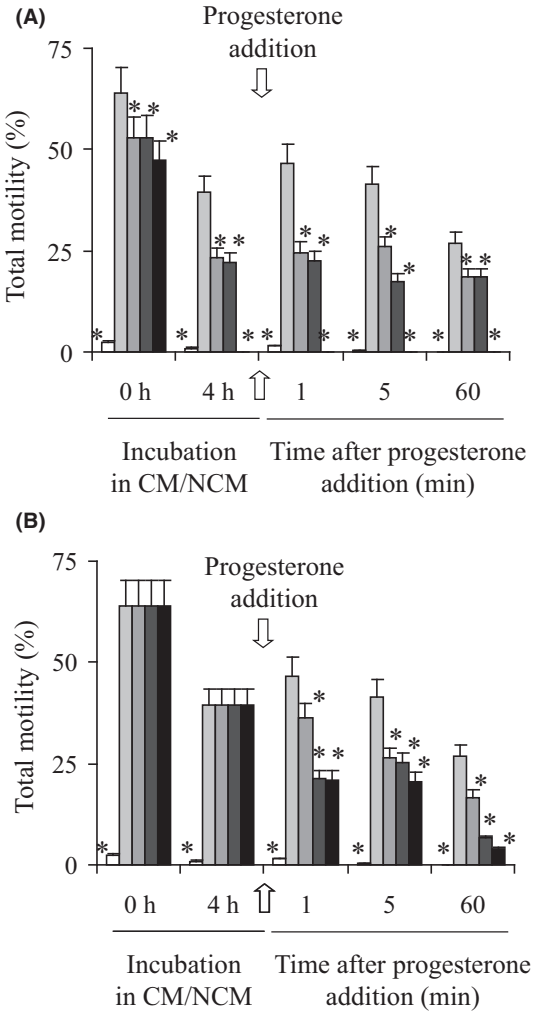


Table 1 Effects of melatonin on curvilinear velocity (VCL) and average path velocity (VAP) of boar spermatozoa subjected to in vitro capacitation and subsequent, progesterone-induced in vitro acrosome exocytosis

Incubation time	0 h	4 h	1 min	5 min	60 min
VCL (μm/sec)					
C−	77.0 ± 2.9^{a*}	39.0 ± 1.1^{b*}	39.1 ± 0.9^{b*}	38.5 ± 1.2^{c*}	0^{d*}
C+	65.7 ± 2.1^{a}	72.9 ± 2.9^{b}	74.1 ± 3.2^{b}	81.4 ± 3.7^{b}	81.6 ± 4.3^{b}
0.5 μM melatonin	67.5 ± 2.3^{a}	55.6 ± 1.5^{b*}	57.9 ± 1.7^{b*}	57.0 ± 2.2^{b*}	44.5 ± 1.2^{c*}
1 μM melatonin	56.2 ± 1.8^{a*}	60.7 ± 2.5^{a*}	59.1 ± 2.6^{a*}	61.2 ± 3.5^{a*}	41.2 ± 2.8^{b*}
5 μM melatonin	62.5 ± 2.5^{a}	0^{b*}	0^{b*}	0^{b*}	0^{b*}
0.5 μM melatonin+PG	65.7 ± 2.1^{a}	72.9 ± 2.9^{b}	68.2 ± 2.7^{a}	90.8 ± 4.7^{c}	76.5 ± 3.9^{b}
1 μM melatonin+PG	65.7 ± 2.1^{a}	72.9 ± 2.9^{b}	67.7 ± 2.6^{a}	89.9 ± 4.5^{c}	76.8 ± 4.2^{b}
5 μM melatonin+PG	65.7 ± 2.1^{a}	72.9 ± 2.9^{b}	71.4 ± 3.1^{ab}	81.1 ± 4.3^{c}	66.8 ± 4.0^{a*}
VAP (μm/sec)					
C−	35.5 ± 1.4^{a*}	25.1 ± 1.1^{b*}	27.5 ± 1.6^{b*}	33.6 ± 0.9^{b*}	0^{c*}.
C+	46.0 ± 2.4^{a}	62.6 ± 3.2^{b}	59.8 ± 2.7^{b}	56.7 ± 2.8^{b}	56.8 ± 3.3^{b}
0.5 μM melatonin	48.1 ± 2.0^{a}	44.6 ± 2.2^{a*}	47.0 ± 2.7^{a*}	62.6 ± 3.7^{b}	85.6 ± 5.4^{c*}
1 μM melatonin	39.8 ± 1.4^{a*}	37.4 ± 1.2^{a*}	47.2 ± 2.6^{b*}	67.2 ± 3.6^{c*}	92.4 ± 6.1^{d*}
5 μM melatonin	39.8 ± 1.6^{a*}	0^{b*}	0^{b*}	0^{b*}	0^{b*}
0.5 μM melatonin+PG	46.0 ± 2.4^{a}	62.6 ± 3.2^{b}	40.8 ± 2.0^{a*}	52.2 ± 4.1^{a}	49.1 ± 3.0^{a}
1 μM melatonin+PG	46.0 ± 2.4^{a}	62.6 ± 3.2^{b}	40.9 ± 2.0^{a*}	51.9 ± 3.9^{a}	50.1 ± 3.1^{a}
5 μM melatonin+PG	46.0 ± 2.4^{a}	62.6 ± 3.2^{b}	38.8 ± 1.8^{c*}	35.4 ± 3.1^{c*}	39.5 ± 1.7^{c*}

Spermatozoa were subjected to IVC and further IVAE as described in the Materials and Methods section. Determination of motion parameters through CASA and statistical analyses has been also described in the Material and Methods section. Spermatozoa were incubated in a non-capacitating medium (NCM, C−) or in capacitating medium without (CM, C+) or with melatonin at final concentrations of 0.5 μM (0.5 μM melatonin), 1 μM (1 μM melatonin) and 5 μM (5 μM melatonin). After 4 h of incubation, progesterone (PG) was added. Simultaneously, three more aliquots were incubated in capacitating medium and, after 4 h of incubation, were added with progesterone and 0.5 μM melatonin (0.5 μM melatonin+PG), progesterone with 1 μM melatonin (1 μM melatonin+PG) and progesterone with 5 μM (5 μM melatonin +PG). In all cases, spermatozoa were subsequently incubated, and aliquots were taken after 1, 5 and 60 min of progesterone addition. Different superscript letters (a–d) indicate significant differences ($p < 0.05$) between columns within a given row. Asterisks indicate significant differences ($p < 0.05$) when compared with C+ (CM) at the same time point. Results are shown as means ± SEM for seven separate experiments.

Table 2 Effects of melatonin on linearity (LIN) and straightness (STR) coefficients of boar spermatozoa subjected to in vitro capacitation and subsequent, progesterone-induced in vitro acrosome exocytosis

Incubation time	0 h	4 h	1 min	5 min	60 min
LIN (%)					
C−	23.6 ± 1.1^{a*}	35.1 ± 1.3^{b*}	36.3 ± 1.6^{b}	33.6 ± 1.5^{b*}	0^{c*}
C+	36.2 ± 1.4^{a}	44.8 ± 2.4^{b}	39.9 ± 1.7^{a}	47.9 ± 2.3^{b}	37.1 ± 2.0^{a}
0.5 μM melatonin	42.0 ± 1.9^{a}	34.8 ± 1.8^{b*}	33.5 ± 1.6^{b}	43.2 ± 2.2^{a}	33.2 ± 2.1^{b}
1 μM melatonin	36.2 ± 1.7^{a}	30.8 ± 2.1^{a*}	33.6 ± 1.4^{a}	44.9 ± 2.4^{b}	35.3 ± 1.8^{a}
5 μM melatonin	39.8 ± 2.2^{a}	0^{b*}	0^{b*}	0^{b*}	0^{b*}
0.5 μM melatonin+PG	36.2 ± 1.4^{a}	44.8 ± 2.4^{b}	26.8 ± 1.2^{c*}	36.5 ± 1.8^{a*}	32.7 ± 1.5^{a}
1 μM melatonin+PG	36.2 ± 1.4^{a}	44.8 ± 2.4^{b}	25.7 ± 1.6^{c*}	36.3 ± 1.9^{a*}	33.2 ± 1.8^{a}
5 μM melatonin+PG	36.2 ± 1.4^{a}	44.8 ± 2.4^{b}	26.0 ± 1.3^{c*}	30.1 ± 1.9^{c*}	28.2 ± 1.2^{c*}
STR (%)					
C−	55.8 ± 2.3^{a*}	48.5 ± 2.4^{b*}	44.6 ± 2.0^{b*}	43.6 ± 1.9^{b*}	0^{c*}
C+	63.3 ± 2.4^{a}	70.9 ± 3.0^{b}	67.7 ± 2.8^{ab}	71.0 ± 2.9^{b}	76.2 ± 3.3^{b}
0.5 μM melatonin	64.7 ± 2.3^{a}	83.9 ± 3.4^{b*}	67.1 ± 3.0^{a}	69.0 ± 3.6^{a}	81.2 ± 4.5^{b}
1 μM melatonin	62.1 ± 2.4^{a}	52.1 ± 1.5^{b*}	68.6 ± 2.5^{ab}	79.1 ± 3.6^{c}	73.9 ± 3.1^{b}
5 μM melatonin	63.1 ± 2.4^{a}	0^{b*}	0^{b*}	0^{b*}	0^{b*}
0.5 μM melatonin+PG	63.3 ± 2.4^{a}	70.9 ± 3.0^{b}	60.6 ± 2.5^{a}	76.1 ± 3.7^{b}	77.1 ± 4.4^{b}
1 μM melatonin+PG	63.3 ± 2.4^{a}	70.9 ± 3.0^{b}	58.1 ± 1.8^{a}	70.5 ± 2.6^{b}	76.0 ± 4.5^{b}
5 μM melatonin+PG	63.3 ± 2.4^{a}	70.9 ± 3.0^{b}	55.6 ± 1.6^{b*}	64.6 ± 1.9^{a*}	64.8 ± 2.9^{a*}

Spermatozoa were subjected to IVC and further IVAE as described in the Material and Methods section. Determination of motion parameters through CASA and statistical analyses has been also described in the Material and Methods section. Spermatozoa were incubated in a non-capacitating medium (NCM, C−) or in capacitating medium without (CM, C+) or with melatonin at final concentrations of 0.5 μM (0.5 μM Melatonin), 1 μM (1 μM melatonin) and 5 μM (5 μM Melatonin). After 4 h of incubation, progesterone (PG) was added. Simultaneously, three more aliquots were incubated in capacitating medium and, after 4 h of incubation, were added with progesterone and 0.5 μM melatonin (0.5 μM melatonin+PG), progesterone with 1 μM melatonin (1 μM melatonin+PG) and progesterone with 5 μM (5 μM melatonin +PG). In all cases, spermatozoa were subsequently incubated, and aliquots were taken after 1, 5 and 60 min of progesterone addition. Different superscript letters (a–c) indicate significant differences ($p < 0.05$) between columns within a given row. Asterisks indicate significant differences ($p < 0.05$) when compared with C+ (CM) at the same time point. Results are shown as means ± SEM for seven separate experiments.

continued and reached values of about 80–85% at 4 h, when more than a hundred spermatozoa were observed in a single agglutination (Figure S6H). Similar results were found throughout the remaining experimental period (Fig. 2A, Figure S6I).

Regarding the percentage of agglutinated spermatozoa with appreciable tail beating, melatonin induced a significant ($p < 0.05$) decrease in all the tested concentrations, reaching minimal values at 4 h (melatonin μM: 12.8% ± 1.9% vs. CM: 43.2% ± 3.2%; Fig. 3A). After progesterone addition, a similar

decreasing pattern was observed. Finally, the addition of both 1 and 5 μM melatonin at 4 h did counteract the decreases in the percentages of agglutination and of agglutinated spermatozoa with tail beating observed in control samples 60 min after progesterone addition (Fig. 3; Figure S6J).

Effects of melatonin on intracellular ROS levels

Incubation of boar spermatozoa in CM induced a slight, but significant ($p < 0.05$) increase in the percentage of viable spermatozoa with high intracellular H_2O_2 levels, which went from 1.6% ± 0.2% at 0 h to 5.8% ± 1.3% at 4 h (Fig. 5A,B). This was in contrast with sperm cells incubated in NCM in which the extent of that increase was higher (9.4% ± 2.5% at 4 h; Fig. 4). The subsequent addition of progesterone did not significantly modify the percentage of high-H_2O_2 cells in spermatozoa incubated in CM, whereas those incubated in NCM showed a slight and gradual increase, reaching values of 13.6% ± 2.9% after 60 min of progesterone addition (Fig. 4).

The addition of melatonin at 0 h did not significantly affect the pattern observed in CM, except in the case of melatonin 5 μM, where there was a significant ($p < 0.05$) decrease in the percentage of cells with high H_2O_2 levels after 1 min of progesterone addition that was not further recovered (Fig. 4A). The addition of melatonin at 1 or 5 μM at 4 h showed a significant ($p < 0.05$) decrease in this percentage at 1 min post-progesterone addition (Fig. 4B).

Percentages of viable spermatozoa with high intracellular $O_2^{-\bullet}$ levels slightly decreased throughout incubation time and went from 1.4% ± 0.2% at 0 h to 6.2% ± 1.7% at 4 h (Fig. 5A,B). Subsequent addition of progesterone did not have a remarkable

Figure 2 Effects of melatonin on the percentage of agglutinated cells of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis. (A): Melatonin added at 0 h. (B): Melatonin added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM medium (C−). Light grey bars: spermatozoa incubated in CM medium (C+). Medium grey bars: spermatozoa incubated in CM added with 0.5 μM melatonin. Dark green bars: spermatozoa incubated in CM added with 1 μM melatonin. Black bars: spermatozoa incubated in CM added with 5 μM melatonin. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.

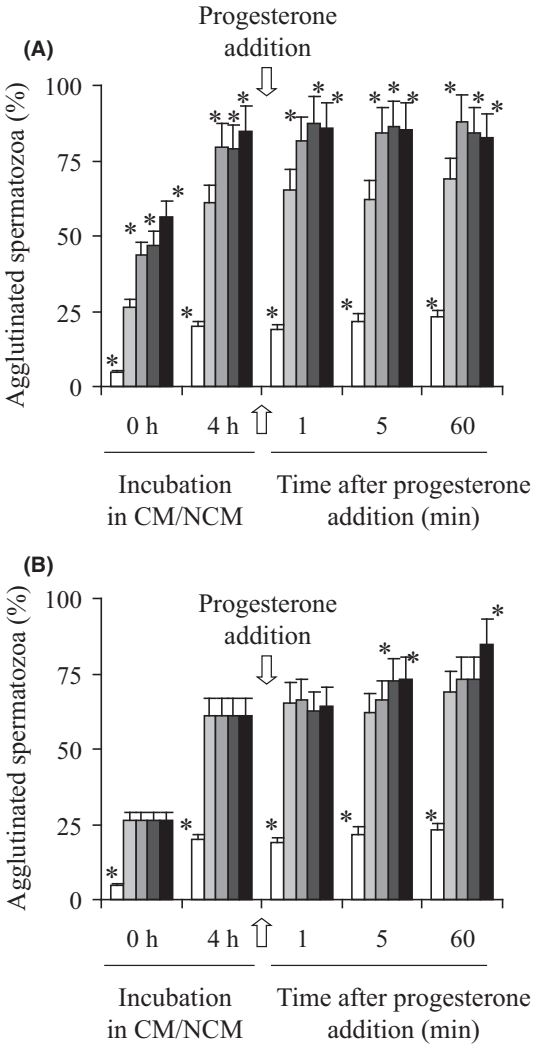


Figure 3 Effects of melatonin on the percentage of agglutinated cells with beating tails of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis. (A): Melatonin added at 0 h. (B): Melatonin added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM medium (C−). Light grey bars: spermatozoa incubated in CM medium (C+). Medium grey bars: spermatozoa incubated in CM added with 0.5 μM melatonin. Dark green bars: spermatozoa incubated in CM added with 1 μM melatonin. Black bars: spermatozoa incubated in CM added with 5 μM melatonin. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.

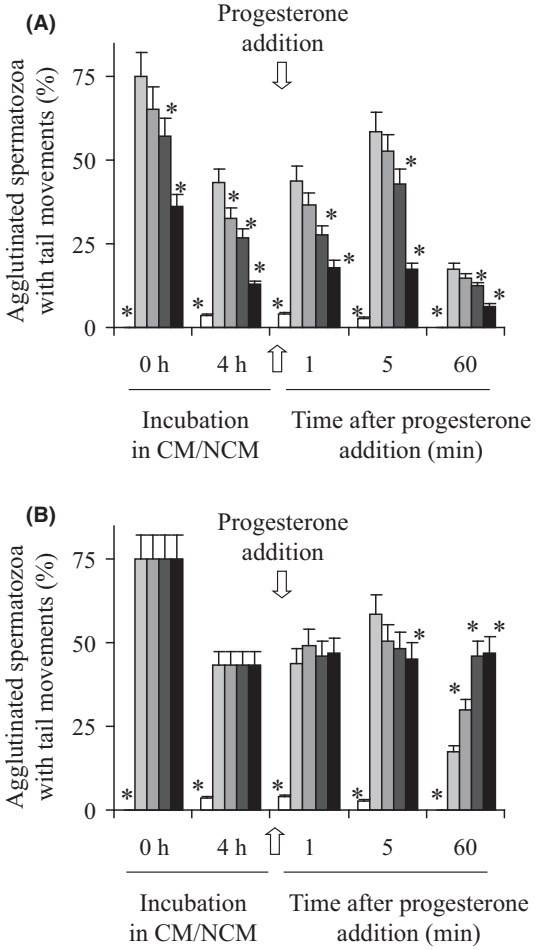
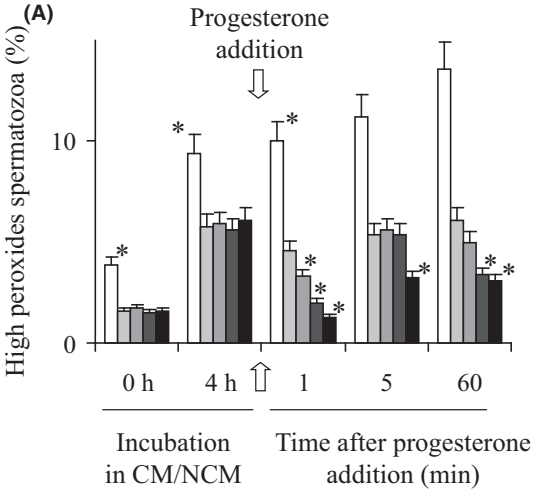


Figure 4 Effects of melatonin on the percentage of viable spermatozoa with high intracellular peroxide levels of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis. (A): Melatonin added at 0 h. (B): Melatonin added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM medium (C−). Light grey bars: spermatozoa incubated in CM medium (C+). Medium grey bars: spermatozoa incubated in CM added with 0.5 μM melatonin. Dark green bars: spermatozoa incubated in CM added with 1 μM melatonin. Black bars: spermatozoa incubated in CM added with 5 μM melatonin. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.



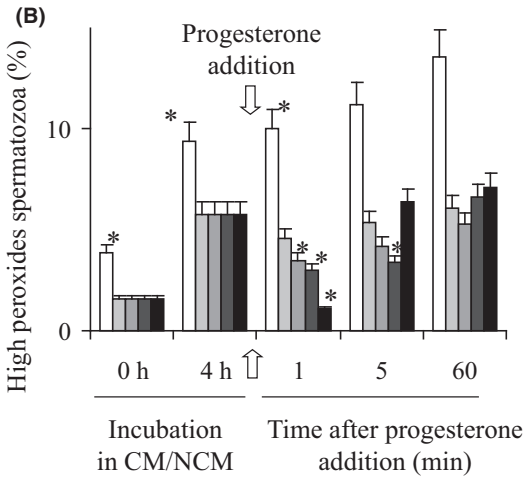
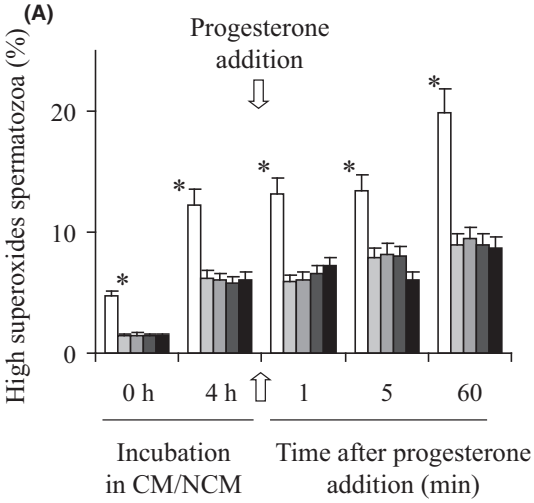
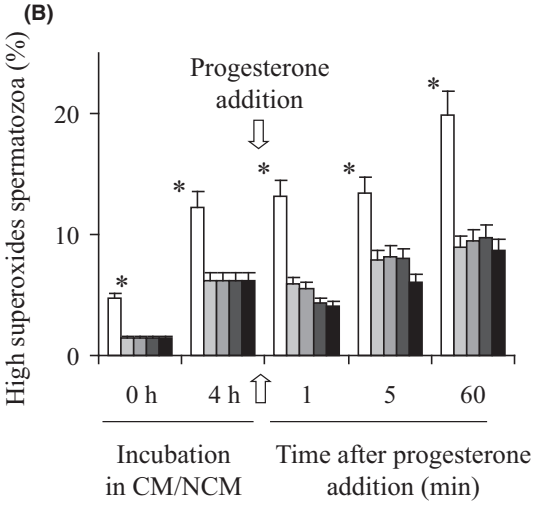


Figure 5 Effects of melatonin on the percentage of viable spermatozoa with high intracellular superoxide levels of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis. (A): Melatonin added at 0 h. (B): Melatonin added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM medium (C−). Light grey bars: spermatozoa incubated in CM medium (C+). Medium grey bars: spermatozoa incubated in CM added with 0.5 μM melatonin. Dark green bars: spermatozoa incubated in CM added with 1 μM melatonin. Black bars: spermatozoa incubated in CM added with 5 μM melatonin. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.





effect on this parameter, and a slight increase was seen after 60 min of progesterone addition (9.0% ± 2.8%; Fig. 5A,B). In the case of incubation in NCM, the values were significantly higher, reaching values of 19.8% ± 4.1% after 60 min of progesterone addition. The addition of melatonin at any of the tested concentrations either at 0 h or at 4 h did not differ from spermatozoa incubated in CM (Fig. 5A,B).

Effects of melatonin on the free cysteine residues in both head and tail sperm extracts

Incubation of boar spermatozoa in CM induced a progressive increase in the free cysteine levels from head extracts which went from 3.9 nmol/g protein ± 0.3 nmol/g protein at 0 h to 17.2 nmol/g protein ± 2.3 nmol/g protein at 4 h (Fig. 6A,B). This increase was not observed in sperm cells incubated in NCM. Subsequent addition of progesterone to spermatozoa incubated in CM did not increase these levels, and there was a slight gradual decrease with values of 11.3 nmol/g protein ± 1.7 nmol/g protein after 60 min of progesterone addition (Fig. 6A,B).

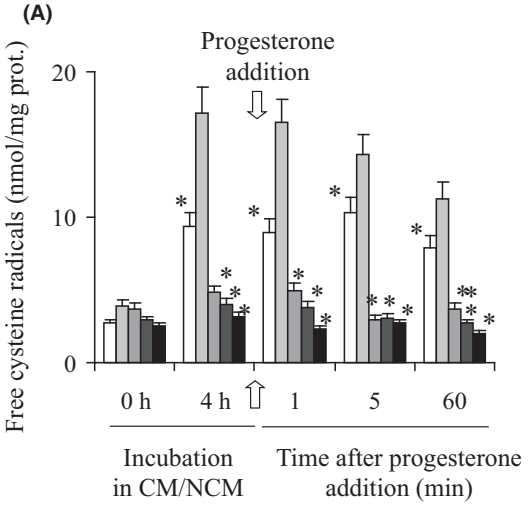
Addition of melatonin at 0 h had a dramatic effect on free cysteine levels from head extracts, as almost abolished the increase observed in CM (Fig. 6A). This effect was maintained after the addition of progesterone. When melatonin was added at 4 h, a similar decreasing effect on free cysteine levels of sperm head extracts was immediately observed (i.e. 1 min after melatonin and progesterone addition), especially at the highest concentrations.

Free cysteine levels of sperm tail extracts also increased in spermatozoa incubated in CM. Values went from 3.7 nmol/g protein ± 0.6 nmol/g protein at 0 h to 9.3 nmol/g protein ± 1.9 nmol/g protein at 4 h (Fig. 7A,B). These values were roughly maintained after the addition of progesterone. The addition of melatonin at 0 h almost abolished that increase at any concentration tested (Fig. 7A). On the contrary, the addition of melatonin at 4 h had no clear effect on this parameter before 60 min after progesterone addition, when free cysteine levels of sperm tail extracts were found to increase in a melatonin dose-dependent manner (5 μM: 12.8 nmol/g protein ± 2.4 nmol/g protein vs. CM: 8.9 nmol/g protein ± 1.7 nmol/g; Fig. 7A).

Effects of melatonin on distribution of lectins over sperm membrane

At the beginning of incubation in CM, WGA signal was located at the sperm head and the whole tail, although the maximal intensity of the signal was observed at the acrosomal edge (Figure S7). Spermatozoa incubated in NCM showed a similar staining, but the acrosomal signal was much less intense. After 4 h of incubation in CM, the intensity of the acrosome-located signal increased and uniformly distributed throughout the entire acrosome (Figure S7). These changes were not detected in spermatozoa incubated in NCM for 4 h. The subsequent addition of

Figure 6 Effects of melatonin on the head intracellular free cysteine radicals levels of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis. (A): Melatonin added at 0 h. (B): Melatonin added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM medium (C−). Light grey bars: spermatozoa incubated in CM medium (C+). Medium grey bars: spermatozoa incubated in CM added with 0.5 μM melatonin. Dark green bars: spermatozoa incubated in CM added with 1 μM melatonin. Black bars: spermatozoa incubated in CM added with 5 μM melatonin. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.



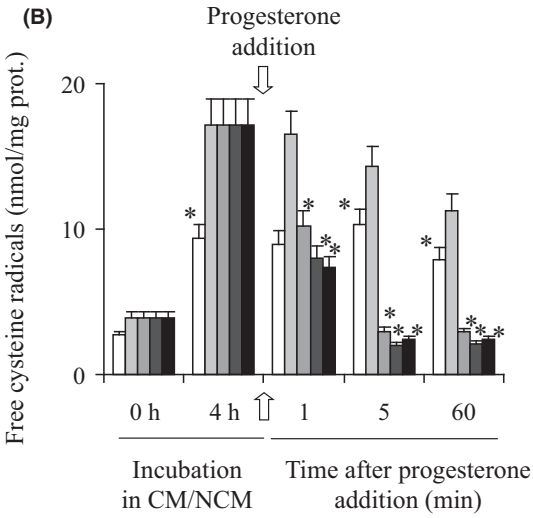
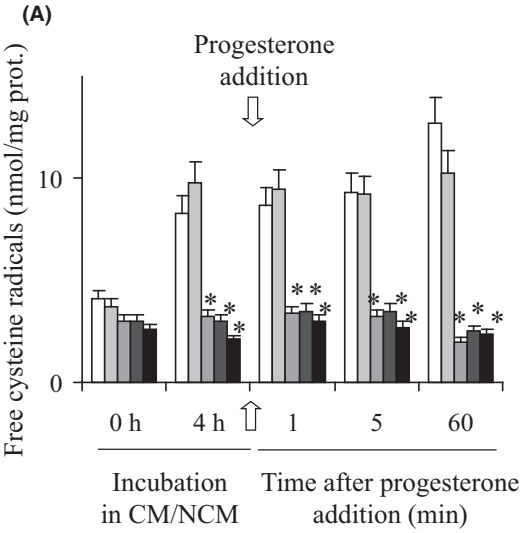
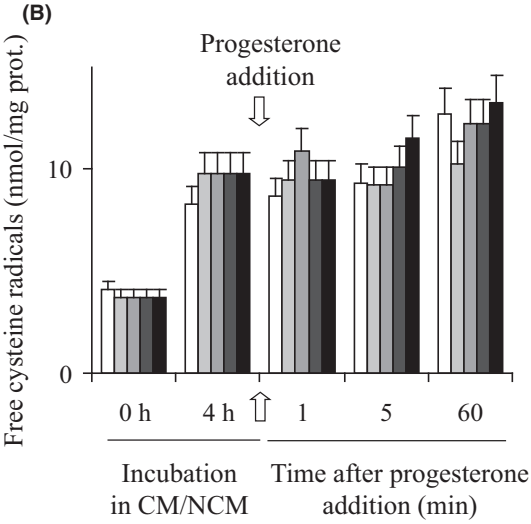


Figure 7 Effects of melatonin on the tail intracellular free cysteine radical levels of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis. (A): Melatonin added at 0 h. (B): Melatonin added together with progesterone at 4 h. White bars: spermatozoa incubated in NCM medium (C−). Light grey bars: spermatozoa incubated in CM medium (C+). Medium grey bars: spermatozoa incubated in CM added with 0.5 μM melatonin. Dark green bars: spermatozoa incubated in CM added with 1 μM melatonin. Black bars: spermatozoa incubated in CM added with 5 μM melatonin. Asterisks indicate significant ($p < 0.05$) differences between a given treatment and C+ samples. Figure shows means ± SEM for seven separate experiments.





progesterone to CM induced further modifications in the acrosome signal of WGA. In effect, although small changes were observed after 1 min of adding progesterone, with some sperm cells losing the inner lectin signal and others showing an irregular acrosome marking, the progesterone-induced changes were much more apparent after 5 min, when a high number of sperm cells showed a diffuse inner or irregular acrosome signal (Figure S7). These patterns were also observed after 60 min of progesterone addition. Addition of melatonin at 0 h induced changes in the WGA-staining pattern. At 4 h, most of the spermatozoa incubated with melatonin showed much more intense acrosome signal than control spermatozoa (CM), but there were also sperm cells that showed no lectin signal in the post-acrosomal area and even spermatozoa with no signal over the head (Figure S7). The addition of progesterone to spermatozoa incubated for 4 h in treatments containing melatonin did not induce immediate changes in WGA distribution. However, the acrosome staining in spermatozoa incubated with melatonin was more intense than that observed in control spermatozoa incubated in CM after 60 min of incubation (Figure S7). In contrast, the addition of 1 μM melatonin at 4 h did not change WGA distribution when compared to control spermatozoa incubated in CM (Figure S7).

Regarding STL, it was mainly found in sperm head and midpiece (in some cells, STL was observed in the entire tail) at 0 h, with a more intense signal in the acrosome area (Figure S8). Following incubation in CM for 4 h, two different STL-staining patterns were observed. Whereas one pattern consisted of an intense and uniform signal throughout the entire acrosome area, STL staining in the other was mainly restricted to the acrosomal edge (Figure S8). In both patterns, STL signal in the midpiece was much decreased or totally absent. The addition of progesterone at 4 h decreased the intensity of acrosome signal in practically all sperm cells (which was already apparent after 1 min of progesterone addition) but increased that of the tail (Figure S8). Addition of 1 μM melatonin at 0 h exhibited similar STL-staining patterns to the control (Figure S8) and there was a clear loss of STL signal in the acrosome following progesterone addition, the STL-staining being restricted to the acrosome edge. While the addition of 1 μM melatonin at 4 h did not clearly affect the STL pattern observed in spermatozoa incubated in CM, a high proportion of spermatozoa showed a clearly intense acrosome signal after 1 min of the addition of melatonin and progesterone, which differed from spermatozoa incubated in CM (Figure S8).

At 0 h, PSA staining was observed in the whole cell (spermatozoa incubated in CM), although the most intense marking was detected in the entire acrosomal area. In the case of spermatozoa incubated in NCM, the acrosomal signal was much less intense and restricted to the acrosome edge (Figure S9). Incubation in CM for 4 h induced an increase in the PSA-staining of the sperm head and tail, despite the post-acrosomal region being devoid of PSA signal. Subsequent addition of progesterone induced a rapid loss of PSA signal in the acrosome, which was evident in a high percentage of spermatozoa after 1 min of the addition of the hormone (Figure S9). In spite of this, there were other spermatozoa that showed an intense acrosome signal. The addition of 1 μM melatonin either at 0 h or at 4 h did not have a clear effect on PSA localisation during IVC and IVAE (Figure S9).

Regarding the localisation of PNA, it was exclusively found at the whole acrosome surface and there were no changes after 4 h of incubation either in CM or in NCM (Figure S10). As expected, the addition of progesterone at 4 h increased the proportion of spermatozoa with less intensity of PNA marking and faint staining restricted to the acrosome edge (Figure S10). These changes were very rapid, as they were already observed after 1 min of progesterone addition. Addition of melatonin either at 0 h or at 4 h showed no differences when compared to spermatozoa incubated in CM (Figure S10).

Effects of melatonin on the ability of in vitro capacitated boar spermatozoa to adhere and penetrate pig oocytes

As shown in Table 4, the number of spermatozoa attached to the ZP (78.4 ± 1.8), the total penetration rate (90.6%) and the percentage of monospermy (69.8%) were higher in the control group (CM) than in the other treatments. Previous incubation of spermatozoa with 1 μM melatonin significantly ($p < 0.05$) decreased the number of spermatozoa adhered to the ZP (68.2 ± 2.7 vs. 78.4 ± 1.8 in CM). Furthermore, incubation with melatonin at 1 μM significantly ($p < 0.05$) decreased the proportion of polyspermic oocytes (14.5% vs. 20.8% in CM). The addition of 1 μM melatonin after in vitro capacitation for 4 h had no effect on polyspermy, but increased the number of spermatozoa attached to the ZP (88.9 ± 1.7 vs. 78.4 ± 1.8 in CM; Table 4).

DISCUSSION

The results shown herein suggest that one of the most important effects of melatonin during the achievement of boar spermatozoa IVC is the increase in cell adhesiveness. The increase in sperm adhesiveness would influence important aspects of boar sperm capacitation such as sperm motility and sperm–zona pellucida interaction through the activation of sperm agglutination, as the results suggested (Fig. 2 and Tables 1–3). Additionally, melatonin did not affect several of the most important capacitation markers, such as membrane lipid disorder, tyrosine phosphorylation levels of P32 and the ability to reach acrosome exocytosis after progesterone stimulation. This lack of additional effects could suggest that the melatonin action of IVC is mainly focused on motility and/or sperm adhesiveness. Regarding the relationship between sperm motility and agglutination, it is worth noting that IVC in species such as the monkey, cattle, sheep and pig leads to an increase in the percentage of agglutinated spermatozoa (Boatman & Bavister, 1984; Ehrenwald *et al.*, 1990; Funahashi & Day, 1993; Lefebvre & Suarez, 1996; Leahy *et al.*, 2016). In fact, sperm agglutination does not only result from IVC, but also from other factors, such as the presence of antibodies (Yakirevich & Naota, 1999) or in response to cell degeneration (Harayama *et al.*, 1998). Furthermore, several components of capacitation media, such as heparin in cattle, BSA in horse, and bicarbonate and calcium in pigs, monkeys and cattle increase sperm agglutination (Lindahl & Sjöblom, 1981; Boatman & Bavister, 1984; Ehrenwald *et al.*, 1990; Funahashi & Day, 1993; Lefebvre & Suarez, 1996; Harayama *et al.*, 1998; Harayama & Kato, 2002). Therefore, in our experimental conditions, melatonin could have enhanced the agglutination-promoting effect of CM components. This hypothesis could explain why the addition of melatonin at concentrations lower than those assayed in this work (100 pM) does not agglutinate ram spermatozoa (Casao *et al.*, 2009). Based on CM composition, one could

suggest that bicarbonate is the agglutinating factor whose action is potentiated by the addition of melatonin. This hypothesis is based on previous works from our laboratory, in which IVC of boar spermatozoa was achieved in a medium without bicarbonate and not much agglutination was observed (Ramió *et al.*, 2008; Ramió-Lluch *et al.*, 2011; Ramió-Lluch *et al.*, 2014). While we cannot determine the exact mechanism through which melatonin could enhance sperm agglutination, our results suggest it is unlikely to be related to changes in the glycocalix composition of membrane surface, as lectin-binding assays did not show apparent changes (Figures S7–S10). Therefore, we could propose two explanations. The first one would be related to a cAMP-mediated mechanism via the activation of the bicarbonate-sensitive adenylyl cyclase and PKA (Harayama & Kato, 2002). Another possible mechanism would involve the maintenance of disulphide bonds, as melatonin induced a clear decrease in the intracellular free cysteine levels of boar spermatozoa. In this respect, it is worth noting that penicillamine has a potent action against agglutination in ram spermatozoa subjected to IVC (Leahy *et al.*, 2016), and one of the mechanisms through which it exerts that effect is linked to its direct action on disulphide radicals, which are converted into sulfhydryl groups (Talevi *et al.*, 2007; Gualtieri *et al.*, 2009). These data would be in concordance with the results obtained in this study, suggesting that the effects of melatonin on sperm agglutination and free cysteine levels could be linked. However, more work is needed to further elucidate this point.

The results observed following sperm–oocyte co-incubation could also be a consequence of melatonin action on sperm agglutination rather than on other capacitation-related changes such as the ability to trigger acrosome exocytosis following the appropriate stimuli, namely progesterone. Thus, the reduction in the number of spermatozoa adhered to the ZP when they were previously capacitated in the presence of melatonin could be a consequence of an increased degree of sperm agglutination, thereby lowering the number of free spermatozoa able to adhere the ZP. Furthermore, as shown in Table 4, the addition of melatonin after IVC significantly increased the number of spermatozoa bound to the ZP. The combined analysis of these results suggests that the melatonin-induced agglutinating effect in IVC conditions could be linked to an unspecific, increased adherence ability of boar spermatozoa to other sperm cells or the oocyte. In turn, the observed decrease in polyspermy in spermatozoa capacitated in the presence of melatonin could result from the decrease in the number of adhered spermatozoa.

Regarding the effects of melatonin on the proportions of boar spermatozoa with high intracellular ROS levels, results displayed in Figs 4 and 5 indicate that the antioxidant action of melatonin does not seem to play a prominent role in the effects observed during IVC and IVAE and, specifically, in the increase in sperm agglutination. While this conclusion could be surprising at first glance, one should note that boar spermatozoa are characterised by a very low ROS production rate, even when subjected to treatments that, such as freeze-thawing, induce the generation of high intracellular ROS in other species (Bilodeau *et al.*, 2000; Guthrie & Welch, 2006; Yeste *et al.*, 2013, 2015a). This feature is different from other species, such as the horse, in which ROS production is more intense (see Gibb & Aitken, 2016; as a review). As a consequence, in species such as equine, bovine and human, melatonin has a clear antioxidant effect and affects membrane lipid peroxidation and ROS levels (Gadella *et al.*, 2008; Rao & Gangadharan, 2008; Du Plessis *et al.*, 2010; Jang *et al.*, 2010; Da Silva *et al.*, 2011; Najafi *et al.*, 2018). In fact, the antioxidant effect of melatonin in species such as the horse is not only related to a direct action on the oxidative potential but also to intracellular Na^+ concentrations, which also affect the overall redox status (Ortega Ferrusola *et al.*, 2017). Taking all of these data into consideration, an expected antioxidant effect of melatonin on boar spermatozoa should be very subtle, if detectable. A possible reason to explain why boar spermatozoa do not accumulate high ROS levels, and thus why melatonin would exert a slight action on this parameter, could be linked to a species-specific mitochondria function. Previous reports have suggested that, despite being important to maintain crucial sperm functions, such as motility, mitochondrial-produced energy is low in boar spermatozoa (Rodríguez-Gil & Bonet, 2016). Indeed, O_2 consumption rate and intracellular ATP levels have been reported to be low during IVC and subsequent IVAE (Ramió-Lluch *et al.*, 2014). In addition to this, induction of IVC in the presence of oligomycin A, a specific inhibitor of the mitochondrial ATP synthase, does not decrease either O_2 consumption rates or ATP levels (Ramió-Lluch *et al.*, 2014), which suggests that boar sperm mitochondria are in an uncoupled status throughout most of their lifespan. A consequence of this uncoupled status is the low rate of ROS production due to substimulation of the electronic chain, which is the most important ROS-synthesising point (Rodríguez-Gil & Bonet, 2016). Interestingly, progesterone-induced acrosome exocytosis is concomitant with a sudden and intense peak in both O_2 consumption rate and intracellular ATP levels (Balis *et al.*, 1999; Gualtieri *et al.*, 2009), which suggests that mitochondria are coupled at this moment. Following this rationale, mitochondrial coupling upon progesterone addition would be associated with a transient increase in ROS generation, especially that of peroxides (Tait & Green, 2012). This hypothesis matches with our observations, as the addition of melatonin together with that of progesterone was found to decrease the proportions of viable spermatozoa with high peroxide levels (see Fig. 4A). However, the fact that such a decrease did not modify the percentage of acrosome-exocytosed spermatozoa induced by progesterone suggests that peroxide levels do not play a vital role for acrosome reaction in boar spermatozoa, at least under our in vitro conditions.

As indicated when discussing the effects on sperm agglutination and adhesiveness, melatonin decreased the intracellular free cysteine levels in both sperm head and tail extracts (see Figs 6 and 7). Free cysteine levels are an indirect marker of the number of disrupted disulphide bonds, as despite not all free cysteine radicals resulting from the breakage of disulphide bonds, a significant percentage has this origin (Yeste *et al.*, 2013, 2014). Our results also indicated that the melatonin-induced decrease in free cysteine residues of sperm head has no impact on achieving the IVC and subsequent progesterone-induced IVAE. Another issue is the observed increase in free cysteine residues in sperm tail extracts during IVC and the melatonin-counteracting effect of that increase. Disulphide bonds are important for the maintenance of a proper sperm flagellum structure (Ijiri *et al.*, 2014). Specifically, disulphide bonds are crucial for a protein associated with the outer dense fibre 1 (ODF1; Cabrillana *et al.*, 2011). Thus, one could suggest that changes in free cysteine residues of sperm tail would affect the structure of the

Table 3 Effects of melatonin on mean amplitude of lateral head displacement (ALH) and frequency of head displacement (BCF) of boar spermatozoa subjected to in vitro capacitation and subsequent, progesterone-induced in vitro acrosome exocytosis

Incubation time	0 h	4 h	1 min	5 min	60 min
ALH (μm)					
C−	2.56 ± 0.05^{a*}	2.57 ± 0.09^{a*}	2.18 ± 0.11^{b*}	2.14 ± 0.10^{b*}	0^{c*}
C+	3.80 ± 0.12^{a}	4.13 ± 0.13^{b}	4.93 ± 0.15^{c}	4.64 ± 0.15^{c}	4.00 ± 0.11^{b}
0.5 μM melatonin	3.31 ± 0.09^{a*}	2.88 ± 0.08^{b*}	4.10 ± 0.12^{c*}	3.63 ± 0.12^{ac*}	5.13 ± 0.21^{d*}
1 μM melatonin	3.50 ± 0.14^{a}	2.72 ± 0.09^{b*}	3.68 ± 0.08^{a*}	3.49 ± 0.11^{a*}	3.85 ± 0.10^{a}
5 μM Melatonin	3.89 ± 0.13^{a}	0^{b*}	0^{b*}	0^{b*}	0^{b*}
0.5 μM melatonin+PG	3.80 ± 0.12^{a}	4.13 ± 0.13^{b}	4.82 ± 0.20^{b}	5.09 ± 0.20^{b}	3.81 ± 0.14^{a}
1 μM melatonin+PG	3.80 ± 0.12^{a}	4.13 ± 0.13^{b}	5.38 ± 0.25^{c}	4.96 ± 0.19^{c}	3.09 ± 0.11^{d*}
5 μM melatonin+PG	3.80 ± 0.12^{a}	4.13 ± 0.13^{b}	5.18 ± 0.20^{c}	4.93 ± 0.18^{c}	2.99 ± 0.06^{d*}
BCF (Hz)					
C−	2.92 ± 0.04^{a*}	3.97 ± 0.26^{b*}	3.88 ± 0.21^{b*}	4.01 ± 0.24^{b*}	0^{c*}
C+	6.68 ± 0.17^{a}	6.54 ± 0.19^{a}	7.18 ± 0.18^{b}	6.28 ± 0.21^{a}	6.03 ± 0.19^{c}
0.5 μM melatonin	6.73 ± 0.15^{a}	6.70 ± 0.16^{a}	7.20 ± 0.12^{b}	6.97 ± 0.24^{b*}	6.85 ± 0.21^{ab*}
1 μM melatonin	6.16 ± 0.11^{a*}	5.95 ± 0.11^{a*}	6.76 ± 0.20^{b*}	6.90 ± 0.24^{b*}	7.20 ± 0.27^{c*}
5 μM melatonin	6.05 ± 0.10^{a*}	0^{b*}	0^{b*}	0^{b*}	0^{b*}
0.5 μM melatonin+PG	6.68 ± 0.17^{a}	6.54 ± 0.19^{a}	6.86 ± 0.21^{a}	5.46 ± 0.09^{b*}	4.37 ± 0.06^{c*}
1 μM melatonin+PG	6.68 ± 0.17^{a}	6.54 ± 0.19^{a}	7.48 ± 0.29^{b}	4.98 ± 0.09^{c*}	3.91 ± 0.08^{d*}
5 μM melatonin+PG	6.68 ± 0.17^{a}	6.54 ± 0.19^{ab}	6.44 ± 0.17^{b*}	4.92 ± 0.08^{c*}	3.85 ± 0.07^{d*}

Spermatozoa were subjected to IVC and further IVAE as described in the Material and Methods section. Determination of motion parameters through CASA and statistical analyses has been also described in the Material and Methods section. Spermatozoa were incubated in a non-capacitating medium (NCM, C−) or in capacitating medium without (CM, C+) or with melatonin at final concentrations of 0.5 μM (0.5 μM melatonin), 1 μM (1 μM melatonin) and 5 μM (5 μM melatonin). After 4 h of incubation, progesterone (PG) was added. Simultaneously, three more aliquots were incubated in capacitating medium and, after 4 h of incubation, were added with progesterone and 0.5 μM melatonin (0.5 μM melatonin+PG), progesterone with 1 μM melatonin (1 μM melatonin+PG) and progesterone with 5 μM (5 μM melatonin +PG). In all cases, spermatozoa were subsequently incubated, and aliquots were taken after 1, 5 and 60 min of progesterone addition. Different superscript letters (a–c) indicate significant differences ($p < 0.05$) between columns within a given row. Asterisks indicate significant differences ($p < 0.05$) when compared with C+ (CM) at the same time point. Results are shown as means ± SEM for seven separate experiments.

Table 4 Effects of melatonin on the adherence and penetration abilities of boar spermatozoa subjected to co-incubation with in vitro maturated porcine oocytes

	Control ($n = 53$)	Capacitation with 1 μM melatonin ($n = 55$)	Co-incubation with 1 μM melatonin ($n = 57$)
Adhered spermatozoa/oocyte	78.4 ± 1.8^{a}	68.2 ± 2.7^{b}	88.9 ± 1.7^{c}
Total penetration rate (%)	90.6^{a}	89.1^{a}	93.0^{a}
Monospermy (%)	69.8^{a}	74.5^{a}	70.2^{a}
Polyspermy (%)	20.8^{a}	14.5^{b}	22.8^{a}

Control: Spermatozoa subjected to a previous standard in vitro capacitation procedure through incubation at 38.5 °C and 5% CO_2 for 4 h. Capacitation with 1 μM melatonin: Spermatozoa subjected to in vitro capacitation in a medium added with 1 μM melatonin. Co-incubation with 1 μM melatonin: Spermatozoa subjected to a previous standard in vitro capacitation procedure for 4 h. Melatonin at 1 μM was added when spermatozoa and oocytes were co-incubated. Different superscript letters between columns within a given row indicate significant ($p < 0.05$) differences between groups.

flagellum which could, in turn, induce subtle changes in sperm motion. Related to this, cleavage of disulphide bonds in mouse hexokinase-I isozyme 1 (HK1) is related to the initiation of sperm motility (Nakamura *et al.*, 2008). Thus, it could be hypothesised that changes in the number of disrupted disulphide bonds along the flagellum, specifically in flagellum-bond-related proteins such as sperm hexokinase-1 (Cabrillana *et al.*, 2011, 2016), could also be related to the changes in motion parameters observed during IVC. Herein, melatonin at 0.5 and 1 μM was found to abolish the IVC-linked changes in kinetic parameters such as VCL, VAP and ALH (see Tables 1–3). While this effect could also be due to other mechanisms, such as sperm agglutination, the possibility that melatonin affects sperm motility through regulating the number of disrupted disulphide bonds should not be dismissed.

It is worth noting that the addition of melatonin together with progesterone after 4 h of IVC has almost no effects on subsequent acrosome exocytosis or on the sperm ability to adhere and penetrate oocytes in vitro. Taking into account that the main difference of spermatozoa before and after their incubation for 4 h in CM is the achievement of a feasible capacitation status, these results suggest that the observed effects of melatonin on parameters such as the adhesiveness and free cysteine levels of the tail are linked to the precise sperm status. Thus, and as indicated by membrane lipid disorder, sperm motility and sensitivity to progesterone, capacitated differs from uncapacitated spermatozoa in their response to melatonin. At this moment, it is not possible to ascertain which the basis for these differences in melatonin action is. Further research focusing on the function of specific melatonin receptors MT1 and MT2, which are present in boar spermatozoa (González-Arto *et al.*, 2016), is warranted.

In conclusion, melatonin modulates the achievement of IVC and subsequent progesterone-induced IVAE in boar spermatozoa via mechanism/s involved in the control of sperm motion through changes in the number of tail disulphide bridges, adhesiveness and further oocyte penetration ability. In addition, prevention of the IVC-induced increase in the disulphide bonds of sperm head proteins mediated by melatonin could also be relevant. Remarkably, melatonin effects on IVC/IVAE in boar spermatozoa do not seem to be related to a direct action on intracellular ROS levels, thus opening up alternative, perhaps

receptor-mediated, pathways to explain the effects of this hormone upon sperm capacitation.

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CONFLICT OF INTEREST

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

AUTHORS' CONTRIBUTIONS

M.R. and R.B. carried out the majority of the experimental work, collaborated in the design of the experiments and wrote the manuscript. A.Pl., M.S., A.Pe. and T.R. collaborated with M.R. and R.B. in performing laboratory work. J.M.F.N. conducted confocal analysis of lectin location. T.M.B., A.C. and J.A.C.P. collaborated in designing the experiments and critically revised the manuscript. S.B. and M.C.M. helped conduct flow cytometry analyses and IVF experiments. As joint senior author, M.Y. was involved in flow cytometry analyses and IVF experiments, designed the experiments, analysed the data, wrote the manuscript and gave his final approval. J.E.R.G. designed the experiments, analysed the data, wrote the manuscript and gave his final approval.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information section at the end of the article.

Figure S1 Effects of melatonin on the percentage of viability of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis.
Figure S2 Effects of melatonin on the percentage of true acrosome exocytosis of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis.
Figure S3 Effects of melatonin on the percentage of cells with capacitation-like membrane lipid disorder of boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis.
Figure S4 Effects of melatonin added at 0 h on tyrosine phosphorylation levels of P32 protein in boar sperm subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis.
Figure S5 Effects of the addition of melatonin to CM at 4 h on tyrosine phosphorylation levels of the P32 protein in boar spermatozoa subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis.
Figure S6 Images showing the effect of 1 μM melatonin on the formation of cell agglutinations in boar sperm subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis.
Figure S7 Effects of melatonin (1 μM) on the distribution of WGA lectin in boar sperm subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis.
Figure S8 Effects of 1 μM melatonin on the distribution of STL lectin in boar sperm subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis.
Figure S9 Effects of 1 μM melatonin on the distribution of PSA lectin in boar sperm subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis.
Figure S10 Effects of 1 μM melatonin on the distribution of PNA lectin in boar sperm subjected to in vitro capacitation and subsequent progesterone-induced acrosome exocytosis.
Figure S11 Examples of sperm-oocyte complexes considered as monospermic and polyspermic.