F4-Neuroprostanes: A Role in Sperm Capacitation
Cinzia Signorini, Elena Moretti, Daria Noto, Simona Mattioli, Cesare Castellini, Nicola Antonio Pascarelli, Thierry Durand, Camille Oger, Jean-Marie Galano, Claudio de Felice, et al.

To cite this version:
Cinzia Signorini, Elena Moretti, Daria Noto, Simona Mattioli, Cesare Castellini, et al..
F4-Neuroprostanes: A Role in Sperm Capacitation. Life, MDPI, 2021, 11 (7), pp.655. 10.3390/life11070655. hal-03378836
**Article**

**F₄-Neuroprostanes: A Role in Sperm Capacitation**

Cinzia Signorini ¹, Elena Moretti ¹,* ¹, Daria Noto ³, Simona Mattioli ², Cesare Castellini ², Nicola Antonio Pascarelli ¹, Thierry Durand ³, Camille Oger ³, Jean-Marie Galano ³, Claudio De Felice ⁴,⁵, Jetty Chung-Yung Lee ⁶ and Giulia Collodel ¹

---

1 Department of Molecular and Developmental Medicine, University of Siena, Policlinico Santa Maria Alle Scotte, Viale Bracci 14, 53100 Siena, Italy; cinzia.signorini@unisi.it (C.S.); daria.noto92@gmail.com (D.N.); n.pascarelli@libero.it (N.A.P.); giulia.colodel@unisi.it (G.C.)

2 Department of Agricultural, Environmental, and Food Science, University of Perugia, Borgo XX Giugno 74, 06123 Perugia, Italy; simona.mattioli@unipg.it (S.M.); cesare.castellini@unipg.it (C.C.)

3 Institut des Biomolecules Max Mousseron (IBMM), UMR 5247, CNRS, Université de Montpellier, ENSCM, 34090 Montpellier, France; thierry.durand@umontpellier.fr (T.D.); camille.oger@umontpellier.fr (C.O.); jean-marie.galano@umontpellier.fr (J.-M.G.)

4 Child Neuropsychiatry Unit, Azienda Ospedaliera Universitaria Senese, 53100 Siena, Italy; geniente@gmail.com

5 Neonatal Intensive Care Unit, Azienda Ospedaliera Universitaria Senese, 53100 Siena, Italy

6 School of Biological Sciences, The University of Hong Kong, Hong Kong, China; jettylee@hku.hk

* Correspondence: elena.moretti@unisi.it; Tel.: +39-577-233511

---

**Abstract:** F₄-neuroprostanes (F₄-NeuroPs), derived from the oxidative metabolization of docosahexaenoic acid (DHA), are considered biomarkers of oxidative stress in neurodegenerative diseases. Neurons and spermatozoa display high DHA content. The aim of this in vitro study was to investigate the biological effects of chemically synthesized 4-F₄-NeuroPs and 10-F₄-NeuroPs in human sperm. Total progressive sperm motility (p < 0.05) and linearity (p = 0.016), evaluated by a computer-assisted sperm analyzer, were significantly increased in samples incubated with 7 ng F₄-NeuroPs compared to non-supplemented controls. Sperm capacitation was tested in rabbit and swim-up-selected human sperm by chlorotetracycline fluorescence assay. A higher percentage of capacitated sperm (p < 0.01) was observed in samples incubated in F₄-NeuroPs than in the controls. However, the percentage of capacitated sperm was not different in F₄-NeuroPs and calcium ionophore treatments at 2 h incubation. The phosphorylated form of AMPKα was detected by immunofluorescence analysis; after 2 h F₄-NeuroP incubation, a dotted signal appeared in the entire sperm tail, and in controls, sperm were labeled in the mid-piece. A defined level of seminal F₄-NeuroPs (7 ng) showed a biological activity in sperm function; its addition in sperm suspensions stimulated capacitation, increasing the number of sperm able to fertilize.

**Keywords:** capacitation; CASA; human sperm motility; F₄-neuroprostanes; Phospho-AMPKα

---

1. **Introduction**

F₄-neuroprostanes (F₄-NeuroPs) are prostaglandin-like molecules generated by non-enzymatic oxidation of docosahexaenoic acid (DHA, 22: 6 n-3). DHA may be non-enzymatically oxidized into different classes of neuroprostanes (F₄-, D₄-, E₄-, A₄-, and J₄-NeuroPs) [1–3], where each class is composed of different isomeric molecules. Oxidation of DHA will generate eight groups of F₄-NeuroPs (4-, 7-, 10-, 11-, 13-, 14-, 17-, or 20-series) [4–6]. Musiek and colleagues [7] reported that the quantification of F₄-NeuroPs provides a highly selective quantitative window for neuronal oxidative damage in vivo. Mainly, measurement of F₄-NeuroPs supplies valuable data in exploring the role of oxidative stress in neurodegenerative diseases [8], and F₄-NeuroPs are considered accurate biomarkers of oxidative damage in Parkinson’s disease [9].

Human spermatozoa and neurons display a high DHA content; it has been reported that sperm motility, morphology, and concentration are positively associated with DHA...
levels [10]. As previously reported, the presence of seminal DHA and measurement of the n-6/n-3 polyunsaturated fatty acid (PUFA) ratio [11] are important to assess semen quality [12]. In addition, oxidative stress and polyunsaturated fatty acid oxidation have been reported to be relevant to the semen quality [10–14]. Therefore, it is practical to assess the relevance of F₄₄-NeuroPs in male infertility as a promising tool to indicate reproductive capacity.

In this regard, F₄₄-NeuroPs were measured in seminal plasma to assess their role in male infertility. No significant differences were found in infertile patients (idiopathic infertility and presence of varicocele) compared to fertile men; however, F₄₄-NeuroPs correlated positively with sperm necrosis and negatively with normal sperm morphology, suggesting a relevant role of F₄₄-NeuroPs as a potential marker of semen quality [13].

Moreover, in a rabbit model, it has been shown that F₄₄-NeuroPs in seminal plasma are affected by diet [15]. They were reported to be present in low levels in the rabbits fed with the control diet compared to those fed a high α-linolenic acid (ALA)-enriched diet, or with a fish oil diet (directly suppling n-3 PUFA such as ALA, which is the precursor of eicosapentaenoic acid (EPA), docosapentaenoic acid [DPAn-3], and DHA in human metabolism). Rabbit buck is the smallest and less expensive laboratory animal model in which almost all the reproductive and toxicological endpoints of humans can be measured.

It should also be taken into account that lipid metabolites have a role not only as biomarkers of oxidative stress in humans but also as biological mediators. On this matter, the products of the IsoP or NeuroP pathway were found to have strong biological actions and may therefore participate as physiological mediators of different diseases [16,17]. In particular, n-6 PUFA derivatives resulting from arachidonic acid (ARA) have prothrombotic and pro-aggregatory properties, whereas n-3 PUFA metabolites resulting from EPA and DHA have anti-inflammatory, antiproliferative, and anti-atherosclerotic activities [18,19]. Furthermore, cardiac antiarrhythmic properties have been reported for 4(RS)-4-F₄₄t-neuroprostane [20].

Possible effects of F₄₄-NeuroPs on sperm are herein investigated. In particular, the aim of this in vitro study was to examine the biological effects of chemically synthetized F₄₄-NeuroPs (4-F₄₄t-NeuroP and 10-F₄₄t-NeuroP) in human sperm. Sperm motility was assessed in the presence of different concentrations of F₄₄-NeuroPs. Sperm motion characteristics were acquired by a computer-assisted sperm analyzer (CASA). The immunofluorescence analysis allowed the localization of Phospho-AMPKα (Thr172), a protein that regulates energy balance and metabolism, which are key functional sperm processes for fertilization. Sperm capacitation was recorded using a rabbit sperm model.

2. Materials and Methods

2.1. Samples

Semen samples were obtained from 15 healthy male donors (aged 29 to 35 years) recruited at the Department of Molecular and Developmental Medicine, University of Siena, Italy. All participants were informed about the study protocol. In addition, we guaranteed their privacy and that the spermatozoa will not be used for genetic studies or fertilization. All volunteers signed an informed consent form for the in vitro study. As it is an in vitro study, ethical approval was not required by the institution.

Ejaculated human spermatozoa were collected in sterile containers after 3–5 days of sexual abstinence. Briefly, after complete liquefaction of the semen samples at 37 °C for 30 min, macroscopic and microscopic examinations were performed [21].

Healthy New Zealand white rabbit bucks of the same age (8 months) and weight (about 4.5 kg) were raised in the experimental farm of the Department of Agriculture, Food and Environmental Science of Perugia (Italy) and used for semen collection. Specific guidelines for the rabbit bucks and the International Guiding Principles for Biomedical Research Involving Animals [22] were followed. The experiment did not require specific authorization by the ethical committee because the animals were not subjected to stressful treatment causing pain and suffering. Semen collection was performed by using a domelike
dummy and an artificial vagina maintained at 37 °C internal temperature and used for chlortetracycline (CTC) fluorescence pattern assay.

2.2. 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP Chemical Synthesis

As 4- and 10-F<sub>4t</sub>-NeuroPs are not commercially available, they were in-house synthesized for the study. [23–25] is summarized in Scheme 1.

![Scheme 1. The synthesis of the two series of 4- and 10-F<sub>4t</sub>-NeuroPs.](image)

The two key bicyclic compounds 1 and 2 were obtained in 10 steps and in 8.7% and 10.3% yield, respectively, from commercially available 1,3-cyclooctadiene. The α and ω chains were introduced by using regioselective protections/deprotections and oxidations, followed by a Wittig elongation and cross-metathesis coupling reactions as the main steps for the 10-F<sub>4t</sub>-NeuroP. The free acids were obtained by saponification of the methyl esters in the presence of LiOH. The 4-F<sub>4t</sub>-NeuroP was obtained starting from intermediate 2 in 12 more steps of synthesis leading to a 23% yield, while 10-F<sub>4t</sub>-NeuroP was obtained in 13 steps from intermediate 1 in 4.5% lower yield.

The confirmation of the structures and the purity of these two neuroprostanes were in agreement with the 1H- and 13C NMR, HRMS, and IR experiments, and all these data can be found at doi:10.1002/chem.201002304 and doi:10.1002/chem.201400380.

2.3. Neuroprostane Preparation and Determination of Optimal Amount for the In Vitro Study

A 3 ng/mL stock solution of F<sub>4</sub>-NeuroPs (1:1 mixture of 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP) was prepared in ethanol and stored at −20 °C. Fresh working solutions were prepared for each experiment. The human semen samples were incubated with different concentrations of F<sub>4</sub>-NeuroP solution (0.7, 7, 70 ng) in Sperm Washing Medium IrvineScientific® (Santa Ana, CA, USA) for 2 h at 37 °C and 5% CO<sub>2</sub>; then, sperm motility was evaluated using a Bürker-Türk hemocytometer (BT, Brand, Wertheim, Germany) counting chamber. Determination of the sperm motility was carried out by simultaneously counting the number of motile spermatozoa (fast progressive, slow, and non-progressive motility) and immotile sperm in 10 fields of a Bürker-Türk counting chamber (volume of one field: 0.004 mm<sup>3</sup>).

Samples without F<sub>4</sub>-NeuroP treatment were used as controls, and samples without F<sub>4</sub>-NeuroP treatment but in the presence of the same percentage of ethanol did not show any difference (data not shown). Specifically, the final percentage of ethanol in the incubation mixture was no more than 0.07%. The experiments were carried out in five basal semen samples.

2.4. Swim-Up

The swim-up technique was used to obtain the motile human sperm fraction: 0.5 mL of each semen sample was placed in a sterile conical centrifuge tube and gently layered with 0.5 mL of Sperm Washing Medium. The tubes were inclined at a 45° angle and incubated for 45 min at 37 °C in 5% CO<sub>2</sub>. Then, 0.5 mL of the uppermost medium that contained
motile sperm fraction was collected and used for the experiments. Approximately $10 \times 10^6$ swim-up-selected sperm/mL were divided into 2 aliquots: one was incubated with F$_4$-NeuroPs and another without F$_4$-NeuroPs (control). All of the aliquots were kept at 37 °C in 5% CO$_2$ for 2 h, and the sperm motility was evaluated as described below.

2.5. Computer-Assisted Sperm Analysis

The motion patterns of the human semen samples treated with F$_4$-NeuroPs were analyzed using a computer-assisted sperm analyzer (CASA model ISAS, Valencia, Spain). The setup parameters were based on a previous report [26]. For each semen sample, two drops and six microscopic fields were recorded for a minimum of 300 sperm tracks. All semen samples were recorded at 100 Hz frames for 1 s; thus, 12–200 successive images were recorded.

The following sperm motion parameters were evaluated: motility percentage, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN = VSL/VCL), amplitude of lateral head displacement (ALH), and beat cross frequency (WOB). The experiments were repeated in eight samples.

2.6. Sperm Capacitation Patterns in Rabbit Sperm and Swim-Up-Selected Human Sperm

The rabbit semen samples collected in an artificial vagina heated to 37 °C with water were immediately transferred to the laboratory [22] and divided into three aliquots—control, calcium-ionophore-induced, incubation with F$_4$-NeuroPs—and evaluated after 2 and 4 h. Swim-up-selected human sperm were incubated with F$_4$-NeuroPs and with Sperm Washing Medium alone for 2 and 4 h.

The chlortetracycline (CTC) fluorescence assay was performed as reported by Kaul et al. [27]. The CTC solution was made by dissolving 7.5 M CTC-HCl in a buffer containing 20 mM Tris–HCl, 130 mM NaCl, and 5 mM cysteine-HCl at pH 7.0. A sperm suspension (100 µL) was put into a 1.5-milliliter foil-wrapped Eppendorf tube, to which 100 µL CTC stock was added. The cells were immediately fixed by adding 8 µL of 12.5% paraformaldehyde to 0.5 mM Tris–HCl buffer (pH 7.5). The slides were prepared by combining 10 µL of the fixed solution with one drop of 1, 4-diazabicyclo [2.2.2] octane dissolved in glycerol/phosphate-buffered saline (PBS) to retard the fading of fluorescence.

A coverslip was placed on top of the slide, and the sperm cells were gently compressed to allow removal of excess fluid. The slides were examined under an epifluorescence microscope (OLYMPUS, USA) using a CH$_2$ excitation filter set at 335–425 nm. Three distinct sperm fluorescence patterns were detected: fluorescence over the entire head, which is characteristic of intact cells (non-capacitated); a non-fluorescent band in the post-acrosomal region of the sperm head, which is characteristic of capacitated acrosome-intact cells; and dull or absent fluorescence on the sperm head, which is characteristic of acrosome-reacted cells. Three hundred sperms per sample were analyzed. The experiments were repeated in five sets of human and rabbit samples.

2.7. Immunofluorescence Analysis for the Localization of Phospho-AMPKα (Thr172)

Swim-up-selected human sperms, incubated or not with F$_4$-NeuroPs, were washed in PBS, smeared on glass slides, air-dried, and fixed in 4% paraformaldehyde for 15 min. After treatment with a blocking solution (PBS–bovine serum albumin (BSA) 1% Normal Goat Serum (NGS) 5%) for 20 min, the slides were incubated overnight at 4 °C with a primary antibody anti-Phospho-AMPKα (Thr172) (Cell Signaling Technology, Danvers, MA, USA) diluted at 1:100; reaction was revealed by an anti-rabbit antibody raised in a goat Alexa Fluor® 488 conjugate (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA), diluted at 1:100. Incubation without the primary antibodies was used as the control. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) solution (Vysis, Downers Grove, IL, USA). Observations were made with a Leica DMI 6000 Fluorescence Microscope (Leica Microsystems, Germany), and images were acquired using the Leica AF6500 Integrated...
System for Imaging and Analysis (Leica Microsystems, Germany). Two hundred sperms per sample were evaluated. Five samples were analyzed.

2.8. Statistical Analysis

Statistical analysis was performed with the statistical software package SPSS Version 23.0 for Windows (SPSS Inc., Chicago, IL, USA). The Kolmogorov–Smirnov test was used to examine if variables were normally distributed. Levene’s test was employed to check the assumption of homoscedasticity. The unpaired two-sample t-test was used to compare the means of two independent groups. For multiple comparison, the differences among the variables examined were checked using a one-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test. A significance level of $p \leq 0.05$ was adopted.

3. Results

3.1. Determination of Human Sperm Motility after $F_4$-NeuroP Incubation

Different amounts of $F_4$-NeuroPs (0.7, 7, 70 ng) were incubated with human sperm at 37 °C and 5% CO$_2$ for 2 h (Table 1). A range from 0.7 to 70 ng of $F_4$-NeuroPs was investigated to test the effect of different amounts of $F_4$-NeuroPs, whose biological effects on human semen are currently unknown.

Table 1. Means ± standard deviation (SD) of total progressive and rapid sperm motility percentages after incubation with different $F_4$-NeuroP amounts (0.7 ($F_4$), 7 ($F_4$), 70 ($F_4+$) ng) and without $F_4$-NeuroPs (control). Five semen samples were analyzed. Statistics are also reported: $F_4$, $F_4$, $F_4+$ vs. control. $p < 0.05$ is considered significant.

|                         | Control | $F_4$  | $F_4$  | $F_4+$ | Statistics                      |
|-------------------------|---------|--------|--------|--------|---------------------------------|
| Total progressive       | 29.4 ± 12| 27.4 ± 11.3| 34.4 ± 12.1| 13.6 ± 5.9 | Control vs. $F_4$, $F_4$, $F_4+$ vs. control. $p < 0.05$ |
| motility %              |         |        |        |        |                                 |
| Rapid progressive       | 10.4 ± 4.0| 9.8 ± 3.8| 16.6 ± 4.2| 2.4 ± 1.1  | Control vs. $F_4$, $F_4$, $F_4+$ vs. control. $p < 0.01$ |
| motility %              |         |        |        |        |                                 |

In the 7 ng $F_4$-NeuroP aliquots, the total and rapid progressive motility were higher than those observed in the non-supplemented control ($p < 0.05$ and $p < 0.01$, respectively); sperm motility was slightly decreased in the presence of 0.7 ng $F_4$-NeuroP treatment, while 70 ng $F_4$-NeuroP treatment led to significantly decreased total and rapid progressive motility (both $p < 0.01$) compared to those observed in controls. Therefore, in this study, we selected the level of 7 ng $F_4$-NeuroPs for subsequent experimentation.

To better evaluate the sperm motility parameter, aliquots of swim-up-selected sperm were analyzed by CASA after 2 h incubation with 7 ng $F_4$-NeuroPs. Total progressive sperm motility was significantly increased after incubation with $F_4$-NeuroPs compared to the control (50.6 ± 20.4% vs. 39.9 ± 14.5%; $p < 0.05$). Among the sperm motility characteristics, the percentages of curvilinear velocity (VCL, 66.7 ± 13.0% vs. 64.6 ± 12.5%), straight-line velocity (VSL, 43.1 ± 9.6% vs. 38.7 ± 9.6%), straightness (STR, 87.6 ± 7.5% vs. 80.9 ± 4.8%), and wobble (WOB, 74.1 ± 10.7% vs. 70.2 ± 9.0%) were increased after $F_4$-NeuroP incubation than those detected in the control, and linearity (LIN) significantly increased ($p = 0.016$) after $F_4$-NeuroP incubation compared to the control.

3.2. Assessment of Capacitation in Rabbit Sperm, Used as Model, and in Swim-Up-Selected Human Sperm

In order to explain the increase in and type of sperm motility observed in the aforementioned experiments, an induction of capacitation in rabbit sperm was used in the animal model. Aliquots of rabbit semen samples were incubated for 2 or 4 h with calcium ionophore and 2 and 4 h with 7 ng $F_4$-NeuroPs. The capacitation rate was evaluated using the CTC fluorescence assay (Table 2).
Table 2. Means and standard deviations (SD) of capacitated or non-capacitated rabbit sperm percentages under different experimental conditions (incubation for 2 or 4 h with calcium ionophore or incubation for 2 or 4 h with F₄-neuroprostanes (F₄-NeuroPs, 7 ng)). Five semen samples were analyzed. Statistics are also reported. *p* < 0.05 is considered significant.

| Samples (n = 5) | Time 0     | Time 2 h    | Time 4 h    | Statistics                                      |
|----------------|------------|-------------|-------------|------------------------------------------------|
| Non-capacitated sperm % |            |             |             |                                                |
| Control        | 95.0 ± 1.4 | 59.4 ± 1.8  | 34.5 ± 3.2  |                                                |
| Calcium ionophore | 95.6 ± 0.5 | 22.1 ± 1.3  | 20.7 ± 0.6  |                                                |
| F₄-NeuroPs     | 95.9 ± 1.2 | 31.6 ± 2.1  | 12.6 ± 0.9  |                                                |
| Capacitated sperm % |            |             |             |                                                |
| Control        | 4.9 ± 1.4  | 29.3 ± 2.5  | 53.8 ± 1.9  |                                                |
| Calcium ionophore | 4.4 ± 0.5  | 71.7 ± 0.9  | 58.0 ± 0.3  |                                                |
| F₄-NeuroPs     | 4.1 ± 1.2  | 57.8 ± 1.9  | 61.7 ± 1.5  |                                                |
| Acrosome-reacted sperm % |            |             |             |                                                |
| Control        | 0          | 11.3 ± 4.2  | 11.6 ± 1.3  |                                                |
| Calcium ionophore | 0          | 6.3 ± 0.6   | 21.3 ± 0.9  |                                                |
| F₄-NeuroPs     | 0          | 10.9 ± 1.2  | 25.7 ± 0.8  |                                                |

After 2 h of incubation with calcium ionophore, the percentage of capacitated sperm (71.7 ± 0.9%) was significantly higher than that observed in the control (29.3 ± 2.5%, *p* < 0.001), whereas at 4 h, the percentage of capacitated sperm (58.0 ± 0.3%) was similar to that in the control (53.8 ± 1.9%). The 2-h incubation with F₄-NeuroPs determined a significant increase in sperm percentage compared to the control (57.8 ± 1.9% vs. 29.3 ± 2.5%; *p* < 0.01). The percentage of capacitated sperm after 2 h of F₄-NeuroP incubation (57.8 ± 1.9%) was similar to that detected after 4 h in the control (53.8 ± 1.9%).

A significant increase in sperm showing acrosome reaction was detected after 4 h of calcium ionophore incubation (21.3 ± 0.9%, *p* < 0.05) compared to the 4-h control (11.6 ± 1.3%).

Afterwards, an evaluation of capacitation was carried out by CTC analysis in swim-up-selected human sperm. The percentage of capacitated human sperm after incubation with F₄-NeuroPs (2 h) was significantly higher (42.2 ± 2.5%, *p* < 0.01) than that in the control sample (10.9 ± 1.5%); after 4 h incubation, as found in the rabbit model, a significant increase in acrosome-reacted sperm (34 ± 1.2%) was detected compared to the control (15.2 ± 2%).

3.3. Localization of Phospho-AMPKα in Swim-Up-Selected Human Sperm

In control samples, after swim-up selection and 2 h of incubation in Sperm Washing Medium, the phosphorylated form of AMPKα was localized in the mid-piece of a very high percentage of spermatozoa (66 ± 1.8%, Table 3; Figure 1a). This localization was significantly decreased (*p* < 0.01) in samples incubated with F₄-NeuroPs (2 h, 13.7 ± 1.7%), in which a dotted label along the tail in a significantly increased percentage of sperm (64.7 ± 1%) (Table 3; Figure 1b) compared to the control (0.5 ± 0.5%, *p* < 0.001) was observed. Finally, an intense signal in the acrosomal region of the sperm (57%) was detected after 4 h of F₄-NeuroP incubation (Figure 1c,d) with respect to the control (17%, *p* < 0.01).
Table 3. Means and standard deviations (SD) of percentages of different Phospho-AMPKα localization in swim-up-selected human sperm. Sperm were incubated for 2 or 4 h with F<sub>4</sub>-neuroprostanes (F<sub>4</sub>-NeuroPs, 7 ng). Five semen samples were analyzed. * p < 0.05, ** p < 0.01, *** p < 0.001.

|                           | Control (2 h) | F<sub>4</sub>-NeuroPs (2 h) | Control (4 h) | F<sub>4</sub>-NeuroPs (4 h) |
|---------------------------|--------------|-----------------------------|--------------|-----------------------------|
| Mid-piece labeling %      | 66 ± 1.8 *   | 13.7 ± 1.7 **               | 17.5 ± 1.7   | 3.2 ± 1.2                   |
| Dotted tail labeling %    | 0.5 ± 0.5    | 64.7 ± 1 ***                | 51 ± 2.6     | 15 ± 0.8                    |
| Acrosomal labeling %      | 1.2 ± 0.5    | 18.7 ± 2.2                  | 17.5 ± 2.1   | 57.2 ± 1.7 **               |
| Any signal %              | 33.5 ± 1.3   | 3.7 ± 1.2                   | 14 ± 1.6     | 24.5 ± 0.6                  |

Figure 1. UV micrographs of swim-up-selected sperm incubated with Sperm Washing Medium for 2 h (a) or with F<sub>4</sub>-NeuroPs for 2 (b) or 4 h (c,d). In (a), the labeling is evident in the mid-piece; in (b), it is dotted along the entire tail; in (c,d), the acrosomes and the mid-piece are fluorescent. Bars: 5 µM.

4. Discussion

Although NeuroPs have been named “neuro” due to the relative abundance of their PUFA precursor (i.e., DHA) in the brain, NeuroPs have been detected and quantified in plasma [28–30], urine [31], and semen [13].

Few data are available about the role of these molecules in seminal fluid. Previously, Longini et al. [13] reported that infertile patients with varicocele had higher levels of seminal F<sub>4</sub>-NeuroPs (154.75 ng/mL) compared to fertile controls (20 ng/mL). In addition, Castellini’s group observed that rabbits supplemented with an n-3 PUFA diet showed improved sperm motion traits and higher levels of F<sub>4</sub>-NeuroPs in their seminal fluid compared to controls [15].

These papers suggest that F<sub>4</sub>-NeuroPs have a biological role in male fertility as well as being markers of oxidative damage, as previously reported in other human diseases [6,28].
It is known that sperm cells are rich in PUFAs, which can undergo auto-oxidation through free-radical-initiated mechanisms in vivo, releasing key products related to pathophysiological events.

NeuroPs, as a class of isoprostanes originating from non-enzymatic oxygenation of DHA, consist of eight regioisomer series (4-, 7-, 10-, 11-, 13-, 14-, 17-, or 20-series), and thus far, among all the F_4-NeuroP molecules, 4-F_4t-NeuroPs and 10-F_4t-NeuroPs are the most represented [6,28,29].

Our hypothesis was that NeuroPs may also possess biological activities. Isoprostanes derived from ARA are vasoconstrictors in many species and induce proliferation of endothelial and smooth muscle cells; they mediate their biological effects by activation or inhibition of several prostanoid receptors, including the thromboxane receptor (TP), prostaglandin F_2α receptor (FP), prostaglandin E_2 subtype 3 receptor (EP3), and prostaglandin D_2 subtype 2 receptor (DP2), and by activation of peroxisome proliferator-activated receptor gamma (PPARγ) [32]. Although less evidence of biological activity is available for F_4-NeuroPs than for F_2-IsoPs [6], anti-arrhythmic properties and the ability of heart protection against reperfusion injury have been reported for the non-enzymatic oxidized metabolite of DHA [20,33,34]. More recently, Lee et al. [16] reported a neuroprotective effect of 4-F_4t-NeuroP through the regulation of pro-resolving lipid mediators and regulation of Nrf/HO-1 in SH-SY5Y cells.

The aim of this paper was to study the in vitro effect of F_4-NeuroPs on sperm function. We tested three F_4-NeuroP concentrations on human ejaculated sperm and different results were obtained: an amount of 7 ng enhanced the progressive sperm motility, an amount of 0.7 ng did not influence sperm motility, and a quantity of 70 ng reduced it. At present, the biological effects of F_4-NeuroPs on human semen are currently unknown, and the chosen doses are within the range of F_4-NeuroP levels measured in human sperm samples. Our data suggest that high levels of F_4-NeuroPs in semen are toxic and an indicator of oxidative stress taking place, while a moderate level of F_4-NeuroPs may have a role in physiological activities as an inducer of the capacitation process.

In this study, CASA showed a significant increase in progressive sperm motility and the LIN motion parameter after 2 h incubation with 7 ng F_4-NeuroPs. It is reported that seminal molecules associated to the capacitation process influence sperm motility and trajectory [35,36]; for example, in porcine epididymal spermatozoa, bicarbonate (HCO_3⁻) activated a more linear and rapid motility [37].

In vitro studies showed that other molecules such as astaxanthin [38] or fertilization-promoting peptide, angiotensin II, and calcitonin, present in seminal plasma [39], ameliorate sperm functioning by accelerating capacitation and acrosome reactions.

Capacitation [40] includes cholesterol loss from the plasma membrane, a change in intracellular ion concentration, an increase in intracellular pH and hyperpolarization of the plasma membrane, activation of flagellum movement, and changes in the pattern of movement, i.e., hyperactivation, phosphorylation of proteins in tyrosine (Tyr) residues [41,42], and the ability to carry out the acrosome reaction stimulated by a physiological agonist.

Using rabbit as the animal model, the percentage of capacitated sperm after F_4-NeuroP incubation was compared with that obtained with calcium ionophore and in controls by CTC analysis. The results indicated that the used F_4-NeuroP concentration in rabbit sperm accelerated the time of capacitation almost similarly to calcium ionophore.

Accordingly, in the experiments carried out with human sperm, the incubation with F_4-NeuroPs allowed a faster increase in capacitated sperm as compared to the experimental conditions in which F_4-NeuroPs were not used.

A study on neuroprostanes opened up new perspectives for products of non-enzymatic oxidized DHA as potent mediators in diseases involving ryanodine complex destabilization [20]. Ryanodine receptors (RyR) are present in spermatogenic cells, and ryanodine can modulate the flagellar bend amplitude [43], one of the important characteristics of sperm hyperactivation also linked to Ca^{2+} influx [44,45]. Interestingly, lipid molecules (ceramides)
activate RyR and calcium influx through the calcium channel of sperm [46]. Thus, a role for F₄-NeuroPs in sperm motility might be hypothesized.

Incubation for 4 h in F₄-NeuroPs led to a significant change in acrosomal status. The acrosome reaction is considered the endpoint of the capacitation process. This is plausible, as we observed a significant difference in reacted sperm with CTC after 4 h. Interestingly, all capacitation events are regulated through the cyclic AMP (cAMP)/protein kinase A (PKA) system [47,48]. Therefore, we performed immunocytochemistry localization of the phosphorylated form of AMPK, which plays an important role in cellular energy homeostasis. In the control samples, after 2 h of incubation, the spermatozoa showed an evident Phospho-AMPKα signal in the mid-piece; at the same time, in the presence of F₄-NeuroPs, a dotted labeling along the entire sperm tail was detected. Finally, after 4 h of F₄-NeuroP incubation, a high percentage of sperm highlighted an intense signal in the acrosomal region compared to the control, suggesting that this compound might play a role in increasing AMPK phosphorylation. In human sperm, an increase in the phosphorylation of PKA substrates during capacitation has been described [49,50], while in chicken sperm, AMPK phosphorylation occurs in the flagellum and acrosome [51]. These findings agree with the fact that ATP production is not limited to mitochondrial respiration in the sperm mid-piece but may also have other origins through anaerobic glycolysis [52]. The active phosphorylated form of AMPK results in increased sperm quality, antioxidant ability, and sperm–zona-pellucida binding capacity [53].

5. Conclusions

In conclusion, a seminal concentration of 7 ng F₄-NeuroPs showed a biological activity in sperm function and was able to induce capacitation. These molecules, as suggested for others [39], could be used in a clinical setting. Spermatozoa from infertile men may present a kind of ‘capacitation difficulty’ due to an altered lipid composition of the plasma membrane, which has been described in infertile men [10]. The addition in sperm suspensions would stimulate capacitation, resulting in a high proportion of sperm to enable fertilization. Further studies need to explain the mechanism involved by using germinal cell cultures treated with isoprostanoids to better investigate the potential receptors and putative molecular signaling involved in the F₄-NeuroP biological activity hypothesized here.

Author Contributions: Conceptualization, G.C., C.S., and E.M.; methodology, T.D., C.O., J.-M.G., C.D.F., D.N., S.M., G.C., E.M., C.S., and C.C.; formal analysis, D.N., S.M., G.C., E.M., C.S., and C.C.; investigation, D.N., N.A.P., and G.C.; resources, G.C., E.M., and C.S.; data curation, G.C. and C.S.; writing—original draft preparation, G.C. and C.S.; writing—review and editing, S.M., E.M., C.S., T.D., C.O., J.-M.G., G.C., C.D.F., C.C., and J.C.-Y.L.; supervision, J.C.-Y.L., C.S., and G.C.; project administration, G.C., C.S., and E.M.; funding acquisition, G.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Department of Molecular and Developmental Medicine funding, University of Siena, PSR 2021, 2267-2021-SC-PAR_001.

Institutional Review Board Statement: Ethical review and approval were waived for this study due to the in vitro protocol.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are available on request from the authors.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Fam, S.S.; Murphey, L.J.; Terry, E.S.; Zackert, W.E.; Chen, Y.; Gao, L.; Pandalai, S.; Milne, G.; Roberts, L.J.; Porter, N.A.; et al. Formation of highly reactive A-ring and J-ring isoprostane-like compounds (A4/J4-neuroprostanes) in vivo from docosahexaenoic acid. J. Biol. Chem. 2002, 277, 36076–36084. [CrossRef] [PubMed]
2. Brooks, J.D.; Milne, G.L.; Yin, H.; Sanchez, S.C.; Porter, N.A.; Morrow, J.D. Formation of highly reactive cyclopentenone isoprostane compounds (A 3/J3-isoprostanes) in vivo from eicosapentaenoic acid. J. Biol. Chem. 2008, 283, 12043–12055. [CrossRef] [PubMed]
3. Barden, A.E.; Corcoran, T.B.; Mas, E.; Durand, T.; Galano, J.M.; Roberts, L.J.; Paech, M.; Muchatuta, N.A.; Phillips, M.; Mori, T.A. Is there a role for isofurans and neuroprostanes in pre-eclampsia and normal pregnancy? Antioxid. Redox Signal. 2012, 16, 165–169. [CrossRef] [PubMed]

4. Nourooz-Zadeh, J.; Liu, E.H.; Angggård, E.; Halliwell, B. F4-isoprostanes: A novel class of prostanoids formed during peroxidation of docosahexaenoic acid (DHA). Biochem. Biophys. Res. Commun. 1998, 242, 338–344. [CrossRef]

5. Yin, H.; Musiek, E.S.; Gao, L.; Porter, N.A.; Morrow, J.D. Regiochemistry of neuroprostanes generated from the peroxidation of docosahexaenoic acid in vitro and in vivo. J. Biol. Chem. 2005, 280, 26600–26611. [CrossRef]

6. Galano, J.M.; Lee, Y.Y.; Oger, C.; Vigor, C.; Vercauteren, J.; Durand, T.; Giera, M.; Lee, J.C. Isoprostanes, neuroprostanes and phytoprostanes: An overview of 25 years of research in chemistry and biology. Prog. Lipid Res. 2017, 68, 83–108. [CrossRef]

7. Musiek, E.S.; Cha, J.K.; Yin, H.; Zackert, W.E.; Terry, E.S.; Porter, N.A.; Montine, T.J.; Morrow, J.D. Quantification of F-ring isoprostane-like compounds (F4-neuroprostanes) derived from docosahexaenoic acid in vivo in humans by a stable isotope dilution mass spectrometric assay. J. Chromatogr. Analyt. Technol. Biomed. Life Sci. 2004, 795, 95–102. [CrossRef]

8. Miller, E.; Morel, A.; Saso, L.; Saluk, J. Isoprostanes and neuroprostanes as biomarkers of oxidative stress in neurodegenerative diseases. Oxid. Med. Cell. Longege. 2014, 2014, 572491. [CrossRef]

9. Seet, R.C.; Lee, C.Y.; Lim, E.C.; Tan, J.J.; Quek, A.M.; Looi, W.F.; Huang, S.H.; Wang, H.; Chan, Y.H.; et al. Oxidative damage in Parkinson disease: Measurement using accurate biomarkers. Free Radic. Biol. Med. 2010, 48, 560–566. [CrossRef]

10. Zerbinati, C.; Caponecchia, L.; Rago, R.; Leoncini, E.; Bottaccioli, A.; Ciacciarelli, M.; Pacelli, A.; Salacone, P.; Sebastianelli, A.; Pastore, A.; et al. Fatty acids profiling reveals potential candidate markers of semen quality. Andrology 2016, 4, 1094–1101. [CrossRef]

11. Collodol, G.; Moretti, E.; Noto, D.; Iacopioni, F.; Signorini, C. Fatty Acid Profile and Metabolism Are Related to Human Sperm Parameters and Are Relevant in Idiopathic Infertility and Varicocele. Mediat. Inflamm. 2020, 2020, 3640450. [CrossRef]

12. Signorini, C.; Moretti, E.; Collodol, G. Role of isoprostanes in human male infertility. Syst. Biol. Reprod. Med. 2020, 66, 291–299. [CrossRef]

13. Longini, M.; Moretti, E.; Signorini, C.; Noto, D.; Iacopioni, F.; Collodol, G. Relevance of seminal F2-dihomo-IsOps, F2-IsOps and F4-NeuroPs in idiopathic infertility and Varicocele. Prostagland. Lipid Mediat. 2020, 149, 106448. [CrossRef]

14. Collodol, G.; Castellini, C.; Lee, J.C.; Signorini, C. Relevance of Fatty Acids to Sperm Maturation and Quality. Oxid. Med. Cell. Longege. 2020, 2020, 7038124. [CrossRef]

15. Castellini, C.; Mattioli, S.; Signorini, C.; Cotozzolo, E.; Noto, D.; Moretti, E.; Brecchia, G.; Del Bosco, A.; Belmonte, G.; Durand, T.; et al. Effect of Dietary n-3 Source on Rabbit Male Reproduction. Oxid. Med. Cell. Longege. 2019, 2019, 3279670. [CrossRef]

16. Lee, Y.Y.; Galano, J.M.; Leung, H.H.; Balas, L.; Oger, C.; Durand, T.; Lee, J.C. Nonenzymatic oxygenated metabolite of docosahexaenoic acid, 4(RS)-4-F4-neuroprostane, acts as a bioactive lipid molecule in neuronal cells. FEBS Lett. 2020, 594, 1797–1808. [CrossRef]

17. Roy, J.; Galano, J.M.; Durand, T.; Le Guennec, J.Y.; Lee, J.C. Physiological role of reactive oxygen species as promoters of natural defenses. FASEB J. 2017, 31, 3729–3745. [CrossRef]

18. Crescente, M.; Armstrong, P.C.; Kirkby, N.S.; Edin, M.L.; Chan, M.V.; Lih, F.B.; Jiao, J.; Maffucci, T.; Allan, H.E.; Mein, C.A.; et al. Profiling the eicosanoid networks that underlie the anti- and pro-thrombotic effects of aspirin. FASEB J. 2020, 34, 10027–10040. [CrossRef]

19. Paniagrahy, D.; Gilligan, M.M.; Serhan, C.N.; Kashfi, K. Resolution of inflammation: An organizing principle in biology and medicine. Pharmacol. Ther. 2021, 26, 107879. [CrossRef]

20. Roy, J.; Oger, C.; Thireau, J.; Roussel, J.; Mercier-Touzet, O.; Delinger, F.; Pinot, E.; Farah, C.; Taber, D.F.; Cristol, J.P.; et al. Nonenzymatic lipid mediators, neuroprostanes, exert the antiarrhythmic properties of docosahexaenoic acid. Free Radic. Biol. Med. 2015, 86, 269–278. [CrossRef]

21. World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen, 5th ed.; WHO: Geneva, Switzerland, 2010.

22. Boiti, C.; Castellini, C.; Theau Clement, M.; Besenfelder, U.; Liguori, L.; Renieri, T.; Pizzi, F. Guidelines for the handling of rabbit bucks and semen. World Rabbit Sci. 2005, 13, 71–91.

23. Grillo, E.; Lo Rizzo, C.; Bianciardi, L.; Bizzarri, V.; Baldassarri, M.; Spiga, O.; Furini, S.; De Felice, C.; Signorini, C.; Leoncini, S.; et al. Revealing the complexity of a monogenic disease: Rett syndrome exome sequencing. PLoS ONE 2013, 8, e56599. [CrossRef] [PubMed]

24. Oger, C.; Brinkmann, Y.; Bouazzouari, S.; Durand, T.; Galano, J.M. Stereoregulated access to isoprostanes via a bicyclo[3.3.0]octene framework. Org. Lett. 2008, 10, 5087–5090. [CrossRef] [PubMed]

25. Oger, C.; Bultel-Poncé, V.; Guy, A.; Balas, L.; Rossi, J.C.; Durand, T.; Galano, J.M. The handy use of Brown’s catalyst for a skipped diyne deuteration: Application to the synthesis of a d4-labeled-F4-neuroprostane. Chem. Eur. J. 2010, 16, 13976–13980. [CrossRef]

26. Castellini, C.; Del Bosco, A.; Ruggeri, S.; Collodol, G. What is the best frame rate for evaluation of sperm motility in different species by computer-assisted sperm analysis? Fertil. Steril. 2011, 96, 24–27. [CrossRef]

27. Kaul, G.; Sharma, G.S.; Singh, B.; Gandhi, K.K. Capacitation and acrosome reaction in buffalo bull spermatozoa assessed by chlortetracycline and pism sativum agglutinin fluorescence assay. Theriogenology 2001, 55, 1457–1468. [CrossRef]
28. Signorini, C.; De Felice, C.; Durand, T.; Galano, J.M.; Oger, C.; Leoncini, S.; Ciccoli, L.; Carone, M.; Ulivelli, M.; Manna, C.; et al. Relevance of 4-F_{2α}-neuroprostane and 10-F_{4α}-neuroprostane to neurological diseases. *Free Radic. Biol. Med.* 2018, 115, 278–287. [CrossRef]

29. Yen, H.C.; Wei, H.J.; Lin, C.L. Unresolved issues in the analysis of F_{2α}-isoprostanes, F_{4α}-neuroprostanes, isofurans, neurofurans, and F_{2α}-dihomo-isoprostanes in body fluids and tissue using gas chromatography/negative-ion chemical-ionization mass spectrometry. *Free Radic. Res.* 2015, 49, 861–880. [CrossRef]

30. Signorini, C.; De Felice, C.; Leoncini, S.; Giardini, A.; D’Esposito, M.; Filosa, S.; Della Ragione, F.; Rossi, M.; Pecorelli, A.; Valacchi, G.; et al. F_{4α}-neuroprostanes mediate neurological severity in Rett syndrome. *Clin. Chim. Acta* 2011, 412, 1399–1406. [CrossRef]

31. Medina, S.; Miguel-Elizaga, I.D.; Oger, C.; Galano, J.M.; Durand, T.; Martínez-Villanueva, M.; Castillo, M.L.; Villegas-Martínez, I.; Ferreres, F.; Martínez-Hernández, P.; et al. Dihomo-isoprostanes-nonenzymatic metabolites of AdA are higher in epileptic patients compared to healthy individuals by a new ultrahigh pressure liquid chromatography-triple quadrupole-tandem mass spectrometry method. *Free Radic. Biol. Med.* 2015, 79, 154–163. [CrossRef]

32. Galano, J.M.; Mas, E.; Barden, A.; Mori, T.A.; Signorini, C.; De Felice, C.; Barrett, A.; Opere, C.; Pinot, E.; Schwedhelm, E.; et al. Isoprostanes and neuroprostanes: Total synthesis, biological activity and biomarkers of oxidative stress in humans. *Prostagland. Lipid Mediat.* 2013, 107, 95–102. [CrossRef]

33. Roy, J.; Fauchonnier, J.; Oger, C.; Farah, A.; Angebault-Prouteau, C.; Thireau, J.; Bideaux, P.; Scheuermann, V.; Bultel-Poncè, V.; Demion, M.; et al. Non-enzymatic oxidized metabolite of DHA, 4(RS)-4-F_{4α}-neuroprostane protects the heart against reperfusion injury. *Free Radic. Biol. Med.* 2017, 102, 229–239. [CrossRef]

34. Galano, J.M.; Roy, J.; Durand, T.; Lee, J.C.; Le Guennec, J.Y.; Oger, C.; Demion, M. Biological activities of non-enzymatic oxygenated metabolites of polyunsaturated fatty acids (NEO-PUFAs) derived from EPA and DHA: New anti-arrhythmic compounds? *Mol. Asp. Med.* 2018, 64, 161–168. [CrossRef]

35. Holt, W.; Harrison, R. Bicarbonate stimulation of boar sperm motility via a protein kinase A-dependent pathway: Between cell and between-eculated differences are not due deficiencies in protein kinase a activation. *J. Androl.* 2002, 23, 557–565.

36. García Herreros, M.; Aparicio, I.M.; Núñez, I.; García-Marin, L.J.; Gil, M.C.; Peña Vega, F.J. Boar sperm velocity and motility patterns under capacitating and non-capacitating incubation conditions. *Theriogenology* 2005, 63, 795–805. [CrossRef]

37. Soriano-Úbeda, C.; Romero-Aguirregomezcorta, J.; Matás, C.; Visconti, P.E.; García-Vázquez, F.A. Manipulation of bicarbonate concentration in sperm capacitation media improves in vitro fertilisation output in porcine species. *J. Anim. Sci. Biotechnol.* 2019, 10, 19. [CrossRef]

38. Andrisani, A.; Dona, G.; Tibaldi, E.; Brunati, A.M.; Sabbadin, C.; Armanini, D.; Alvisi, G.; Ambrosini, G.; Ragazzi, E.; et al. Astaxanthin Improves Human Sperm Capacitation by Inducing Lyn Displacement and Activation. *Mar. Drugs* 2015, 13, 5533–5551. [CrossRef]

39. Fraser, L.R.; Osigwayu, O.O. Human sperm responses to calcitonin, angiotensin II and fertilization-promoting peptide in prepared semen samples from normal donors and infertility patients. *Hum. Reprod.* 2004, 19, 596–606. [CrossRef]

40. Attken, R.J.; Nixon, B. Sperm capacitation: A distant landscape glimpsed but unexplored. *Hum. Reprod.* 2013, 19, 785–793. [CrossRef]

41. de Lamirande, E.; Leclerc, P.; Gagnon, C. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol. Hum. Reprod.* 2011, 17, 161–168. [CrossRef]

42. Visconti, P.E.; Krapf, D.; de la Vega-Beltrán, J.L.; Acevedo, J.J.; Darszon, A. Ion channels, phosphorylation and mammalian sperm capacitation. *Asian J. Androl.* 2012, 14, 395–405. [CrossRef]

43. Darszon, A.; Nishigaki, T.; Beltran, C.; Treviño, C.L. Calcium channels in the development, maturation, and function of spermatozoa. *Physiol. Rev.* 2011, 91, 1305–1355. [CrossRef]

44. Lishko, P.V.; Botchkina, I.L.; Kirichok, Y. Progesterone activates the principal Ca^{2+} channel of human sperm. *Nature* 2011, 471, 387–391. [CrossRef]

45. Brenker, C.; Goodwin, N.; Weyand, I.; Kashikar, N.D.; Naruse, M.; Krähling, M.; Müller, A.; Kaupp, U.B.; Strünker, T. The CatSper channel: A polyomodal chemosensor in mammalian sperm. *EMBO J.* 2012, 31, 1654–1665. [CrossRef]

46. Vaquer, C.C.; Suhamain, L.; Pavarotti, M.A.; De Blas, G.A.; Belmonte, S.A. Ceramide induces a multicomponent intracellular calcium increase triggering the acrosome secretion in human sperm. *Biochim. Biophys Acta Mol. Cell Res.* 2020, 18, 118704. [CrossRef]

47. Signorelli, J.R.; Diaz, E.S.; Fara, K.; Barón, L.; Morales, P. Protein phosphatases decrease their activity during capacitation: A new requirement for this event. *PLoS ONE* 2013, 8, e81286. [CrossRef]

48. Martínez-León, E.; Osyska-Salut, C.; Signorelli, J.; Pozo, P.; Pérez, B.; Kong, M.; Morales, P.; Pérez-Martínez, S.; Diaz, E.S. Fibronectin stimulates human sperm capacitation through the cyclic AMP/protein kinase A pathway. *Hum. Reprod.* 2015, 30, 2138–2151. [PubMed] [CrossRef]

49. O’Flaherty, C.; de Lamirande, E.; Gagnon, C. Phosphorylation of the Arginine-X-X-(Serine/Threonine) motif in human sperm proteins during capacitation: Modulation and protein kinase A dependency. *Mol. Hum. Reprod.* 2004, 10, 355–363. [CrossRef]

50. Bedu-Addo, K.; Lefèvre, L.; Moseley, E.L.; Barratt, C.L.; Publicover, S.J. Bicarbonate and bovine serum albumin reversibly ‘switch’ capacitation-induced events in human spermatozoa. *Hum. Reprod.* 2005, 11, 683–691. [CrossRef]

51. Nguyen, T.M.; Alves, S.; Grasseau, I.; Métayer-Coustard, S.; Praud, C.; Froment, P.; Blesbois, E. Central role of 5’-AMP-activated protein kinase in chicken sperm functions. *Biol. Reprod.* 2014, 91, 121. [CrossRef]
52. Nguyen, T.M.; Froment, P.; Combarnous, Y.; Blesbois, É. L’AMPK, régulateur de l’énergie et des fonctions des spermatozoïdes [AMPK, regulator of sperm energy and functions]. Med. Sci. (Paris) 2016, 32, 491–496. [CrossRef] [PubMed]
53. Feng, T.Y.; Lv, D.L.; Zhang, X.; Du, Y.Q.; Yuan, Y.T.; Chen, M.J.; Xi, H.M.; Li, Y.; Han, N.; Hu, J.H. Rosmarinic acid improves boar sperm quality, antioxidant capacity and energy metabolism at 17°C via AMPK activation. Reprod. Domest. Anim. 2020, 55, 1714–1724. [CrossRef] [PubMed]