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SYNERGISTIC INHIBITION OF HUMAN SPERM FUNCTION BY FUCOIDAN AND OUABAIN SUGGESTS A PROMISING NON-HORMONAL CONTRACEPTIVE STRATEGY

INIBIÇÃO SINÉRGICA DA FUNÇÃO ESPERMÁTICA HUMANA POR FUCOIDAN E OUABAÍNA REVELA UMA POTENTE ESTRATÉGIA CONTRACEPTIVA NÃO HORMONAL

INHIBICIÓN SINÉRGICA DE LA FUNCIÓN ESPERMÁTICA HUMANA POR FUCOIDAN Y OUABAÍNA REVELA UNA POTENTE ESTRATEGIA ANTICONCEPTIVA NO HORMONAL

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ABSTRACT

The development of safe and effective non-hormonal contraceptive options is a global priority, particularly given the mucosal irritation and inflammatory responses associated with chemical spermicides such as Nonoxinol-9 and Sodium Lauryl Sulfate. Natural bioactive compounds have emerged as promising alternatives due to their diverse biological properties and potentially improved safety profiles. This study investigated the *in vitro* effects of Fucoidan, a sulphated polysaccharide from brown algae, and Ouabain, a cardiotonic glycoside that selectively inhibits the sperm-specific Na^+/K^+-ATPase α4 isoform, on human sperm motility and vitality. Semen samples from healthy volunteers (n=15) were exposed to saline (negative control), Sodium Lauryl Sulfate (positive control), Fucoidan, Ouabain, or a Fucoidan + Ouabain combination, and evaluated at 0 and 15 minutes following WHO (2021) guidelines. All treatments reduced motility and vitality compared with the negative control. Fucoidan alone produced modest reductions, whereas Ouabain induced a more pronounced inhibitory effect. The combined treatment resulted in the greatest decline, reducing progressive motility and vitality by approximately 28% at both time points, indicating a synergistic interaction. Statistical analysis (Kruskal–Wallis and Wilcoxon test) confirmed significant differences

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between treatments ($p < 0.05$). These findings support the potential of Fucoidan and Ouabain, particularly in combination, as candidates for the development of natural, non-hormonal contraceptive formulations. Further studies are required to assess cytotoxicity, mucosal safety, and long-term applicability.

KEYWORDS: Ouabain. Glycoconjugates. Sperm Motility.

RESUMO
O desenvolvimento de opções contraceptivas não hormonais seguras e eficazes é uma prioridade global, especialmente considerando a irritação da mucosa e as respostas inflamatórias associadas a espermicidas químicos como Nonoxinol-9 e Lauril Sulfato de Sódio. Compostos bioativos naturais surgiram como alternativas promissoras devido às suas diversas propriedades biológicas e a perfis de segurança potencialmente superiores. Este estudo investigou os efeitos *in vitro* de Fucoidan, um polissacarídeo sulfatado de algas marrons, e Ouabaína, um glicosídeo cardiotônico que inibe seletivamente a isoforma α4 da Na^+/K^+-ATPase específica de espermatozóides, sobre a motilidade e a vitalidade de espermatozóides humanos. Amostras seminais de voluntários saudáveis (n=15) foram expostas à solução salina (controle negativo), Lauril Sulfato de Sódio (controle positivo), Fucoidan, Ouabaína ou à combinação Fucoidan + Ouabaína, sendo avaliadas nos tempos 0 e 15 minutos segundo o WHO *Laboratory Manual for the Examination and Processing of Human Semen* (2021). Todos os tratamentos reduziram a motilidade e a vitalidade em comparação ao controle negativo. O Fucoidan isolado produziu reduções modestas, enquanto a Ouabaína induziu um efeito inibitório mais pronunciado. A combinação resultou no maior declínio, reduzindo a motilidade progressiva e a vitalidade em cerca de 28% em ambos os tempos, indicando interação sinérgica. As análises estatísticas (Kruskal–Wallis test e Wilcoxon test) confirmaram diferenças significativas entre os tratamentos ($p < 0,05$). Esses achados apoiam o potencial de Fucoidan e Ouabaína, especialmente em combinação, como candidatos para o desenvolvimento de formulações contraceptivas naturais e não hormonais. Estudos adicionais são necessários para avaliar citotoxicidade, segurança mucosal e aplicabilidade prolongada.

PALAVRAS-CHAVE: Ouabaína. Glicoconjugados. Motilidade dos espermatozóides.

RESUMEN
El desarrollo de opciones anticonceptivas no hormonales seguras y eficaces es una prioridad global, especialmente considerando la irritación de la mucosa y las respuestas inflamatorias asociadas con espermicidas químicos como el Nonoxinol-9 y el Lauril Sulfato de Sodio. Los compuestos bioactivos naturales han surgido como alternativas prometedoras debido a sus diversas propiedades biológicas y a perfiles de seguridad potencialmente superiores. Este estudio investigó los efectos in vitro de Fucoidan, un polisacárido sulfatado derivado de algas pardas, y Ouabaína, un glucósido cardiotónico que inhibe selectivamente la isoforma α4 de la Na^+/K^+-ATPasa específica de los espermatozoides, sobre la motilidad y la vitalidad de espermatozoides humanos. Muestras de semen de voluntarios sanos fueron expuestas a solución salina (control negativo), Lauril Sulfato de Sodio (control positivo), Fucoidan, Ouabaína o a la combinación Fucoidan + Ouabaína, y evaluadas a los 0 y 15 minutos según las directrices de la OMS (2021). Todos los tratamientos redujeron la motilidad y la vitalidad en comparación con el control negativo. El Fucoidan produjo reducciones moderadas, mientras que la Ouabaína generó un efecto inhibitorio más pronunciado. La combinación resultó en el mayor descenso, reduciendo la motilidad progresiva y la vitalidad en aproximadamente un 28% en ambos tiempos, lo que indica una interacción sinérgica. Los análisis estadísticos (Kruskal–Wallis y Wilcoxon prueba) confirmaron diferencias significativas entre los tratamientos ($p < 0,05$). Estos hallazgos respaldan el potencial de Fucoidan y Ouabaína, especialmente en combinación, como candidatos para el desarrollo de formulaciones anticonceptivas naturales y no hormonales. Se requieren estudios adicionales para evaluar citotoxicidad, seguridad mucosal y aplicabilidad a largo plazo.

PALABRAS CLAVE: Ouabaína. Glicoconjugados. Motilidad Espermática.

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INTRODUCTION

The search for contraceptive methods that combine efficacy, safety, and mucosal tolerability has intensified in recent years, driven by the limitations of hormonal contraceptives and conventional chemical spermicides. Nonoxynol-9 (N-9), belongs to the class of surfactant membrane disruptors, a group characterized by non-specific disruption of cellular and microbial membranes (Hifnawy *et al*., 2021). In addition, Sodium Lauryl Sulfate (SLS) is a member of this same class and exhibits an identical membrane-disrupting mechanism, having been evaluated as a potent sperm inhibitor and membrane destabilization (Zhernov *et al*., 2024). Although N-9 is the most widely used surfactant spermicide, it is frequently associated with mucosal irritation, inflammatory responses, and increased susceptibility to sexually transmitted infections (STIs) (Hifnawy *et al.,* 2021; Zhernov *et al.,* 2024). These adverse effects highlight the need for safer alternatives, particularly for individuals who cannot or prefer not to use hormonal methods.

In addition, hormonal contraceptives, although effective, are associated with increased risks of venous thromboembolism, arterial thrombosis, stroke (Practice Committee of the American Society for Reproductive Medicine, 2017; Yonis *et al*., 2024). Their systemic effects, combined with the high failure rates of barrier methods under typical use, 13% for male condoms and 21% for female condoms (Hatcher, 2018), underscore the importance of developing non-hormonal contraceptives with improved safety and reliability. Moreover, diaphragms often require the concomitant use of N-9, which can induce epithelial microlesions and inflammatory cytokine release, further compromising mucosal integrity (Hifnawy *et al*., 2021; Xu *et al*., 2022).

In response to these challenges, natural compounds with spermicidal or spermiostatic potential have gained attention. Plant-derived compounds and seaweed extracts are being investigated for their spermicidal activity with lower toxicity and as promising candidates (Qiu *et al*., 2022). Reversible effects of topical spermicides have been demonstrated in recent animal studies. The antimicrobial peptide 17BIPHE2 showed strong *in vitro* spermicidal activity and effective contraception in mice, with fertility returning after treatment withdrawal, indicating reversibility without damage to the reproductive tract (Lee *et al*., 2022). Similarly, the serine protease inhibitor AEBSF reduced sperm motility and fertilization in mice, producing reversible spermicidal and contraceptive effects without altering vaginal epithelial viability in vivo (Barton *et al*., 2020). Natural antimicrobial peptides such as LL-37 and subtilosin also exhibit selective spermicidal and microbicidal activity, preserving healthy microbiota and showing potential for safe use in topical formulations (Tanphaichitr *et al*., 2016; Sutyak *et al*., 2008). In cattle, gossypol induced temporary sperm alterations that were fully reversed after exposure ceased, with no permanent histological changes in the testes (Hassan *et al*., 2004). Overall, these findings indicate that certain topical spermicides can act effectively yet

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transiently, allowing fertility recovery after discontinuation, while avoiding permanent reproductive tissue damage in the animal models tested.

Considering the adverse effects of chemical spermicides such as N-9 and SLS, and systemic effects of hormonal contraceptives, it is essential to expand research on bioactive alternatives with an appropriate safety profile. In this context, Fucoidan and Ouabain emerge as promising candidates due to their therapeutic potential, which justifies them *in vitro* analysis, both individually and in combination, as proposed in this study.

1. THEORETICAL FRAMEWORK

1.1. Scientific Basis

1.1.1. Fucoidan

Fucoidan, a sulfated polysaccharide extracted from brown algae such as *Fucus vesiculosus*, exhibits diverse biological activities, including antioxidants, anti-inflammatory, anticoagulant, and antitumor effects (Li *et al.*, 2008; Pradhan *et al.*, 2020; Zahariev *et al.*, 2023; Chollet *et al.*, 2016; Asanka Sanjeewa *et al.*, 2021). In reproductive contexts, Fucoidan reduces sperm motility and interferes with sperm–zona pellucida interaction due to its sulfated fucose structure, which mimics natural ligands and competes for binding sites (Patankar et al., 1993; Song et al., 2007; Woo et al., 2000; McDermott et al., 2012). Although this mechanism was not evaluated in the present study, its spermiostatic potential makes it a promising candidate for a combination contraceptive development.

1.1.2. Ouabain

Ouabain, a cardiotonic glycoside derived from *Strophanthus gratus,* selectively inhibits the Na^+/K^+-ATPase α4 isoform (NKAα4), a sperm-specific pump essential for maintaining membrane potential, intracellular ion homeostasis, and flagellar motility (Woo *et al.*, 2000; McDermott *et al.*, 2012; McDermott *et al.*, 2021). Inhibition of NKAα4 disrupts sodium and calcium gradients, leading to impaired motility and altered signaling pathways involved in capacitation (Zhu *et al.*, 2019; Syeda *et al.*, 2020; Chianese & Pierantoni, 2021; Thundathil *et al.*, 2018; Yang *et al.*, 2014). Experimental studies demonstrate that Ouabain at micromolar concentrations of 10^{-6} M, resulted in the inhibition of total, progressive, and hyperactivated sperm motility. This dosage selectively inhibits NKAα4 without affecting NKAα1, which plays a less relevant role in sperm motility (McDermott *et al.*, 2021; Yang *et al.*, 2014). The α4 isoform is present in the flagellum of mature spermatozoa and its inhibition results in the loss of sperm motility.

The Figure 1 illustrates the cascade of reactions that lead to the inhibition of NKAα4 by Ouabain, consequently promoting an increase in intracellular calcium ($[Ca^{2+}]_i$) and cytoplasmic acidification, which compromise several parameters of flagellar movement and, consequently, alter

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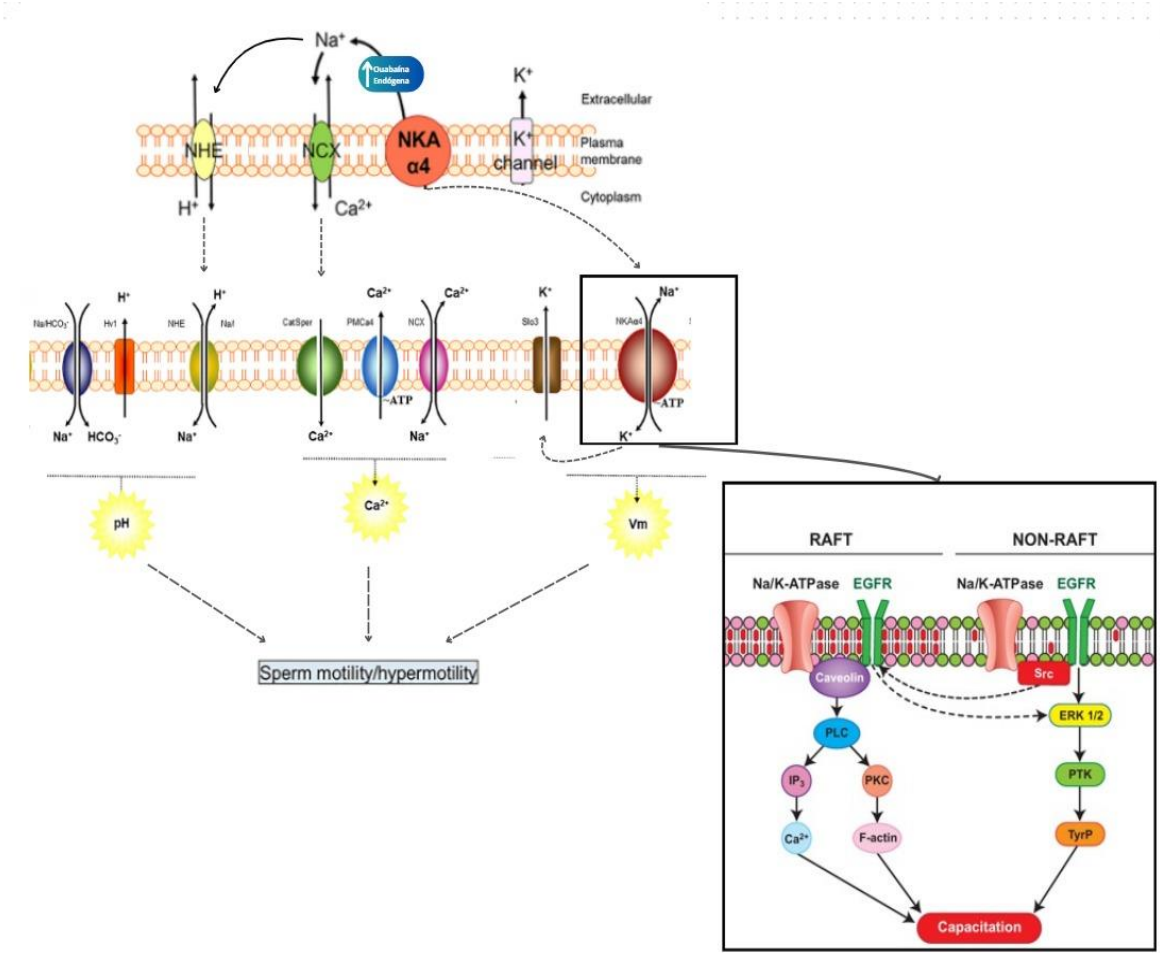
male fertility. In addition, the Na^+ gradient generated by NKAα4 is used by secondary transport mechanisms to maintain adequate cytosolic levels of H^+ and Ca^{2+} (Syeda *et al.,* 2020; Lastra-Vargas *et al.,* 2021). A proper membrane potential is vital for sperm motility, and plasma membrane hyperpolarization is essential for sperm capacitation (Syeda *et al.,* 2020).

Figure 1. Schematic representation of the cascade of reactions leading to the inhibition of NKAα4 by Ouabain, the main ion transport systems involved in sperm physiology, and the relationship of NKAα4, along with a hypothetical model presenting NKAα4-mediated signaling pathways in specific membrane microdomains of bovine sperm during capacitation (Syeda *et al*., 2020; Lastra-Vargas *et al*., 2021; Thundathil *et al*., 2018, adapted)



Furthermore, it illustrates the ion transport systems that act synergistically with NKAα4 during sperm capacitation, such as PMCA4, NCX, SLO3, NHE, Na^+/HCO_3^-, and Hv1, all of which are essential for regulating intracellular pH and calcium levels (Syeda *et al*., 2020; Lastra-Vargas *et al*., 2021; Thundathil *et al*., 2018).

NKAα4 participates in distinct signaling pathways in membrane microdomains: in raft domains, it interacts with caveolin-1 and activates PLC, promoting cytoskeletal reorganization; in

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non-raft domains, it associates with Src and EGFR, activating ERK1/2 and triggering protein phosphorylation by tyrosine kinases (Thundathil *et al*., 2018).

Proteomic analysis has revealed proteins such as plakoglobin, ADAM, and annexin A2 in these microdomains, all involved in gamete adhesion and fusion, with plakoglobin being particularly relevant due to its interaction with NKAα4 in the equatorial segment of capacitated spermatozoa (Syeda et al., 2020; Thundathil *et al.*, 2018).

1.1.3. Sodium Lauryl Sulfate (SLS)

Surfactants such as N-9 and SLS share a non-specific membrane-disrupting mechanism, rapidly compromising cellular integrity. N-9, although historically used in topical contraceptives, was shown to cause epithelial irritation, microabrasions, and increased local inflammation, including the upregulation of pro-inflammatory cytokines, which ultimately increased susceptibility to HIV infection (Zhernov *et al.,* 2024). SLS exhibits a comparable toxicological profile, acting as a potent protein-denaturing agent capable of inducing immediate sperm immobilization and membrane lysis, in 279.2 µg/mL, as demonstrated experimentally by Haineault and collaborators (2003). SLS reproduces the same pattern of cytotoxicity, epithelial damage and reproducible effects, even at the low concentrations, it is associated with mucosal hypersensitivity, ulcerations, delayed wound healing, and inflammatory responses (Sabri *et al*., 2023).

2. METHODOLOGY

Experimental Design

The study was structured using a randomized block experimental design, in which time was defined as the blocking factor (T0 and T15 minutes), following the methodological rationale described by Rithaporn *et al.,* (2003). This approach was adopted because sperm motility can be significantly influenced by exposure duration to bioactive compounds, including fucoidan, ouabain, and the positive control sodium lauryl sulfate (SLS). By treating time as a block, variability associated with incubation duration was minimized, thereby increasing statistical analysis and experimental reliability.

All bioactive compounds were prepared in 0.9% physiological saline (NaCl) as the vehicle solution. Ouabain (Sigma-Aldrich) was used at a final concentration of 10^{-5} mol/L, based on Yang *et al*. (2014). Fucoidan (Sigma-Aldrich), a sulfated polysaccharide derived from *Fucus vesiculosus*, was prepared at a concentration of 1 mg/mL according to Zayed *et al*. (2019). Sodium lauryl sulfate (SLS) was used as the positive control at 279.2 µg/mL, following Haineault *et al*. (2003). All concentrations were selected based on previously published experimental evidence demonstrating biological activity within these ranges.

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The randomized block design divided the samples into five experimental groups: (1) Negative Control (0.9% physiological saline), (2) Positive Control (SLS at 279.2 µg/mL), (3) Fucoidan (1 mg/mL), (4) Ouabain (10^{-5} mol/L), and (5) Fucoidan–Ouabain (FU–OUA).

Sperm motility was evaluated in a humid chamber under optical microscopy immediately after exposure (T0) and after 15 minutes of incubation (T15). Standardization of procedures was conducted with the technical support of embryologists and andrologists from the Assisted Human Reproduction Clinic, who contributed to the development and validation of the serial semen analysis protocol.

Semen Collection and Sample Selection

Eighteen semen samples were initially collected; however, three were excluded based on predefined inclusion criteria. Therefore, the final sample size consisted of fifteen (n=15) normozoospermic samples. Semen was obtained by masturbation following 72–120 hours of controlled sexual abstinence and collected in sterile containers for immediate transport to the laboratory. For every volunteer, five individual slides were prepared per time point, corresponding to the five treatment groups. For each slide, a double-verification protocol was implemented to mitigate observational bias: an initial manual count was performed in the laboratory, followed by a second manual recount of the digital fields recorded using the Leica DM 2500 and LAS EZ software.

The study was approved by the Research Ethics Committee of the Federal University of Espírito Santo (CEP; Opinion No. 6.885.109; CAAE: 79783124.5.0000.5060) and by the National Research Ethics Commission (CONEP; Opinion No. 7.003.256), Brazil.

Semen Analysis According to WHO (2021)

All laboratory procedures were performed in accordance with the WHO Laboratory Manual for the Examination and Processing of Human Semen (6th edition, 2021), ensuring methodological standardization, reproducibility, and comparability.

Only ejaculates presenting total motility ≥ 40%, sperm concentration ≥ 15 × 10^6 spermatozoa/mL, and normal morphology ≥ 4% were included. All participants were non-smoking. These criteria ensured the use of normozoospermic samples, minimizing baseline variability across treatment comparisons.

After ejaculation, semen samples were allowed to liquefy at room temperature (20–28 °C). Sperm concentration was determined using a Neubauer hemocytometer chamber following a 1:20 dilution protocol. Specifically, 50 µL of semen were diluted in 950 µL of 0.9% saline solution. After homogenization, 10 µL of the diluted sample were loaded into the Neubauer chamber, and sperm cells were counted under a conventional optical microscope. Concentration was calculated using the WHO formula for sperm count determination.

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Macroscopic evaluation included pH, viscosity, appearance, and odor. Motility assessment was performed manually in a wet preparation under optical microscopy, classifying spermatozoa into progressive motility, non-progressive motility, and immotile categories (A, B, C, D classification system).

Samples were prepared immediately at T0 and observed again at T15, analyzed under optical microscopy to determine membrane integrity, distinguishing viable (unstained) from non-viable (stained) spermatozoa.

Techniques for Assessing Sperm Motility Under Treatments

For experimental treatments, 20 µL of semen were mixed with 20 µL of the respective bioactive compound (1:1 ratio), resulting in the final working concentrations described above. The mixture was homogenized for 30 seconds to ensure uniform exposure. Subsequently, 10 µL were placed in a humid chamber slide and evaluated immediately (T0) and again after 15 minutes of incubation (T15).

Semen Analysis

Sperm concentration was subsequently calculated using the formula shown below:

$$sperm\ concentration = \frac{a}{\frac{1}{10} \times \frac{n}{25} \times \frac{1}{b}}$$

where:

a – number of spermatozoa counted in 5 grids (80 squares)

b – dilution factor (1:20)

n – number of large squares counted (5)

1/10 – chamber depth (0.1 mm)

1/25 – total area of one grid

That is:

$$sperm\ concentration = \frac{(number\ of\ spermatozoa\ counted) \times 5 \times 20.000}{80}$$

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Distribution across treatments

Semen samples were analyzed after liquefaction (30 minutes), and sperm concentration per milliliter, motility, and vitality were determined. The doses of the bioactive compounds were prepared using the split sample technique, meaning that the same semen sample was used for all different treatments.

The standardization of the technique and protocol was developed after approval by CEP/CONEP and consisted of the following steps: the sterile collection container was weighed before being handed to the volunteer and again after sample collection.

In the microscopic analysis of motility, 10 µl of the content were placed on a glass slide, covered with a coverslip, and immediately analyzed under all experimental conditions. Sperm vitality was assessed using eosin–nigrosin staining to verify the integrity of the cell membrane, employing a 1:1:1 proportion with equal aliquots (10 µl) of sperm suspension, eosin–nigrosin dye, and the bioactive compound (Rithaporn *et al*., 2003). After mixing, 10 µl were pipetted onto a slide, covered with a coverslip, and examined immediately to assess sperm vitality at 0 and 15 minutes.

Statistical analysis

The results were expressed as mean ± standard deviation. The reliability of the manual observations was ensured through a double-verification protocol: an initial count was performed in the laboratory, followed by a digital re-verification using recorded videos captured via a Leica DM 2500 photomicroscope and LAS EZ software. Statistical analysis was conducted using the arithmetic mean of these two counts. Significance was assessed using the Kruskal-Wallis and Wilcoxon tests ($p<0.05$) with p-value adjustment (Benjamini; Yekutieli, 2001) within the R statistical environment.

3. RESULTS AND DISCUSSION

All compounds reduced motility and vitality compared with the negative control, with the FU–OUA combination being the most effective. Table 1 presents a descriptive analysis of progressive motility and vitality values for each treatment at T0 and T15. The data are presented as mean ± standard deviation.

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Table 1. Distribution of progressive motility and vitality across different times and treatments

Treatment	Motility at T0	Motility at T15	Vitality at T0	Vitality at T15
Negative control	68.3 ±7.80	60.4 ±10.7	65.5 ±6.78	59.1 ±7.48
Positive control (SLS)	55.5 ±11.2	50.7 ±10.3	45.9 ±10.5	40.3 ±12.5
FU	55.1 ±8.95	53.0 ±11.1	49.2 ±12.1	44.4 ±10.4
OUA	54.1 ±15.3	48.2 ±14.4	46.6 ±14.4	43.9 ±15.1
FU–OUA	49.1 ±10.3	43.3 ±15.4	46.2 ±9.84	38.7 ±12.6

Values are expressed as Mean ± Standard Deviation (SD). While Table 1 presents raw data (Mean ± SD), the percentage reductions reported in the text reflect relative variations compared to the negative control at each time point. These derived indices highlight the synergistic and proportional impact of the treatments, even though they are not explicitly listed in the table's columns. This relative calculation allows for a clearer understanding of the proportional impact of agents like Fucoidan and Ouabain on sperm function.

Across all treatments, reductions in motility and vitality were observed at both time points, with the FU–OUA combination consistently showing the greatest decrease.

At time T0, a decrease in sperm motility was observed in all treatments, when compared with negative control: SLS to 55,5 ± 11,2 (n= -18,74%), FU to 55,1 ± 8,95 (n= -19,32%), OUA to 54,1 ± 15,3 (n= -20,8%) e FU–OUA to 49,1 ± 10,3 (= -28,1%). At T15, the reduction was also pronounced: SLS (n= -16,06%), FU (n= -12,25%), OUA (n= -20,19%) e FU– OUA (n= -28,3%).

Regarding sperm vitality, the data reveals a severe decline following the treatments, with the most critical impact observed in the FU–OUA combination at both time intervals. At time T0, vitality decreased from 65.5 ± 6.78 (negative control) to 46.2 ± 9.84, representing a 29.4% reduction in cell survival. By T15, the same treatment evidenced a vitality of 38.7 ± 12.6 compared to the negative control of 59.1 ± 7.48, corresponding to a further reduction of 34.5%. While all treatments caused significant reductions compared to the control, the combination of Fucoidan and Ouabain exhibited the strongest influence on the loss of sperm vitality.

Motility

Statistical significance and comparison vs negative control

Statistical analysis using the Kruskal-Wallis test confirmed significant differences between treatments at both T0 ($p<0.001$) and T15 ($p=0.003$). At time T0, the post-hoc test revealed that all treatments significantly reduced progressive motility compared to the negative control (68.3 ± 7.80), as illustrated in Figure 2. Specifically, isolated treatment with Ouabain (OUA) reduced motility to 54.1 ± 15.3, representing a 20.8% decrease. By T15, however, the post-hoc test indicated that only the

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FU–OUA combination maintained a statistically significant reduction relative to the negative control (60.4 ± 10.7), a trend further detailed in Figures 2 and 3.

Figure 2. Boxplot of motility at T0 and T15

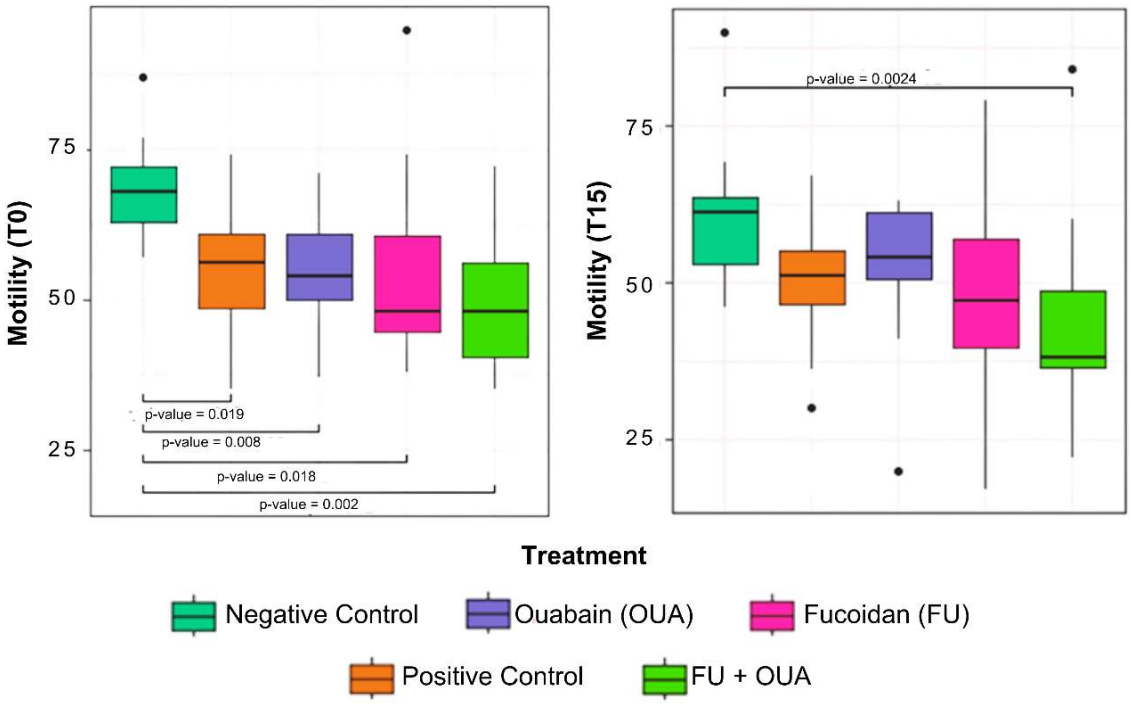

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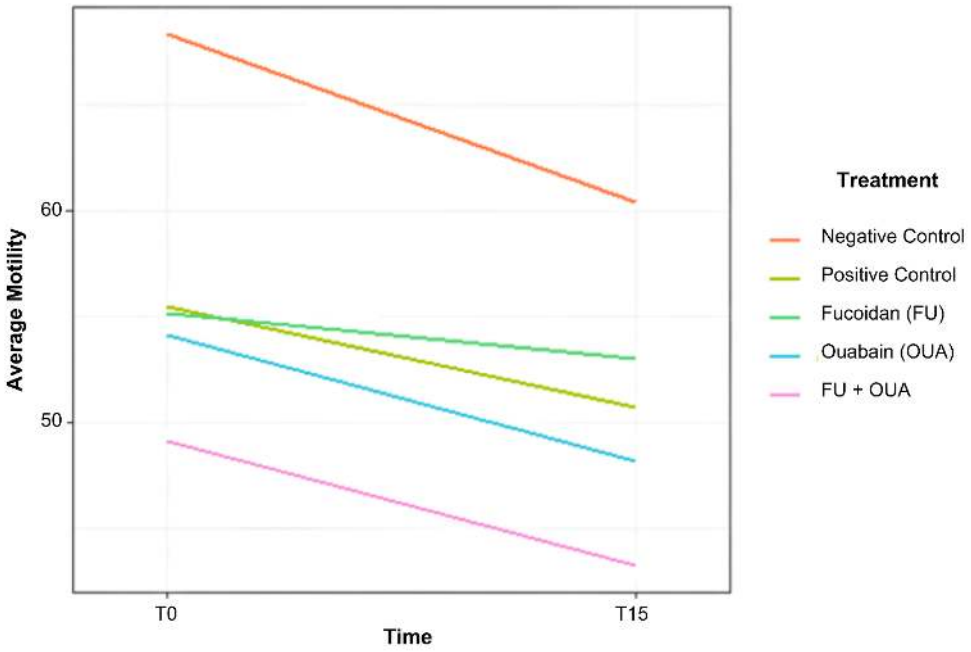
Figure 3. Average motility in T0 and T15.



Efficacy of the combined FU–OUA treatment

The combination of Fucoidan and Ouabain consistently produced the most substantial decline in sperm motor function at both incubation periods. At T0, motility in the combined group dropped to 49.1 ± 10.3, reflecting a 28.1% reduction compared to the negative control. This inhibitory effect remained sustained at T15, with motility values reaching 43.3 ± 15.4, which corresponds to a 28.3% reduction. These results highlight the potent and synergistic spermiostatic action of the combined bioactive compounds.

Comparison with the positive control (SLS)

When evaluated against the positive control (SLS), the FU–OUA demonstrated superior inhibitory efficacy. At T0, the combined treatment elicited a ~11.5% greater reduction than SLS (55.5 ± 11.2), surpassing the more modest isolated effects of FU (~0.7%) and OUA (2.52%). By T15, the combined treatment achieved a ~14.6% greater reduction in motility compared to SLS (50.7 ± 10.3), exceeding the 4.93% reduction observed for OUA alone. This ability to significantly outperform a conventional chemical surfactant underscores the potential of this interaction for the development of multifunctional, non-hormonal contraceptive formulations.

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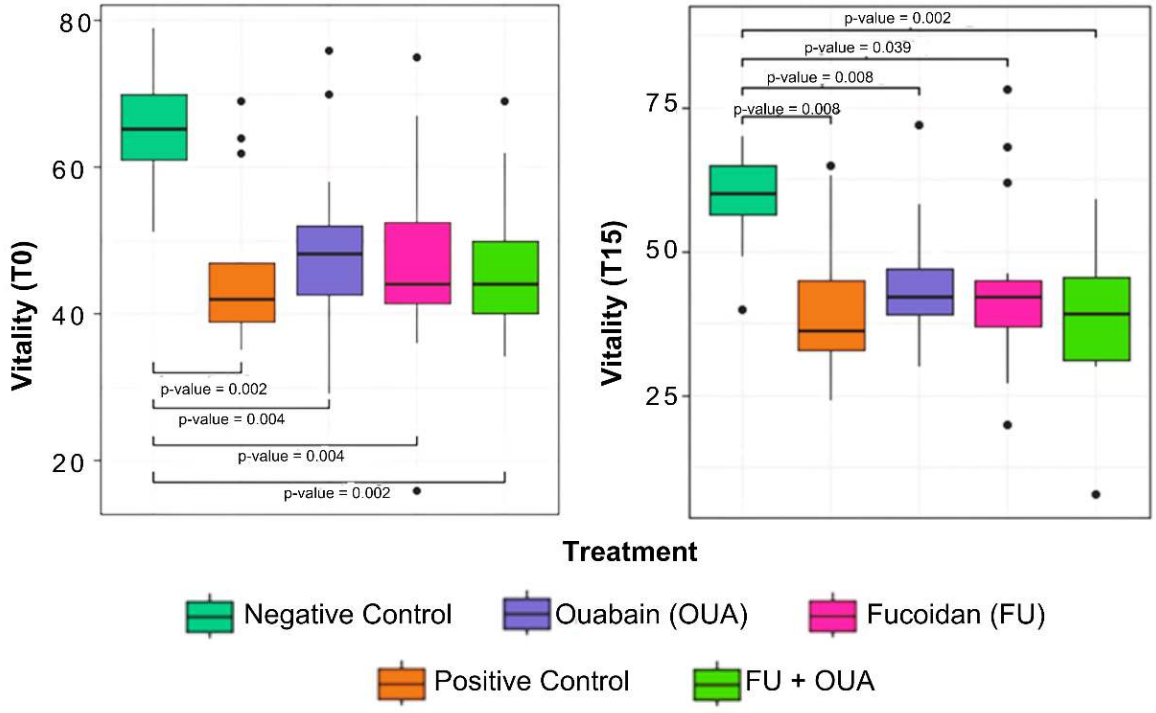
Vitality

Statistical significance and comparison vs negative control

Statistical analysis using the Kruskal-Wallis test ($p<0.05$) confirmed that all treatments significantly reduced sperm vitality compared to the negative control at both time points. At T0, the post-hoc test demonstrated that every intervention notably decreased cell survival, with Ouabain (OUA) alone reducing vitality to 46.6 ± 14.4, which represents a 28.9% reduction relative to the negative control (65.5 ± 6.78). By T15, the decline became even more pronounced and time-dependent, as shown in Figure 4, with all treated groups maintaining survival levels significantly lower than the negative control (59.1 ± 7.48).

Figure 4. Boxplot of vitality at time T0 and T15 when compared with the negative control



Efficacy of the Combined FU–OUA Treatment

The combination of Fucoidan and Ouabain (FU–OUA) demonstrated the most substantial overall impact on sperm integrity. At T0, the combined treatment lowered vitality to 46.2 ± 9.84, a 29.4% reduction compared to the negative control. By T15, the effect reached its most critical point, with vitality dropping to 38.7 ± 12.6—a total reduction of 34.5%. These findings suggest that the interaction between these two bioactive compounds increasingly compromises the sperm cell membrane over time.

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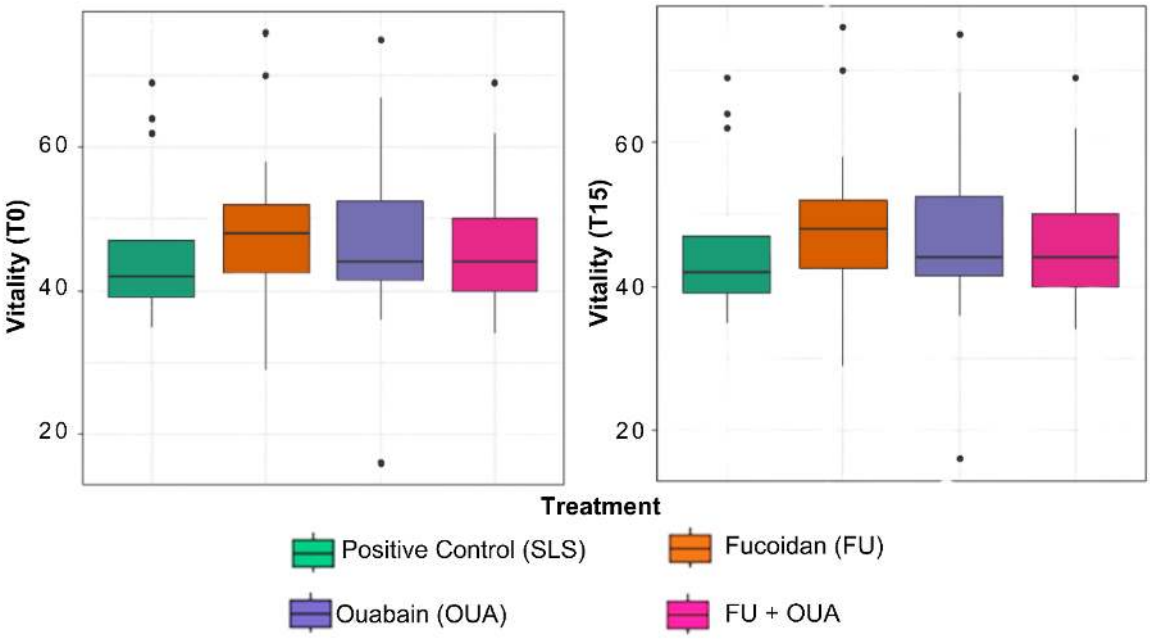
Comparison with the Positive Control (SLS)

When compared to the positive control (SLS), the statistical analysis in Figure 5 revealed no significant differences between the isolated treatments (FU or OUA) and the SLS at either T0 or T15. While SLS remained the most effective single agent at the initial time point (T0), being roughly 0.65% more effective than the combination, the FU–OUA treatment surpassed the efficacy of SLS by T15. At this final interval, the combined treatment achieved a ~4% greater reduction in vitality than the positive control.

Figure 5. Boxplot of vitality at time T0 and T15 when compared with the positive control (SLS)



Biological basis: NKAα4 as central axis of sperm motility

The Na^+/K^+-ATPase is a major ATP-consuming enzyme responsible for maintaining ionic gradients and plasma membrane potential, and the NKAα4 isoform represents the sperm-specific variant of this pump (Zhang *et al*., 2025). NKAα4 is predominantly localized to the flagellum and, to a lesser extent, the sperm head, displaying a highly organized distribution pattern (Oishee *et al.,* 2025). Its activity is essential for sustaining membrane potential, Na^+ and Ca^{2+} homeostasis, and the regulation of flagellar motility, thereby supporting capacitation and male fertility (Syeda *et al.,* 2025; Lee; Hwang, 2024). Under capacitating conditions, NKAα4 redistributes toward the acrosomal region, whereas under non-capacitating conditions it remains mainly in the flagellum (Milewski; James, 2025).

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Pharmacological inhibition of NKAα4 by ouabain induces membrane depolarization, cytosolic acidification, and increased intracellular Ca^{2+}, rapidly suppressing progressive motility and potentially contributing to oxidative disturbances, as suggested by previous studies (Syeda *et al.*, 2020; Lastra-Vargas *et al*., 2021; Syeda *et al*., 2025). Complementarily, Zhang, Wang and Li (2025) demonstrated that sperm motility and vitality depend on adequate bioenergetic and redox balance, including mitochondrial potential, ATP availability, and ROS control, indicating that disruptions in these axes compromise sperm function. Selective inhibition of NKAα4 reduces progressive motility by ~50% *in vitro* without immediate loss of viability and can block fertilization, supporting NKAα4 as a potential target for nonhormonal male contraception (Syeda *et al.*, 2025; Woo; James; Lingrel, 2000; McDermott *et al.,* 2012).

From a physicochemical perspective, inhibition of NKAα4 by ouabain alters electrochemical gradients essential for flagellar beating, including membrane depolarization, cytoplasmic acidification, and increased $[Ca^{2+}]i$, which interact with Ca^{2+} influx through CatSper channels and the maintenance of transmembrane potential (Abe *et al.*, 2024).

Beyond ion transport, some studies propose that NKAα4 may participate in signaling processes when associated with lipid microdomains, potentially interacting with structural organizers such as caveolin-1 (Heredero-Jiménez, 2024). These interactions have been suggested as hypothetical mechanisms linking pump activity to downstream pathways that regulate sperm function (Syeda *et al.*, 2020; Lastra-Vargas *et al.*, 2021; Thundathil *et al.*, 2018), although such pathways were not directly assessed in the present study and should therefore be interpreted as potential mechanistic explanations rather than experimentally demonstrated effects.

Biophysical dimension: lipid microdomains and cytoskeletal reorganization

In this study, the primary observable outcome was a reduction in sperm motility when FU and OUA were combined. Although direct measurements of membrane biophysics were not performed, the combined FU–OUA treatment resulted in a clear reduction in sperm motility, a functional outcome that aligns with mechanisms previously associated with disturbances in NKAα4 activity and raft-dependent signaling.

The observed motility impairment is consistent with the hypothesis that perturbations in NKAα4 activity may alter membrane organizational platforms, offering a coherent explanatory framework for the functional reduction observed. This mechanistic framework suggests that the FU+OUA combination may impair motility by disrupting the integration of membrane microdomains, a pathway previously associated with the mislocalization of signaling elements (Thundathil; Rajamanickam; Kastelic, 2018; Lastra-Vargas *et al.,* 2021).

Integrating these concepts suggests the mechanistic hypothesis that targeted perturbation of NKAα4 can destabilize raft-dependent signaling, ultimately compromising progressive motility and

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fertilizing capacity. Although the present study focused on motility analysis, the literature supports the hypothesis that agents such as Fucoidan and Ouabain may influence broader biophysical and signaling networks that regulate sperm function (Thundathil; Rajamanickam; Kastelic, 2018; Lastra-Vargas *et al.,* 2021; Tanghe *et al.,* 2004). These pathways warrant further investigation to confirm if they extend to other processes essential for fertilization, such as gamete adhesion and the acrosome reaction (Geng *et al.*, 1997; Novoyatleva *et al.*, 2019).

ROS in sperm function and their biphasic role

Reactive Oxygen Species (ROS) exhibit a well-established biphasic role in sperm physiology. At physiological levels, they participate in essential processes such as capacitation, hyperactivation, the acrosome reaction, and fertilization; however, excessive ROS generation leads to oxidative stress, reduced motility, DNA damage, and reproductive failure (Mo *et al.*, 2025; Xu *et al.*, 2025). In this context, when ROS production surpasses the antioxidant capacity, oxidative stress becomes established, resulting in lipid peroxidation, DNA fragmentation, and protein oxidation, ultimately compromising motility and fertility (Wang; Fu; Li, 2025; Sengupta *et al.,* 2024). Elevated mitochondrial ROS further impair sperm DNA integrity and negatively affect early embryonic development, even when fertilization occurs (Mateo-Otero *et al.,* 2024). Consistent with these observations, animal models reinforce this pattern, showing that ROS overproduction induces premature capacitation, early acrosome reaction, and reduced fertilizing ability (Karanwal *et al.*, 2024).

Although ROS function as indispensable redox messengers during capacitation, excessive levels disrupt axonemal protein structure, diminishing motility, effective capacitation, and subsequent embryonic development (Mo *et al.*, 2025; Karanwal *et al.*, 2024). Within this mechanistic framework, the motility reduction observed under FU–OUA treatment may reflect an amplification of oxidative stress, given that simultaneous interference with redox signaling and ionic transport has the potential to exacerbate ROS-driven functional impairment.

Glycan–protein interaction and functional blockade of fertilization

Fucoidan is a sulfated polysaccharide found in invertebrates and brown seaweeds, composed mainly of sulfated L-fucose (Sanjeewa *et al.*, 2021). It exhibits beneficial biological activities in humans, such as antitumor (Park *et al*., 2013), anti-inflammatory (Sanjeewa *et al.*, 2021), anti-obesity (Kim; Jeon; Lee, 2014), hypolipidemic (He *et al*., 2023), anticoagulant/antithrombotic (Ye *et al.*, 2016), antioxidant (Yuan; Macquarrie, 2015), among others (Sanjeewa *et al.*, 2021). These activities are attributed to its unique structure and its ability to modulate key intracellular pathways, regulate the accumulation of ROS, and maintain cell survival and death pathways (Pradhan *et al.*, 2020). Studies have demonstrated that initial sperm–oocyte binding is structurally dependent on

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sperm surface proteins that recognize specific ZP glycans, which comprise a highly N-glycosylated and fucosylated glycoprotein complex (Wang *et al*., 2024; Wu *et al.*, 2025).

Furthermore, literature reports highlight that Fucoidan can interact with P-selectin and modulate critical intracellular signaling pathways, such as PI3K/Akt and Wnt/β-catenin (Xue *et al*., 2013). This mechanistic framework suggests a correlation between the glycobiological blockade and the reduction in progressive motility observed in the combined treatment at T15 and reflects an integrated collapse of sperm architecture. Fucoidan's interaction with P-selectin may destabilize membrane microdomains.

4. CONSIDERATIONS

The synergistic inhibition of sperm function by the FU–OUA combination suggests a convergence of two integrated inhibitory levels: ionic (membrane depolarization and Ca2+ overload via selective NKAα4 inhibition) and redox (hypothetical ROS-mediated damage to membrane integrity). This cooperative interaction likely disrupts the NKAα4 signaling hub within membrane microdomains, providing a coherent mechanistic hypothesis for the rapid and sustained loss of both progressive motility and vitality.

The treatments, both isolated and combined, demonstrated efficacy equivalent to the positive control (SLS) and superior to the negative control. They show strong potential as a basis for the development of new spermicidal or spermiostatic agents. Statistical analyses revealed significant differences between treatments at T0 and T15, with the Fucoidan–Ouabain combination showing the greatest decrease in motility and demonstrating a prolonged effect over time. Additionally, the interaction between the investigated compounds and ROS suggests a possible mechanism of action that may compromise cellular integrity and sperm function. These findings reinforce the potential of the treatments studied for future biotechnological applications, although cost considerations remain essential.

Contraceptive hormonal methods for men alter the hypothalamic–pituitary–gonadal axis, generating systemic effects and requiring continuous suppression of spermatogenesis. In contrast, sperm-specific targets allow inhibition of motility or fertilization without hormonal interference, with greater reversibility and lower toxicity (Mariani *et al.*, 2023; Khamamkar *et al.*, 2025). Animal models reinforce this approach by showing reversible infertility without behavioral impairment, although challenges remain, such as ligand selectivity, long-term safety, and clinical standardization (Mariani *et al*., 2023).

Any non-hormonal agent has yet achieved clinical approval, and greater collaboration between academia and industry is needed to overcome the challenges of translating preclinical findings into safe and effective therapies for human use (Johnston; Goldberg, 2020).

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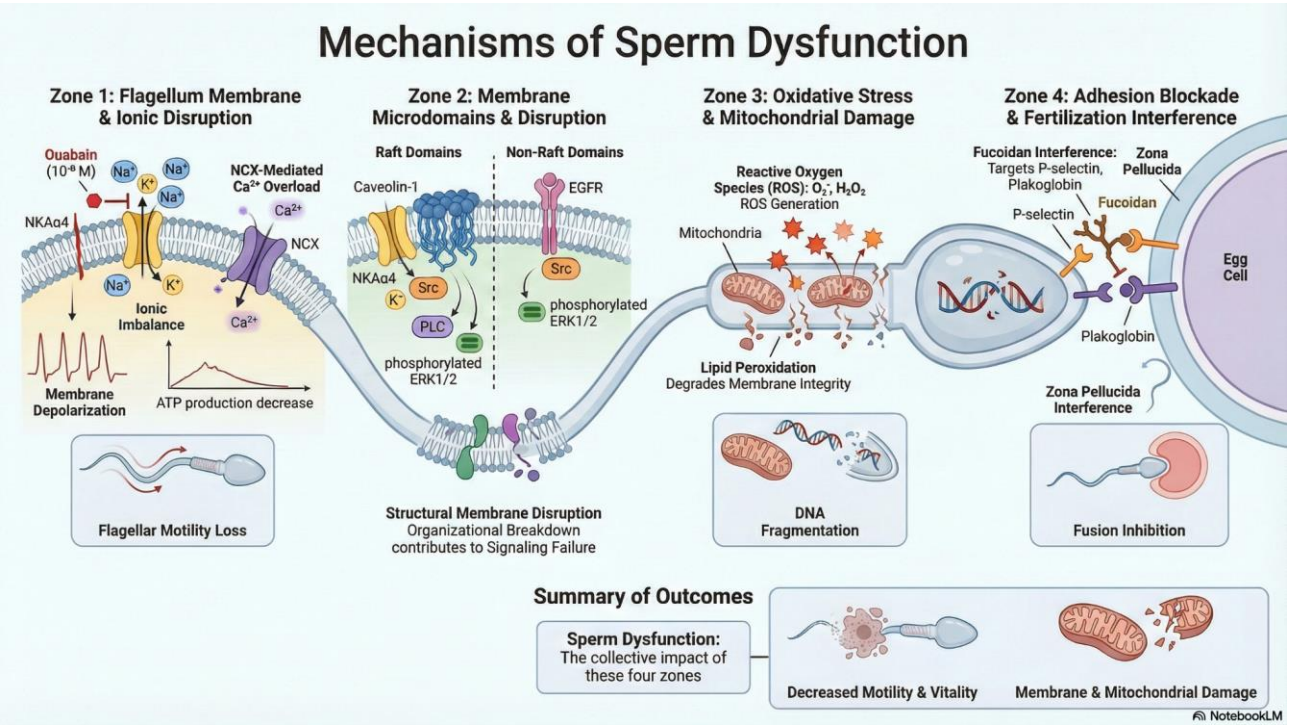
The findings support the potential of FU–OUA as components of novel non-hormonal contraceptive formulations, as their rapid inhibitory effects and distinct mechanisms of action make them attractive alternatives to conventional spermicides. Building on these results, future research should investigate the safety of topical formulations in vaginal and cervical epithelial models, the potential synergistic interaction, optimal concentrations for maximal efficacy with minimal cytotoxicity, formulation strategies suitable for pericoital use, and in vivo validation in translational models. These steps are essential for advancing these compounds toward clinical application.

Figure 6 summarizes the integrated ionic, oxidative, and adhesive disruptions possibly triggered by the treatments, illustrating how these combined mechanisms ultimately impair sperm motility (Wang *et al*., 2024; Wu *et al*., 2025; Novoyatleva *et al*., 2019). Integrated, these processes may explain the reduction in sperm motility (Syeda *et al*., 2020; Mo *et al.*, 2025).

Figure 6. Hypothetical mechanisms of sperm dysfunction: ionic imbalance and adhesion blockade



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