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ORIGINAL PAPER

Effect of isotretinoin on sperm quality in humans: An *in vitro* model

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Summary ***Objective: Isotretinoin is a retinoid widely used for severe acne, known for its teratogenicity, but with an unclear impact on male fertility and the risk of fetal exposure through semen. This study evaluated in vitro the effect of different concentrations of isotretinoin (therapeutic and extremely high) on the motility, vitality, and integrity of human sperm DNA.***

Methods: For this purpose, human semen samples (n = 17) were exposed to isotretinoin concentrations of 22 nM, 660 nM, 66,000 nM, and 660,000 nM. Sperm motility and vitality were assessed up to 5 hours post-exposure, while DNA fragmentation was evaluated at 2 hours.

Results: Concentrations of 22 nM, 660 nM, and 66,000 nM did not significantly affect the sperm assessed parameters. However, the highest concentration (660,000 nM) induced immediate cytotoxicity in human sperm, resulting in 100% immotile and nonviable sperm cells, as well as increased sperm DNA fragmentation.

Conclusions: Knowing that the concentrations achieved clinically in semen and blood are considerably lower than the cytotoxic concentration, these findings suggest that treatment with therapeutic doses of isotretinoin does not compromise human sperm function in vitro.

KEY WORDS: Isotretinoin; Sperm; Motility; Vitality; Fertility.

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INTRODUCTION

Isotretinoin (13-cis-retinoic acid) is a vitamin A derivative known commercially as *Roaccutane*, *Isoface*, or *Acneral* (1), which has been used as a treatment for severe acne because it inhibits hyper keratinization and the formation of small, clogged bumps on the skin and induces apoptosis in sebocytes, which in turn decreases sebum production and the size of sebaceous ducts (2, 3). Despite being so effective in treating these types of conditions, adverse effects associated primarily with teratogenicity, psychiatric symptoms, and metabolic abnormalities have been attributed (4). Specifically, during pregnancy, there is a high risk of spontaneous abortions occurring, and in cases where gestation continues, it is estimated that there is a 20% to 35% risk of congenital disabilities in fetuses exposed to the drug, including craniofacial, cardiovascular, neurological, and thymic malformations (5). In addition, 30-60% of children prenatally exposed to isotretinoin show neurocognitive impairment, even in the absence of physical defects (5, 6). In the reproductive field, research is mainly focused on teratogenic effects during gestation (6), leaving aside the possible consequences of this drug on male fertility. However, during sexual intercourse, semen can reach the female reproductive tract and potentially carry isotretinoin concentrations that trigger adverse sequelae in the fetus due to exposure to this drug (7). The presence of isotretinoin in human semen has been reported, with detected concentrations ranging from 9.6 nM to 14 nM (8, 9), with a maximum of 22 nM (8), approximately 30 times less than the concentration of isotretinoin reported in blood (660 nM) (8). Although the concentrations detected in semen are relatively low, their potential for teratogenicity remains a concern.

It is important to note that there are contradictory results on the consequences of systemic therapy with isotretinoin on sperm quality, since it has been shown that the administration of therapeutic doses (0.5-1. 0 mg/kg/day) in humans increases sperm concentration, motility and vitality (8, 10-12), while studies in other animal models, mainly rats, show opposite results, reporting decreased sperm concentration, motility, vitality and morphology, accompanied by testicular degeneration, increased apoptosis and reduced populations of Sertoli and Leydig cells which play an essential role in the spermatogenesis (13-15).

Therefore, the present study aimed to determine the *in vitro* effect of isotretinoin on motility, vitality, and DNA integrity in human spermatozoa.

MATERIALS AND METHODS

Semen simples

The present experimental *in vitro* study included 17 semen samples obtained from healthy individuals with normal seminal parameters (normozoospermic). The samples were collected by masturbation in a sterile container, after a period of sexual abstinence of 2 to 7 days, following the guidelines established in the manual for seminal analysis published by the *World Health Organization* in 2021 (16).

The Bioethics Committee approved the protocol and informed consent form for *Research in Humans at the*

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Of the 17 samples, five were used to standardize the procedure and to determine the optimal concentrations of the isotretinoin treatments. Six samples were used to evaluate the effect of the treatments on sperm motility and vitality, while the remaining six samples were used to assess sperm DNA integrity.

Isotretinoin concentrations
A stock solution of isotretinoin was prepared at a concentration of 4.4 x 107 nM by dissolving 0.013226 g isotretinoin (*Jiangxi Huazhou Technology Co., Ltd., China. Molecular weight = 300.44 g/mol*) in 1000 µL of 100% dimethyl sulphoxide (*DMSO, Carlo Erba, Rome, Italy*). From this stock solution, serial dilutions were performed in phosphate buffer solution to obtain the working solutions: 4.4 x 105 nM, 4.4 x 103 nM, and 4.4 x 102 nM.
For the experimental treatments, different final dilutions of isotretinoin were prepared by mixing the working solutions with the semen samples. The experimental groups were exposed to final concentrations of 22 nM, 660 nM, 66,000 nM, and 660,000 nM isotretinoin in a final volume of 120 µL of semen, while 120 µL of unexposed semen was considered the control.

Methods for sperm motility and vitality assessment
Initially, the initial sperm motility and vitality (time 0) were quantified in each semen sample, which were then exposed to isotretinoin treatments and incubated for 5 hours at 37°C. During this period, semen parameters (motility and vitality) were evaluated every hour post-exposure in duplicate.
Briefly, sperm motility was determined by direct observation of each sample at 40x, counting at least 200 spermatozoa per plate. Sperm motility was classified into rapid progressive motile (A), slow progressive motile (B), non-progressive spermatozoa (C), and immotile (D). At the same time, sperm vitality was evaluated by mixing the semen sample with 0.5% eosin-Y (*Sigma Chemical Co, USA*), quantifying at least 200 spermatozoa, and discriminating between those that excluded (live) or did not exclude the dye (live).

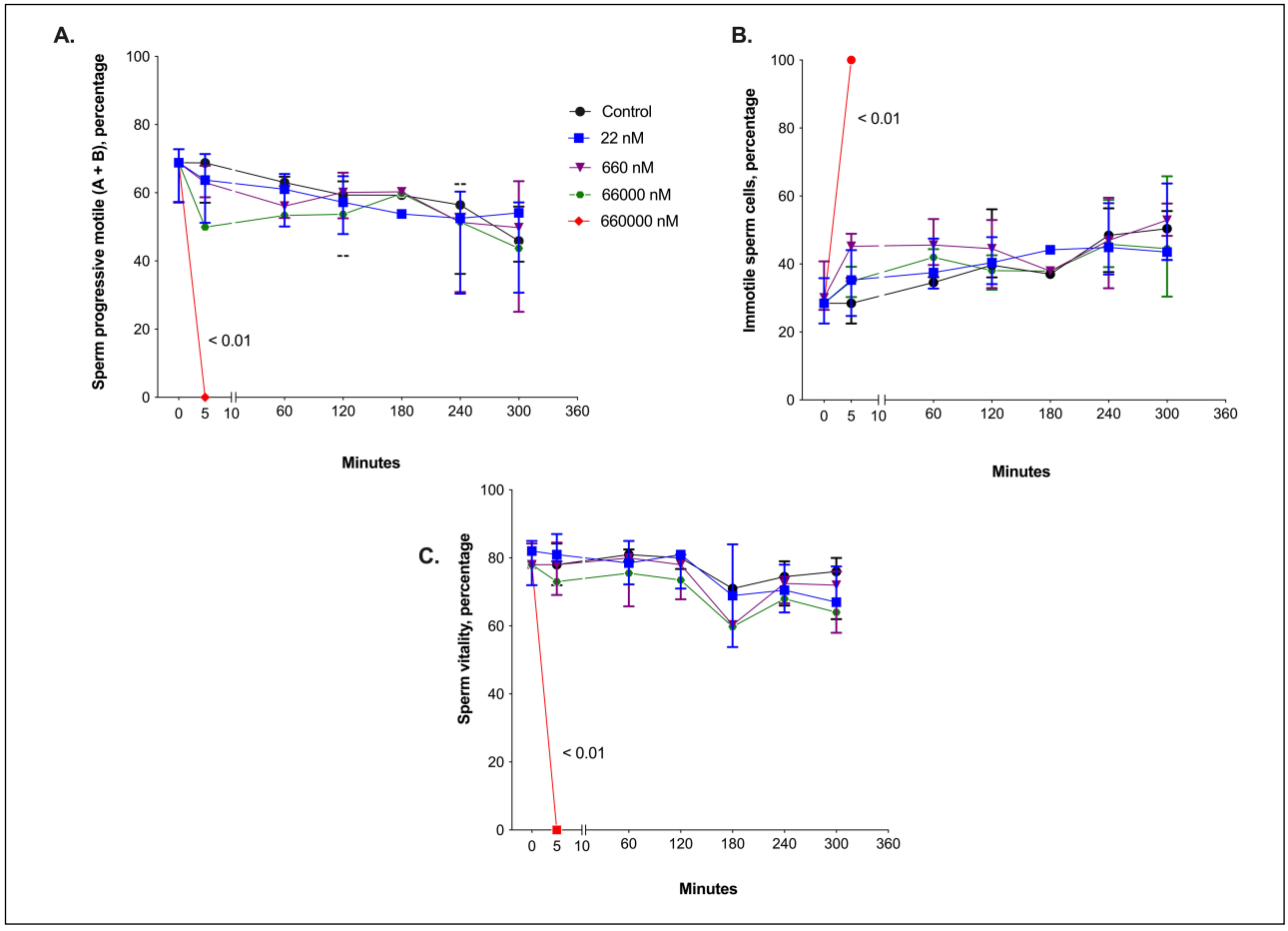
Sperm DNA integrity assessment
The determination of *sperm DNA fragmentation* (SDF) was evaluated by the *sperm chromatin dispersion test* (SCD), following the protocol previously described (17). SDF determination was performed two hours after incubation with each treatment. A minimum of 500 spermatozoa per sample were counted, and SDF was classified according to their halo formation: large and medium halo formation (normal sperm DNA), whereas damaged sperm DNA showed small, no halo, or degraded halos.

Statistical analysis
Results are expressed as median and interquartile range. The difference between seminal parameters before and after each time of incubation with isotretinoin was evaluated by a nonparametric ANOVA (*Kruskal-Wallis*) with a Dunn's post-test or a nonparametric t-test (*Mann-Whitney*) using the statistical program Prism 9.0 (*GraphPad Software, San Diego, CA, USA*). A value of $p < 0.05$ was considered statistically significant.

Results
Isotretinoin concentrations (22 nM, 660 nM, and 66,000 nM) did not significantly affect conventional sperm parameters (motility and vitality) relative to samples without isotretinoin (Figure 1), and SDF was not compromised after exposure to these isotretinoin concentrations (Figure 2). However, the highest concentration of isotretinoin (660,000 nM) had an immediate adverse effect, in which there was a drastic decrease in vitality (< 0.01), progressive motility (< 0.01), and an increase in immotile sperm (< 0.01) at 5 minutes post-exposure (Figure 1). Additionally, this concentration significantly increased (< 0.001) the percentage of fragmented spermatozoa two hours post-exposure (Figures 2, 3).

Discussion
The findings of the present *in vitro* study, in which the effect of isotretinoin on human seminal parameters was evaluated, show that isotretinoin concentrations reported in blood and semen during acne treatment (22 nM and 660 nM) (8) do not significantly affect the conventional and functional sperm parameters (motility, vitality, and DNA integrity) evaluated. Even when increasing the concentration of isotretinoin to 66,000 nM (one hundred times the concentration reported in blood), no adverse effects on sperm function were observed throughout the exposure time.
A key finding of this study was that, although exposure to a concentration a thousand times higher than that reported in blood (660,000 nM) produced an immediate cytotoxic effect, resulting in the death of 100% of spermatozoa (Figure 1), not all showed DNA damage after two hours of exposure (Figure 2). However, a significant increase in SDF was observed compared to the control group, suggesting a differential effect on sperm vitality and genomic integrity. This can be attributed to the fact that isotretinoin is a fat-soluble compound (1), that in extremely high concentrations it could integrate directly into the plasma membrane of the sperm, altering its fluidity and permeability. In addition, spermatozoa are sensitive to oxidative stress due to their high content of polyunsaturated fatty acids in the plasma membrane. Isotretinoin has an impact on the production of *reactive oxygen species* (ROS), thereby inducing oxidative stress by decreasing plasma vitamin E levels and *superoxide dismutase* (SOD) levels (18); excess ROS can cause lipid peroxidation, which compromises the integrity of the sperm membrane, leading to cell death even before damage to the sperm's DNA is evident, as it is highly compacted and protected by protamines (19). Therefore, even in the event of massive damage to the membrane, the nucleus with its compacted DNA may be more resistant and not fragment as easily.
It is important to note that, under normal therapeutic conditions, isotretinoin does not accumulate in semen in significant amounts. Previous articles have reported an average concentration of 9.6-14 nM in semen after treatments with cumulative doses of up to 120 mg/kg in six months (8, 9). Additionally, one study evaluated blood accumula-

Figure 1.
Effect of isotretinoin at different concentrations (22 nM, 660 nM, 66,000 nM, and 600,000 nM) on progressive sperm motility (A), immotile sperm (B), and sperm vitality (C).



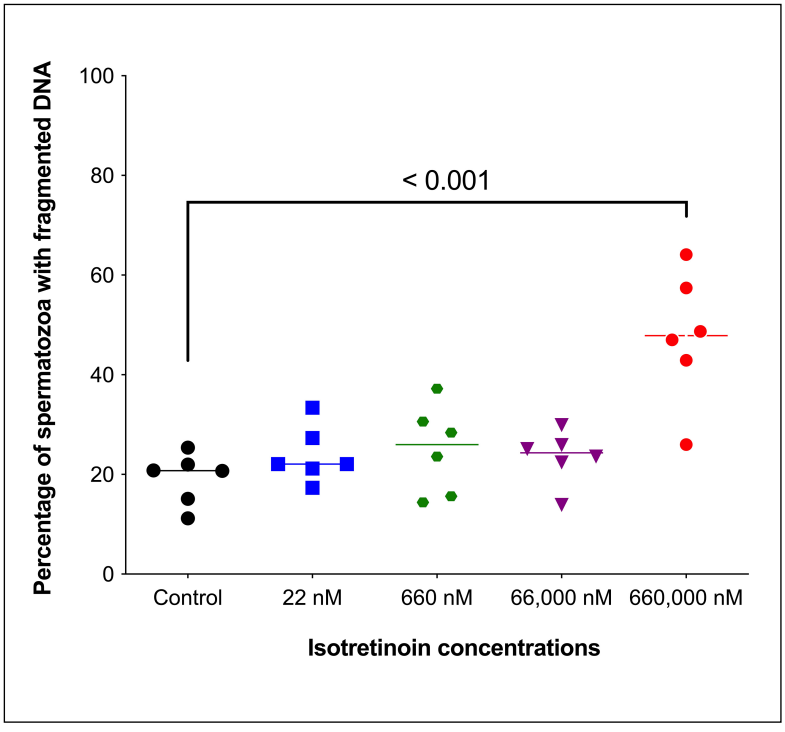
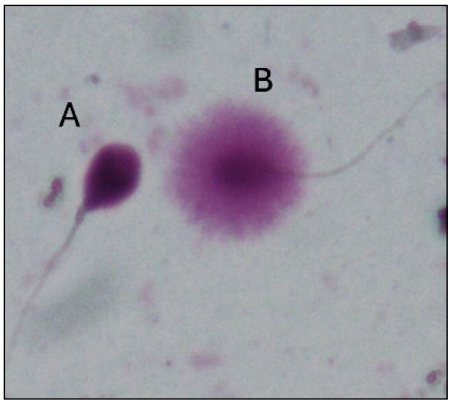


Figure 2.
Effect of isotretinoin at different concentrations (22nM, 660nM, 66,000nM, and 600,000nM) on sperm DNA integrity.

Figure 3.
Fragmented (A) and normal sperm cell (B).



tion after three months of treatment, demonstrating a 30% increase in isotretinoin concentration from 320 nM (in the first month) to approximately 1250 nM (20). The literature data indicate that therapeutic doses of isotretinoin do not generate seminal or blood concentrations close to the maximum concentrations evaluated in the present *in vitro* study. This implies that the adverse effects observed *in vitro*, especially at the higher concentrations, are unlikely to be replicated in vivo at current clinical doses.

However, although isotretinoin usually induces prolonged remission of acne and may even be permanent in some patients (21), the need to resort to one or more additional cycles of isotretinoin has sometimes been described because the recommended cumulative doses (120-150 mg/kg) were not reached in the initial treatment (22). In addition, pharmacokinetic studies have reported that the mean elimination half-life of isotretinoin and its primary metabolite (4-oxo-isotretinoin) ranges from 10 to 20 hours and 24 to 29 hours, respectively (23, 24); however, one study observed an increase in the elimination half-life of two patients (from 5.3 hours to 7 days), which implies greater variability in the elimination of this drug. This is attributed to hepatic recirculation of the drug, thus allowing its elimination to be delayed (25). Considering that approximately five half-lives are needed to allow for the total elimination of a drug, and that a patient has delayed elimination of isotretinoin, it would take more than a month for the levels of this drug to return to baseline values.

On the other hand, the need to resort to multiple cycles of isotretinoin in patients with severe acne relapses, coupled with the possibility of the drug being consumed without medical supervision, poses a potential risk. In these cases, if the recommended waiting period between cycles is not observed, and if the patient is one of those with prolonged drug elimination due to hepatic recirculation, a significant accumulation of isotretinoin in the body could occur. Although studies have shown that usual therapeutic doses do not result in elevated seminal concentrations (8, 9), the scenario of excessive use could create an in vivo environment with higher concentrations than those reported. Therefore, under these conditions, seminal quality could be compromised.

Despite these findings, it is essential to further investigate several aspects through other studies, especially considering the interspecies differences that have been observed. Studies in different animal models have reported adverse effects of isotretinoin on sperm parameters (13, 14, 26-28), which is contrary to what was observed in human spermatozoa (8, 10-12). This difference highlights the importance of direct research in human cells, as findings in other animal models may not be extrapolated to the human species, probably due to differences in drug metabolism.

Furthermore, although the concentration of 660,000 nM induced sperm cytotoxicity, the fact that not all dead sperm showed DNA fragmentation suggests a dissociation between membrane damage and immediate genomic damage. Therefore, it is essential to investigate the specific molecular mechanisms responsible for the cytotoxicity of isotretinoin at high concentrations in human spermatozoa. It would be pertinent to investigate other sperm functional parameters further to ensure that isotretinoin is not affecting human spermatozoa in any way at the currently used therapeutic doses.

Finally, the findings of this study suggest that, although therapeutic concentrations of isotretinoin do not appear to affect semen quality, excessive and unsupervised use of the drug could result in dangerous systemic accumulation. Although the highest concentration of isotretinoin (660,000 nM) induces cytotoxicity, the dissociation between cell death (vitality) and immediate genomic damage (SDF) gives rise to new approaches for future studies, which should focus on clarifying the molecular mechanisms of this cytotoxicity and evaluating other functional sperm parameters, thus ensuring that the use of isotretinoin at therapeutic doses does not compromise long-term male reproductive health.

Admittedly, the current study has several limitations. While the design is adequate, the number of samples and the limited monitoring period of only 5 hours are limitations. Furthermore, similar studies are needed to evaluate other parameters and functional mechanisms related to sperm DNA damage, including mitochondrial membrane potential and the production of reactive oxygen species.

Conclusions

In conclusion, our *in vitro* study demonstrates that isotretinoin concentrations present in blood and semen during acne treatments do not adversely affect the motility, vitality, or DNA integrity of human spermatozoa. While extremely high concentrations showed a cytotoxic effect, the effects on genomic integrity were differential, and these concentrations are unlikely to be reached in vivo. Therefore, these findings suggest that current clinical doses of isotretinoin do not pose a risk to sperm function. This study contributes to a clearer understanding of the effects of isotretinoin in men. Furthermore, it highlights the need for future research on the molecular mechanisms of action to provide a more comprehensive picture.

Declarations

Ethical approval and consent for participate: *We obtained ethical approval for this study, Research in Humans, at the Institute of Medical Research, School of Medicine, University of Antioquia. Written informed consent was obtained from all participants.*

Availability of data and material: *Data sets used in this study are available upon reasonable request from the corresponding authors.*

Competing interests: *The authors declare that they have no conflicts of interest.*

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Authors' contributions: *All authors made a significant contribution to the work, during conception, study design, execution, acquisition of data, analysis, interpretation, writing-review and editing. All authors read and approved the final manuscript.*

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