Experimental Research

ANNALS OF MEDICINE & SURGERY

OPEN

Investigation of the effects of thymoquinone (TQ) and polyvinylpyrrolidone (PVP) on the motility, viability, and chromatin status of sperm in normozoospermic semen samples

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Objective: Adverse effects of polyvinylpyrrolidone (PVP) on sperm membrane and chromatin have been proven in many studies. Among the natural products proposed as an alternative for PVP, thymoquinone (TQ)-a major constituent of *Nigella sativa* plant- has been suggested as a potential natural spermostasis. Therefore, this study aimed to compare the effects of TQ with PVP for sperms motility, survival, DNA denaturation, and DNA fragmentation in normozoospermic men (men with a normal or healthy sperm profile).
Methods: An experimental trial was carried out on 30 normozoospermic men of the Andrology Unit of (Shahid Beheshti Hospital, Isfahan, Iran). Each washed semen samples were divided into four fractions and was randomly treated with TQ (50 µg/ml), %5 PVP, and %10 PVP (M/V) which was compared to untreated fraction (control).
Results: There was a significant difference between the four groups in terms of motility, viability, DNA denaturation, and fragmentation (P <0.05). TQ caused sperm immobility, while 5% PVP and 10% PVP decreased (98 and 99%, respectively) sperm motility compared to control. TQ did not affect sperm viability compared to the control group, but PVP decreased it. Besides, TQ did not affect DNA denaturation and fragmentation, but PVP increased it.
Conclusion: TQ could be used as an alternative natural spermostasis with less adverse effects rather than PVP which causes more efficient immobilization and isolation of individual sperm cells.

Keywords: chromatin, motility, Polyvinylpyrrolidone, sperm, Thymoquinone, viability

Introduction

Infertility is a pervasive problem that affects about 10% of couples according to the WHO[1]. In normozoospermic men (men with a normal or healthy sperm profile)., the total number, of progressively motile and morphologically normal spermatozoa is equal to or greater than the minimum. But, in some cases like female factor infertility, assisted reproductive techniques such as in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are necessary. polyvinylpyrrolidone (PVP) is usually used in routine ICSI cycles to immobilize and isolate individual sperm cells before ICSI. Also, in IVF laboratories, PVP 5% and 10% are used to adjust sperm viability[2]. There is some evidence of destructive effects of PVP on sperm membrane and chromatin[3].

The aqueous extract of Nigella sativa (N. sativa) plant (Ranunculaceae family) has a valuable active ingredient named thymoquinone (TQ) and its derivate[4]. Also, *N. sativa* oil prevents DNA damage. In-vivo studies on reproductive systems of male rats have revealed that *N. sativa* extract increases the sexual organ weight, sperm count, and fertility indices[5].

PVP, a synthetic polymer of N-vinylpyrrolidone monomers[3], is commonly used in industry and medicine for subcutaneous and intravenous injections[6]. In recent years, PVP has been used for human oocyte culture during embryonic development[3]. PVP, known as Percoll, is a selective compound for sperm and a

HIGHLIGHTS

- Polyvinylpyrrolidone (PVP) on sperm membrane and chromatin has been proven in many studies.
- Thymoquinone (TQ) major constituent of *Nigella sativa* plant- has been suggested as a potential natural spermostasis.
- TQ could be used as an alternative natural spermostasis with fewer adverse effects rather than PVP.
- it was recommended that PVP be used carefully in the sperm culture medium and TQ could be used as alternative spermostasis.

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Key finding: Thymoquinone (TQ) effectively immobilized sperm without compromising viability or inducing DNA damage, making it a potentially safer alternative to Polyvinylpyrrolidone (PVP) for assisted reproductive techniques.

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Annals of Medicine & Surgery (2024) 86:826–830

Received 8 September 2023; Accepted 21 November 2023

Published online 3 January 2024

http://dx.doi.org/10.1097/MS9.0000000000001572

freeze-protection compound for embryo and sperm storage[7]. In microinjection, PVP has been successfully used to increase the sperm solution viscosity to facilitate working on sperm. During the injection into mouse embryos, PVP decreased the integrity of bull sperm membranes and induced cellular toxicity[7]. Also, in-vitro studies represented that PVP has a deleterious effect on the ultrastructure of human sperm, plasma membrane, and the chromosomal membrane in ICSI embryos[8]. Sadeghnejad and colleagues stated that *N. sativa* extract significantly decreased total motility and sperm count with fast and slow progressive and non-progressive motilities while, the percentage of viable, apoptotic, and necrotic sperms did not significantly change in normozoospermic men[9].

Adding 50 μg/ml of TQ does not decrease the percentage of viable sperms[10]. In human sperm cultures, unlike the oral administration of *N. sativa* extract and TQ, direct application of quinones in the medium inactivates sperms, induces immotility, immobilizes, decreases the fertilization rate and development of embryos after IVF and ICSI[10]. Instant sperm immobilization is important to increase ICSI success. Sperm immobilization facilitates the release of egg-activating factors associated with sperm when the sperm goes inside the oocyte. The use of immobilized sperms via PVP is clinically controversial due to its membrane destruction before injection[3]. Therefore, efforts to replace PVP with natural plant spermostatic compounds such as TQ are essential. So, this study aimed to investigate in-vitro effects of TQ as a natural compound on sperm motility, viability, and chromatin status in normozoospermic men as compared with synthetic PVP.

Materials and methods

Chemicals

All chemicals were supplied by Sigma-Aldrich unless stated otherwise.

Ethics

The ethical committee of (Payame Noor University) approved all procedures performed in this study involving human participants (ethical code No. 44769). Also, informed consent was obtained from all individual participants included in this study.

Study design

This study was an experimental trial on the population referring to Andrology Unit of (Shahid Beheshti Hospital, Isfahan, Iran). The population was randomly sampled from 19 to 47 years old normozoospermic men. The inclusion criteria were: healthy, young fertile, normozoospermic men with three up to 7-day periods of sexual abstinence. The patients had an average age of 33 years. Also, the exclusion criteria were poor motility of sperm, azoospermia, oligozoospermia, low semen volume, any disorder in sperm morphology, and a short abstinence period.

Semen collection

Semen samples were collected by masturbation in sterile containers after three to 7 days of sexual abstinence from 30 normozoospermic men (Andrology Unit of Shahid Beheshti Hospital, Isfahan, Iran) and classified as normozospermic according to WHO criteria[11]. The samples were incubated to liquefy at 37°C for 20–30 min. After routine semen analysis, the samples were washed twice in Modified Ham's F10 medium with %5 albumin (Irvine Scientific)[10].

Each sample was divided into four fractions (0.5 ml) which were randomly treated with TQ (50 μg/ml) as well as %5 PVP and %10 PVP (M/V) and compared to untreated fraction (control). The four groups were incubated for 1hr and then compared for motility and viability rate as well as DNA denaturation and fragmentation of the spermatozoa.

Sperm motility

Sperm motility is determined based on total percentage of motile sperm using an optical microscope equipped with a computer-assisted sperm analysis system (CASA, VT-Sperm Test, 2.3 model-Company of Video Test-Finland). In each treated sample, 200 sperms were evaluated.

Sperm viability

The percentage of viable spermatozoa was evaluated by Eosin Y staining[11]. In this method, viable and non-viable cells are white and red, respectively. Because viable sperms do not allow vital stain to enter the cells. In brief, 0.05% Eosin Y solution was prepared in 0.9% NaCl (M/V). Five microlitres of eosin solution was added to five μl of the sample on a slide. Then, the slides were examined under an optical microscope (Olympus Microscope), with a magnification of 400×. At least 200 sperm per slide were examined and the percentage of non-stained (viable) was calculated. Viable sperm had light white or pink heads and nonviable sperm had red or dark pink heads.

DNA denaturation

DNA denaturation was evaluated using acridine orange (AO) staining[12]. Ten mL of AO solution (1% M/V in distilled water) was added to a mixture containing 40 ml of citric acid (0.1 M) and 2.5 ml of Na_2HPO_4, $7H_2O$ (0.3 M), pH—2.5. The 1% AO stock solution was stored at 4°C in dark for 4 weeks. A thick smear of the washed sample was prepared and air-dried for 20 minutes. Then, the slides were fixed in Carnoy's solution (3:1 of methanol/acetic acid solution) and further stained with AO solution. The stained smears were examined for up to 24 h under a fluorescent microscope (Olympus Microscope) equipped with 490 nm excitation and 530 nm barrier filters. Normal and denatured DNA sperms represented green colour and orange colours, respectively[13].

DNA fragmentation

DNA fragmentation was evaluated using the Sperm DNA Fragmentation Assay (SDFA) Kit. Generally, the chromatin and DNA were denaturalized in a microgel substrate under an acidic treatment. Next, DNA strands were spread as far as possible around the sperm head by the removal of chromatin proteins. It is visible as a halo around the sperm head during staining. However, DNA fragmentation results in a lack of spread of the DNA strands and a lack of aura, or a very small halo around the sperm head. At least 200 sperms were counted per slide and reported as a percentage of sperm with halo (normal) or without halo (fragmented) with 1000× magnification under a light microscope (Olympus)[14].

Statistics

All statistical calculations were carried out by Statistical Package for Social Studies software (SPSS version 20, SPSS Inc.). Data analysis was performed using analysis of variance (ANOVA) and mean values were compared via Duncan's multiple range tests at 95% and 99% CI ($P < 0.05$ and $P < 0.01$).

The methods have been stated in accordance with STROCSS 2021 guidelines[15].

Results

Sperm motility

The percentage of sperm motility was significantly different in different groups ($P < 0.01$) (Fig. 1). Furthermore, the sperm motility in TQ, PVP 5%, and PVP 10% groups were significantly lower than control ($P < 0.01$). Also, sperm motility in PVP 10% was less than PVP 5% ($P = 0.05$) and in TQ group, less than PVP 10% ($P = 0.01$). TQ caused spermostasis (motility of zero) compared to control ($P < 0.01$). Finally, PVP 5% and 10% PVP significantly reduced sperm motility (98 and 99%, respectively) compared to control ($P < 0.01$).

Sperm viability

All groups were different from control in terms of mean percentage of sperm viability ($P < 0.01$) except for TQ ($P = 0.39$) (Fig. 2). Sperm viability in the PVP groups was less than TQ and control groups. Also, sperm viability in 5% PVP group was higher than 10% PVP ($P < 0.01$). 5% PVP and 10% PVP reduced sperm viability compared to control group (20% and 33%, respectively).

DNA denaturation

The mean of DNA denaturation was significantly affected by different treatments ($P < 0.01$) except for TQ group ($P = 0.054$) compared to control (Fig. 3). PVP groups showed higher DNA denaturation than control as well as TQ group ($P < 0.01$). Adversely, PVP 10% and PVP 5% increased DNA denaturation (68% and 43%, respectively) compared to control group.

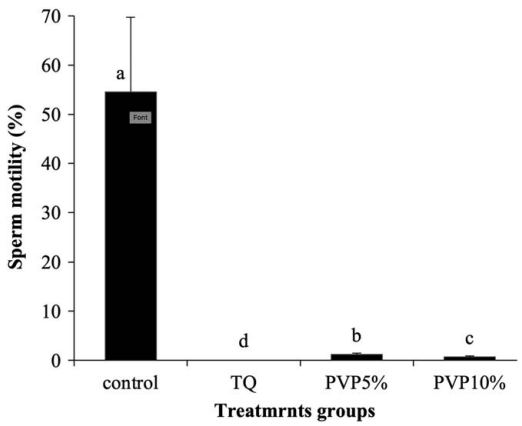

Figure 1. The effect of thymoquinone (TQ), polyvinylpyrrolidone (PVP)5% and PVP10% on sperm motility (%). Data represent mean ± SD ($n = 30$). Different letters indicate difference between the means (Duncan's multiple range tests, $P \leq 0.01, 0.05$).

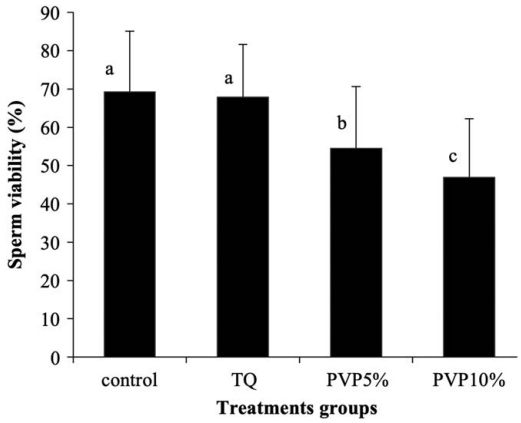

Figure 2. The effect of thymoquinone (TQ), polyvinylpyrrolidone (PVP)5% and PVP10% on sperm viability (%). Data represent mean ± SD ($n = 30$). Different letters indicate difference between the means (Duncan's multiple range tests, $P \leq 0.01, 0.05$).

DNA fragmentation

Although there was a significant difference between the mean percentage of DNA fragmentation in different groups ($P < 0.01$), TQ groups were like control ($P = 0.052$) (Fig. 4). Also, DNA breakage in the TQ group was significantly less than PVP groups ($P < 0.01$). In addition, different concentrations of PVP significantly increased DNA fragmentation compared to control (52% and 80% for PVP 5% and PVP 10%, respectively) ($P < 0.01$).

Discussion

Consistent with previous findings, TQ (50 μg/ml) caused sperm immobilization[9,10,14]. Fazelian and colleagues found that 5 and 10 μg/ml of TQ increased sperm motility[12]. Other studies also indicated quinones can induce spermostasis in human rat sperms[14]. In this study, when PVP concentration is increased, sperm motility is further decreased. Although sperm motility is

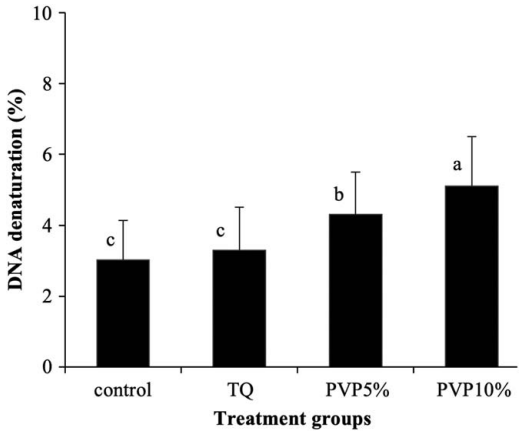

Figure 3. The effect of thymoquinone (TQ), polyvinylpyrrolidone (PVP)5% and PVP10% on DNA denaturation (%). Data represent mean ± SD ($n = 30$). Different letters indicate difference between the means (Duncan's multiple range tests, $P \leq 0.01, 0.05$).

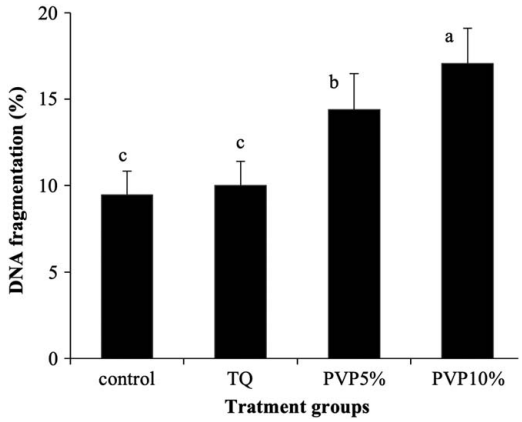

Figure 4. The effect of thymoquinone (TQ), polyvinylpyrrolidone (PVP)5% and PVP10% on DNA fragmentation (%). Data represent mean ± SD ($n = 30$). Different letters indicate difference between the means (Duncan's multiple range tests, $P \leq 0.01, 0.05$).

essential for successful fertilization, partial spermostasis is necessary to keep sperm in the injection needle during ICSI[16,17]. According to our findings, TQ can stop spermatozoa in culture medium. In this study, TQ did not affect sperm viability and could preserve spermostasis which agreed with other studies[9,10,12]. Based on Sadeghnejad and colleagues, sperm viability did not significantly change after using *N. sativa* extract containing TQ[9]. Also, TQ application has not changed sperm viability[12]. We found sperm viability decreased by PVP and it was more decreased by PVP concentration. This was supported by Kato and Nagao and Nel[3,18]. In this research, TQ inhibited sperm motility without decreasing sperm viability in agreement with others[10]. According to Sheikh Bahai *et al.*[19], TQ as a potential antioxidant counteracted the effects of methotrexate toxicity on sperm motility and viability.

Thereby, TQ is suggested as a spermostatic compound rather than spermicide because it did not change DNA denaturation of the sperms[11]. Any damage to sperm DNA causes oxidative stress and induces gene mutations, DNA denaturation, and fragmentation[20,21]. TQ via strong antioxidant properties prevents oxidative stress by scavenging reactive oxygen species (ROS) which protects sperm against DNA damage. Inversely, PVP caused more DNA denaturation using higher doses of PVP. TQ is also suggested as a chromatin stabilizing compound because it prevents DNA denaturation and maintains normal chromatin compaction. *N. sativa* extract preserves normal chromatin compaction which protects sperm DNA from denaturation[22]. TQ regulates meiotic G1 phase of gametogenesis via P53 protein[23]. However, the use of PVP should be revised because of increasing DNA denaturation. TQ did not affect DNA fragmentation, in this study. Although there is no comprehensive study on the effect of TQ on sperm DNA fragmentation, the results of this study were in agreement with other studies. Oxidative stress has been stated as a major cause of sperm DNA breakage[20]. Our findings on PVP effect on DNA fragmentation were not consistent with Nel who had worked with a single semen sample. So, further research was needed to confirm the findings[18]. Additionally, our findings were in line with other studies on the harmful effects of PVP on sperm features and DNA damage[3].

The potential mechanisms by which Thymoquinone might affect sperm motility, viability, and DNA integrity are shown in some studies. A study by Inanc *et al.*[24] provides insights into the potential mechanisms by which Thymoquinone (TQ) may affect sperm motility, viability, and DNA integrity. In the context of frozen-thawed ram semen, the study revealed that TQ supplementation at concentrations of 50 and 100 μg/ml significantly improved total and progressive motility, highlighting TQ's potential to enhance sperm movement. Furthermore, TQ was found to increase plasma membrane acrosome integrity (PMAI), a vital factor for sperm viability, particularly at 50 μg/ml. The study also observed a notable reduction in DNA damage in TQ-treated groups, suggesting TQ's ability to protect sperm DNA integrity, a crucial factor for fertility. These findings, while conducted on ram semen, provide valuable insights into the potential benefits of TQ for sperm quality and fertility, with implications for broader reproductive research and potential applications in human reproductive medicine.

One notable limitation is the relatively small sample size used in this research, which included ejaculates from a group of five Sonmez rams. While the study yielded valuable insights into the effects of TQ on sperm parameters, the small sample size could affect the generalizability of the results.

Clinical implications of the findings

The findings of this study have significant clinical implications, particularly for fertility treatments. In assisted reproductive techniques such as IVF and ICSI, the immobilization of sperm before injection is a critical step. The traditional choice for this purpose has been PVP. However, the study results highlight the potential drawbacks of using PVP, particularly at higher concentrations. The findings of this study advocate for a reevaluation of the use of PVP and the exploration of alternatives like TQ that may offer a more favourable balance between immobilization efficiency and the maintenance of sperm health.

Conclusion

TQ induced partial spermostasis in the culture medium and had a comparative advantage on viable, denatured, and fragmented sperms compared to PVP. TQ has shown the potential to effectively halt sperm motility. Regarding the role of DNA damage, especially DNA denaturation and sperm fragmentation in the successful sperm culture, fertilization, and healthy embryos from ICSI, it was recommended that PVP be used carefully in the sperm culture medium and TQ could be used as alternative spermostasis.

Ethical approval and consent to participate

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Ethical committee of Payame Noor University of Tehran approved all procedures performed in this study involving human participants (ethical code No. 44769). Also, informed consent was obtained from all individual participants included in this study.

Consent

Consent to participate: from the under 16 years old was given by a parent or legal guardian.
Consent for publication: not applicable.

Source of funding

No funding was secured for this study.

Author contribution

T.K. and S.A.F.: conceptualized and designed the study, drafted the initial manuscript, and reviewed and revised the manuscript. A.R.V.d.: designed the data collection instruments, collected data, carried out the initial analyses, and reviewed and revised the manuscript. S.A.E. and F.G.I.: coordinated and supervised data collection, and critically reviewed the manuscript for important intellectual content.

Conflicts of interest disclosure

The authors deny any conflicts of interest in any terms or by any means during the study.

Research registration unique identifying number (UIN)

Unique identifying number is: researchregistry7599.

Guarantor

Tayebeh Khosravi.

Availability of data and material

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Provenance and peer review

Not commissioned, externally peer-reviewed.

Acknowledgements

Financial support by the Isfahan University of Medical Sciences is gratefully acknowledged. The authors thank Dr. Imaneh Dehghani for revising the manuscript.

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