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Impact of Long-term Polyvinylpyrrolidone Exposure on Gene Expression, Chromatin Structure, and Malondialdehyde Levels in Human Sperm

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ABSTRACT

In an intracytoplasmic sperm injection (ICSI) procedure, sperm must be immobilized due to its manipulation; therefore, Polyvinylpyrrolidone (PVP) has been used for this purpose. Based on many studies, fertilization rates may be reduced by incubation of sperm in PVP as it changes sperm mitochondria, nucleus, and membrane structures. Since PVP is used widely in infertility centers, it is noteworthy to investigate the side effects of this agent. This study was designed to evaluate the effects of this component on chromatin quality with acridine orange (AO) staining, Malondialdehyde (MDA) levels, and *BAX*, *BCL2*, *HSP70* (*HSPA2*), and *SOD2* genes expression in different time intervals (0, 15, 30, and 60 minutes). In this research, 25 normal sperm specimens were applied after preparation with the swim-up method. Results showed that incubation of sperm in PVP for 15, 30, and 60 min significantly increased MDA levels and AO percentage rate compared to unexposed spermatozoa to PVP. Furthermore, the expression of *BAX*, *BCL2*, *HSP70*, and *SOD2* genes were altered after incubation in a PVP medium at different time intervals. Incubation of sperm in PVP significantly increased the adverse effects after 15 min. Therefore, sperm should not be exposed to PVP for more than 15 minutes during the ICSI procedure. Altogether, results showed that incubation of sperm in a PVP medium during an ICSI procedure for more than 15 min may result in significant changes in the biological features of sperm. Therefore, expediting the ICSI procedure could mitigate the potential adverse effects of PVP on sperm parameters.

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Introduction

Infertility is a global health burden affecting approximately 15% of reproductive-aged couples. Male factor is responsible for almost half of all infertility cases, which affect an estimated 7% of all men across the world (Karavolos *et al*., 2020). In humans, male infertility may be affected by various risk factors, including obesity, aging, varicocele, endocrine dysfunction, testicular cancer, environmental or occupational exposure to pollutants, alcohol, tobacco, and oxidative stress (Sciorio *et al*., 2024). These factors can lead to abnormal sperm production and, ultimately, unsuccessful fertilization. Since 1992, infertile men have been able to receive treatment through assisted reproductive techniques (ART). In ART, Intracytoplasmic sperm injection (ICSI) is applied to manage male factor infertility. In the

ICSI procedure, a single spermatozoon with normal morphology is injected into the oocyte cytoplasm (Esteves *et al*., 2017; Hosseini *et al.*, 2018). Before the injection, the spermatozoon is immobilized under light microscopy to observe the most suitable cell. For this purpose, Polyvinylpyrrolidone (PVP) is applied by embryologists to manipulate sperm more easily (Barmaki *et al.*, 2021; Kato and Nagao, 2012).

For many years, PVP was used for handling sperm in ICSI procedures, and the obtained fertility is 20 to 80%. PVP is used widely in infertility centers; therefore, it is noteworthy to investigate the side effects of this agent. Based on many studies, fertilization rates may be reduced by incubation of sperm in PVP as it changes sperm mitochondria, nucleus, and membrane structures (Ding *et al.*, 2020) Hence, selecting a suitable spermatozoon is one of the great concerns during ICSI treatment. Additionally, it is unavoidable that injected PVP remains with sperm in the oocyte for an extended period, potentially negatively affecting fertilization, embryonic development, and pregnancy outcome (Jean *et al.*, 2001). Moreover, it has been reported that exposing human spermatozoa to PVP for more than 15 minutes decreases viability and morphology while increasing DNA fragmentation and abnormal chromatin status. Moreover, PVP affected spermatozoa's mitochondrial membrane potential and increased the acrosome-reacted spermatozoa percentage (Sabour *et al.*, 2022). The side effects of PVP are a major concern for ART centers; however, its hazardous effects on sperm should be examined.

There are various mechanisms that are detrimental to spermatozoa. Small amounts of reactive oxygen species (ROS) are necessary for different procedures, including capacitation, acrosome reaction, and fertilization. Overproduction of ROS and decreased antioxidant levels cause oxidative stress (OS). The generation of ROS results in lipid peroxidation, and the byproduct of this phenomenon is malondialdehyde (MDA). The breakdown of phospholipids leads to the accumulation of MDA, which is preferred as a surrogate marker of oxidative stress. Spermatozoa are susceptible to the OS because of plasma membrane polyunsaturated fatty acids. Superoxide dismutase (SOD) is enzymatic antioxidants that detoxify superoxide anion radicals. *SOD* genes have the main function of balancing the amount of ROS production (Forsberg *et al.*, 2001). *SOD2* is one of the isoforms of *SOD* that has a specific genetic organization. *SOD2* has a vital role in regulating cell function in aerobic situations. It was demonstrated that there is a relationship between activities of seminal *SOD*, sperm fertilization capacity, and male infertility (Miao and Clair, 2009). Germ cell apoptosis is an important procedure in eliminating germ cells with abnormal functions to maintain the ratio of germ cells to Sertoli cells. In pathological conditions, extensive germ cell apoptosis causes male infertility. *BCL2* is a gene that inhibits apoptosis and preserves cells from ROS production. Activation of different genes, such as *BAX* and caspase 3 causes DNA fragmentation and decreases sperm motility, which finally contributes to male infertility (Maliheh *et al.*, 2022). Heat shock proteins (HSP) are a group of proteins that are activated during heat stress. HSPs preserve cellular functions by protecting cells from the adverse effects of heat and stress. The expression of *HSPs* related to stress resistance and its thresholds has a relationship with stress level (Rajoriya *et al.*, 2014). This study proposed to investigate the effects of PVP on chromatin quality (Acridine orange staining), MDA level and gene expression at different time intervals to access an ideal time that sperm can be safely incubated in PVP during ICSI with less possible damage.

Materials and Methods

Study participants

In this study, semen samples were obtained from patients at the Yazd Reproductive Sciences Institute. Samples were collected by masturbation into sterile specimen cups after 2-5 days of sexual abstinence. For liquefaction, the samples were incubated at 37 °C for 20 min. Sperm parameters were evaluated based on the World Health Organization guidelines (WHO, 2021). Subsequently, 25 normozoospermia samples were selected from 65 couples with female factor infertility who were less than 35 years old and without a history of varicocele and

smoking. This study was approved by the Ethics Committee and Deputy of Research and Development of Shahid Sadoughi University of Medical Sciences (IR.SSU.RSI.REC.1398.035).

Sperm preparation

Sperm samples were prepared using the direct swim-up method. The sperm culture medium was Ham's F10 supplemented with 5 mg/mL human serum albumin (HSA). A sterile conical tube was used to place 1 mL of semen under 1.2 mL culture media and then was incubated at 37 °C for one hour. After that, the upper level of the medium was removed, and a 2 mL culture medium was added to dilute the medium, followed by centrifugation at 400 g for 5 min. The pellet was resuspended in 0.5 mL Ham's F-10 and warmed to 37 °C. 0.5 mL of the culture medium was added to wash spermatozoa, and the tube was closed tightly and placed in a 37 °C incubator, which was then used to assess parameters. Samples were kept at 70°C for MDA assessment and gene expression.

PVP preparation

In this study, a 10% PVP solution, routinely used in infertility centers, was utilized. PVP powder (1 gr, Sigma-Germany) was dissolved in 10 mL Ham's F-10 with HEPES (Gibco-Denmark), then filtered and stored in the refrigerator (Nasresfahani *et al.*, 2008).

Study design

The swim-up technique was used for sample preparation. Each sample was divided into four groups and incubated with PVP at room temperature for 15, 30, and 60 minutes, respectively, at a ratio of (1:1) (Nasresfahani *et al.*, 2008). Then each part was assessed at specified times for chromatin quality (Acridine orange staining), MDA level and genes expression. The schematic of the study design is presented in (Fig 1).

Acridine orange staining

This fluorescent dye is used to differentiate healthy double-stranded DNA from denatured single-stranded DNA. For this staining, the smear of each sample was prepared and dried in the air. The slides were fixed with Carnavi fixative at 4°C for 2 hours. After that, slides were stained by adding 2-3 mL of Acridine orange (AO) dye (Merk-Germany) in 19% citrate-phosphate buffer (pH 2.5) for 10 min in the dark place. One hundred sperm were evaluated by filter (460 nm) at the ×100 magnification fluorescent microscope (Olympus BX5, Japan). Sperm with normal DNA stained bright green and sperm with abnormal DNA stained red or orange (Afsari *et al.*, 2022).

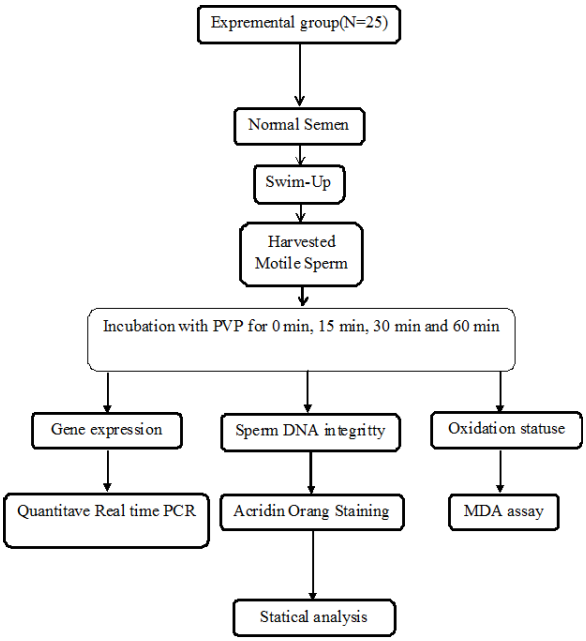

Fig. 1. Schematic of study.

Assessment of sperm MDA level

MDA is the end product of lipid peroxidation, which reacts with thiobarbituric acid (TBA) and produces a pink complex with a high absorbance of 530-540 nm. This test was performed using the Teb Pazhouhan Razi (TPR) (Tehran, Iran) kit. 100 µL of sperm samples were added to 100 µL of standard samples in a microtube. Then, 100 µL of reagent (R4) and 200 µL of chromogen were added to the microtube. The tube was then placed in a hot water bath for 1 hour and on ice for 10 min. After centrifuging at 4 °C for 15 min, 200 µL of samples were placed in plate wells, and the absorbance was recorded by a plate reader in 530-540 nm (Tofighi Niaki *et al.*, 2023).

Quantitative real time-PCR

To assess *HSPA2*, *BAX*, *BCL2*, *SOD2*, and *HSPA2* expressions in spermatozoa samples, the

Trizol Kit (Invitrogen, USA) was used to extract total RNA. External DNA contaminants were removed from RNA samples by using DNase 1 (Fermentase). A spectrophotometer (NanoDrop, Thermo Fisher) was utilized to evaluate RNA purity and concentration. Materials for quantitative real-time PCR (Q-PCR) included constructed cDNA, master mix, forward and reverse primers, and DNase/RNase-free water. A thermal cycler (Applied Biosystem, ABI, Step One Plus, USA) was used for gene expression (Tofighi Niaki *et al.*, 2023). Oligonucleotide primer sequences are reported in (Table 1).

Table 1. Oligonucleotide primer sequences.

Genes*	Primer sequences (5'-3')	Accession No.	Product size (bp)
HSPA2	F: GAGCTCAATGCCGACCTCTT R:CTTGGGGATACGAGTGGAGC	NM_021979.3	126
SOD2	F: AAGGAACGGGGACACTTACA R:CACACATCAATCCCCAGCAG	NM_001322819.2	95
BAX	F:AGATCATGAAGACAGGGGCC R:AGACACTCGCTCAGCTTCTT	NM_001291428	136
BCL2	F:GCCCTGTGGATGACTGACTA R: GAAATCAAACAGAGGCCGCA	NM_000633	117
GAPDH	F:CAAGAGCACAAGAGGAAGAGAGAG R: TCTACATGGCAACTGTGAGG	NM001289746	103

* *HSPA2*= Heat shock protein A2; *SOD2*= Superoxide dismutase 2; *BAX*= BCL2 Associated x- protein; *BCL2*= B-Cell lymphoma protein family; *GAPDH*= Glyceraldehyde 3- phosphate dehydrogenase; bp= base pairs.

Statistical analysis

After data collection, SPSS version 19 was used for data analyses. The normality of the data was assessed with the one-sample Kolmogorov-Smirnov test, revealing that all data were normally distributed. Independent t-test, ANOVA, and Mann-Whitney test were used to compare the groups, and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PVP impacts on chromatin structure

After the preparation of sperm, AO staining was performed to differentiate healthy double-stranded DNA from denatured single-stranded DNA (Fig. 2). In control, the percentage was 21.80 ± 4.76. In 15, 30, and 60 min after PVP incubation, this rate was 53.24 ± 6.99 ($P < 0.001$), 64.12 ± 7.34 ($P<0.001$), and 75.12± 7.17 ($P < 0.001$), respectively. Thirty min ($P < 0.001$) and 60 min ($P < 0.001$) PVP incubation causes a significant increase in the AO percentage rate compared to the 15 min. Moreover, this rate was significantly changed at 60 min than 30 min (Table 2).

PVP impacts on sperm MDA level

After the swim-up method (0 min), the sperm MDA level percentage was 0.061 ± 0.009. At 15, 30, and 60 min after PVP incubation, this rate was 0.097± 0.029, 0.16± 0.037, and 0.19 ± 0.033, respectively. Fifteen min ($P < 0.001$), 30 min ($P < 0.001$), and 60 min ($P < 0.001$) after sperm incubation with PVP, this rate was significantly increased compared to the 0 min. Thirty min ($P < 0.001$) and 60 min ($P<0.001$) PVP incubation caused a significant increase in MDA level percentage compared to 15 min. Moreover, this rate was not significantly altered in 60 min compared to 30 min (Table 2).

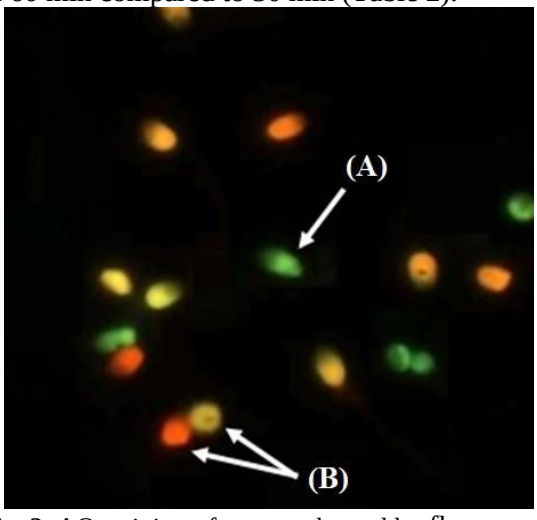

Fig. 2. AO staining of sperms showed by fluorescent microscope: A= Double strand DNA, which showed green; B= Single strand DNA, which showed yellow and orange.

PVP impacts on gene expressions

HSPA2, *BAX*, *BCL2*, and *SOD2* gene expressions are explained in (Fig. 2). The expression of the *HSPA2* gene increased significantly in 15, 30, and 60 min incubation in PVP compared to the control group (P < 0/001). *BAX* expression increased remarkably after 15, 30, and 60 min exposure in PVP compared to the control group (P < 0/001). *BCL2* significantly decreased after 15, 30, and 60 min incubation in PVP compared to the control group (P < 0/001). It was observed that *SOD2* gene expression increased noticeably in different incubation times compared to the control group (P < 0/001), which is shown in (Figure 3).

Table 2. The acridine orange assessment and MDA level in different groups (mean± SD).

Variables	0 min. (Control)	After 15 min	After 30 min	After 60 min
AO (+)	21.80±4.76	53.24±6.99	64/12±7.34	75.12±7.17
p-value	-	< 0/001*	< 0/001*	< 0/001*
MDA (nmol/ml)	0.061±0.009	0.097±0.029	0.16±0.037	0.19±0.033
p-value	-	< 0/001*	< 0/001*	< 0/001*

AO= Acridine orange; MDA= Malondyaldehyde; *= p<0.01 significant difference

Discussion

The results obtained from staining techniques indicated that PVP increases chromatin damage over time. These results were consistent with the results reported in previous studies (Rougier *et al*., 2013). Apoptosis and oxidative stress are underlying mechanisms that impair DNA structure. ROS affects sperm cell plasma membrane due to many polyunsaturated fatty acids (Dorostghoal *et al*., 2017; Romeo and Santoro, 2014; Jabbari, 2017; Wolski and Slowikowska-Hilczer, 2013).

MDA is a byproduct of lipid peroxidation that alters sperm biological features (Walczak-Jedrzejowska *et al.*, 2013; Yazdinejad *et al.*, 2020). Dorostghoal *et al.* examined the role of seminal oxidative stress and DNA fragmentation in the etiology of male infertility in the southwest of Iran. They found that fragmented DNA and MDA levels were significantly higher in infertile men compared with fertile men. The increased level of MDA in the spermatozoa was significantly correlated with DNA fragmentation values and negatively correlated with sperm motility and morphology (Dorostghoal *et al.*, 2017). From these findings, it can be concluded that the evaluation of seminal oxidative stress and DNA integrity can be helpful in men referring to infertility clinics during infertility treatment strategies. There are limited studies on the relationship between PVP incubation and oxidative stress. Roychoudhury et al. reported that PVP suppresses sperm oxidative stress and should be preferred for sperm handling during ICSI treatment. MDA levels were 0.097 after 15 minutes, 0.16 after 30 minutes, and 0.19 after 60 minutes in comparison to MDA levels for control sampling, which was 0.061mol/ml. They suggested that PVP may be advantageous in the context of male factor infertility. The results of the present study are contrary to the findings of Roychoudhury 's study (Roychoudhury *et al.*, 2018).

The results showed that sperm MDA levels increased significantly after 15, 30, and 60 min incubation of sperm with PVP. It seems that this contradiction needs further investigation in the sperm oxidant and antioxidant systems. Furthermore, in this study, *SOD2* expression significantly increased after incubation in different time intervals with PVP. Mitochondrial *SOD*, or Mn *SOD*, also called *SOD2*, is a gene that is expressed in high levels, and the *NRF2-ARE* signaling pathway regulates it in human semen and preserves sperms from oxidative stress indices. It was reported that SOD maintained H_2O_2 and $O2^-$ balanced and disrupted enzyme expression that affected sperm function. In male infertility, SOD has low activity, and its antioxidant capacity is decreased in seminal plasma. It was revealed that male infertility is associated with SOD activity of seminal and sperm fertilization potential (Fallahi *et al*., 2021). HSPA2 protein is detected in normal testis and also in spermatocytes and spermatids. HSPA2 has a main role in human spermatogenesis and is placed in the peri-acrosomal region, which contributes to oocyte activation. In oligozoospermic and azoospermia cases, the level of *HSPA2* expression decreased compared to the fertile cases (Nixon *et al.*, 2015).

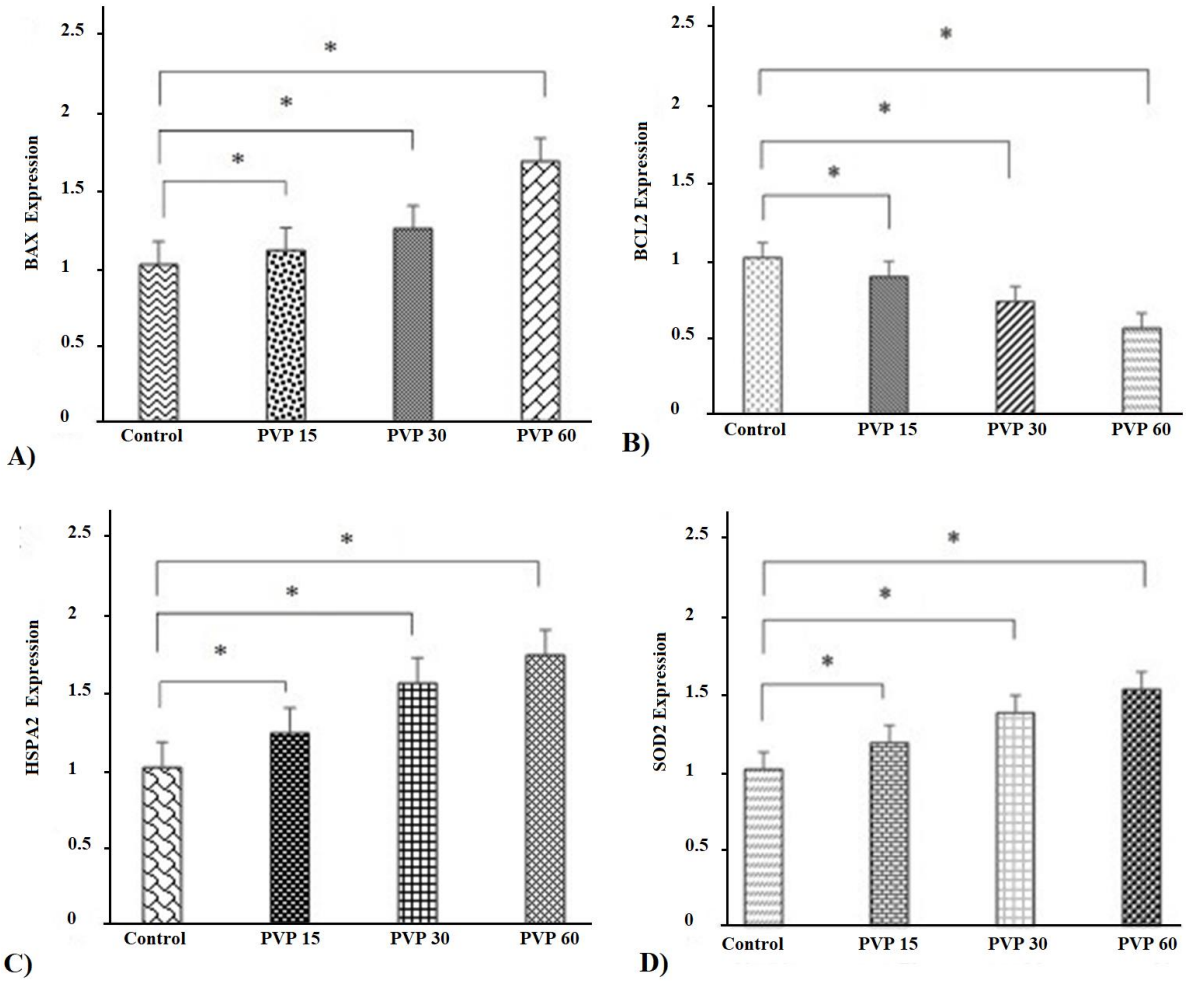

Fig. 3. Gene expression in different groups and times (15, 30, and 60 min): A) BAX; B) Bcl2; C) HSPA2; D) SOD2 expressions. Data was exhibited mean±SD, and $p<0.05$ is set as statically significant. HSPA2= Heat shock protein A2; SOD2= Superoxide dismutase 2; BAX= BCL2 Associated x- protein; BCL2= B-Cell lymphoma protein family; GAPDH= Glyceraldehyde 3- phosphate dehydrogenase; *= P<0.001.

In this study, PVP exposure after different time intervals increased gene expression. Another mechanism that may have deleterious effects on sperm biological parameters is apoptosis. Apoptosis is activated due to heat shock, stressors, ROS, ultraviolet radiation, drugs, synthetic peptides, and toxins. There are various genes involved in this process, such as *BAX*, *BCL2*, etc. (Asadi *et al.*, 2021). In this study, *BAX* expressed significantly increased in all time intervals compared to the control group and the *BCL2* as an anti-apoptotic gene decreased in different time intervals compared to the control group. It can be concluded that apoptosis can occur due to prolonged incubation of PVP and affected biological aspects of spermatozoa; therefore, it is better to perform the ICSI procedure with professional embryologists in order to avoid the activation of detrimental mechanisms.

Chemical and harmful agents must be removed from ART program. Therefore, another substance with fewer side effects is required. For instance, components like sodium hyaluronate may decrease sperm movements and prevent sperm from sticking to the dish or pipette. However, there are differences in the sperm motility in these two media while no difference was detected in zygotes growth in both of these media (BM *et al.*, 2016).

Conclusion

Altogether, results showed that incubation of sperm in PVP medium during ICSI procedure 15 min may result in significant changes in biological features of sperm. Thus, performing ICSI procedure faster would reduce the possible impacts of PVP on sperm parameters.

Conflict of interests

Authors declare no conflict of interest.

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