

Original Article:

The Effect of Fe₂O₃ Nanoparticles on Sperm Parameters and the Expression of Mitochondrial Apoptotic Pathway Genes in Rats With Scrotal Hyperthermia



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Abstract

Introduction: Hyperthermia can cause infertility in men following an increase in testicular temperature. Oxidative stress has been found to be one of its major causes. In the present study, the effects of iron superoxide nanoparticles on the expression of Bcl-2 and Bax family genes were studied.

Materials and Methods: A total of 48 adult rats were purchased from the Pasteur Institute of Iran. The rats were later divided into 4 groups: control group, control group receiving superoxide nanoparticles (Fe₂O₃), hyperthermia group, h:yperthermia group receiving superoxide nanoparticles (Fe₂O₃). After RNA extraction, evaluating the sperm parameters and the expression of Bax and Bcl-2 genes was examined using RT-PCR technique.

Results: Exposure to iron superoxide nanoparticles (Fe₂O₃) decreased sperm parameters, increased proapoptotic BAX gene and decreased expression of BCL2 anti-apoptotic gene.

Conclusion: Exposure to nanoparticles by reducing sperm parameters and increasing apoptosis has a negative effect on fertility. The association between infertility and testicular hyperthermia is becoming increasingly apparent; administration of iron superoxide (Fe₂O₃) nanoparticles can have significant effects on male infertility. Moreover, green synthesis of nanoparticles is also recommended in this field.

Keywords: Rat, Bax, Bcl-2, Scrotal Hyperthermia.

1. Introduction

Infertility is a sexual disorder that is active. Infertile couples are those who are unable to conceive within a year without the use of contraception [1]. Infertility affects roughly 15% of couples, with idiopathic infertility ac-

counting for 10-15% of cases. Male factors are to blame for half of all infertility cases [2, 3].

The most significant causes of male infertility are increased scrotal temperature, genetic, congenital or acquired anomalies, malignancies, endocrine disorders, immunological and idiopathic causes. Despite extensive studies on male infertility, the etiology of many

infertile men remains unclear [4]. This might be due to a variety of factors such as pollution, reactive oxygen species (ROS), genetic and epigenetic disorders, to name but a few [5].

Testicular hyperthermia is one of the reasons of infertility in males, as previously stated. The lack of scrotal temperature control is the main cause of testicular hyperthermia, which consequently leads to thermal stress [6]. This can cause spermatogenesis to be significantly inhibited, eventually leading to infertility. Lifestyle and behavioral variables, occupational and environmental factors and clinical issues induced by other comorbidities are all potential causes of thermal imbalance [7]. Overproduction of free radicals is one of the main causes of cell damage, and these substances can affect gene expression [8]. Whereas lower levels of ROS in the testicles have been found to increase spermatogenic activities such as capacitation, acrosome response, hyperactivation, and sperm-oocyte fusion, excessive levels of ROS have been proven to cause significant damage to testicular tissue [9]. Antioxidant compounds in the testicles are important to maintain ROS at a sustainable level [10].

Nanoparticles (NPs) are particles with a size of fewer than 100 nanometers [11]. The significance of nanoparticles was increased when researchers discovered that particle size may affect a substance's attributes. In the industrial and biomedical fields, NPs are frequently utilized [12]. Nanoparticles' distinct chemical and physical properties have led to their extensive adoption in processes such as vaccination, drug delivery, diagnosis or treatment of a wide range of diseases, cancer cell heat therapy, immune-metrics, tissue regeneration, excretion of biological fluids intoxication, dental alloys, bladder catheters, etc. [13]. Some nanoparticles can prevent hormone disruption through a variety of methods, including antioxidant capabilities. Due to their capacity to penetrate through cell membranes, these nanoparticles exhibit antioxidant effects in low doses and poisonous effects in high quantities. Furthermore, researches have demonstrated that combining nanoparticles with antioxidants boost the antioxidant impact and quantity [14].

Accordingly, the purpose of current study was to identify the sperm response to temperature-induced stress and to investigate the effect of Fe_2O_3 nanoparticles in order to provide more detailed information on cell damage as new methods for treatment of male infertility.

2. Materials and Methods

Materials

In this study, Fe_2O_3 nanoparticles (Sigma Aldrich, German Formula Weight: 159.69 g/mol) were purchased. One mg of the nanoparticle was diluted in 2000 μl of distilled water and then injected subcutaneously into mice with a 100 μl insulin syringe. The size of the synthesized particles was estimated to be 4.8-5.6 nm using an electron microscope. Nanoparticles were injected in 6 doses (0.01, 0.02, 0.03, 0.04, 0.05, 0.06). The dose of Fe_2O_3 nanoparticles was determined based on LD50; namely, the concentration that caused the death of half of the rats was set at 0.03. Accordingly, LD50 was determined as 0.03 mg/kg body weight. Thus, in the present study, this concentration was utilized.

Animals

In the current study, 48 adult male rats weighing 25-30 g were purchased from the Pasteur Institute in Tehran for this experiment. The animals were kept under standard conditions of 12 hours of light and 12 hours of darkness, humidity (65.5%) and relative humidity of 50% to 10% and at a temperature of $22 \pm 1^\circ \text{C}$. Rats were fed the same diet and all animals were given the same amounts of corn, wheat, barley and pellets, all of which had free access to water.

Induction of Scrotal Hyperthermia

A hot water bath was used for induction of Scrotal Hyperthermia, the lower part of body (consisting of scrotum and hind legs) at temperature of 43°C for 30 min once a day for five consecutive weeks; For control rats, hot water bath at temperature of 22°C was used. Then, the rats were dried and examined for any damage on scrotal and next placed in cages. The animals were anesthetized by administering ketamine (100 mg/kg) and xylazine (5 mg/kg) through intraperitoneal injection. Studies have shown that no animals were harmed. After scrotal hyperthermia induction, the rats were randomly divided into 3 groups.

- 1) Control group
- 2) Rats with scrotal hyperthermia
- 3) Rats that received Fe_2O_3 nanoparticles

After the treatments were completed, all of the animals were killed by an overdose of anesthesia, and the testicu-

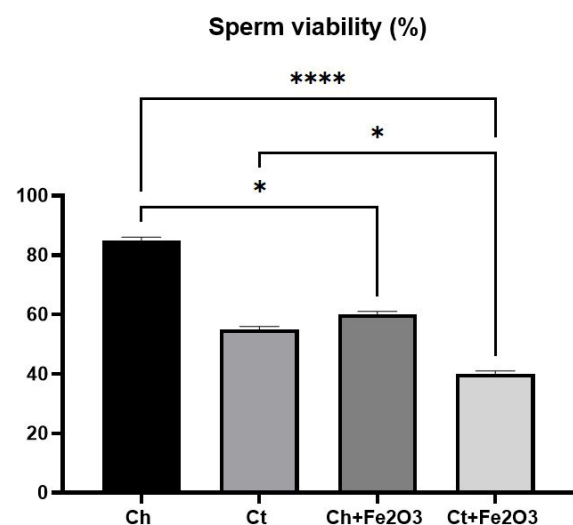
Table 1. The sequence of primers used in this study

Primers	Sequence	Tm
BAX-F	5'-GAAGGTGAAGGTCGGAGT CA-3'	60.2 C°
BAX-R	5'-TTGAGGTCAATGAAG GGG TC-3'	59.9 C°
BCL-2 F	5'-GAGCTGCAGAGGATGATTGC-3'	61.1 C°
BCL-2 R	5'-AAGTTGCCGTCAGAAACATG- 3'	60.2 C°
B- actin F	5- CGGTCCGATGCCCTGAGGCTCTT-3	61.7 C°
B- actin R	5- CGTCACACTTCATGATGGAATTGA-3	60.9 C°

lar tissue was taken for tissue testing, and the epididymis semen was collected.

Sperm Parameters

Four attributes were examined for sperm analysis including sperm concentration, viability, morphology, and motility. First, the sperm was extracted from the epididymal tissue and the sperm (10 microliters) was transferred to a homo cytometer, and then the sperm count was performed under an optical microscope at 40X magnification. According to the recommendations of the World Health Organization, microscopes were used to assess sperm motility in ten fields.

**Figure 1.** The effect Fe₂O₃ nanoparticles on sperm viability percentage.

*The different letters indicate significant differences between the groups according to the Tukey test at the probability level of 0.001

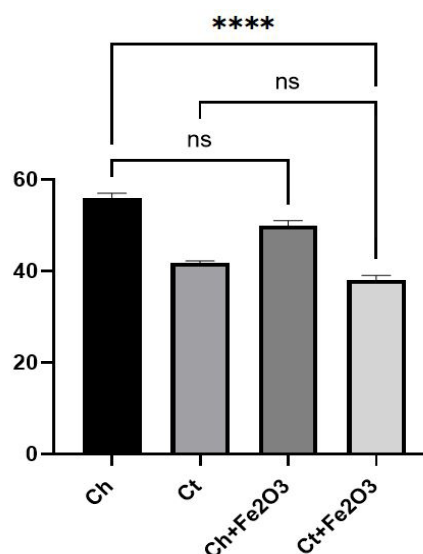
Ch: healthy control; Ch+Fe₂O₃: healthy control receiving Fe₂O₃ nanoparticles; Ct: rats with scrotal hyperthermia; Ct+ Fe₂O₃: rats with scrotal hyperthermia receiving Fe₂O₃ nanoparticles.

cDNA synthesis

cDNA synthesis was proceeded as what follows:

For cDNA synthesis, the following components were added to the tube: 0.2 µg / µl (random hexamer), MMULV enzyme 100 units, 0.5 µg / µl (oligo dT primer), 10 µl of RNA, and finally the final reaction volume. It reached 20 µl. The sample was placed at 65 ° C for 5 minutes and after that frozen rapidly. Housekeeping genes B-actin were used in technique Rt-PCR. The gene transcription

Sperm concentration(M/ml)

**Figure 2.** The effect Fe₂O₃ nanoparticles on sperm viability percentage.

*The different letters indicate significant differences between the groups according to the Tukey test at the probability level of 0.001

Ch: healthy control; Ch+Fe₂O₃: healthy control receiving Fe₂O₃ nanoparticles; Ct: rats with scrotal hyperthermia; Ct+ Fe₂O₃: rats with scrotal hyperthermia receiving Fe₂O₃ nanoparticles.

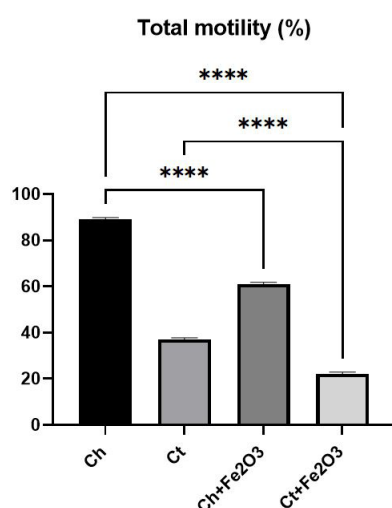


Figure 3. The effect Fe₂O₃ nanoparticles on sperm viability percentage.

*The different letters indicate significant differences between the groups according to the Tukey test at the probability level of 0.001

Ch: healthy control; Ch+Fe₂O₃: healthy control receiving Fe₂O₃ nanoparticles; Ct: rats with scrotal hyperthermia; Ct+ Fe₂O₃: rats with scrotal hyperthermia receiving Fe₂O₃ nanoparticles.

sequence was obtained from www.ncbi.nlm.nih.gov and then the forward and reverse oligonucleotide primers were designed by Primer3 software. (Table 1).

Statistical analysis

To find significant differences in the investigated features across the rat groups, one-way analysis of variance (ANOVA) was performed. The data were analyzed using SPSS software version 22. Statistical significance was considered as a p value of less than 0.001.

3. Results

Semen Parameters

Sperm Viability

Scrotal Hyperthermia had a significant impact on sperm viability in rats, with a significant reduction in this parameter. In addition, the results revealed that Fe₂O₃ nanoparticles have a toxic effect on sperm viability in both control and Scrotal Hyperthermia rats. Therefore, the results of the current study indicate the negative effect of Fe₂O₃ nanoparticles on increasing sperm viability (Figure 1).

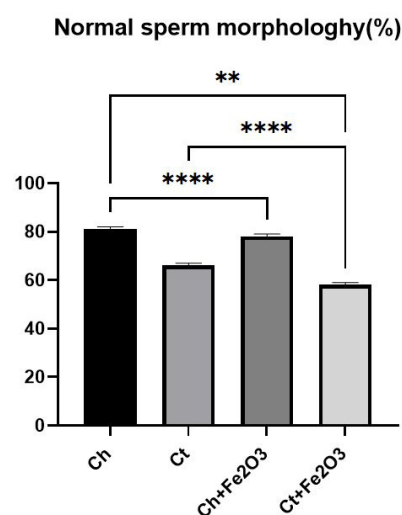


Figure 4. The effect Fe₂O₃ nanoparticles on sperm viability percentage.

*The different letters indicate significant differences between the groups according to the Tukey test at the probability level of 0.001

Ch: healthy control; Ch+Fe₂O₃: healthy control receiving Fe₂O₃ nanoparticles; Ct: rats with scrotal hyperthermia; Ct+ Fe₂O₃: rats with scrotal hyperthermia receiving Fe₂O₃ nanoparticles.

Sperm concentration

The current research found that the concentration of sperm in different groups of rats differed significantly ($P < 0.001$). A relative reduction in sperm concentration was found under scrotal hyperthermia conditions. Furthermore, Fe₂O₃ nanoparticles reduced sperm concentration in control and scrotal hyperthermia rats. The negative effect of Fe₂O₃ nanoparticles on decreasing sperm concentration in rats with scrotal hyperthermia was observed in the current study. (Figure 2).

Sperm motility

Scrotal hyperthermia caused a significant reduction in sperm motility due to scrotal Hyperthermia. Fe₂O₃ nanoparticles were also shown to be toxic to sperm motility. The negative effect of Fe₂O₃ nanoparticles on decreasing sperm motility was observed in Scrotal hyperthermia rats in the present study (Figure 3).

Normal Sperm Morphology

A relative reduction in the percentage of sperm with normal morphology was found because of scrotal hyperthermia in rats. Although treatments used in the current

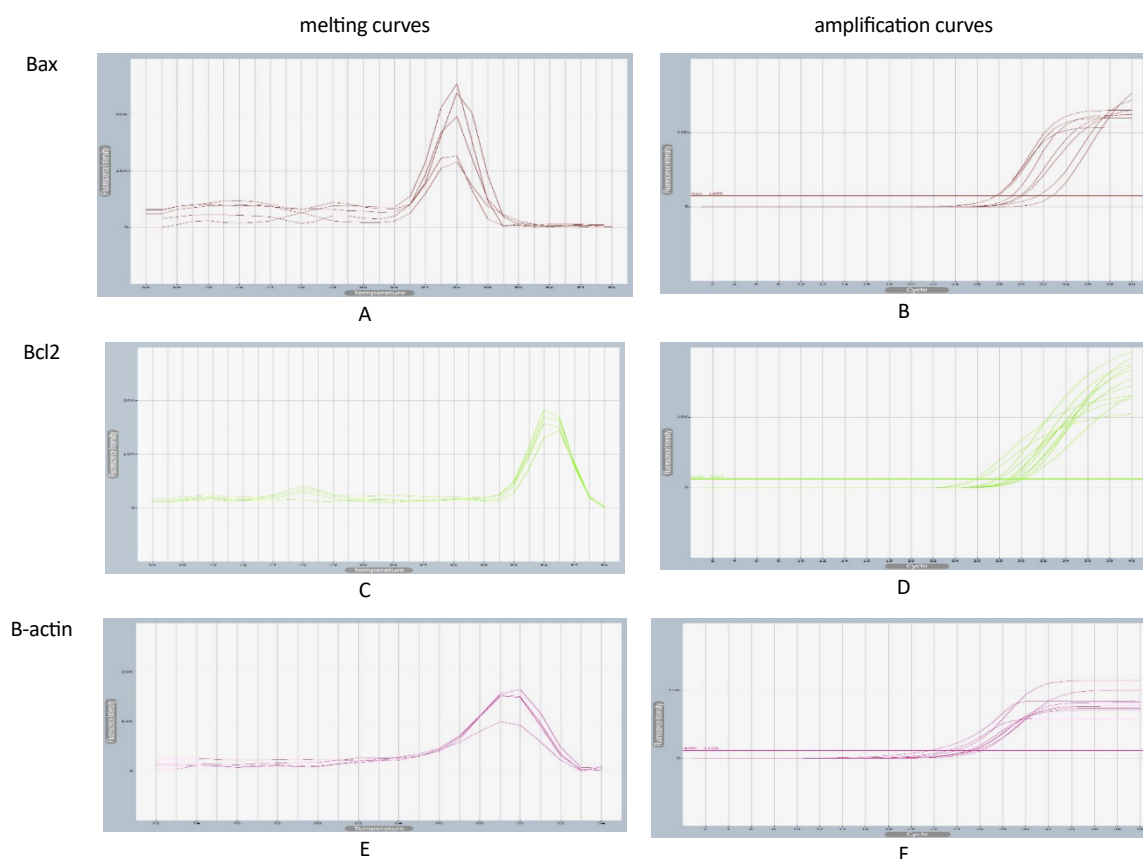


Figure 5. Diagram of melting and amplification curves of Bax, Bcl-2 B-actin genes. A: Diagram of melting curves of Bax gene; B: Diagram of replication curves of Bax gene; C: Diagram of melting curves of Bcl2 gene; D: Diagram of replication curves of Bcl2 gene; E: Diagram of melting curves of B-actin gene; F: Diagram of replication curves of B-actin gene

study had an important effect on the percentage of sperm with normal morphology ($P < 0.001$), Fe₂O₃ nanoparticles reduced normal sperm in control and scrotal hyperthermia rats. Therefore, the results of the present study show that exposure administration of Fe₂O₃ nanoparticles have negative effects on Normal Sperm Morphology (Figure 4).

Results of gene expression

Diagram of melting and amplification curves of Bax, Bcl-2 B-actin genes

The following diagrams show the melting curves of the Bax and Bcl-2 genes, showing the specific binding of the primer. B-actin has been used as a reference. Since the diagram is a single peak, the primers are especially attached. The following diagram shows the replication curves of Bax, Bcl-2 and B-actin genes, demonstrating in which product cycle it has doubled. As can be seen in the following curves, the Bcl-2 gene in the nanoparticle-receiving samples reached the exponential phase in a lower cycle than the control samples, as well as B-actin, and since the cycle in which the reaction entered the

exponential phase It depends on the amount of DNA in the original pattern, so the amount of Bcl-2 gene in the samples tested has increased. Amplification curves also showed a decrease in Bax gene expression in nanoparticle-receiving hyperthermia samples. Hence, it represents the contribution of nanoparticles to the induction of apoptosis (Figure 5 A-F).

Bax and Bcl-2 expression

The results of real-time PCR experiment showed that the effect of Fe₂O₃ nanoparticles, increased Bax expression in control samples and Scrotal hyperthermia samples are observed and this increase in expression in hyperthermia samples is more than that of the control samples. The expression of Bcl-2 also decreased compared to Bax, indicating that this nanoparticle has an apoptotic effect on the samples (Figure 6).

4. Discussion

Birth rate has decreased rapidly in recent decades [15]. Male infertility accounts for nearly half of all causes of infertility [16]. The results of many studies have shown

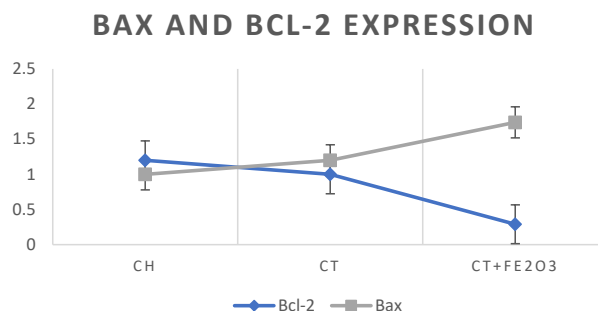


Figure 6. Comparison of Bax and Bcl-2 gene expression in germ cells in different treatments.

CH: Healthy control; CT: rats with scrotal hyperthermia; CT+ Fe₂O₃: rats with scrotal hyperthermia receiving Fe₂O₃ nanoparticles.

that the effective cause of infertility in 50% of couples is the male factor. Male infertility can be related to a variety of causes, including congenital, acquired, or idiopathic factors that impair sperm production, and requires a thorough evaluation of lifestyle and advanced diagnostic sets to assess sperm quality and function [17]. High temperature, which affects the process of spermatogenesis, is one of the reasons behind male infertility, and studies have revealed that scrotal hyperthermia causes significant disruption of this physiological process, ultimately leading to infertility [18]. However, there are few clinical studies [19-21].

This study was performed to investigate the effect of heat stress on male infertility. We made dynamic changes in sperm parameters and investigated the effects of iron superoxide nanoparticles on genes Bax and Bcl-2. Previous studies on Bax and Bcl-2 genes have shown that sperm parameters and Bcl-2 levels significantly decrease, while the expression of inflammatory factors and Bax proteins is significantly increased [22]. The previous study showed that transient and recurrent hyperthermia of the scrotum severely impairs sperm production. Induction of hyperthermia caused by repeated exposure to scrotum heat showed that Bcl-2 levels were significantly reduced [23].

Many studies have been conducted to investigate the effect of hyperthermia on spermatogenesis and its mechanism in animal models [20-23]. Scrotal hyperthermia has been related to the death of germ cells and spermatogenesis disruption. Cell apoptosis is the mechanism by which germ cells die as a result of scrotal hyperthermia. The testicles, prostate, and penis are among the organs that experience cell death. Apoptosis has been reported to play an important function in the removal of undesirable cells, in addition to contributing to the development of a variety of diseases. Short term exposure of the tes-

ticles to temperatures above 43°C for 15 to 20 minutes additionally killed cells due to apoptosis [24, 25].

Studies have shown that drug delivery by iron oxide nanoparticles, although their potential biomedical application can alter gene expression, impair iron homeostasis, oxidative stress as well as altered cellular responses [26]. In a previous study, the systemic effect of iron oxide nanoparticles on the homeostasis of major organs in male rats was investigated and the dynamics of elemental changes in tissues obtained at different intervals from nanoparticle injection were evaluated [27]. In the current study, the damaging effect of Fe₂O₃ NPs on sperm parameters in healthy and scrotal hyperthermia rats was attributable to an increase in oxidative stress and ROS production. The results are consistent with those of other studies in this area. Previous research has shown that nanoparticles hurt male reproductive cells [28]. The nanoparticle sensitivity of spermatogonial stem cells appears to play a significant function. Damage to reproductive cells is caused by the production of inflammation or edema in the interstitial tissue, as well as oxidative stress [29]. Nanoparticles have also been associated with cellular DNA damage and cell dysfunction in other studies. Autophagy-induced cell death appears to play a key function [30]. However, more research is needed in this regard.

The green synthesis of nanoparticles by plants has received a lot of attention in recent years and is considered an alternative to chemical methods for the synthesis of nanoparticles [31]. In addition, the green synthesis of nanoparticles is very affordable. Therefore, the green synthesis of Fe₂O₃ nanoparticles is recommended to reduce symptoms in patients with scrotal hyperthermia. Nowadays, the bio-fabrication of iron oxide nanoparticles using various plant sources, plant parts and microbial cells has received much attention due to its environmen-

tally friendly nature. Due to the stabilizing and masking agents in biologically compatible, stable and non-toxic biological resources, its use for various biomedical applications has many benefits. Plants and plant components contain a variety of phytochemicals that play a key role in the synthesis and biosynthesis of nanoparticles [32]. The findings are consistent with the results of other studies. The use of nanoparticles for drug delivery is a very important goal in the treatment of diseases. However, by reducing the toxic effects of nanoparticles on the function of sperm parameters and regulating the expression of Bax and Bcl-2 genes, it is very important to do this with the green synthesis of nanoparticles.

5. Conclusion

Male infertility is one of the major challenges facing modern fertility medicine today, and it is crucial to provide couples with an opportunity to have children. In addition, the association between infertility and scrotal hyperthermia is becoming increasingly apparent. The present study showed that Fe₂O₃ nanoparticles can greatly affect the parameters of sperm genes, Bax, Bcl-2. Today, green synthesis of nanoparticles has helped to reduce their toxic effects. Therefore, green synthesis of nanoparticles has also been proposed in future studies.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Islamic Azad University, Iran (IR.IAU. PS.REC.1399.209).

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Author's contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declare no conflict of interest.

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