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Vitamins A, C, and E Exert Anti-apoptotic Function in the Testis of Rats After Exposure to Zinc Oxide Nanoparticles

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Some reports emphasize that zinc oxide nanoparticles (ZnO NPs) are detrimental to the reproductive organs of animals. As such, this research aimed at exploring the apoptotic potential of ZnO NPs on testis along with the beneficial role of Vitamins (V) A, C, and E against ZnO NP-induced damage. To this aim, a population of 54 healthy, male Wistar rats were used in this work and then assigned into nine groups of 6 rats as G1: Control 1 (Water); G2: Control 2 (Olive oil); G3: VA (1000 IU/kg), G4: VC (200 mg/kg), G5: VE (100 IU/kg), G6: ZnO NPs exposed animals (200 mg/kg); and G7, 8 and 9: ZnO NPs-exposed animals that were pre-treated with either VA, C, or E. Apoptosis rates were estimated by measuring the level of apoptotic regulatory markers including Bcl-2-associated X (Bax) and B-cell lymphoma protein 2 (Bcl-2) using western blotting and qRT-PCR assays. The data indicated that ZnO NPs exposure elevates the level of Bax protein and gene expression, whereas the protein and gene expression of Bcl-2 was reduced. Further, the activation of caspase-3,7 occurred after exposure to ZnO NPs, while the above alterations were significantly alleviated in the rats that were co-treated with VA, C, or E and ZnO NPs relative to the rats in the ZnO NPs group. In summary, VA, C, and E exerted anti-apoptotic functions in the testis of rats following administration of ZnO NPs.

Key Words: *Apoptosis; Antioxidants; Rats; Zinc Oxide; Nanoparticles*

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INTRODUCTION

The increasing use of zinc oxide nanoparticles (ZnO NPs) in various products and even medical fields indicates their broad applications in human life. The ever-expanding usages of ZnO NPs are linked with widespread human exposure, which eventually results in health risks.[1] Ample *in-vitro* studies[2-4] have reported ZnO NPs as toxic agents in various cell types. Those studies have reported various mechanisms through which ZnO NPs can induce a considerable cytotoxic impact including induction of oxidative stress which in turn leads to apoptosis or cell death.

Indeed, the tiny size of nanoparticles helps them easily enter the body and even they may accumulate in tissues such as testis, which subsequently can affect several normal functions of the male reproductive tract. It has also been found that NPs exert negative effects on the male reproductive system through activation of apoptosis.[5] For instance, it has been revealed that AgNPs induce testicular damage and apoptosis in rats.[6]

Apoptosis is a programmed form of cell death which is classified into two major forms: extrinsic and intrinsic. This type of cell death is involved in many diseases such as cancer,[7] infertility,[8] etc. Intrinsic apoptosis contributes to the ZnO NPs' toxicity in which B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax) proteins, as well as the caspase family play important roles in triggering apoptosis.[9] In this respect, several research works have demonstrated that the potential and permeability of the mitochondrial membrane might be controlled by adjusting Bcl-2 family proteins. Bax, as one of the important components of Bcl-2

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family, enters the mitochondrial membrane after its activation, enhancing membrane permeability and then giving rise to release of cytochrome c, activation of several caspases, and eventually cell death.[10]

Furthermore, some reports have indicated that when tissues are damaged by a toxicant, apoptosis can occur.[11,12] This has motivated researchers to investigate the apoptotic mechanisms of harmful agents. Additionally, further attention has been directed towards studies on the protective properties of antioxidants against damages caused by chemicals.[13,14]

Several roles of vitamins (V) in improving health conditions of the male reproductive system have been well demonstrated.[15,16] For example, VC and E are reported as strong antioxidants, which can reduce testicular injury.[17]

Vit A, as a potent antioxidant, contributes to cell growth as well as mitigating many of the detrimental effects of xenobiotics in the reproductive system.[18,19]

Since it is still unclear whether consumption of vitamins would be effective in mitigating the apoptotic activity of ZnO NPs in the male reproductive tract, this research was performed to assess the probable ameliorative influence of VA, C, and E on apoptosis-associated factors in the rat's testis exposed to ZnO NPs.

MATERIALS AND METHODS

1. Chemicals and reagents

Powder of ZnO NPs was bought from Nanosany Company, (Mashhad, Iran). The NPs were 20 nm in size and had spherical morphology. Characterization of the purchased ZnO NPs were reported in the former publication.[20]

Olive oil and powder of VA, C and E were bought from Sigma-Aldrich. After that, we dissolved the VA and VE in olive oil to prepare the desired concentrations as suggested in former studies.[21,22] Also, the solution of VC (200 mg/kg) was made in distilled water.[23] The suspension of NPs (200 mg/kg body) was prepared in fresh distilled water according to a previous work.[24]

2. Experimental design and animal grouping

A population of 54 male healthy Wistar rats with an average weight of 200±15 g were procured from the Animal Care Center-Hamadan University of Medical Sciences (Hamadan, Iran) and maintained under standard conditions according to our previous study.[25]

The animals were randomly distributed into 9 groups of 6 rats per group. It is noteworthy that the ZnO NPs administration was started one week after consumption of vitamins. This work was performed over 3 weeks. The nine experimental groups were as follows: Control 1: treatment with 1 ml/day bi-distilled water, Control 2: treatment with 1 ml/day olive oil, VA: treatment with VA (1000 IU/kg), VC: treatment with VC (200 mg/kg), VE: treatment with VE (100 IU/kg) by gavage for 21 days, ZnO: treatment with bi-distilled water for 7 days followed by administration of ZnO NPs (200 mg/kg) by gavage for 14 days. ZnO+VA: co-treatment with ZnO NPs (200 mg/kg) and VA (1000 IU/kg), ZnO+VC: co-treatment with ZnO NPs (200 mg/kg) and VC (200 mg/kg), ZnO+VE: co-treatment with ZnO NPs (200 mg/kg) and VE (100 IU/kg) by gavage for 14 days following administration of vitamins for 7 days.

3. Evaluating apoptosis-associated genes using the reverse transcription-polymerase chain reaction (RT-PCR)

The RNA from the testis tissues was isolated through Kiazol solution (Kiazist, Iran) following the instructions of the Kiazist kit. After checking the purity and quality of the RNAs, we made cDNA with a commercial kit (BioFact™ RT Series, Korea) according to manufacturer's protocols.

A LightCycler®96 System (Roche, Germany) was used to run a qRT-PCR reaction by using SYBR Green method (Ampliqon, Denmark). It should be mentioned that the sequences of primers including β-actin (reference gene), Bax and Bcl-2 were designed by primer 3 software (Table 1). Lastly, we calculated the relative mRNA expression by using a $2^{-\triangle\triangle Ct}$ assay.[26] The β-actin gene expression was analyzed as an endogenous control to normalize the data.

4. Evaluating protein expression by western blot analysis

The lysates of the testis tissue were prepared with a RIPA buffer including protease inhibitor cocktail (Kiazist, Iran) and then kept at −20℃ until use. In brief, an identical amount of protein from each sample was selected and then electrophoresed on SDS-PAGE. Subsequently, the protein bands were transferred onto the nitrocellulose membrane. Next, the membrane was blocked with fat-free milk in a tris-buffered saline medium with Tween 20 (TBST). Incubation with primary antibodies of Bax (sc-23959), Bcl-2 (sc-7382), and β-actin (ab8227) was done for 24 hours at 4℃. Then, we washed the membrane using TBST solution which was then then was revealed with HRP-conjugated antibodies (ab97040). The target protein bands were visualized with the aid of X-ray films and an ECL kit. Finally, we quantified the bands using Image J software.

TABLE 1. Primer sequences used for qRT-PCR experiments

Gene	Forward	Reverse	Product size (bp)
β-actin	ATCAGCAAGCAGGAGTACGAT	AAAGGGTGTAAAACGCAGCTC	94
Bax	ACTAAAGTGCCCGAGCTGA	ACTCCAGCCACAAAGATGGT	161
Bcl-2	GAGCGTCAACAGGGAGA	GCCAGGAGAAATCAAACA	164

5. Assessment of caspase 3,7 activation by colorimetric reaction

In brief, the testis homogenate was extracted following the Kit recommendations (Kiazist, Iran). The standard curve of p-nitroaniline (pNA) was drawn to examine the activation of caspase 3,7 according to the manufacture's instruction.

6. Statistical analysis

All data of this study were analyzed with a one-way analysis of variance (ANOVA) by using GraphPad Prism 9 software. The mean difference comparison between groups was calculated by a post hoc Tukey's test in which the significant level was regarded as $p < 0.05$. The values were shown as mean±standard error of mean (SEM).

RESULTS

1. Gene expression

Results obtained from the qRT-PCR analysis are depicted in Fig. 1. ZnO NPs could elevate the gene expression of Bax, accompanied by a significant decrease in Bcl-2 ($p < 0.05$). Pre-treatment with VA, C and E could mitigate the alterations of gene expression since the relative expression of Bax and Bcl-2 in ZnO NPs+VA, C and E groups is nearly closed to the animals in the control value. Specifically, the gene expression of Bax to Bcl-2, as an important indicator of apoptosis, was markedly augmented in rats receiving ZnO NPs ($p < 0.001$). Meanwhile, VA, C and E meaningfully attenuated the ZnO NPs-induced apoptosis as a remarkable reduction was observed in Bax to Bcl-2 ratio ($p < 0.01$ as compared to the ZnO NPs group). As detected by qRT-PCR data, it seems that VC and E have more protective potency than VA against ZnO NPs.

2. Protein expression

As it is presented in Fig. 2, compared to the control rats, the Bax expression was markedly elevated in the ZnO NPs-intoxicated rats ($p < 0.01$) (Fig. 2B). Also noteworthy, the anti-apoptotic Bcl-2 protein was reduced in rats receiving ZnO NPs only ($p < 0.01$) (Fig. 2C). Importantly, these alterations were noticeably improved in rats receiving ZnO NPs+VA, C and E compared to those that merely received ZnO NPs. Moreover, Bax to Bcl-2 protein expression of ZnO NPs group was meaningfully higher than in rats of control group ($p < 0.001$) (Fig. 2D). Remarkably, the protective function of VA, C and E resulted in a significant decline in Bax to Bcl-2 values as compared with the rats in ZnO NPs group ($p < 0.001$).

3. Alterations of caspase 3,7 activity

Administration of ZnO NPs meaningfully enhanced the activation of caspase 3,7 relative to the rats in the control group ($p < 0.001$) (Fig. 3). However, comparing these with the ZnO NPs group, treatment with VA, C and E considerably declined the negative influence of ZnO NPs at sig-

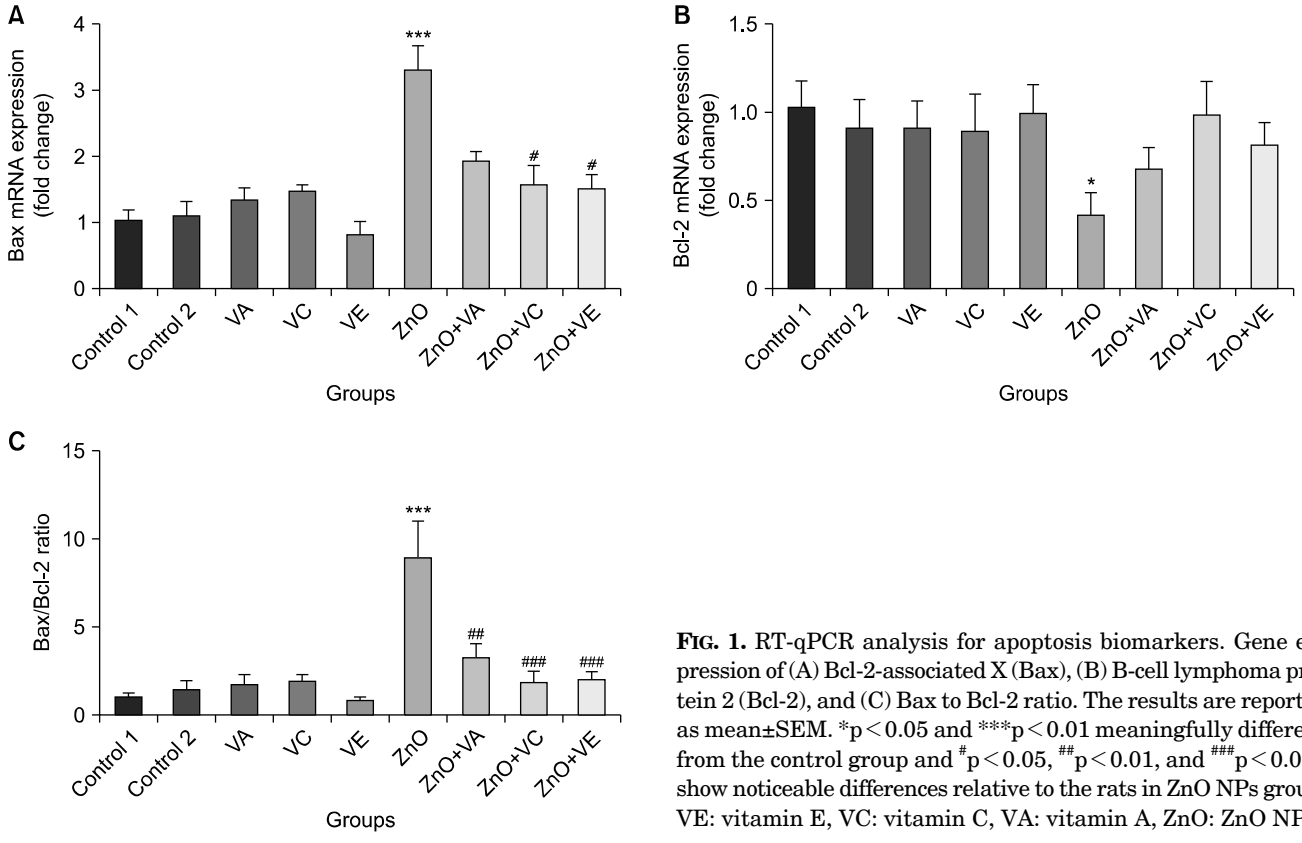

FIG. 1. RT-qPCR analysis for apoptosis biomarkers. Gene expression of (A) Bcl-2-associated X (Bax), (B) B-cell lymphoma protein 2 (Bcl-2), and (C) Bax to Bcl-2 ratio. The results are reported as mean±SEM. $^{*}p < 0.05$ and $^{***}p < 0.01$ meaningfully different from the control group and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ show noticeable differences relative to the rats in ZnO NPs group. VE: vitamin E, VC: vitamin C, VA: vitamin A, ZnO: ZnO NPs.

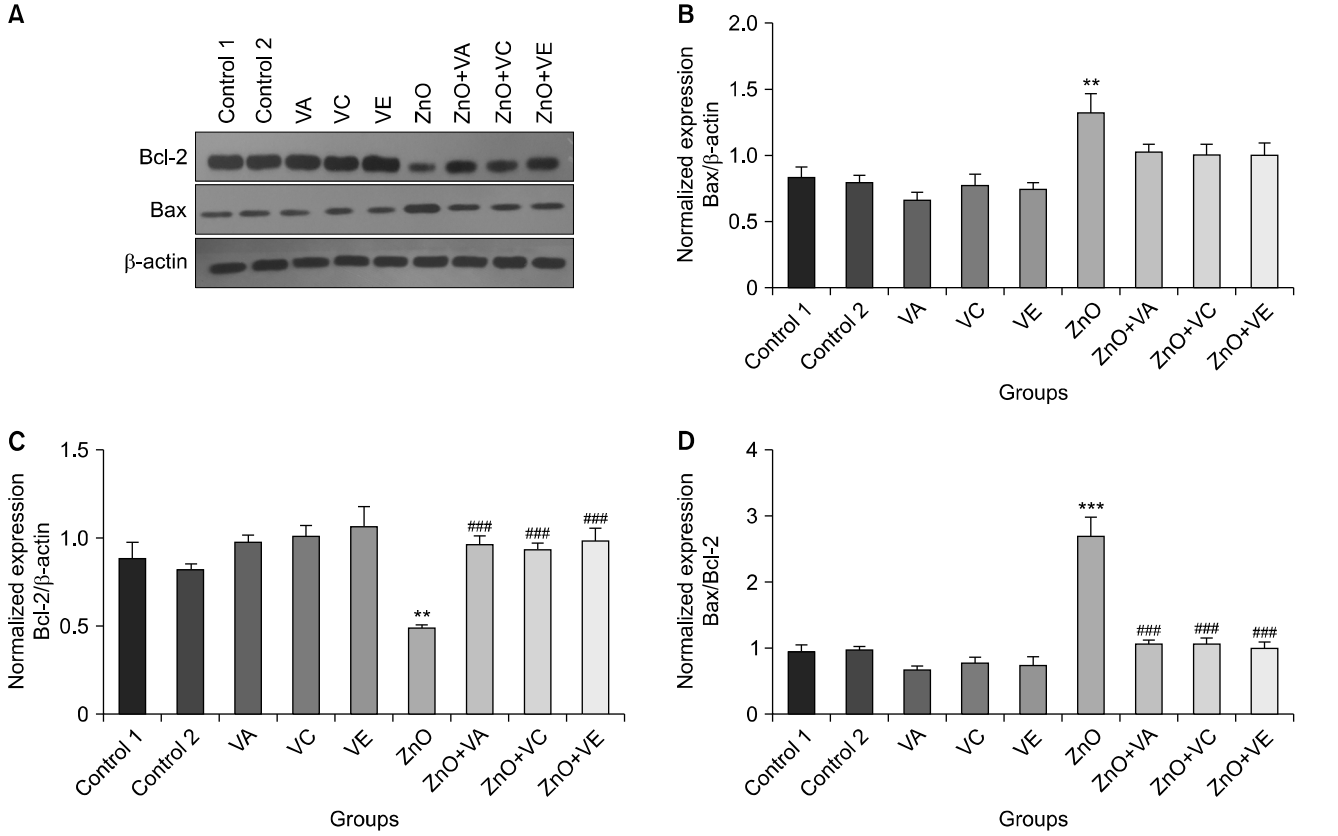

FIG. 2. Western blotting analysis for apoptosis biomarkers. Protein expression of (B) Bcl-2-associated X (Bax), (C) B-cell lymphoma protein 2 (Bcl-2), and (D) Bax to Bcl-2 ratio (A). The data are reported as mean±SEM. $^{**}p < 0.01$ and $^{***}p < 0.01$ significantly different from the control group and $^{###}p < 0.001$ show noticeable differences relative to the rats in ZnO NPs group. VE: vitamin E, VC: vitamin C, VA: vitamin A, ZnO: ZnO NPs.

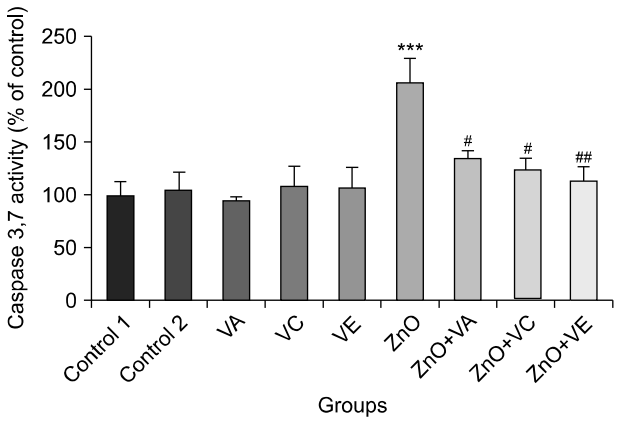

FIG. 3. Alterations of caspase 3,7 activity in experimental and control groups. Data are expressed as % relative to the control (100 %) and reported as mean±SEM. $^{***}p < 0.01$ significantly different from control group and $^{#}p < 0.05$, $^{##}p < 0.01$ show noticeable differences relative to the rats that only received ZnO NPs (ZnO group). VE: vitamin E, VC: vitamin C, VA: vitamin A, ZnO: ZnO NPs.

nificant levels of 0.04, 0.01 and 0.003, respectively. It is important to note that VE had the most ameliorative effects as compared to the VC and A.

DISCUSSION

Our study evaluated the beneficial functions of VA, C, and E in mitigating apoptosis in rat's testis after receiving ZnO NPs. According to the results derived from qRT-PCR and western blotting assays, the ZnO NPs cause apoptosis through elevation of Bax to Bcl-2 protein and gene expression (an indicator of apoptosis). On the other hand, supplementation of vitamins could demonstrably reduce apoptosis in rats.

A majority of previous *in-vitro* research have demonstrated that ZnO NP exposure make Leydig and Sertoli cells, as two important cell types in the testes of mammalians, undergo apoptosis.[27-29] Further, in a former report presented by Tang et al.,[30] ZnO NPs caused testicular damage, as revealed by increased expression of Bax/Bcl-2. Our observations are in agreement with the mentioned report. In order to explain the Bax/Bcl-2 pathway, Bcl-2 is known as an anti-apoptotic protein embedded in the outer mitochondrial membrane. It can also protect mitochondrial membrane from injuries and prevent translocation of cytochrome-c to the cytosol.[31] In addition, Bax, as an important marker of apoptosis, involves in ZnO NPs-induced apoptosis. In a previous report, we found that ZnO NPs promote apoptosis in rats' liver, where the NPs altered the pro-

tein and gene expression of Bax and Bcl-2.[25] In addition to ZnO NPs, it was reported that nano-copper could affect the expression of apoptotic genes in the rat testicular tissue as the mRNA expression of caspase 3 and Bax was increased, and Bcl-2 expression was downregulated in the 175 mg/kg-treated group.[11]

In addition to the mentioned alterations, our results indicated that ZnO NPs stimulate apoptosis through induction of caspase 3,7. Similar findings were obtained by Tang et al.[30] who reported that ZnO NPs enhance the expression of caspase-3 in the testis of mice, which indicates the activation of apoptosis.

In order to investigate whether VA, C, and E consumption ameliorate the apoptotic potential of ZnO NPs, we explored the apoptosis level in the rat's testicular tissue after co-exposure to ZnO NPs and one of the VA, C, and E. The findings of this study revealed that pre-supplementation with VA, C, and E can attenuate the apoptotic activity of ZnO NPs. In line with these results, Rahimi et al.[32] observed that VE mitigates the incurred testicular injury by inhibiting apoptosis in rats. It was also determined that VE can modulate the impaired ratio of Bax to Bcl-2 in rats' testis treated with cadmium chloride.[33] The results of these reports, together with our results, emphasize that VE can prevent testicular damage in the presence of harmful agents.

The results highlight that VC, as a vital antioxidant, has a preventive influence toward testis injury. This finding concurs with some recent reports which emphasize VC or ascorbic acid has the ability to protect organs from injury by reducing apoptosis-inducing effects of toxicant agents.[34,35]

In the present investigation, the administration of VA exerted ameliorative activity against apoptosis in the rats treated with ZnO NPs. It has been reported that treatment with all-trans retinoic acid (an active derivation of VA) suppresses arsenic-mediated apoptosis. Indeed, the lipophilic nature of the vitamin helps it permeates into the cell membrane and then reduces oxidative stress as well as apoptosis.[36] These findings for vitamin therapy were in agreement with former investigations[37-39] on the efficiency of vitamins under stress conditions. For example, a research study[35] suggested that VC can mitigate the reproductive toxicities caused by nickel NPs.

Taken together, according to the present research and resent papers,[20,25] treatment with VA, C, and E may prevent ZnO NPs-caused toxicity by reducing oxidative damage and apoptosis.

Although some reports have stated the adverse influence of ZnO NPs on the testicular tissue, this research was the first to elucidate the beneficial function of VA, C, and E against apoptosis caused by ZnO NPs in testis of rats. Our results revealed that ZnO NPs could provoke apoptosis in the testis tissue by enhancing the protein and gene expression of Bax as well as a significant decline in Bcl-2. Further, the activation of caspase 3,7 was augmented in ZnO NPs group. Specifically, the administration of VA, C, and E could satisfactorily mitigate these apoptotic effects caused by ZnO NPs. However, further investigations are still required to investigate the possible apoptotic pathways activated by ZnO NPs accompanied by the protective role of vitamins.

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CONFLICT OF INTEREST STATEMENT

None declared.

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