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Evaluation of DNA repair capacity in parents of pediatric patients diagnosed with autism spectrum disorder using the comet assay procedure

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ABSTRACT

Background: Autism Spectrum Disorder (ASD) is characterized by impairments in social communication, limited repetitive behaviors, impaired language development, and interest or activity patterns, which include a group complex neurodevelopmental syndrome with diverse phenotypes that reveal considerable etiological and clinical heterogeneity and are also considered one of the most heritable disorders (over 90%). Genetic, epigenetic, and environmental factors play a role in the development of ASD.
Aim: This study was designed to investigate the extent of DNA damage in parents of autistic children by treating peripheral blood mononuclear cells (PBMCs) with bleomycin and hydrogen peroxide (H2O2).
Methods: Peripheral blood mononuclear cells (PBMCs) were isolated by the Ficoll method and treated with a specific concentration of bleomycin and H2O2 for 30 min and 5 min, respectively. Then, the degree of DNA damage was analyzed by the alkaline comet assay or single cell gel electrophoresis (SCGE), an effective way to measure DNA fragmentation in eukaryotic cells.
Results: Our findings revealed that there is a significant difference in the increase of DNA damage in parents with affected children compared to the control group, which can indicate the inability of the DNA molecule repair system. Furthermore, our study showed a significant association between fathers' occupational difficulties (exposed to the influence of environmental factors), as well as family marriage, and suffering from ASD in offspring.
Conclusion: Our results suggested that the influence of environmental factors on parents of autistic children may affect the development of autistic disorder in their offspring. Subsequently, based on our results, investigating the effect of environmental factors on the amount of DNA damage in parents with affected children requires more studies.

1. Introduction

Autism Spectrum Disorder (ASD) is characterized by impairments in social communication, unusually limited repetitive behavior, interest, or activity patterns, and impaired language development. It includes a group complex neurodevelopmental syndrome with diverse phenotypes that reveal considerable etiological and clinical heterogeneity and is also considered one of the most heritable disorders (over 90%) (Shen et al., 2010).

According to a study by the Center for Disease Control and Prevention's (CDC) Autism and Developmental Disease Monitoring (ADDM) Network, ASD affects approximately one in 59 children by the age of 8. The researchers state that genetic, epigenetic, and environmental factors contribute to the development of ASD (C. Lintas 2008; Kern et al., 2016). In alternative scientific studies, ASD has been represented as a heritable disorder with complex inheritance, genetic heterogeneity, and a remarkable risk of recurrence in siblings (Shen et al., 2010, Markkanen et al., 2016). Nearly 20% of ASD children had genomic abnormalities (chromosomal aberrations, copy number variations, and disruptions of single genes), and 80% of them had balanced

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genomes, indicating that nongenetic elements take precedence in the etiology of ASD (Ivanov et al., 2015; Markkanen et al., 2016). Despite numerous appreciable research efforts, the etiology of ASD is still poorly understood (Lebrun et al., 2018).

However, generally, genetic risk factors and epigenetic dysregulations (microRNA, DNA methylation, and histone modification), and environmental risk factors (drug and toxin exposure, environmental pollutants, and socioeconomic status) are implicated in the etiology of ASD. (Hussain et al., 2019).

Gene expression and DNA damage repair are influenced by both genetic and environmental factors (Azqueta and Collins, 2013). DNA damage repair is the primary stage of cellular defense that protects the integrity of the genome (Singh, 2016, Lebrun et al., 2018). To detect DNA damage (DNA strand breaks), single-cell gel electrophoresis or the comet assay is a sensitive and suitable method. The comet assay provides a rapid, sensitive, visual, and versatile technique for measuring DNA damage in eukaryotic cells (Clingen et al., 2000).

Singh et al. have described an alkaline version of the CA for detecting single-strand breaks (SSBs), double-strand breaks (DSBs), and alkali-labile sites (ALSs; in the alkaline state, ALSs convert to SSBs) in single eukaryotic cells (Gafter-Gvili et al., 2013, Wang et al., 2013).

Although the association between ASD and paternal age is now well established, the effect of parental DNA repair ability and chromosomal aberrations, considering the environmental conditions that they are under the influence of, has not yet been fully understood. (Moeinian et al., 2021).

The present study was established to compare the frequencies of DNA damage in parents with affected children and parents without diagnosed disorders in their offspring using the comet assay.

2. Material and Methods

2.1. Subjects and samples

The case-control study sample comprised 60 parents with an ASD child (25–60 CARS score, maternal mean age: 28.86 ± 4.86 years, and paternal mean age: 34.3 ± 5.21 years) and 30 unrelated healthy parents without diagnosed disorders in their offspring (maternal mean ages: 30.2 ± 6.16 years, and paternal mean age: 34.8 ± 6.43 years) who were referred to the Psychiatric Department of Ali Asghar Hospital, Tehran, Iran, from December 2017 to August 2018. The study was approved by the ethical advisory board of the Iran University of Medical Sciences (IR.IUMS.REC13950.9411294003). All subjects were informed of the aim and experimental details of the study and volunteered to donate blood for sample collection. All blood donors completed a standardized questionnaire that included their age, current health status (e.g., exposure to diagnostic X-rays, particularly during pregnancy in mothers), lifestyle (e.g., smoking, drug dependence, and alcoholic beverages), environmental factors affecting them, and occupational difficulties (e.g., stress, radiation exposure, chemical agent exposure, as well as exposure to indoor and outdoor environmental pollutants).

2.2. Isolation and treatment of human lymphocytes

Isolation and treatment of human lymphocytes Peripheral blood mononuclear cells (PBMCs) were isolated from 3 ml of peripheral blood samples by centrifugation at 2500 RPM for 25 min using 3 ml of Ficoll-Paque with the standard method. The PBMC layer was carefully isolated and washed with phosphate-buffered saline (PBS). Then, isolated PBMCs were counted, and their viability was determined after treatment with trypan blue.

A volume of 75 µl freshly prepared PBMCs (approximately 2 ×105 cells) was transferred to an epi-tube, and PBMCs were then treated with bleomycin (BLM) (25 µl BLM at a concentration of 20 µl/1 ml PBS) and H2O2 (25 µl H2O2 at a concentration of 75 µl/L). BLM, which is a glycopeptide antibiotic used as a chemotherapeutic, induces DNA damage and has antitumor effects. The BLM activity mechanism is the induction of single- and Double-Strand Breaks (SSB and DSB) (Singh et al., 1988).

H2O2 is considered to be a Reactive Oxygen Species (ROS)-inducing DSB (Posar and Visconti, 2017).

2.3. Alkaline comet assay

The alkaline comet assay provides a suitable method for assessing DNA fragmentation and was performed according to the protocol of Singh et al. with modifications (Vorstman et al., 2017).

The Comet tail moments (% DNA in tail × tail length) were determined by fluorescence intensity analysis using CometScore software (TriTek Corp., Sumerduck, VA) to quantify the amount of DNA damage.

2.4. Statistical analysis

We performed data analysis using the Shapiro-Wilk test to assess the normality of the distributions, followed by the Mann-Whitney-U test. Furthermore, we performed a chi-square test to compare the effects of the parents' occupational difficulties, environmental factors, and family marriage on the incidence of autism in children between the case and control groups. $P < 0.05$ level of significance was considered for comparison between groups. All statistical analyses were performed using Microsoft Excel 2010 (Microsoft Corporation) and SPSS version 25 (IBM Corporation).

3. Results

3.1. Effect of treatment on DNA damage

In order to evaluate the DNA repair capacity in parents with an ASD child, in lymphocytes treated with bleomycin and H2O2, DNA damage increased significantly after treatment in cases compared to the control group. Fig. 1 shows the photomicrograph of the comet assay belonging to cell suspensions in controls and patients treated with bleomycin and H2O2. As shown in Fig. 1, DNA damage is visible in the tail. The mean tail moments were collected for each treated sample and normalized to the control value to determine the degree of DNA damage. Our findings indicated a notable difference in tail moments in patients compared to controls. This evidence demonstrated that the DNA damage increased in the case group (Fig. 2). Descriptive statistics of comet tail moment values are shown in Table 1.

3.2. Assessment of the normality of the data distribution

To test the normality assumption for the t-test, the Shapiro-Wilk test was performed (Table 1). Considering 0.05 level of significance, the normality assumption in the control group was rejected for both fathers treated with bleomycin and H2O2 (p-value of 0.025 and 0.010, respectively). Considering the same significance level of 0.05, the normality assumption in the control by mothers treated by bleomycin and H2O2 was also rejected (p-value of 0.006 and 0.002, respectively), which may be due to the small sample size of the control group.

Considering 0.05 level of significance, the normality assumption in the case group is rejected only for the father-bleomycin score (p-value = 0.13). The normality assumption could not be rejected in both control and case groups only for the sum-bleomycin score. Therefore, the sum-bleomycin score is the only variable, fulfilling the necessary assumption of normality for the t-test. For the other scores, nonparametric Mann-Whitney-U test was carried out.

3.3. Evaluating the difference between Tail Moment in the case and control group

Due to the variance heterogeneity of the sum-bleomycin score in two

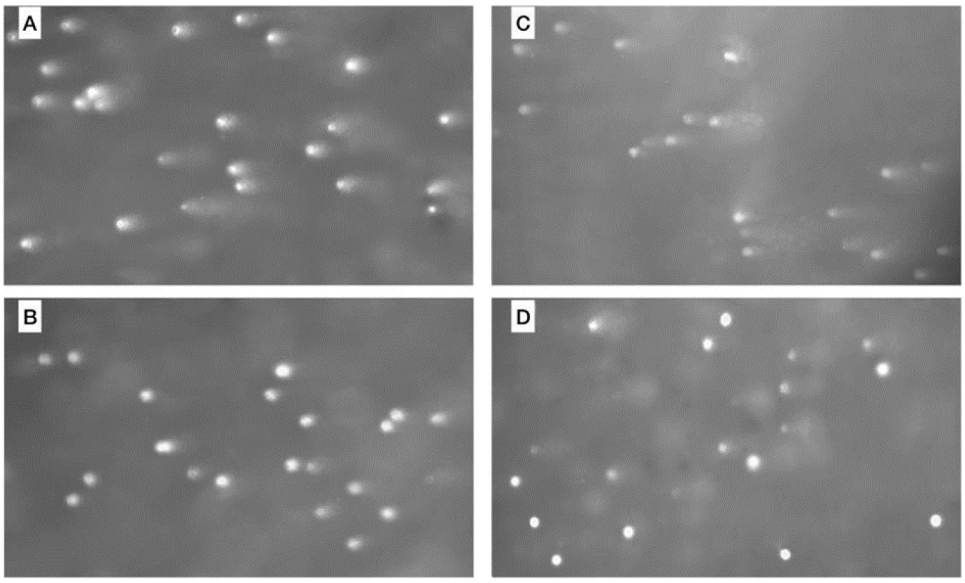

Fig. 1. Photomicrograph of alkaline comet assay of suspended cells under fluorescence microscope. A. Patient, treated with bleomycin. B. Control, treated with bleomycin. C. Patient, treated with H2O2. D. Control, treated with H2O2.

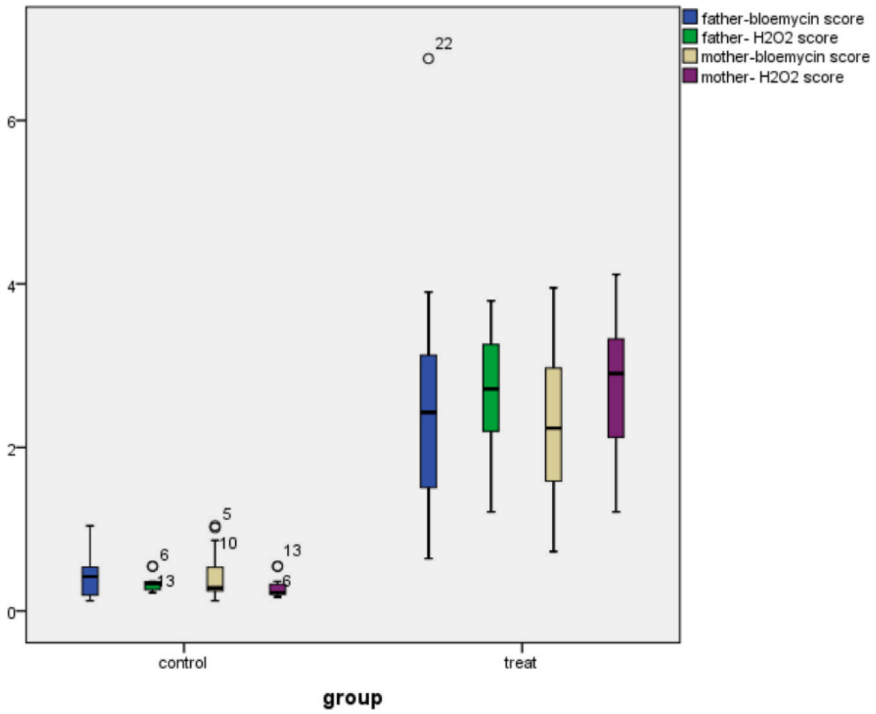

Fig. 2. The effect of bleomycin and H2O2 treatment on case and control groups. The tail moments of the patients are significantly higher than those of the control group.

groups of cases and controls, Welch's t-test was carried out, which is an adaptation of the student's t-test when two samples have unequal variances, as in this study. The sum-bleomycin score mean in the case group was significantly larger than that in the control group (p-value < 0.0001). For the other five variables' scores, which did not follow a normal distribution, the non-parametric Mann-Whitney-U test was performed. As seen in Table 1, all p-values are less than the significance level of 0.05. Therefore, the distributions of all scores were significantly different in two groups (Table 1). We conducted a non-parametric Mann-Whitney-U test for all six variables scores. In this case, we had the following results in Table 1.

3.4. Assessing the association between fathers' occupational difficulties and environmental factors with comet tail moment in the case group

Our study investigated the relationship between fathers' career hardships and environmental factors using the Comet Tail Moment. Based on the obtained results, an independent samples t-test with a P-value of 0.011 and a Spearman rank correlation with a P-value of 0.402 showed a significant relationship between fathers' occupational difficulties (fathers treated with bleomycin) and tail moment.

Table 1
Tail Moment Mean ± standard deviation and standard error mean values of groups after treatment. P value obtained from Mann-Whitney-U test and Shapiro-Wilk test.

	group	Sample size	Tail Moment (Mean)	Std. Deviation	Std. Error Mean	p-value (Shapiro-Wilk test)	p-value (Mann-Whitney-U test)
Father-Bleomycin score	control	15	0.445380	0.3108623	0.0802643	0.025	< 0.0001
	treatment	30	2.483784	1.2471266	0.2276931	0.013	
Father- H2O2 score	control	15	0.332437	0.1014084	0.0261835	0.010	< 0.0001
	treatment	30	2.726142	0.6852050	0.1251008	0.518	
Mother-Bleomycin score	control	15	0.437212	0.3067997	0.0792153	0.006	< 0.0001
	treatment	30	2.242298	0.8816470	0.1609660	0.370	
Mother- H2O2 score	control	15	0.282174	0.1227006	0.0316812	0.002	< 0.0001
	treatment	30	2.733705	0.8072381	0.1473808	0.252	
Sum-Bleomycin score	control	15	0.8826	0.52018	0.13431	0.113	< 0.0001
	treatment	30	4.7261	1.91530	0.34968	0.794	
Sum-H2O2 score	control	15	0.6146	0.21374	0.05519	0.001	< 0.0001
	treatment	30	5.4598	1.31778	0.24059	0.365	

3.5. The association of fathers' career difficulties and environmental factors with the onset of autism in children

To analyze the association between the comet tail moment in parents and the incidence of autism in children in two groups treated with Bleomycin and H2O, a Mann-Whitney-U test was conducted between case and control groups of parents with occupational difficulties. Since p-values were less than 0.001, there was a significant association between parents' occupational difficulties and environmental factors with occurrence of autism in offspring (Fig. 3).

4. Discussion

Autism spectrum disorders (ASDs) are neurodevelopmental conditions characterized by impairments in social interactions and communication and restricted, repetitive and stereotyped patterns of behavior that typically.

emerge in the first few years of life. Autism typically manifests by the age of three and persists throughout a person's lifetime. (Tanner and Dounavi, 2021).

Despite studies have been conducted on, the etiology of ASD has not yet been fully determined. Etiopathogenesis may be caused by a wide range of factors, including genetic, epigenetic, and environmental factors (Posar and Visconti, 2017). Genetic studies have shown that hundreds of genes and DNA repair variants play a role in ASD (Vorstman et al., 2017). As the impact of new mutations leading to autism spectrum disorder has been reported, the reduction of repair mechanisms in DNA molecules in parents accelerates the possibility of new mutations in offspring. Gene expression and DNA damage repair are under the influence of these genetic and environmental factors (Lebrun et al., 2018). DNA damage can result from exposure to either exogenous (environmental) or endogenous (oxidative) factors (Markkanen et al., 2016).

Oxidative stress is known to be a major genotoxic agent, inducing single- and double-stranded DNA breaks (Porokhovnik et al. 2016).

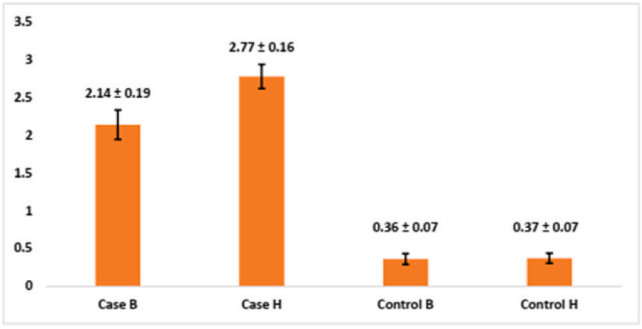

Fig. 3. Tail moment in case and control group fathers experiencing occupational difficulties and under the influence of environmental toxins.

The pathogenesis of some serious diseases, such as ASD, depends on the extent of DNA damage and ineffective repair mechanisms. The comet assay is a suitable, rapid, sensitive, visual, and versatile method for assessing DNA damage and repair (Burlinson et al., 2007), and investigating oxidative damage in peripheral blood mononuclear cells (Collins, 2002, Collins, 2014).

In the current study, we aimed to investigate the degree of DNA damage in parents of autistic children, and considering that previous studies demonstrated that a significant percentage of parents with autistic children in Iran are exposed to factors that cause DNA damage, the Comet assay has been approved to explore the amount of damage.

In 2016, Russian researchers investigated the extent of DNA damage in mothers with affected children (Porokhovnik, Kostyuk et al., 2016). The result showed that the degree of DNA damage increased significantly after treatment in parents of autistic children compared with control subjects. In other words, the repair system in control subjects is more efficient than in the case group. Our results have similarity with other studies conducted previously. Several studies investigated the association of DNA repair, genotoxic, and environmental factors and autism and found some DNA repair-related genes known to be associated with autism spectrum phenotype, such as *OGG1, BRCA2, FAN1, MBD4, MUTYH*, and *XRCC4* (Li et al., 2005, Sajdel-Sulkowska et al., 2009, Cukier et al., 2010, Ionita-Laza et al., 2014, Shpyleva et al., 2014, 2016, Markkanen et al., 2016, Benitez-Burraco, 2018). Muraleedharan et al. examined DNA damage and DNA repair efficiency in schizophrenia using the Comet assay, and their results showed significant differences between study groups (Muraleedharan et al., 2015). The result of a study conducted by Bjørge et al. using a mouse model in which the DNA glycosylases Ogg1 and MUTYH play a role in the repair of 8-oxo-guanine (8-oxo-g) DNA damage lacked expression; stated lack of expression of these two glycosylases induces anxiety behaviors (Markkanen et al., 2016).

The obtained results from collected DNA samples from the cerebellum of autistic mouse models and autistic individuals Both showed increased levels of 8-oxo-hydroxyguanine, 5-methylcytosine, and 5-hydroxymethylcytosine. The increase in 8-oxo-hydroxyguanine and 5-methylcytosine levels is significantly related to the decrease in *8-oxo-guanine DNA-glycosylase 1 (Ogg1)* gene expression. These findings demonstrate that further research is needed to determine *OGG1* role in autism pathogenesis (Shpyleva et al., 2014).

Investigating oxidative damage by measuring 8-hydroxydeoxyguanosine (8-OH-dG) using the ELISA method showed 8-OH-dG was significantly increased in autistic individuals (Sajdel-Sulkowska et al., 2009).

The association between oxidative stress and DNA damage is well established. Besides, examination of autistic brain tissues showed more oxidative stress compared to controls. Consequently, a study of polymorphisms in *XPD* and *XRCC4* (repair system) genes in the Turkish population indicated a significant association between the *XRCC4* gene and autism, but no significant association was found between *XPD* and

autism patients (Dasdemir et al., 2016).

At different stages of human development, the nervous system is exposed to various types of damage, which is repaired by repair systems. In a study conducted by Khan et al., a four-year-old Korean child with xeroderma pigmentosa had a mutation of the *XPC (Xeroderma pigmentosum Group C-Complementing Protein*) gene that was examined but did not show the neurological symptoms that were part of the disease phenotype. Neurological examinations revealed that this patient shows autistic features with hyperactivity. Results obtained from fibroblast cell culture showed a repair system defect, which shows an association between mutation of the *XPC* gene and autistic features (Khan et al., 1998, Quackenbush et al., 1999).

Microcephalin (MCPH1) is another gene that plays a role in DNA repair and DNA damage checkpoints and, based on reports, is involved in the occurrence of autistic characteristics (Singh et al., 2012).

Disparate studies show different etiologies of autism-causing factors. Examination of microdeletions in 15q13.3 region, which contained *FAN1*, which plays a role in DNA interstrand cross-link repair, by using *FAN1* knocked-out mouse models revealed mice represent autism-related phenotypes (Forsingdal et al., 2016). Another study included two different whole-exome sequencing datasets in autism spectrum disorder patients who identified variants in *FAN1*, concluded this region is related to neurological phenotypes and mental disorders (Ionita-Laza et al., 2014). *MBD4* is another gene that plays a role in both the repair process and the development of autism and belongs to the methyl-CpG binding domain (*MBD*) gene family (which includes *MECP2, MBD1, MBD2, MBD3*, and *MBD4*). Studies in different populations have proven the association of this gene family with autism (Li et al., 2005, Cukier et al., 2010). *BRCA2* is another gene involved in maintaining the stability of the genome, especially in the repair of double-strand breaks through the homologous recombination pathway. Studies have shown mutations in this gene are related to autism phenotypes (Neale et al., 2012, Benitez-Burraco, 2018).

4.1. Conclusion

As we mentioned earlier, oxidative stress is one of the genotoxic factors that play a role in the pathogenesis of autism as single- and double-strand breaks are causative in DNA. In a study conducted in 2016 on autistic patients and their mothers, the amount of DNA damage was determined by the Comet test. The results showed that autistic patients have more DNA damage than healthy children of the same age, and the mothers of autistic patients have more DNA damage than healthy mothers who are at the right age for pregnancy. According to results, there are genotoxic factors in mentally healthy mothers with autistic children, which is known as the maternal effect, which can determine the etiology process in the fetus and is one of the ASD-causative factors in offspring (Porokhovnik et al. 2016).

Other authors have found a correlation between the degree of DNA damage and autism. As earlier stated, oxidative stress is considered a genotoxic agent that plays a role in the pathogenesis of ASD. In 2016, Porokhovnik et al. investigated the degree of DNA damage caused by oxidative stress in autistic children and their parents using comet assay. Their result showed significantly increased DNA damage in autistic children and their mothers compared to controls (Porokhovnik et al. 2016).

In general, it can be concluded that lifestyle, workplace, father's occupational difficulties, and exposure to genotoxic and chemical agents lead to increased DNA damage or decreased capacity of the DNA repair system that may be considered pathogenic in autistic children.

CRediT authorship contribution statement

Mansoureh Akouchekian: Supervision, Conceptualization, Methodology, Data curation. **Rasoul Alizadeh:** Investigation, Writing – original draft. **Fatemeh Beiranvandi:** Writing - original draft. **Masoumeh Dehghan Manshadi:** Writing – review & editing. **Fatemeh Taherizadeh:** Data curation, Software, Validation. **Mitra Hakim Shooshtari:** Visualization, Investigation.

Declaration of Competing Interest

There is no conflict of interest between authors.

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Compliance with ethical standards

The study was approved by ethical advisory board of Iran University of Medical Sciences (IR.IUMS.REC 1395.28660).

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