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Antioxidant Enzyme Responses in Cladoceran *Moina macrocopa* under Oxidative Stress

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Oxygen is the cornerstone of the life of all living organisms, as it plays a pivotal role in regulating the bio-physiological functions of the organism that are necessary to sustain its existence. Oxidative stress/ hypoxia is a condition of inadequate oxygen that disrupts the harmony of an organism's life by impairing its growth, puts the organism's survival at risk, and destabilizes the ecosystem. Like all other organisms *Moina,* a small cladoceran is susceptible to the alteration in dissolved oxygen levels. Here we report that, in response to hypoxia, *Moina* adjusts their antioxidant enzyme activities as a mechanism of adaptation. Significant changes were observed in the levels of LDH, GST, and SOD antioxidant enzymes of *Moina* under hypoxia *(p=0.001053,* GST *p=0.0010053,* SOD *p=0.0015).* These results can contribute to wider research on environmental stress tolerance by aquatic organisms and will assist in the conservation of species like *Moina. Moina,* being an ecological indicator, this research will help in environmental monitoring using it as a model organism.

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Keywords: Hypoxia; Moina; antioxidant enzymes; oxidative stress; aquatic ecology.

ABBREVIATIONS

DCW : Dechlorinated Water
DO : Dissolved Oxygen
MOC : Mustard Oil Cake
ROS : Reactive Oxygen Species)
BSA : Bovine Serum Albumin)
LDH : Lactate Dehydrogenase)
NADH : Nicotinamide Adenine Dinucleotide Hydrogen)
CAT : Catalase
H_2O_2 : Hydrogen Peroxide
NBT : Nitro Blue Tetrazolium
EDTA : Ethylene Diamine Tetra Acetic Acid
GSH : Reduced Glutathione)
CDNB :1-chloro2,4 Dinitrobenzene
S-2,4 : Dinitrophenyl Glutathione (DNP-SG)
O.D : Optical Density
ε : Molar Extinction Coefficient
mM :Millimolar
nm : Nanometer

1. INTRODUCTION

Oxygen plays a key role in various physiological processes like cellular respiration, metabolism, blood circulation, etc., and therefore is a vital molecule for the survival of all living organisms (Wang, J, & Zhan, Z. 2014; Semenza, G. L. 2011). Terrestrial organisms can easily avail the oxygen available in the atmosphere. But aquatic organisms often have to confront the challenge called “oxidative stress” or “hypoxia”; as they rely on the Dissolved Oxygen (DO- Oxygen Dissolved In water) for their survival (Diaz, R. J., & Rosenberg, R. 2008). Oxidative stress or hypoxia is a condition that can result from the imbalance between the production of reactive oxygen species and the organism’s inability to neutralize them with antioxidant defenses. This imbalance leads to cellular damage (Davies, 2000; Birben et al., 2012). It has been reported that oxidative stress or hypoxia can stimulate ROS production and lead to oxidative damage (Livingstone, 2001; Lushchak, 2011; Halliwell & Gutteridge, 2007; Moller et al., 2007). To alleviate the effect of oxidative damage, the organisms lean to the array of antioxidant enzymes like Lactate Dehydrogenase (LDH), Catalase (CAT), Glutathione S Transferase (GST), Superoxide Dismutase (SOD). The activities of these antioxidant enzymes play an essential role in maintaining cellular health under stressed conditions (Nakamura et al., 2010; Samarakoon et al., 2023; Samarakoon and Fujino;).

It is known that Lactate Dehydrogenase (LDH), a key enzyme that contributes to anaerobic metabolism by converting the pyruvate to lactate under oxygen depletion conditions and helps the cells to navigate through the challenge of oxidative stress (Meyer et al., 2011). On the other hand, catalase and Superoxide Dismutase (SOD) are involved in neutralizing hydrogen peroxide and superoxide radicals respectively, thereby reducing oxidative damage (Mandel et al., 1998; Do et al., 2024; Samarakoon et al., 2023; Bouchnak and Steinberg, 2014). Glutathione S Transferase (GST) contributes by conjugating the reactive electrophiles to glutathione, facilitates the detoxification of ROS, and prevents cellular damage (Hayes & Pulford, 1995). Collectively, these enzymes constitute an essential defense system against oxidative stress. Therefore, we aimed to study these enzymes and evaluate their activity in *Moina* under normoxic and hypoxic conditions. *Moina*, a freshwater cladoceran, serves as forage for fishes hence commercially used as fish feed (DeMott, 1998). It is also an integral part of the aquatic food chain (Müller-Navarra et al., 2000) and serves as an ecological indicator of the aquatic body as it is sensitive to alterations in abiotic parameters like salinity, pH, temperature, etc. (Barata et al., 2006, Arnot et al., 2008). The model organism *Moina* is commonly used in aquatic toxicity studies because of its sensitivity towards pollutants, toxicants, chemical agents, and drugs (Sharma et al., 2017). Considering the ecological, economical, and research-based significance of *Moina,* it is essential to understand the effect of hypoxia on the antioxidants and their regulation. This will aid in evaluating how the organism adapts to environmental challenges. We have estimated the activities of LDH, CAT, GST, and SOD in *Moina* under oxidative stress conditions that indicate the roles of antioxidant enzymes in protecting the organism from oxidative damage. This research is important to study *Moina’*s biochemical responses to stress and offers valuable insights into broader ecological implications of oxidative stress in aquatic ecosystems. It will also help in environmental monitoring using *Moina* as a model organism.

2. MATERIALS AND METHODS

2.1 Chemicals

NADH, Sodium pyruvate, Tris base, Hydrogen Peroxide, Potassium phosphate buffer, EDTA (Ethylene Diamine Tetra Acetic Acid), NBT (Nitro Blue Tetrazolium), Riboflavin, GSH (Reduced glutathione), CDNB (1-chloro2,4 Dinitrobenzene). All the chemicals were of analytical grade and were obtained from SRL Chemicals, Mumbai.

2.2 Culturing *Moina.*

Moina macrocopa were procured from CUBE, HBCSE, Mumbai, and species identification was confirmed by Bhanushali et al. (2021). *Moina* were cultured in an earthen pot with a capacity of fifteen liters. Dechlorinated water (DCW), (obtained by exposing tap water to the atmosphere for 2-3 days), with an average pH of 7.9 ± 1.5, was used for culturing *Moina*. *Moina's food* was prepared by mixing dung manure and mustard oil cake (MOC) in a 4:1 ratio. This mixture was decomposed for seven days and filtered before being used as food (Chakrabarti, 2017). In an earthen pot containing ten liters of dechlorinated water, approximately 200 *Moina* were inoculated and fed with 500 mL of the prepared food. The experimental setup was maintained under a 12:12 hour light-dark cycle. Organisms were separated based on their size using sieves with varying pore sizes as and when needed and were subsequently used for the experiment.

2.2.1 Induction and optimization of hypoxia and normoxia

To establish normoxic conditions, culture media was aerated using an aerator (RS390, 220volt). In contrast, hypoxia was induced in the culture by stopping aeration, Dissolved oxygen (DO) levels were monitored daily using a digital DO meter (Spectrum, SI214). To maintain the desired DO level, a small portion of the culture medium was replaced with fresh DCW. For normoxia, the DO levels were maintained at 8.0-8.5 mg/lit whereas for hypoxia, it was maintained at 1.5-2.2 mg/lit.

2.3 Sample Preparation

Adult *Moina* were isolated from the culture using a sieve/fine mesh strainer of approximate pore size 0.2mm to 0.5mm. The collected organisms were pooled for subsequent analysis. Approximately 0.04 to 1 g of *Moina* were homogenized in a 100 mM phosphate buffer (pH 7.5) containing 100 mM KCl and 1 mM EDTA (1:4, w/v). The homogenate was centrifuged at 10,000 x g for 10 minutes at 5°C. The supernatant was collected and immediately used for enzyme assays (Barata et al., 2005). All the biochemical experiments were conducted at 25^{o}C ±2^{o}C, unless stated otherwise.

2.4 Antioxidant Enzyme Analysis

Biochemical measurements were performed using a single-beam spectrophotometer (Bio-era). The experiment was performed in quintuplicate to minimize variability and ensure statistical robustness. The chicken liver homogenate was a positive control to confirm the functionality of the assay. Protein concentrations in the samples were determined using the Lowry et al. (1951) method with Bovine Serum Albumin (BSA) as the standard.

2.4.1 Lactate Dehydrogenase Assay (LDH)

Lactate dehydrogenase (LDH) activity was determined by monitoring the decrease in absorbance at 340 nm, corresponding to the oxidation of NADH (ε340 = 6220 $M^{-1}cm^{-1}$) as described by Worthington (1972). One unit of LDH activity is defined as the amount of enzyme required to oxidize one micromole of NADH per minute. The reaction mixture of 3.0 ml consisted of 0.2 M Tris buffer (pH 7.3), 1.6 mM NADH, and homogenate prepared from normoxic and hypoxic *Moina*. Absorbance changes at 340 nm were recorded using a single-beam spectrophotometer (Bio Era) for both normoxic and hypoxic conditions. Enzyme activity (U/mL) and specific activity (U/mg protein) were calculated, and the results were compared.

2.4.2 Catalase Assay (CAT)

Catalase (CAT) activity was determined by monitoring the decrease in absorbance at 240 nm, which corresponds to the consumption of hydrogen peroxide (H_2O_2) (Ni et al., 1990). Here, one unit of CAT activity is defined as the amount of enzyme required to decompose one micromole of H_2O_2 per minute under specified conditions at 25°C. The reaction mixture prepared was 3.0 ml, containing 0.05 M phosphate buffer (pH 7.2), 50 mM hydrogen peroxide, and the homogenate. The change in the absorbance was monitored and enzyme activity and specific activity were calculated.

2.4.3 Superoxide Dismutase (SOD) Assay

Superoxide dismutase (SOD) activity was measured based on the enzyme's ability to inhibit the reduction of Nitro Blue Tetrazolium (NBT). The absorbance was measured at 560 nm. Here, One unit of SOD activity is defined as the amount of enzyme required to achieve 50% inhibition of NBT reduction (Worthington 1993). Enzyme activity was calculated based on the percentage of inhibition of reduction of NBT. The control (enzyme blank) consisted of 1.5 mM NBT, 0.1 M EDTA containing 0.3 mM sodium cyanide, and 0.067 M phosphate buffer (pH 7.8). The total reaction mixture (3.0 mL) was exposed to sunlight for 10 minutes, and the absorbance was measured. Similarly, the assay was carried out for the samples obtained from normoxic and hypoxic *Moina*. Percent inhibition was calculated using the formula;

O.D Blank- O.D of Sample (Normoxic/hypoxic) ×100 / O.D Control

Based on the percent inhibition obtained, the enzyme activity was calculated by using the formula;

% Inhibition / 50 × Dilution Factor

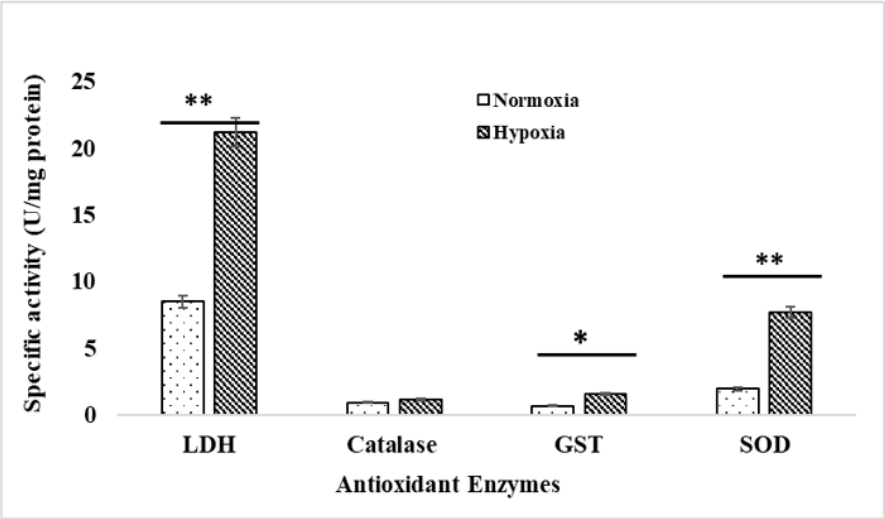

Fig. 1. Effect of oxidative stress on antioxidant enzyme activities in normoxic and hypoxic *Moina*

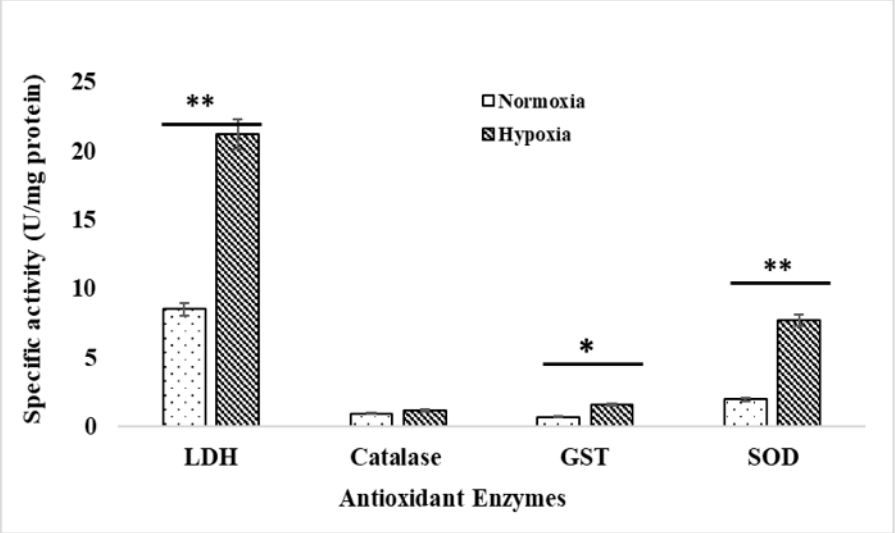

Fig. 2. Effect of oxidative stress on the specific activity of antioxidant enzymes in normoxic and hypoxic

LDH p=0.001053 (p < 0.01), catalase p=0.0044(p < 0.01), SOD p=0.0015(p < 0.01); GST p=0.0010053 (p<0.01)

2.4.4Glutathione-S-Transferase Assay (GST)

The activity of Glutathione S-transferase (GST) towards its substrate 1-chloro-2,4-dinitrobenzene (CDNB) was determined using a method established by (Borgeraas & Hessen, 2002). Here, The formation of the product, S-2,4-dinitrophenyl glutathione (DNP-SG) conjugate, was monitored by measuring the increase in absorbance at 340 nm. The reaction mixture 3.0 ml contained a high concentration (100 mM) of CDNB and a lower concentration (1 mM) of its co-substrate, reduced glutathione (GSH). Further, based on a change in the absorbance, the enzyme activity and specific activity were calculated.

2.5 Statistical Analysis

All measurements were performed in quintuplet and the results reported as means ± S.E.M. analysis of variance (one way ANOVA) followed by Tukey's post-hoc multiple comparison tests were performed to determine the effect of hypoxia on each antioxidant enzyme studied. Significant differences were established at $P<0.05$.

3. RESULTS AND DISCUSSION

We observed that *Moina macrocopa* exhibits notable biochemical adaptability to low-oxygen environments. Under hypoxia, the activities of antioxidant enzymes, specifically enzymes LDH, GST, and SOD levels were substantially increased. In this section, results are presented and discussed thoroughly.

LDH, the crucial enzyme involved in metabolism and responsible for the interconversion of pyruvate to lactate. This key reaction Pyruvate□lactate and vice versa plays a vital role in anaerobic metabolism and LDH levels increase under low oxygen concentrations (Sawada et.al 2023). LDH is known to demonstrate the highest change in the cells kept for several days under hypoxic conditions (Marti et al 1994). Our results are consistent with these established ideas as LDH activity under hypoxic conditions (12.69 U/ml) was 28% higher than that in normoxic condition (12.69 U/ml) (Fig. 1). The specific activity of LDH increased by approximately 2.5 fold in the hypoxic *Moina* (21.25 U/min/mg protein) as compared to that in the normoxic *Moina* (8.53 U/min/mg protein). A similar effect of hypoxia has also been reported in studies using root tissue (Hoffman et al., 1986). Hence, we can conclude that mechanistic response to hypoxia involving LDH is a universal phenomenon in plants and animals. In contrast, it was found to be increased to 21.25 U/min/mg protein in hypoxic *Moina* (Fig. 2). Tukey's post-hoc test revealed significant differences in LDH activity between normoxic and hypoxic conditions*, p=0.001053* ($p < 0.01$). This elevation in the enzyme activity and specific activity indicates cellular stress in *Moina.* A rise in LDH activity occurs as a result of oxidative stress, signifying a transition to anaerobic metabolism (Hochachka et al., 1993, Gorr et al., 2010). Our results are consistent with similar observations in aquatic species like *Daphnia magna (Malek, 2022).* Therefore, we say that To survive under oxygen-deprived conditions, it is essential for organisms to undergo such metabolic adaptations, which aid in energy conservation and production when oxygen is scarce.

Catalase, an essential antioxidant enzyme that plays a vital role in protecting cells from oxidative damage caused due to oxidative stress (Becker et al., 2011). We observed that normoxic *Moina* exhibited catalase activity of 1.40 U/ml, as compared to 1.48 U/ml (Fig. 1) in *Moina,* under hypoxic conditions. However, the marginal difference in the catalase activity under both conditions was not reflected in the specific activity of the enzyme. The specific activity was 0.98 U/min/mg and 1.5 U/min/mg for normoxic and hypoxic *Moina* respectively (Fig. 2). Tukey's post-hoc test revealed moderately significant differences in catalase activity between normoxic and hypoxic conditions*, p=0.044* ($p < 0.05$). Increased catalase activity under hypoxia, indicates *Moina's* ability to neutralize hydrogen peroxide thereby minimizing the oxidative damage caused by the production of Reactive Oxygen Species (ROS). It is consistent with widely noticed adaptive responses across different species as a strategy to maintain cellular redox balance (Ahmad, 1995; Chance & Greenstein, 1992). For eg. In marine crab *Scylla serrata* and freshwater crustacean *Daphnia magna* showed a rise in CAT activity when exposed to hypoxia and thermal stress *(Im* et al*., 2020, Becker* et al*., 2011)*. This highlights a shared antioxidant defense mechanism to combat oxidative stress and also shows that the upregulation of catalase is vital for neutralizing ROS, which ensures the continued function of vital physiological processes under oxygen-deprived conditions.

Superoxide dismutase (SOD), is a crucial antioxidant enzyme that catalyzes the conversion

of superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) to prevent cells from oxidative damage caused by Superoxide radicals (ROS: Reactive Oxygen Species) produced during hypoxia (Lee et al., 2024). We found that normoxic *Moina* exhibited the SOD activity of 10.2U/ml whereas in hypoxic *Moina* it was found to be 18.6 U/ml (Fig 1) which indicates an approximate 80% rise in the enzyme activity. This rise in the SOD activity indicates that the inhibition of ROS produced was high in hypoxic *Moina* as compared to the normoxic *Moina*. The specific activity of SOD in normoxic *Moina* was found to be 2U/min/mg protein, in contrast, in hypoxic *Moina* it was observed as 7.75 U/min/mg protein (Fig. 2). Tukey's post-hoc test revealed significant differences in specific activity of SOD between normoxic and hypoxic conditions, $p=0.0015$($p < 0.01$). The notable difference in SOD activity between hypoxic and normoxic *Moina* suggests an adaptive response in low-oxygen environments. The enhanced antioxidant activity likely plays a key role in safeguarding the cellular structure from oxidative damage caused by the ROS. Our results align with the reports where elevated SOD activity has been suggested as a mechanism to confront the effect of oxidative stress (Snyder et al., 2004, Im et al., 2020). This ensures that in *Moina*, elevated SOD activity plays a crucial role in coping with oxidative stress and prevents it from oxidative damage.

Glutathione-S-Transferase, a key antioxidant enzyme that helps to neutralize the ROS and other electrophilic molecules generated during oxidative stress by forming a glutathione adduct (R-SG) (Michalaki et al., 2022). We observed the GST activity of 2.23 U/ml for normoxic *Moina.* In contrast, in hypoxic *Moina* GST activity was found to be 3.39 U/ml (Fig. 1) which indicates an approximate 52% rise. The specific activity of GST in normoxic *Moina* was 0.74 U/min/mg protein and for hypoxic *Moina* it was found to be 1.65 U/min/mg protein (Fig. 2). As per post-hoc Tukey's test, normoxic and hypoxic *Moina* exhibit a significant difference in the specific activity of GST at $p=0.0010053$ ($p<0.01$). An increase in the GST level reflects an organism's response to oxidative stress, as GST plays an essential role in detoxifying reactive metabolites and shields the cell from oxidative damage. An elevated GST level indicates an adaptive response aimed at neutralizing harmful compounds under stressful conditions. Comparable alterations in GST from 29% to 52 % have been reported in cladoceran, *Daphnia magna,* and other species of *Daphnia,* where enhanced GST activity is linked to combined exposure to low food, thermal stress, and pollutants (Michalaki et al., 2022, Sánchez et al., 2008, Im et al., 2020). Similar increased GST levels have been reported earlier in mammals like rats. Wherein, elevated GST levels have been correlated with the reduction in the free radicals and cellular damage (Snyder et al., 2004).

Collectively, We can say that antioxidant enzymes LDH, Catalase, SOD, and GST play vital roles in the adaptation of *Moina* towards hypoxia and aid in its survival.

Protein concentration measurements, performed using the Lowry method (Lowry et al., 1951), revealed that normoxic *Moina* had an average protein concentration of 1.35 mg/ml. In contrast, hypoxic *Moina* exhibited a lower protein concentration of 0.865 mg/ml. The observed reduction in protein concentration under hypoxia (from 1.35 mg/ml to 0.865 mg/ml) aligns with earlier reports that hypoxia often leads to reduced protein synthesis and turnover as part of metabolic suppression in *Daphnia (Im* et al*., 2020)*. Such suppression helps conserve energy for critical survival processes. The altered protein level evidences a clear physiological response to oxygen availability. It also depicts that under normoxia, the organism is efficiently synthesizing the proteins required to accomplish the cellular functions and metabolism of the organism (Görlach et al., 2015). However, the decreased protein concentration under hypoxia could be the result of the reallocation of energy towards survival strategies, like shifts towards anaerobic metabolism. This reduction in protein synthesis can be a consequence of the transition from protein production to the activation of antioxidant defense mechanisms and other protective pathways to cope with oxidative stress (Zhang et al., 2019). Moreover, the diminished protein concentration might signify protein breakdown as an integral aspect of adaptation to conserve energy and achieve cellular equilibrium in oxygen-deficient environments.

4. CONCLUSION

Based on the findings of this study we conclude that under oxidative stress, *Moina* exhibits altered antioxidant enzyme levels and protein concentrations to alleviate the effects of hypoxia. This may eventually help *Moina* to counteract the effects of oxidative damage caused by ROS production, thereby maintaining cellular

homeostasis in *Moina* under oxygen deprived conditions.

5. FUTURE PERSPECTIVE

Future research on *Moina* can be focused on investigating physiological adaptations to low oxygen concentrations. One can scrutinize the mechanism with which an organism is switching from an aerobic metabolic pathway to an anaerobic metabolic pathway, under oxygen-deprived conditions. Moreover, multi-omics approaches like epigenomic, genomic, and proteomic approaches can enhance our understanding of molecular and biochemical networks underlying the tolerance to hypoxia. Lastly, we say that due to the impressive ability of *Moina* to survive under depleted oxygen levels, it has great potential as an indicator species in freshwater ecosystems. Assessing the organism's response to fluctuating oxygen levels allows for accurate evaluation of water quality and provides valuable insights into the health of aquatic ecosystems, this will help in the conservation of both the aquatic body and the organism.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

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Details of the AI usage are given below:

1. Chat Gpt: For sentence framing, vocabulary
2. perplexity: For cross referencing.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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