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Asian Journal of Plant Science and Research, 2014, 4(4):74-81

ISSN : 2249-7412
CODEN (USA): AJPSKY

The effect of melatonin and minocycline on *Drosophila melanogaster* subjected to paraquat-induced oxidative stress

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ABSTRACT

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and the ability of a biological system to readily detoxify the reactive intermediates or to repair the resulting damage. Antioxidants remove free radical reactive intermediates and terminate the oxidation chain reactions. Melatonin is a small indole amine, secreted rhythmically with increased synthesis during the dark phase of the lighting cycle. Melatonin is an antioxidant and can detoxify highly reactive oxygen radicals. Minocycline is a broad spectrum, second generation tetracycline antibiotic, reported to possess antioxidant properties with radical scavenging potency. Paraquat is a widely used herbicide, which catalyzes the formation of reactive oxygen species (ROS), more specifically the superoxide free radical. Drosophila melanogaster is a widely used model organism. It is sexually dimorphic with a high fecundity and short generation time. The wild type flies (p generation) were separated into 4 groups and cultured under the stated conditions. The F1 adults of each group were subjected to treatment with the herbicide, Paraquat and the survival rate was determined. Melatonin in isolation was shown to be more effective in reducing the effects of Paraquat-induced oxidative stress. The female flies of each group were also reported to more resilient to stress and also scored higher in the locomotor assays.

Key words: oxidative stress, melatonin, minocycline, paraquat, reactive oxygen species.

INTRODUCTION

Oxygen is potentially a toxic substance, and its use by aerobes, although necessary for their immediate survival, may also be hazardous to their long-term existence. The phenomenon of oxygen toxicity, sometimes referred to as the "oxygen paradox" is inherent in the atomic structure of oxygen. Molecular oxygen is a biradical that upon single electron additions sequentially generates the partially reduced molecules O_2^-, H_2O_2 , and OH ,which by further reactions can generate an assay of additional reactive oxygen metabolites (ROM) or reactive oxygen species (ROS) and cause extensive oxidative damage to biological macromolecules([1], [2].

1.1. Cellular Defense against ROS

Cells have a variety of defense mechanisms to ameliorate the harmful effects of ROS. Superoxide dismutase (SOD) catalyzes the conversion of two superoxide anions into a molecule of hydrogen peroxide and oxygen. In the peroxisomes located next to the mitochondria of eukaryotic cells, the enzyme catalase converts H_2O_2 to water and oxygen and thus completes the detoxification initiated by SOD.

1.2. Oxidative Stress and Antioxidants.

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. In the other words, oxidative stress is an imbalance of pro-oxidants and antioxidants, which can be quantified in humans as the redox state of plasma GSH/GSSG [3]. Oxidative stress is suspected to be involved in neurogenerative diseases, cardiovascular disease, age-related diseases. However, reactive oxygen species can be beneficial, as they are used by the immune system to attack and kill pathogens [4]. Further, short term oxidative stress may also be important in the prevention of aging by induction of a process named mitohormesis [5].

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen form a substance to an oxidizing agent, resulting in production of free radicals. In turn , these radicals can start chain reactions. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions [6]. Plants and animals maintain complex systems of multiple types of antioxidants such as glutathione, melatonin, vitamin C, vitamin A and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxides.

1.3. MELATONIN

Melatonin, a hormone secreted by the pineal gland, is an antioxidant which protects cytosolic and nuclear macromolecules from free radical cytotoxicity. Melatonin (MEL) (N-acetyl-5-methoxytryptamine) is a small indoleamine with chemical formula $C_{13}H_{16}N_2O_2$ (molecular weight 278g/mol), secreted rhythmically with increased synthesis during the dark phase of the lighting cycle [7].

Melatonin can detoxify highly reactive oxygen radicals, without undergoing redox cycling. Redox cycling may allow other antioxidants(such as vitamin C) to act as pro-oxidants, counter intuitively promoting free radical formation. Melatonin is referred to as a terminal or suicidal antioxidant because once oxidized it cannot be reduced to its former state as it forms several stable end products on reacting with free radicals [8]. Melatonin as a natural antioxidant , can serve as a suitable indicator of the dark period [9]. The production of melatonin, decreases with age [10] and it is suggested that maintaining melatonin at a high level could slow age related alterations. In addition to scavenging reactive oxygen species, melatonin stimulates the activity of antioxidant enzymes superoxide dismutase, glutathione, peroxidase and catalase.

1.4. MINOCYCLINE

Minocycline, a broad spectrum tetracycline antibiotic, is reported to possess antioxidant properties with radical scavenging potency. It is a bacteriostatic antibiotic, classified as a long-acting type. It is the most lipid-soluble of the tetracycline class antibiotics. Minocycline is not a naturally occurring antibiotic, but was synthesized semi-synthetically from natural tetracycline antibiotics by Lederle laboratories in 1972 and marketed under the brand Minocin. [11].

Minocycline is reported to possess antioxidant properties with radical scavenging potency similar to vitamin E [12]. Minocycline along with other known antioxidants such as tocopherol showed direct radical scavenging activity, proposed to be due to the presence of phenolic ring capable of reacting with free radicals, leading to the formation of relatively stable and unreactive phenol derived free radicals [13]. Studies by [14] strongly support a similar ability of minocycline in whole organism studies under variety of oxidative stress paradigms.

1.5. PARAQUAT

Paraquat is the trade name for N,N'-dimethyl-4,4'-bipyridinium dichloride, one of the most widely used herbicides in the world. Paraquat catalyzes the formation of reactive oxygen species (ROS), more specifically the super oxide free radical. It undergoes redox cycling *in vivo* being reduced by an electron donor such as NADPH, before being oxidized by an electron receptor such as dioxygen to produce super oxide, a major reactive oxygen species. The super oxide anion can lead to the synthesis of more toxic reactive oxygen species such as hydroxyl radicals and hydrogen peroxide. On the other hand, the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a consequence of paraquat administration results in the disruption of biochemical process requiring NADPH [14].

1.6. Drosophila melanogaster

Drosophila melanogaster commonly referred to as the fruit fly is used as the model organism. It referred to as the fruit fly is a small insect usually found near unripe and rotted fruit. The wild type of fruit flies are yellow-brown in color with transverse black rings across the abdomen and brick red eyes. The fruit flies exhibit sexual dimorphism. The females are 2.5mm in length, while the males are slightly smaller and distinguished by the presence of a distinct black patch at the tip of a rounded, large abdomen, sex combs and a cluster of spiky hairs(claspers) surrounding the reproducing parts used to attach to the female during mating.

The genome of D.melanogaster contains 4 pairs of chromosomes, an X/Y pair and 3 autosomes labelled 2, 3 and 4. The genome of D.melanogaster was sequenced in 2000 and its 165 million base pairs have been annotated. About 75% of known human disease genes have a recognizable match in the genome of fruit flies and 50% of fly protein sequences have mammalian homologs. Drosophila is being used as genetic model for several human diseases including the neurogenerative disorders Parkinson's, Huntington's, Spinocerebellar ataxia and Alzheimer's disease. Drosophila is also being used to study mechanisms underlying aging and oxidative stress, immunity, diabetes, cancer and drug abuse

2. SCOPE OF THE STUDY

The objective of the present study was to determine the effect of two antioxidants (namely Melatonin and Minocycline) administered in isolation or in combination in the diet of Parental (P) and First filial (F1) of generation on *Drosophila melanogaster*.

The effects analyzed were the role of the two antioxidants in-

1. Increasing the survival rate of F1 generation adult *Drosophila melanogaster* flies subjected to oxidative stress induced by Paraquat.
2. Ameliorating the impact of oxidative stress induced by Paraquat on F2 generation adult flies as assessed by four physiological characteristics involved in *Drosophila melanogaster* survival and fitness. The characteristics assessed included time in motion, negative geotaxis, starvation resistance and desiccation resistance.

MATERIALS AND METHODS

The *Drosophila melanogaster* flies were cultured by employing the standard technique. Melatonin was dissolved in water at a concentration of 100µg/ml. The melatonin was added to the standard food media at the concentration of 1ml/vial/day, when required. Minocycline (500mg) dissolved in 500ml was added to the food media at a concentration of 100µg/ml. The dissolved minocycline was added to the food media at the rate of 1ml/vial/day.

The wild type flies were grouped into the following 4 groups. Each group included a set of 5 pairs of flies (5 male and 5 female) and were treated as the parental (P) generation. The 5 pairs of flies were allowed to mate in the vial. The F1 progeny larvae were subjected to different treatments based on the groups.

Group I- F1 larvae were fed with Standard Food Media (ripe banana smash).
Group II- F1 larvae were fed with Standard Food Media and Melatonin (100µg/ml/vial/day).
Group III- F1 larvae were fed with Standard Food Media and Minocycline (100µg/ml/vial/day).
Group IV- F1 larvae were fed with Standard Food Media, Melatonin (50µg/ml/vial/day) and Minocycline (50µg/ml/vial/day).

The F1 generation adults of each group were divided into 2 subgroups and subjected to the dosages of Paraquat herbicide exposure (20mM and 40mM).

The survival rate was determined after 12hrs, 24hrs, 36hrs, 48hrs, 60hrs and 72hrs.

The F1 male and female flies of each group were allowed to mate and the F2 larvae and adults were treated according to the group protocol.

Testing of physiological characteristics involed in the Survival and Fitness of *Drosophila melanogaster*
The physiological characteristics involved in *Drosophila melanogaster* survival and fitness were tested on 20 day old flies. The F2 adults of each group were tested for 4 physiological parameters namely,

1. Time in Motion
2. Negative Geotaxis
3. Starvation Resistance
4. Desiccation Resistance

1.Time in motion
The time in motion assay determines the amount of time a fly spent in spontaneous motion.

A set of 10 male and 10 female flies from each group were removed from the vial after anaesthetizing by cooling. The flies were replaced individually in 8 dram glass vials and forced to the bottom 1cm of the vial using a sponge. Each flies in each group, time in motion was measured twice by two different experimenters and recorded. The average of the two trials was used in data analysis. The fraction of a two minute time interval in which the fly was moving was recorded with a stop watch.

2.Negative geotaxis
The fruit flies naturally tend to fly upward against gravity, a trait known as negative geotaxis. The negative geotaxis assay tests for the escape response of the fly.

A set of 10 male and 10 female flies from each group were removed from the vial after anaesthetizing by cooling. The flies were replaced individually in 8 dram glass vials and forced to the bottom 1cm of the vial using a sponge. The sponge keeping them at the bottom 1cm of the vial was moved up the vial to allow 8cm of vertical space. This vial were tapped on to the bench top to make the flies fall to the bottom of the vials. The flies were given 1 minute to move up to the 8cm mark. The number of flies that made it to the top of vial was recorded and analyzed.

3.Starvation resistance
The starvation resistance assay determines the amount of time the fly can survive in the absence of food.

A set of 10 male and 10 female flies from each group were removed from the vial after anaesthetizing by cooling. The flies were transferred to experimental vials and a cotton ball moistened with 5ml of distilled water was placed above the sponge that enclosed the flies inside each vial. The vials were then sealed with two layers of parafilm to keep the moisture inside the vial in order to prevent death from desiccation. The number of dead flies was recorded every hour until all the flies were dead.

4.Desiccation resistance
Desiccation resistance was measured by the amount of time a fly survives in the absence of food and water.

A set of 10 male and 10 female flies from each group were removed from the vial after anaesthetizing by cooling. The flies were placed in 8 dram vials, with 4 flies of the same sex in each vial. A sponge was used to create a barrier between the flies and 3.0g of desiccant $CaCl_2$. The vials were then sealed with two layers of para film to prevent outside moisture from entering the vial. The number of dead flies in each vial was recorded every hour until all flies were dead.

RESULTS AND DISCUSSION

The viability studies showed that supplementation with Melatonin increased the quantity, but did not have any role in the survival rate of Drosophila pupae. Supplementation with Minocycline reduced the quantity and survival rate of pupae. The combined supplementation of the two antioxidants did substantially increase the quantity of pupae(TABLE 4.1.).

TABLE 4.1. Study on the viability of the first filial (F1) generation of *Drosophila melanogaster* exposed to antioxidants

PARAMETERS	GROUP I (Std. Food Media)	GROUP II (Std. Food Media + Melatonin)	GROUP III (Std. Food Media + Minocycline)	GROUP IV(Std. Food Media + Melatonin + Minocycline)
No. of pupae/10 pairs of parental flies	87	162	78	187
No. of adults/ 10 pairs of parental flies	74	139	58	161
Survival rate of pupae in (%)	85.05	85.8	74.35	86.09

Minocycline was administered at a dose of - 100µg/ml/vial/ day in group III.
Melatonin was administered at a dose of - 100µg/ml/vial/ day in group II.
Minocycline was administered at a dose of - 50µg/ml/vial/ day in group IV.
Melatonin was administered at a dose of - 50µg/ml/vial/ day in group IV.

TABLE 4.2a. Effect of melatonin and minocycline supplementation on the survival rate of *Drosophila melanogaster* of the F1 generation exposed to 20mM paraquat

% survival after the specified period of exposure	GROUP I (Std. Food Media)		GROUP II (Std. Food Media + Melatonin)		GROUP III (Std. Food Media + Minocycline)		GROUP IV (Std. Food Media + Melatonin + Minocycline)	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
% Survival after 24 hrs.	80	100	100	100	100	100	100	100
% Survival after 36 hrs.	70	100	90	90	100	100	100	100
% Survival after 48 hrs.	50	80	80	90	90	100	100	100
% Survival after 60 hrs.	50	70	80	80	80	90	100	90
% Survival after 72 hrs.	40	70	70	80	80	80	90	90
% Survival after 84 hrs.	30	60	70	80	70	80	80	90

Minocycline was administered at a dose of - 100µg/ml/vial/ day in group III.
Melatonin was administered at a dose of - 100µg/ml/vial/ day in group II.
Minocycline was administered at a dose of - 50µg/ml/vial/ day in group IV.
Melatonin was administered at a dose of - 50µg/ml/vial/ day in group IV.

The study on the % survival after exposure to 20mM and 40mM, paraquat-induced oxidative stress showed that the supplemented groups performed better than the control group. The female % survival rate was higher than the male % survival rate (TABLE 4.2a&b.)

TABLE 4.2b. Effect of melatonin and minocycline supplementation on the survival rate of *Drosophila melanogaster* of the F1 generation exposed to 40mM paraquat

%survival after the specified period of exposure	GROUP I (Std. Food Media)		GROUP II (Std. Food Media + Melatonin)		GROUP III (Std. Food Media + Minocycline)		GROUP IV (Std. Food Media + Melatonin + Minocycline)	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
% Survival after 24 hrs.	80	100	100	100	100	100	100	100
% Survival after 36 hrs.	60	80	100	100	100	100	100	100
% Survival after 48 hrs.	50	80	80	100	90	100	100	100
% Survival after 60 hrs.	50	70	80	90	90	90	80	90
% Survival after 72 hrs.	40	70	60	90	80	80	80	90
% Survival after 84 hrs.	40	60	60	80	80	80	70	90

Minocycline was administered at a dose of - 100µg/ml/vial/ day in group III.
Melatonin was administered at a dose of - 100µg/ml/vial/ day in group II.
Minocycline was administered at a dose of - 50µg/ml/vial/ day in group IV.
Melatonin was administered at a dose of - 50µg/ml/vial/ day in group IV.

An analysis of the physiological characteristic namely time spent in motion during a time interval of 2 minutes, showed that the male flies spent a lesser time in motion than the female flies of their respective groups. Supplementation with combined antioxidants marginally increased the time in motion in male flies (TABLE 4.3.).

TABLE 4.3. Study on the time spent in motion and negative geotactic ability by the F2 generation adults of *Drosophila melanogaster* exposed to antioxidants for previous generations

PARAMETERS	GROUP I (Std. Food Media)		GROUP II (Std. Food Media + Melatonin)		GROUP III (Std. Food Media + Minocycline)		GROUP IV (Std. Food Media + Melatonin + Minocycline)	
	Male	Female	Male	Female	Male	Female	Male	Female
Time Spent in Motion during a time interval of 2 minutes (In seconds)	56.6 ± 1.46	57 ± 0.84	56.9 ± 2.40 ns	58.4 ± 1.79*	52.4 ± 2.14 ns	57.4 ± 1.73 ns	57 ± 1.75 ns	58 ± 1.832*
Percentage of flies that climbed up to the 8cm mark(%)	66.6	70	66.6	76.6	56.6	60	66.6	56.6

Minocycline was administered at a dose of - 100µg/ml/vial/ day in group III.
Melatonin was administered at a dose of - 100µg/ml/vial/ day in group II.
Minocycline was administered at a dose of - 50µg/ml/vial/ day in group IV.
Melatonin was administered at a dose of - 50µg/ml/vial/ day in group IV.

TABLE 4.4. Study on the resistance to starvation stress in the F2 generation adults of *Drosophila melanogaster* exposed to antioxidants for previous generations

%Survival after the specified period of starvation stress	GROUP I (Std. Food Media)		GROUP II (Std. Food Media + Melatonin)		GROUP III (Std. Food Media + Minocycline)		GROUP IV (Std. Food Media + Melatonin + Minocycline)	
	Male	Female	Male	Female	Male	Female	Male	Female
% Survival after 12 hrs.	90	90	100	100	100	100	100	100
% Survival after 24 hrs.	70	80	80	90	90	80	90	100
% Survival after 36 hrs.	50	50	70	70	70	60	70	80
% Survival after 48 hrs.	40	30	40	50	40	50	40	60
% Survival after 60 hrs.	20	10	10	20	20	10	10	40
% Survival after 72 hrs.	---	---	---	---	---	---	---	10

Minocycline was administered at a dose of - 100µg/ml/vial/ day in group III.
Melatonin was administered at a dose of - 100µg/ml/vial/ day in group II.
Minocycline was administered at a dose of - 50µg/ml/vial/ day in group IV.
Melatonin was administered at a dose of - 50µg/ml/vial/ day in group IV.

The female flies in all the supplemented groups except for the control group showed greater resistance to starvation stress (TABLE 4.4.).

TABLE 4.5. Study on the resistance to desiccation stress in the F2 generation adults of *Drosophila melanogaster* exposed to antioxidants for previous generations

%Survival after the specified period of starvation stress	GROUP I (Std. Food Media)		GROUP II (Std. Food Media + Melatonin)		GROUP III (Std. Food Media + Minocycline)		GROUP IV (Std. Food Media + Melatonin + Minocycline)	
	Male	Female	Male	Female	Male	Female	Male	Female
% Survival after 2hrs.	80	90	100	100	100	100	100	100
% Survival after 4 hrs.	60	70	80	90	100	100	80	100
% Survival after 6 hrs.	60	70	70	80	100	90	80	90
% Survival after 8 hrs.	40	50	60	60	80	70	60	80
% Survival after 10 hrs.	20	10	40	50	50	40	40	30

Minocycline was administered at a dose of - 100µg/ml/vial/ day in group III.
Melatonin was administered at a dose of - 100µg/ml/vial/ day in group II.
Minocycline was administered at a dose of - 50µg/ml/vial/ day in group IV.
Melatonin was administered at a dose of - 50µg/ml/vial/ day in group IV.

The study showed that the higher resistance to desiccation stress was observed in the female flies supplemented with Melatonin and the male flies supplemented with Minocycline. The supplemented groups showed a higher percentage of survival among both male and female flies as compared to the control group (TABLE 4.5.).

The study demonstrated that D*rosophila melanogaster* on supplementation with two classic antioxidants administrated in isolation or in combination, revealed interesting results in the testing for certain physiological characteristics involved in survival and fitness.

An interesting result was the increased resistance exhibited by the female flies as compared to the male flies. It is generally reported that female flies are more resistant to stress, than male flies, most likely because females are bigger and can withstand the stress to which they are exposed for a longer duration before dying.

The big difference in size probably explains the observed survival difference between sexes on starvation resistance as compared with the other tested stresses. Starvation resistance relies entirely on the available bodily reserves of the organism, which in females are larger than in male.

4.4.THE ROLE OF MINOCYCLINE SUPPLEMENTATION

The supplementation with minocycline reduced the quantity and percentage of surviving pupae of the F1 generation. This result was also reported by Oxenburg et al., (2012) [15] and it was suggested that minocycline depresses fecundity. It is known that fecundity suppression can result in lifespan extension [16].

The other possible action mechanisms of minocycline effects might include inhibition of nitrite oxide production, modulation of microglial activation, and inhibition of release of inflammatory cytokines [17]. Minocycline is reported to inhibit the production of KYN (Kynurenine) from Tryptophan (TRP). Tryptophan is an essential amino acid participating in the biosynthesis of proteins and methoxyindoles (Serotonin and melatonin).

KYN is a common substrate for the biosynthesis of neuroprotective KYNA and neuro toxic 3-HK. The impairment of TRP conversion into KYN was associated with the increase of KYNA and decrease of 3-HK content in adult head tissues [18]. Therefore, the deficiency of KYN might limit formation of 3-HK to a greater extent than the production of KYNA. Minocycline induced down regulation of KYN production might lead to a shift of post – KYNA metabolism from formation of neurotoxic 3-HK towards production of neuro protective KYNA. In addition inhibition of TRY-KYN metabolism could “save” some tryptophan from degradation and make it available for biosynthesis of serotonin and consequently, N-acetylserotonin , BDNF Trk B receptor agonist [15]. The stimulating effects of Minocycline on vertical climbing is in line with the observation that KYN decreases locomotor activity in a rat model [19].

4.5.THE ROLE OF MELATONIN SUPPLEMENTATION

It was observed that in most of the survival and fitness characteristics assessed equimolar concentration of melatonin was more effective than minocycline and the antioxidant combination.

Dietary paraquat is an established free radical generator. According to the free radical theory of aging, free radicals, or more specifically oxygen radicals give rise to the biological damage that causes the physiological decline characteristic of older organisms.

Further, the levels of defence may be as important as the rate of free radical\production in governing the rate of damage. The efficacy of melatonin in counteracting the toxicity of Paraquat in *Drosophila melanogaster* has been established.

The increased resistance of *Drosophila melanogaster* fed with melatonin to paraquat was a demonstration of the antioxidative protection provided by the hormone [14].

It has recently been suggested that the first metabolite of melatonin in the melatonin antioxidant pathway may be N-acetyl-N-formyl-5-methoxykynuramine(AFMK) rather than the common, excreted 6-hydroxy melatonin sulphate. AFMK alone is detectable in unicellular organisms and metazoans. A single AFMK molecule can neutralize upto 10 ROS/RNS since many of the products of the reaction or derivatives are themselves antioxidants. This capacity to absorb free radicals extends at least to the quaternary metabolites of melatonin, a process referred to as “the free radical scavenging cascade”. This characteristic is not true of other conventional antioxidants [20].

CONCLUSION

1. The present study attempted to look beyond the hype that surrounds ANTIOXIDANTS. Antioxidant supplements represent a $500 milllion dollar industry that continues to grow. Antioxidants are still added to breakfast cereals, sport bars, energy drinks and other processed foods and they are promoted as additives that can prevent heart

disease, cancer, cataracts, memory loss and a host of other conditions. However, a number of recent studies have provided little support to antioxidants as providers of substantial protection against diseases.

2. The present study revealed that of the two Antioxidants studied, namely melatonin and minocycline, Melatonin was singularly effective in reducing the effects of oxidative stress. Another, interesting aspect that came to light was that female flies were more resistant to stress.

3. The F1 adults of each group were subjected to treatment with the herbicide, paraquat and the survival rate was determined.

4. The F2 adults of each group were tested for the following physiological characteristics involved in the survival and fitness namely,

a. Time in motion
b. Negative geotaxis
c. Starvation resistance
d. Desiccation resistance.

5. Melatonin in isolation was shown to be more effective in reducing the effects of Paraquat-induced oxidative stress.

6. The female flies of each group were also reported to more resilient to stress and also scored higher in the locomotor assays.

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