

Melatonin in semen extender did not improve quality of frozen-thawed feline sperm

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Abstract

We evaluated impacts of melatonin addition to a tris-egg-yolk (TEY) semen extender on sperm quality after freezing cat semen. Sperm was obtained via urethral catheterization (UC) after medetomidine treatment and epididymal slices (ES) in 10 cats. Samples were frozen after dilution in a TEY extender with melatonin (2 or 3 mM) or without melatonin (control). Sperm quality was assessed before and after freezing. In raw semen, sperm progressive motility (PM) was greater ($p = 0.004$) in samples obtained by ES than by UC. Sperm PM, vigor and sperm with preserved membrane function significantly decreased in frozen-thawed samples compared to raw semen, without significant differences between collection methods. In postthaw UC samples, no significant differences were observed between control and melatonin (2 and 3 mM) groups, being the overall means as follows: sperm PM: 29.4%, sperm membrane function: 40.8%; sperm with condensed chromatin: 92.5 %, and live acrosome-intact sperm: 28.9%. In postthaw ES samples, no significant differences were observed between control and melatonin (2 and 3 mM) groups, being the overall means as follows: sperm PM: 36%; sperm membrane function: 42%; sperm with condensed chromatin: 86%; and live acrosome-intact sperm: 32%. In conclusion, supplementation of the extender with melatonin (2 mM or 3 mM) did not improve sperm quality after freezing in cats.

Keywords: Cryopreservation, extender, cat, melatonin, oxidative damage

Introduction

Semen cryopreservation enables long-term storage of genetic material, although the freeze-thaw process exposes sperm to thermal, mechanical, osmotic and oxidative stress, ultimately compromising seminal quality. These stressors may alter sperm motility, viability, membrane integrity and fertilizing capacity.¹⁻³ Low postthaw sperm quality in felids has led to continuous efforts to optimize semen extenders to enhance sperm survival. Tris-egg yolk-based (TEY) extenders supplemented with a cryoprotective (glucose or fructose, glycerol) are heavily used for cryopreservation of feline sperm.⁴⁻⁶

A major contributing factor to cryoinjury during sperm cryopreservation is the generation of reactive oxygen species (ROS). Excessive ROS production induces lipid peroxidation, leading to deleterious changes in sperm acrosome and plasma membrane fluidity and integrity, DNA damage, and the promotion of premature sperm cryocapacitation.² Sperm are particularly vulnerable to oxidative stress due to their limited antioxidant enzyme defenses and the high content of polyunsaturated fatty acids in their plasma membrane.⁷⁻⁹ Consequently, semen extenders have been supplemented with antioxidant compounds (i.e. catalase, glutathione peroxidase, superoxide dismutase, vitamin E, cysteine, taurine) and reduction in ROS-induced damage evaluated.^{10,11}

Enzymatic antioxidants (e.g. superoxide dismutase, catalase) have a critical primary role in neutralizing specific ROS cascades. Glutathione peroxidase addition to cat semen extender improved sperm motility, mitochondrial activity, and DNA and membrane integrity, but not acrosome integrity.⁷ Nonenzymatic molecules like Trolox or cysteine inhibit lipid peroxidation within the plasma membrane whereas L-carnitine serves dual functions as a scavenger of oxygen free radicals and promoter of mitochondrial fatty acid transport. Like glutathione peroxidase, Trolox and cysteine addition to freezing extender improved several parameters in frozen thawed cat semen but did not have a positive effect on acrosome integrity¹⁰ whereas L-carnitine supplementation only improved sperm motility.¹¹ Therefore, despite various protective mechanisms, the efficacy of exogenous antioxidants in feline sperm cryopreservation is limited.¹² Although factors (e.g. antioxidant concentration, specific combinations, species-specific membrane characteristics) might have a protective effect,¹² systematic evaluation of single or combined antioxidant strategies that preserve postthaw sperm quality is lacking.

Melatonin, a lipophilic molecule synthesized by the pineal gland, exerts multiple biological functions, including antioxidant protection. In sperm, melatonin reduces oxidative damage, DNA fragmentation and lipid peroxidation and modulates sperm capacitation. These effects are possibly mediated through the enhancement of endogenous antioxidant enzyme activity and the reduction of ROS concentrations.¹³⁻¹⁶ In several species, melatonin addition to semen extenders has been associated with improved sperm quality following cryopreservation.¹⁷⁻¹⁹ However, to authors' knowledge, no studies assessed the protective effect of melatonin on sperm during cryopreservation in cats. Therefore, we evaluated impacts of melatonin addition to a TEY semen extender on postthaw sperm quality in cats.

Materials and methods

Animals

This study was approved by the ethics committee of the School of Veterinary Sciences of Buenos Aires University (CICUAL N° 2022/40). Informed written consent was obtained from owners before enrollment. Male client-owned domestic shorthair cats (n = 10) were included in the study. All cats (2.2 ± 1.1 years) were clinically and reproductively healthy.

Semen collection

Sperm was obtained from cats (n = 10) via urethral catheterization (UC) after medetomidine treatment²⁰ and epididymal slices.^{21,22} Briefly, cats were given 100 µg/kg of subcutaneous medetomidine and 10 minutes later, a catheter (Argyle 5-1/2"-14 cm) was inserted in the urethra. Catheter was gently drawn back and washed with 0.1 ml of physiological saline solution, collecting the suspension in an Eppendorf tube. After this procedure, orchiectomy was performed under general anesthesia. As medetomidine had been previously injected, only 5 mg/kg of intramuscular ketamine chlorhydrate and 0.1 mg/kg of subcutaneous meloxicam were given. After orchiectomy, epididymides were dissected and the cauda epididymides were sliced repetitively in a glass Petri dish containing 1 ml of physiological saline solution at 37 °C. After 10 minutes, the sperm suspension was filtered (CellTrics 30 µm, Partec™) into an Eppendorf tube while tissue fragments were removed.^{21,22}

Extenders and melatonin supplementation

Semen extender used was based on tris, citric acid and fructose (2.42% tris, 1% fructose, 1.38% citric acid, 20% egg yolk and 4% glycerol)⁵; these chemicals were from Sigma Aldrich.

Melatonin powder was first dissolved in dimethyl sulfoxide (DMSO); 2 and 3 mM melatonin concentration (0.001 or 0.0014 grams in 20 µl of DMSO) solutions were prepared and added to 2 ml extender. Melatonin concentrations were chosen based on reports^{18,23} and based on our observation using 1 mM concentration (pilot study [n = 3 cats]) that did not improve postthaw sperm quality.

Experimental design and cryopreservation

Sperm quality (progressive motility [PM], vigor, membrane function, concentration and morphology in raw semen samples (collected by both methods) were evaluated. Samples were split into 3 aliquots: 1 was used as a control sample (diluted in the TEY freezing extender without melatonin) and the other 2 were diluted in the freezing extender with the addition of melatonin at concentrations mentioned above. Aliquots were centrifuged at 600 x g for 5 minutes; supernatant was removed and sperm pellet was resuspended at room temperature in extenders to reach a final sperm concentration of 50 x 10⁶ sperm/ml. Therefore, for each male, 6 tubes were prepared: sperm obtained via UC diluted in the extender without melatonin (Control UC); sperm obtained by UC diluted in the extender with the addition of 2 mM melatonin (UC2); sperm obtained by UC diluted in the extender with the addition of 3 mM melatonin (UC3); sperm obtained via epididymal slices (ES) diluted in the extender without melatonin (Control ES); sperm obtained by ES diluted in the extender with the addition of 2 mM melatonin (ES2); and sperm obtained by ES diluted in the extender with the addition of 3 mM melatonin (ES3). Tubes were placed in glasses filled with water at room temperature and were left in the refrigerator at 4 °C for 120 minutes to ensure slow cooling.⁵ Then, samples were loaded into 0.25 ml straws (previously refrigerated), transferred into a Styrofoam box, frozen by placing horizontally in vapors 4 cm above the surface of liquid nitrogen for 10 minutes and then immersed into liquid nitrogen. Straws were thawed in a water bath at 37 °C for 30 seconds. After thawing, sperm PM, vigor, membrane function, chromatin condensation, viability and acrosome integrity were evaluated.

Sperm quality assessment

To evaluate subjective sperm PM, a 10-µl aliquot of each sperm sample was placed on a prewarmed glass slide, covered with a prewarmed cover slip, and subjectively assessed by 2 skilled independent observers, using a phase contrast microscope (100 x). Sperm vigor was estimated by placing a drop of sample between a slide and coverslip, using a warm stage (37 °C) and phase contrast microscopy (100 x), where 5 = maximum vigor and 0 = no vigor. Two skilled independent observers also assessed this parameter. Concentration was analyzed using a Neubauer chamber, performing the sperm count by observation with an optical microscope.

Sperm morphology was determined using the T15 stain (Biopak), evaluating a minimum of 200 cells per sample using light microscopy (1,000 x).²² Membrane function was evaluated using the hypoosmotic swelling test (HOST); a minimum

of 200 sperm per sample was assessed using phase contrast microscopy (400 x).²⁴ Chromatin condensation was assessed using the toluidine blue (TB) stain.²² Briefly, semen was extended, air-dried and fixed with ethanol 96° for 2 minutes. Staining was done using a working solution of TB diluted 1:3 with pH 4 buffer, prepared on the day of use from a stock solution of 1% TB (preserved at 4 °C protected from light). After 5 minutes, smears were washed with distilled water. A minimum of 200 sperm were evaluated using light microscopy. TB staining patterns were as follows: light blue (condensed chromatin), light violet (moderate chromatin decondensation) and dark blue-violet (high chromatin decondensation). Viability and acrosome integrity were evaluated by the fluorescent dye propidium iodide (PI) (viability) combined with the peanut agglutinin lectin labeled with fluorescein isothiocyanate (FITC-PNA) stain (acrosome integrity).^{25,26} Briefly, samples were incubated with FITC-PNA for 10 minutes at 37°C (final concentration: 20 µg/ml). Afterwards, PI was added, and samples were incubated for another 10 minutes at the same temperature (final concentration: 10 µg/ml). Then, 6 µl of stained sperm was placed onto a glass slide and covered with a coverslip. Samples were evaluated using bright field and epifluorescence microscopy (Leica Microsystems, Wetzlar, Germany). At least 200 sperm were counted. The following sperm populations were defined: live sperm with intact acrosomes (no fluorescence in either head or acrosome cap), live sperm with reacted acrosomes (no fluorescence in the head and intense green fluorescence in the acrosome cap), dead sperm with intact acrosomes (red fluorescent head with no fluorescence in the acrosome cap) and dead sperm with reacted acrosomes (red fluorescent head with spotted or half-moon green fluorescence in the acrosome). The percentage of each population was calculated.

Data analysis

The normality of the data and variance homogeneity were assessed by the Shapiro-Wilk test and Levene's test, respectively. A general linear model with a first-order autoregressive

covariance structure was used (RStudio).²⁷ Post hoc analysis was performed with a Tukey test. The response variables were sperm PM, vigor, percentage of sperm with preserved membrane function, percentage of live sperm with an intact acrosome and percentage of sperm with condensed chromatin. The fixed effects were treatment (with 4 levels: raw samples, frozen-thawed samples diluted in control extender, frozen-thawed samples diluted in the extender with melatonin 2 or 3 mM) and the collection method (with 2 levels: UC and ES). Interaction between treatment and the collection method was assessed. In all cases, the cat was set as a random effect. Values are expressed as mean ± standard deviation. Differences were considered significant when p values were < 0.05.

Results

In raw semen samples, sperm PM was greater in samples obtained via ES than by UC (p = 0.004) whereas the other variables were not different between collection methods (Table 1). In frozen-thawed samples there was no interaction between extender and collection method (p = 0.13) for sperm quality. Sperm PM and vigor were not different between freezing extenders (control extender and with the addition of 2 mM or 3 mM melatonin) both in samples obtained via UC and ES. There were no differences among samples diluted in freezing extenders in the percentage of sperm with condensed chromatin (light blue stain) in the percentage of live sperm with an intact acrosome or in the percentage of sperm with preserved membrane function (Table 2). Sperm PM and vigor decreased (Figure) in the frozen-thawed samples diluted in extenders compared to raw samples, for both collection methods p < 0.0001). Similarly, sperm with preserved membrane function decreased in frozen-thawed samples (p < 0.0001).

Discussion

The protective effect of melatonin on cells has frequently been explained by the antioxidant action of this molecule. In sperm,

Table 1. Sperm quality analysis in raw semen samples obtained by epididymal slices (ES) and urethral catheterization (UC)

	ES	UC
Progressive motility (%)	65 ± 9*	48 ± 13
Vigor	3.5 ± 0.5	2.8 ± 0.8
Sperm with normal morphology (%)	77 ± 12	82 ± 9
Sperm with preserved membrane function (%)	90 ± 6	93 ± 7
Sample concentration (sperm x10 ⁶)	207 ± 130	135 ± 97

*differed between ES and UC (p = 0.004)

Table 2. Sperm quality in frozen-thawed semen obtained by epididymal slices (ES) and urethral catheterization (UC) without melatonin (ES and UC control) or with 2 mM (ES2 and UC2) or 3 mM (ES3 and UC3) melatonin

	ES Control	ES2	ES3	UC Control	UC2	UC3
Progressive motility (%)	21 ± 10	22 ± 10	31 ± 14	20 ± 8	25 ± 6	20 ± 8
Vigor	1.7 ± 0.5	1.9 ± 0.3	2.4 ± 0.7	1.7 ± 0.5	2 ± 0.3	2 ± 0.2
Sperm with preserved membrane function (%)	41 ± 9	40 ± 10	48 ± 7	40 ± 6	42 ± 8	40 ± 6
Sperm with condensed chromatin (%)	84 ± 21	87 ± 13	88 ± 9	96 ± 4	90 ± 7	92 ± 5
Live sperm with an intact acrosome (%)	29 ± 12	30 ± 12	37 ± 12	26 ± 11	31 ± 9	29 ± 11

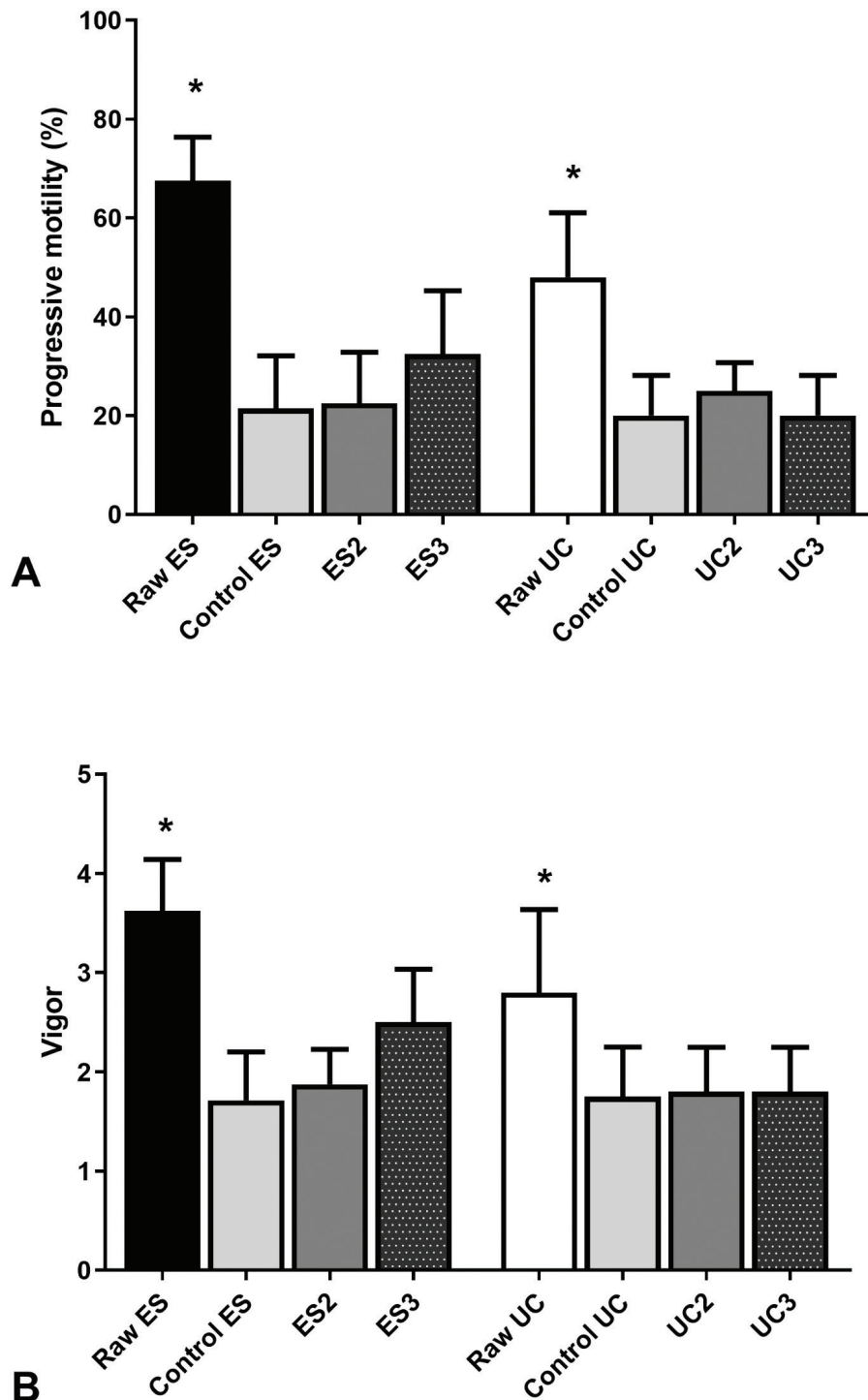


Figure. Sperm progressive motility percentage A. and vigor B. in frozen-thawed semen samples. Control UC: sperm obtained via urethral catheterization (UC) diluted in the freezing extender without melatonin; UC2: sperm obtained via UC diluted in the freezing extender with the addition of melatonin 2 mM; UC3: sperm obtained by UC diluted in the freezing extender with the addition of melatonin 3 mM, Control ES: sperm obtained via epididymis slicing epididymal slices (ES) diluted in the freezing extender without melatonin; ES2: sperm obtained by ES diluted in the freezing extender with the addition of melatonin 2 mM; and ES3: sperm obtained by ES diluted in the freezing extender with the addition of melatonin 3 mM

melatonin reduces oxidative damage, DNA fragmentation and peroxidation of membrane lipids.¹⁶ Several studies have reported that in rams, horses and dogs, certain parameters (e.g. sperm PM, DNA and acrosome integrity) were better

preserved in frozen-thawed samples diluted in extenders containing melatonin.^{13,23,28,29} However, in our study in cats, its addition to semen extender did not improve sperm quality in frozen-thawed samples.

In our study, sperm PM and vigor decreased in frozen-thawed samples compared to raw semen obtained via either collection method. Extender supplementation with melatonin at 2 concentrations (2 and 3 mM) did not improve these parameters in comparison to the control extender (without melatonin). Similarly, in dogs, melatonin addition to the freezing extender at various concentrations (0.5, 1, 2, and 3 mM) did not exert a protective effect on sperm PM, since this variable decreased in frozen-thawed samples, both supplemented or not, without significant differences between them.^{18,23} In contrast, in buffaloes, bulls and rams, sperm diluted in an extender containing melatonin had better sperm PM after thawing compared to sperm diluted in the control extender, although this beneficial effect was dependent on melatonin concentrations.^{13,19,29} Specifically, while low or moderate supplementation enhanced sperm PM maintenance, increasing concentrations beyond an optimal range (variable according to the species: from 100 pM-1,000 nM) was not effective in maintaining this parameter. In light of these results, it is possible that the lack of effect of melatonin on sperm PM and vigor evidenced in our study could be related to the concentration used.

Melatonin preserves membrane integrity and function by preventing lipid peroxidation and may also regulate the sperm capacitation process through binding to its specific receptors.^{16,30} In ram and horse, it has been reported that both membrane and acrosome integrity were better preserved when melatonin was added to the extender.^{29,31} Similarly, in bull, HOST was improved in thawed samples containing melatonin, whereas in buffaloes and dogs, melatonin had a protective effect on acrosome integrity but only at the highest concentrations (2.5-3.5 mM).^{14,17,32} In our study, the percentage of live sperm with an intact acrosome and the percentage of sperm with preserved membrane function were not affected by melatonin supplementation, independent of the concentration used. It is possible that in cats, higher melatonin concentrations may be required to achieve protective effects on membranes.

Cryopreservation can compromise both chromatin packaging stability and DNA integrity.³³ Melatonin has been reported to protect against ROS-mediated DNA fragmentation (Comet assay) in some species (e.g. rams)^{13,34} and dogs (sperm chromatin dispersion test),²³ among others. ROS can affect chromatin condensation by damaging protamine disulfide bonds that may secondarily render the DNA more vulnerable to fragmentation.^{35,36} In our study, chromatin condensation status was evaluated to determine whether melatonin preserves structural chromatin packaging in frozen-thawed samples. However, melatonin supplementation did not improve this parameter, suggesting that it did not protect protamine-mediated chromatin structure under our experimental conditions. Nevertheless, potential protective effects against direct DNA fragmentation cannot be ruled out without specific DNA integrity assays, which remain to be performed.

In general, our results demonstrated that the addition of 2 or 3 mM melatonin to the freezing extender did not provide protection to cat sperm, as no significant differences were observed after freezing-thawing in any of the evaluated parameters compared to the control samples. Selection of these specific concentrations (2 and 3 mM) was based on previous findings in dogs,^{18,23} where doses ranging 1-3.5 mM improved overall postthaw sperm quality whereas lower concentrations (e.g. 0.5 mM) failed to exert a protective effect.

Besides, melatonin is known to exert 'biphasic' effects on various sperm characteristics among species;^{16,19} although excessively low concentrations may be insufficient to scavenge ROS, high concentrations can induce cytotoxic or prooxidative effects.^{13,14} Lack of beneficial effects on cat sperm observed in our study suggests that the concentrations assessed (2 and 3 mM) may not coincide with the optimal supplementation dose for this species. Given the limited volume of feline semen samples, evaluating a broader dose-response curve in a single trial was not implemented, to avoid compromising sperm concentration per straw or the need to pool ejaculates. However, alternative explanations beyond concentration must also be considered. Distinct species-specific membrane lipid compositions (e.g. polyunsaturated fatty acids: cholesterol ratio) may alter susceptibility to cryodamage and responsiveness to exogenous antioxidants.^{1,6,7} Furthermore, melatonin receptor distribution or postreceptor cascades might differ in cat sperm compared to species like rams or bulls, where melatonin supplementation had consistent success.^{15,34} Therefore, future studies assessing a wider range of concentrations and other cellular and molecular studies, will be required to establish whether melatonin can exert any beneficial effect on feline sperm cryopreservation.

It is important to acknowledge that although the central premise of melatonin supplementation relies on its direct and indirect antioxidant properties,¹⁶ we evaluated various sperm quality parameters (motility, acrosome/membrane integrity, chromatin condensation) rather than direct markers of oxidative stress. Specific oxidative biomarkers (e.g. malondialdehyde concentration or total antioxidant capacity^{3,8}) were not measured. Consequently, although 2 and 3 mM melatonin supplementation did not translate into improved postthaw sperm quality, our findings did not rule out potential modulation of intracellular oxidative pathways that were not evaluated. Future research incorporating oxidative stress assays will be essential to elucidate whether melatonin protects sperm from oxidative stress during freeze-thawing in cats.

As reported,³⁷ sperm PM in raw semen was greater in samples obtained via ES than UC; other reports^{21,38} had contradicting observations. Differences in semen collection protocols and media/extendors used when obtaining the samples might explain this discrepancy. Thus, this information should be considered when choosing the collection method to be implemented. In conclusion, melatonin addition to a TEY extender at the studied concentrations did not improve sperm quality in frozen-thawed samples in cats.

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Authors' contribution and declaration

MG designed and performed the study and wrote the manuscript; VA performed the study and collaborated in data analysis; EM and first MM performed the study; MR data analysis and edited the manuscript; DN collaborated in data analysis, performed language translation and critically revised the manuscript; second MM critically revised and edited the manuscript and CA Collaborated in the design of the study, performed the study, and critically revised the manuscript. All authors have read and approved the final version of the manuscript.

Conflict of interest

None of the authors have any conflict of interest to declare.

Data availability

Corresponding author can provide data based on reasonable request.

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