The effect of quercetin on fertility of frozen-thawed ram epididymal spermatozoa

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Abstract

The aim of the present study was to evaluate the effects of quercetin as an antioxidant supplement on frozen-thawed ram epididymal sperm quality. Quercetin is a type of flavonoid antioxidant that is found in plants, with the ability to scavenge free radicals. Twenty testicles from mature rams were collected from a nearby slaughterhouse immediately after slaughter. Epididymal spermatozoa were recovered from the caudal of epididymides by injecting Bracket and Oliphant's (BO) medium retrogradely through the ductus deferens and extended with a tris egg-yolk-based extender and supplemented with 0, 5, 10, 15, 20, and 50 µg/mL quercetin. Following equilibration, the straws were frozen, and then plunged into liquid nitrogen. After thawing, optimized concentrations of quercetin were defined based on their viabilities and used to assess fertilization and developmental potential. The results showed that the viability of frozen-thawed spermatozoa significantly increased by using 5 and 10 µg/mL quercetin in the freezing extender. However, total and progressive motility of frozen-thawed spermatozoa were not affected by 5 and 10 µg/mL quercetin in comparison with control (0 µg/mL). The mean number of zygote, morula, and blastocyst stage embryos increased significantly by using 5 and 10 µg/mL quercetin compared with other frozen-thawed treatments(P <0.05). However, the blastocyst rate of fresh sperm was significantly higher (P <0.05). In conclusion, to improve the quality of frozen-thawed ram epididymal spermatozoa, 5 and 10 µg/mL quercetin appears to be an attractive option. Further studies are suggested to understand the synergistic effect of quercetin with other antioxidants to improve the ram freezing–thawing process.

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Introduction

Sperm cryopreservation is an indispensable tool to conserve the elite germplasm to improve farm animal species (Kar *et al.*, 2015). Sperm recovery from the caudal epididymis can be advantageous after the unexpected death of endangered animals or animals with genetic merit. This procedure is an important tool for the recovery and conservation of germplasm after death (Papa *et al.*, 2015). Additionally, there is no need to maintain male animals for the laborious process of harvesting ejaculated sperm for research, especially in sheep with seasonal reproduction. However, ejaculated sperm differ in many parameters, including the types of proteins that are bound to the plasma membrane and their motion characteristics. It is known that the *in vivo* fertility of epididymal sperm tends to be lower than that of ejaculated ones (Papa *et al.*, 2015).

While cryopreservation has deleterious effects on mammalian sperm, the extent of that damage varies across species and relies heavily on sperm resilience to the freezing-thawing process (Yeste, 2016). Despite the many technical advances in the last decades, artificial insemination (AI) in sheep has not achieved the widespread use that has occurred in the cattle and swine industries. The female size, low economic return, variable fertility rates, and more rapid loss of semen quality with storage time are important obstacles for the routine use of these techniques. Therefore, sperm quality, which is negatively affected by cryopreservation, has a critical impact on the application of AI (Mata-Campuzano *et al.*, 2014; Najafi *et al.*, 2014).

Oxidative stress, owing to the imbalance between the presence of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), and sperm antioxidant activity, is a major cause of spermatozoal damage during the freezing process. The lipid composition of the sperm plasma membrane makes this structure susceptible to oxidative damage owing to the high content in unsaturated fatty acids, especially in ram

spermatozoa, which contain a higher polyunsaturated/saturated fatty acids ratio than other species, such as rabbits, bulls and human beings (Silva *et al.*, 2013; Martínez-Páramo *et al.*, 2012; Akalin *et al.*, 2015).

Sperm protection against oxidative stress is provided mainly by seminal plasma, which contains several antioxidant components. However, the endogenous antioxidative capacity of semen is not sufficient during storage, and dilution in the extender media also reduces the seminal plasma constituents (Cabrita *et al.*, 2011; Daramola *et al.*, 2015). Various strategies have been used to improve the freezing-thawing process, including the usage of cryoprotectants and cryodiluents, altering the cooling and warming rates and, most importantly, supplementing semen freezing media with various antioxidants (Forouzanfar *et al.*, 2013). Antioxidants are the main defence against oxidative stress induced by free radicals. There are preventive antioxidants and scavenger antioxidants. Preventive antioxidants, such as metal chelates and metal-binding proteins, block the formation of new free radicals, whereas scavenging antioxidants remove the free radicals that have already formed (Fathollahi *et al.*, 2013).

In recent years, the use of herbal antioxidants has been gaining attention from researchers. Two-thirds of the world's plant species have medicinal value. In particular, many medicinal plants have great antioxidant potential (Motlagh *et al.*, 2014). Quercetin is a flavonoid polyphenol, with more intensive antioxidant activity than vitamins E and C, as well as less toxicity. Phenolic compounds act as free radical scavengers and, at times, as metal chelates, acting both in the initiation step and in propagation of the oxidative process (Silva *et al.*, 2012).

Because of the antioxidant properties of polyphenols and the need to improve the quality of cryopreserved epididymal ram sperm, the aim of the present study was to assess the effect of various concentrations of quercetin on viability, motility and fertility of frozen-thawed ram epididymal sperm by using *in vitro* fertilization technique.

Material and Methods

All the culture media and other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo, USA) and the plasticwares were purchased from Falcon (Paignton, UK), unless stated otherwise.

Testes with epididymides from 10 chal rams (2.5–3.5 years of age) were collected from a nearby slaughterhouse immediately after slaughter. In the laboratory, the testes were rinsed twice with normal saline and were then trimmed to remove the extra testicular tissue and washed 10 times with saline containing 0.1% streptomycin sulphate. Connective tissue covering the caudal epididymis was removed by dissection with care to avoid rupturing blood vessels or the epididymal duct. Spermatozoa were recovered from the caudal of epididymides by injecting BO medium (containing 10 µg/mL heparin, 137.0 µg/mL sodium pyruvate, 1.942 mg/mL caffeine sodium benzoate, and 10 mg/mL fatty acid free bovine serum albumin (BSA)) retrogradely through the ductus deferens. Collected spermatozoa were washed once by centrifugation with BO medium at 600 g for 7 min. The pellet obtained was suspended in BO medium and evaluated for motility.

The extender in the present study was prepared according to the protocol established by Baghshahi *et al.* (2014). The extender was composed of 2.7 g tris, 2.44 g citric acid, 0.5 g glucose, 7% glycerol (v/v), 15% egg yolk and 100 IU penicillin for 100 mL sterile Milli-Q water. The osmolarity and pH were set at 335 mOsm and 6.5, respectively. The final treatments were prepared using various concentrations of quercetin (0–50 µg/mL) added to prepared extenders. The spermatozoa samples from six testes (one testis from each ram) with more than 80% motility were pooled and diluted with different extenders prepared at 25 °C to a final concentration of 4×10^8 sperm/mL. The samples were drawn into 0.25 mL French straws (IMV, L'Aigle, France) which were maintained at 4 °C for two hours. The straws were then placed 3 cm above the surface of liquid nitrogen, where the temperature was approximately -120 °C. After 15 minutes, the straws were immersed directly into liquid nitrogen (-196 °C) for long-time storage. After one month, three straws were thawed in a 37°C water bath for 30 seconds, pooled, and immediately subjected to analysis and evaluation.

The MTT assay was performed according to the protocol established by Byun *et al.* (2008) with some modification. This assay was used to access sperm viability. For each sample, six wells of the 96-well microplate were used. One hundred mL sperm sample (30×10^6 sperm/mL) plus 10 µL of MTT (Thermo Fisher Scientific, USA,V-13154) stock solution [5 mg MTT/mL Phosphate-buffered saline (PBS)] were placed in each well. Spermatozoa were incubated at 37 °C in a humidified atmosphere for four hours. Then 100 µL of the sodium dodecyl sulphate (SDS) - hydrogen chloride (HCl) solution (10 mL of 0.01 M HCl was added to 1 g SDS) was added to each well and mixed thoroughly using the pipette and spermatozoa were incubated at 37 °C for 16 hours in a humidified chamber. The absorbance was measured in a spectrophotometer (MS2 Reader). The freeze-killed procedure was used to obtain the standard curve and the relationship between the MTT reduction rate and sperm viability. After the dilution of sperm with BO medium, 3 mL of the diluted sperm was divided in two fractions. One fraction was maintained at 37 °C, while the sperm in the other fraction were killed by two cycles of plunging into liquid nitrogen and thawing at 37 °C. Treatments for

analysis were prepared by combining aliquots of viable and freeze-killed spermatozoa at ratios of 10:0, 8:2, 6:4, 4:6, 2:8, and 0:10 v/v, respectively. The prepared samples were analysed by the MTT assay (above). Standard curves, y = 0.0139x-1.0108 (where y and x stand for optical density and viability, respectively) for the relationship between the MTT reduction rate and the percentage of viable spermatozoa were acquired (Figure 1). This standard curve was applied to acquire the percentage of viable sperm in each sample in accordance with the MTT reduction rate.
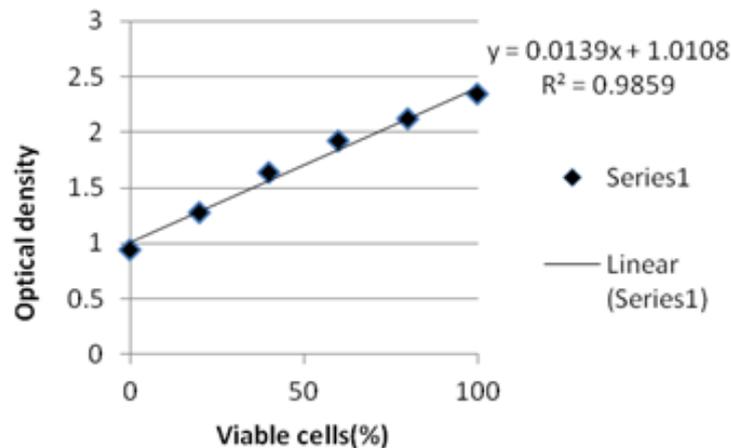

Figure 1 Relation between viability of spermatozoa and their optical density by using MTT assay

Ten mL sperm were placed on a slide (37 °C). Spermatozoa with motility and progressive motility were then assessed by counting 200 spermatozoa in each replicate using bright-field microscopy (400×) (Olympus CX21FS1, Japan). Each sample was counted 10 times. Based on the World Health Organization's (WHO) laboratory manual (2010) the spermatozoa moving actively, either linearly or in a large circle, regardless of speed, were graded as having progressive motility.

In vitro embryo production was performed according to the protocol established by Zandi *et al.* (2014) with some modification. Sheep ovaries collected from a nearby abattoir were brought over to the laboratory in PBS containing 100 IU/mL penicillin and 50 mg/mL streptomycin at 30 to 34 °C within three hours of slaughter. Cumulus-oocyte complexes (COCs) were released from all visible follicles (>2 mm diameter) by slicing the surface of an ovary with a surgical blade. Those with more than three layers of compact unexpanded cumulus cells and homogenous evenly granular ooplasm were transferred to 100 mL droplets (15 to 20 COCs per droplet) of in vitro maturation (IVM) medium, which consisted of TCM-199, containing 10% foetal bovine serum (FBS), 10% buffalo follicular fluid, 5 mg/mL follicle stimulating hormone (FSH), 1 mg/mL oestradiol-17β, 0.81mM sodium pyruvate, and 50 mg/mL gentamicin sulphate, and were cultured under mineral oil in a petri dish in a CO_2 incubator at 38.5°C for 24 hours. For *in vitro* fertilization the oocytes were washed twice with BO medium and were transferred to 50 mL droplets (15 to 20 oocytes per droplet) of capacitation and fertilization BO medium [washing BO medium containing 10 mg/mL fatty acid-free bovine serum albumin (BSA)]. The spermatozoa were prepared for fertilization according to the protocol established by Chauhan *et al.* (1998). Oocytes were then inseminated with a final concentration of 1×10^6/mL motile spermatozoa and incubated under mineral oil in a CO_2 incubator at 38.5 °C for 18 hours. The cumulus cells were then removed from the presumed zygotes, after which these were cultured in mCR2aa containing 0.6% BSA for 48 hours in groups of 15 to20 per droplet. Following this, the cleaved embryos obtained were cultured in the in vitro culture (IVC) medium (mCR2aa+0.6% BSA+10% FBS) for up to 8 days until morulae and blastocysts were obtained, with a change of medium every 48 hours. To study the fertility of fresh and frozen-thawed spermatozoa each treatment was replicated six times.

Data were analysed with a statistical software program (SPSS 16, IBM, USA). Comparisons between multiple numeric datasets were performed using one-way ANOVA followed by Duncan's multiple-range test. Results are expressed as mean ± SEM, and statistical significance was accepted at *P* <0.05.

The statistical model used was:

$Y_{ij} = \mu + a_i + e_{ij}$

Where: Y_{ij} is the trait under study or response variables (viability of fresh spermatozoa or viability, motility or fertility of frozen-thawed spermatozoa)

μ is the overall mean

a_i is the fixed effect of different quercetin concentrations (i = 0, 5, 10, 15, 20, 50 and 100 µg/mL for fresh spermatozoa, ori = 0, 5, 10, 15, 20 and 50 µg/mL for frozen-thawed spermatozoa)

e_{ij} is the random error effect.

Results

In the first experiment, the effects of various concentrations of quercetin (0–100 µg/mL) on viability of fresh spermatozoa were studied. Results showed that by increasing the concentrations of quercetin from 20 µg/mL, the viability of spermatozoa decreased significantly (P <0.05) (Figure 2). However, after the cryopreservation of sperm, 5 and 10 µg/mL of quercetin significantly increased the viability of spermatozoa in compared to 0, 20 and 50 µg/mL treatments (P <0.05) (Figure 3).
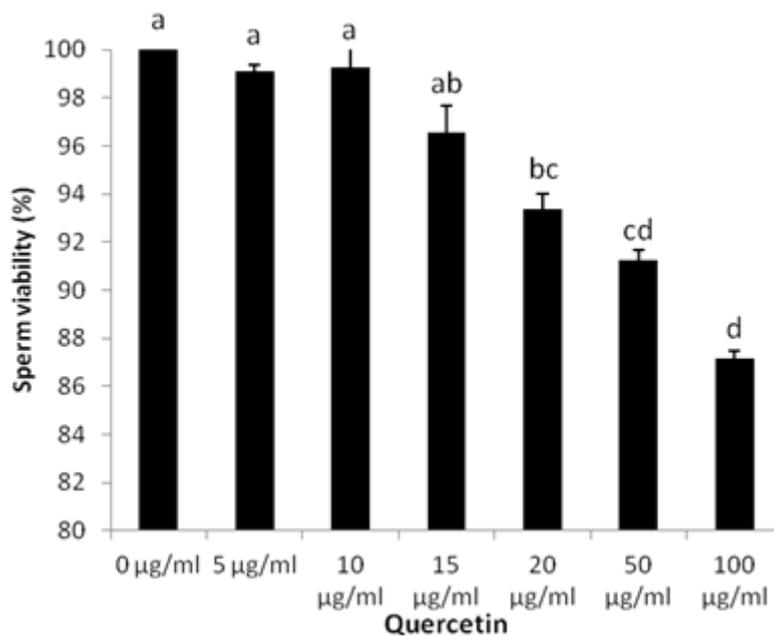

Figure 2 Effects of various concentrations of quercetin on viability of fresh spermatozoa
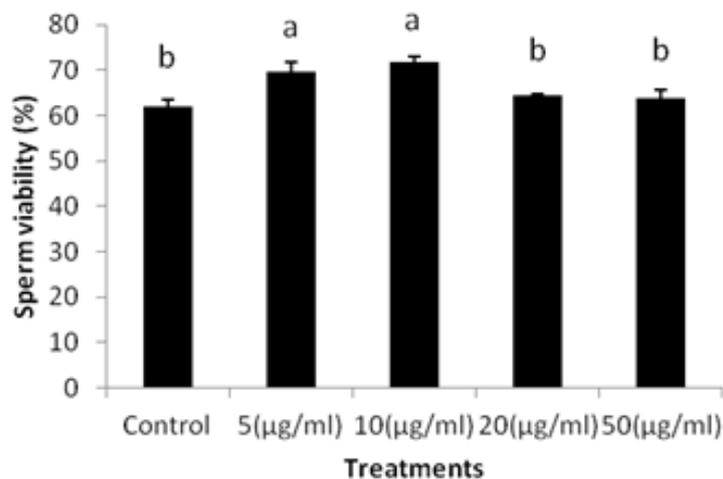

Figure 3 Effects of various concentrations of quercetin on viability of spermatozoa after freezing and thawing

Total motility and progressive motility of frozen-thawed spermatozoa with the addition of 5 and 10 µg/mL of quercetin were not affected in comparison with 0 µg/mL, although they decreased significantly with 20 and 50 µg/mL of quercetin (Figure 4).
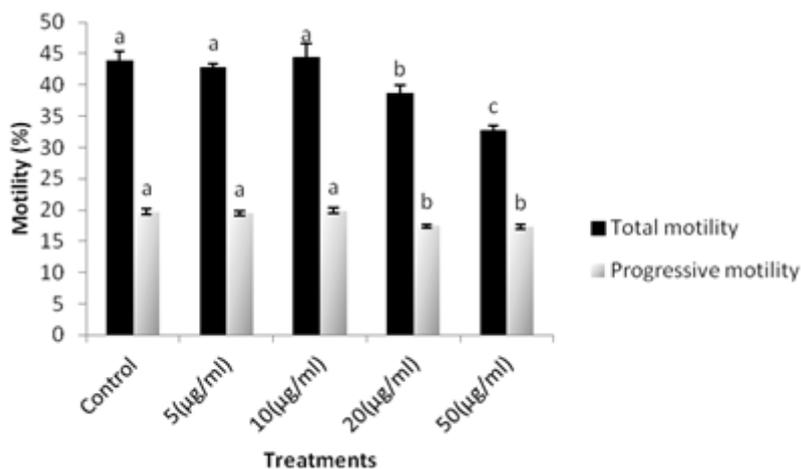

Figure 4 Effects of various concentrations of quercetin on total motility and progressive motility of spermatozoa after freezing and thawing

The fertility of frozen-thawed spermatozoa was evaluated by in vitro fertilization technique (Table 1). The mean number of zygote, morula and blastocyst stage embryos increased significantly with using 5 and 10 µg/mL of quercetin in comparison with other frozen-thawed treatments ($P < 0.05$). However, the blastocyst rate of fresh sperm was significantly higher than for other groups ($P < 0.05$).

Table1 Effects of quercetin in sperm extender on cleavage, morula and blastocyst development of sheep oocytes cultured *in vitro*

Treatments	Cleavage (%)	Morula(%)	Blastocyst (%)
Fresh sperm	83[a] ± 1	74[a] ± 2	37[a] ± 1
CS[*] (0µg/mL quercetin)	62[c] ± 3	49[c] ± 6	21[c] ± 2
CS (5µg/mL quercetin)	76[ab] ± 0.5	66[ab] ± 0.3	32[b] ± 0.1
CS (10µg/mL quercetin)	73[b] ± 2	64[b] ± 2	29[b] ± 1
CS (20µg/mL quercetin)	60[c] ± 2	51[c] ± 2	20[c] ± 3
CS (50µg/mL quercetin)	60[c] ± 1	46[c] ± 1	11[d] ± 1

[*]CS: Cryopreserved sperm
Data were expressed as mean ± SEM
Around 130 oocytes were used in each treatment
[a,b,c] Different letters indicate statistical difference within each column ($P < 0.05$)

Discussion

In vitro embryo production can be a valuable technique in reproductive biotechnology in farm animals. However, the feasibility and the quality of the produced ovine embryos are lower than for other species (Romao *et al.*, 2013).This is mainly because ram spermatozoa are sensitive to the conditions of low-temperature anabiosis and freezing (Sabev *et al.*, 2006). Although semen possesses an antioxidant system, cryopreservation increases the intensity of lipid peroxidation of spermatozoa and their activity is affected (Najafi *et al.*, 2014). Previous results showed that supplementation with antioxidants such as cysteamine,

ergothioneine, vitamin E analogue, Trolox, catalase, and superoxide dismutase mimetic agent reduces lipid peroxidation and H_2O_2 generation (Forouzanfar *et al.*, 2013). Increasing interest is currently being shown in the antioxidant activity of natural antioxidants and synthetic substances, such as clove bud, rosemary, resveratrol, and quercetin (Silva *et al.*, 2012; Baghshahi *et al.*, 2014; Motlagh *et al.*, 2014). Research has shown that the antioxidant activity of polyphenols is more effective and less toxic than vitamins E and C. However, various factors, such as method of administration, dose, and structure of phenolic compounds, affect their activities (Silva *et al.*, 2012). For example, antioxidants might have negative effects owing to excessive scavenging of free radicals, possibly by altering their physiological levels (Najafi *et al.*, 2014). As shown in the current study, viability, motility and fertility of spermatozoa decreased significantly when higher concentrations of polyphenol quercetin (≥20 µg/mL) were used.

The viability of frozen-thawed spermatozoa increased significantly by using 5 and 10 µg/mL quercetin in the freezing extender. However, total motility and progressive motility of frozen-thawed spermatozoa with 5 and 10 µg/mL of quercetin were not affected in comparison with 0 µg/mL (42±19% and 44±19% vs. 43±19%). In agreement to the current results, in a study on red deer epididymal spermatozoa after freezing and thawing, Anel-López *et al.* (2012) showed that despite obtaining good total motility after thawing (60±3%), the progressive motility of the samples was very low, with only 4±0.3% of the spermatozoa being identified as progressive. In the study of Silva *et al.* (2012), the progressive motility on ram ejaculated spermatozoa was 50±3%. These authors showed that there were no differences among experimental groups in progressive motility when using 5, 10, 15 and 20 µg/mL of quercetin. They also reported that there were no differences among experimental groups with regard to vigour, acrosome integrity and plasma membrane integrity. The percentage of sperm with a high mitochondrial membrane potential was greater in 0 µg/mL, then 5 µg/mL quercetin groups than those of 10, 15, and 20 µg/mL($P <0.05$). That mitochondrial membrane potential values decreased with increasing concentrations of resveratrol or quercetin was probably beneficial to sperm. The mitochondrial system is the main source of intracellular ROS, with the respiratory chain acting in an autoxidation process, whereas polyphenols, such as quercetin, have important roles in absorption and neutralization of ROS, because of their oxido reduction properties (Silva *et al.*, 2012). Bucak *et al.* (2007) showed that adding 5 mM of cysteamine to a ram freezing extender yielded a higher percentage of viable spermatozoa after thawing, although they did not find any improvement in plasma membrane functional integrity. However, Çoyan *et al.* (2011) found that 4 mM ergothioneine, despite the good motility results, reduced membrane integrity and mitochondrial activity.

Commonly, fertilization rate is considered the gold standard for assessing the cryopotential of semen cryopreservation media (Forouzanfar *et al.*, 2013). The current results showed that the freezing–thawing process of spermatozoa negatively affected the mean number of cleavage, morula, and blastocyst. However, the mean number of zygote, morula and blastocyst stage embryos increased significantly when using 5 and 10 µg/mL of quercetin compared with other frozen-thawed treatments ($P <0.05$). Peris *et al.* (2004) suggest that the poorer fertilizing efficiency of frozen ram semen might be at least partly due to the abnormal sperm DNA structure, despite having a normal appearance soon after thawing. Additionally, if the antioxidant capacity of the extender is insufficient, excessive ROS could attack sperm membranes, which are very susceptible to oxidants (Najafi *et al.*, 2014). Quercetin inhibits ROS formation by enzymatic and non-enzymatic systems, especially NADPH oxidase and NADH-dependent oxidoreductase, localized in the sperm plasma membrane and mitochondria, and scavenge ROS. This antioxidant induces calcium release into the cell, according to the time and concentrations of polyphenol, with control of intracellular free calcium concentration being crucial for the maintenance of normal cell function (Silva *et al.*, 2012).

Conclusions

To improve the quality of frozen-thawed ram epididymal spermatozoa, 5 and 10 µg/mL quercetin appears as an attractive option. However, the blastocyst rate of fresh sperm was significantly higher. Further studies to understand the synergistic effect of quercetin with other antioxidants in order to improve the ram freezing–thawing process are suggested.

Authors' Contributions

RA was involved in the experimental design, data collection and data analysis. MZ was involved in study design, data analysis, data interpretation and manuscript editing. MRS was involved in study design and data interpretation. All authors have read and approved the final manuscript.

Conflict of Interest Declaration

Authors declare that there is no conflict of interest for this study.

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