IJAS Iranian Journal of Applied Animal Science

Comparative Evaluation of Ascorbic Acid Supplementation in Tris-Egg Yolk Based Extender and Triladyl® Extender on Post-Thaw Kinematics Parameters of Sahiwal Bull Semen

Research Article

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Received on: 1 Mar 2020
Revised on: 30 Apr 2020
Accepted on: 16 May 2020
Online Published on: Mar 2022

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Online version is available on: www.ijas.ir

ABSTRACT

The current study was conducted to see the effects of ascorbic acid supplementation into tris-citric acid-glycerol-yolk (TCGY) extender and comparing it with Triladyl extender for bovine semen cryopreservation. Semen was collected from Sahiwal bulls for three weeks and each ejaculate (N=30) was divided into three aliquots. The first aliquot was extended using TCGY extender (control group), the 2nd aliquot was extended using TCGY supplemented with 2.1 mg/mL of ascorbic acid (AA group) and the 3rd aliquot of the same ejaculate was extended using Triladyl extender (TA group). Following the processing and freezing, thawed semen straws from each treatment were evaluated for sperm motility, viability, acrosome integrity and live/dead ratio. The average path velocity was the highest in TA group (77.99±2.99 µm/s) compared with other two groups (P<0.05). Similarly, values of total motility showed that TA group was superior to the other two groups (93.6±0.94% in TA versus 83.33±1.71% in control and 88.53±1.38% in AA group) (P<0.05). The amplitude of lateral head displacement (8.47±0.27 µm versus 5.00±0.22 µm and 4.74±0.13 µm), length of the average path (50.23±1.79 µm *vs.* 25.33±2.12 µm and 23.51±0.39 µm), length of the curvilinear path (85.01±3.03 µm *vs.* 38.89±1.48 µm and 38.65±0.77 µm), length of the straight-line path (37.28±1.33 µm *vs.* 19.39±0.84 µm and 20.42±0.29 µm) and track speed (130.93±5.33 µm/s *vs.* 89.13±3.47 µm/s and 92.11±1.88 µm/s) were significantly higher (P<0.05) in AA group as compared to TA and control group. Values for plasma membrane integrity (PMI), acrosome membrane integrity (AMI) and eosin were higher in AA and TA groups than in the control group (P<0.05). The addition of ascorbic acid in TCGY extender can serve as a better alternative to commercial extender for cryopreservation of semen.

KEY WORDS antioxidants, ascorbic acid, bovine semen cryopreservation, Triladyl.

INTRODUCTION

Cryopreservation of semen has a long history regarding the development of techniques and procedures for successful freezing to ensure post-thaw sperm quality and fertility. With the passage of time many ingredients have been utilized for the dilution of semen before cryo-preservation. The need for adding diluents or extenders is basically to protect sperms from damage during the process of cryopreservation. Egg yolk has been used due to its protective effect on the cooling of cattle semen and hence use of cooled or chilled semen started (Moussa *et al*. 2002; Thun *et al*. 2002). The effect of different concentrations on semen cooling was investigated (Vishwanath and Shannon, 2000),

thereby contributing towards the development of extenders. Commercial application of semen can be well assessed from the use of cryopreserved doses of semen (Ashrafi *et al.* 2013) while the freeze-thaw system has detrimental effects on the morphometric, kinematics and physical properties of the semen (Munsi *et al.* 2007; Peña *et al.* 2009). Tris-citrate-egg yolk-based extenders have fructose as an energy source; egg yolk has the beneficial role of providing low-density lipoproteins (LDL) to the plasma membrane of sperms to protect during the freezing procedure. It is achieved by incorporating lecithin into the lipid bilayer of the plasma membrane (Moussa *et al.* 2002). But in modern times the concept of biosecurity and the hazard of spreading disease from using animal source lipoproteins led to the development of non-animal origin sources of LDL such as sterile soy lecithin (SL). With these various commercial extenders came to replace locally made extenders. These include the commercial extender, Biociphos Plus and Bioxcell by IMV® France, Triladyl, Optidyl, Andromed and Biladyl by Minitub Germany, Bovipro and Botu-Bov by Biovet Brazil (De Ambrogi *et al.* 2006). While these extenders provide us with the luxury to be available as ready-to-use (RTU) ones, they do raise the cost more than double as compared to extenders made locally by semen cryopreservation labs.

The production of reactive oxygen species (ROS) is a biological process and helpful in many important functions of sperms like capacitation, acrosome reaction and penetration to zonapellucida (Aitken *et al.* 1995; Cocuzza *et al.* 2008). The oxidative stress occurs due to an imbalance between pro-oxidants and antioxidants. For example, with leukocytes contamination in the semen the superoxide radicals are produced (Aitken *et al.* 1996; Griveau and Le Lannou, 1997). ROS production by sperms may be either through the activity of mitochondria or through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme system associated with the plasma membrane (PM) of sperms while still in the testis (Vernet *et al.* 2001). The first mechanism, through the respiratory activity of mitochondria produces ROS physiologically and pathologically, through its electron transport chain system, production of superoxide resulting in H_2O_2 synthesis involving enzymes complexes (Saint-Pierre *et al.* 2002). The physiological role of ROS in the sperms is signal transduction. Many of the physiologically important functions depend upon the levels and types of ROS like hyper activation, capacitation, acrosome reaction and binding to zona pellucida. ROS has an effect of phosphorylation events in capacitation process (Aitken *et al.* 1995; De Lamirande *et al.* 1997; Leclerc *et al.* 1997; Lewis and Aitken, 2001).

Although seminal fluid has endogenous antioxidants (Fejercakova *et al.* 2013) to neutralize these ROS, the process of cooling, freezing and thawing disturbs this balance (Mostafa *et al.* 2009). Consequently, the sperm plasma membrane is prone to lipid peroxidation (LPO) by these ROS as bovine sperms are opulent in polyunsaturated fatty acids (PUFA) which may result in alteration of sperm viability, movement and acrosomal integrity (Bilodeau *et al.* 2000; Ansari *et al.* 2010). This over production needs to be cleansed off with the supplementation of non-enzymatic or synthetic antioxidants. Non-enzymatic antioxidants or synthetic antioxidants are many and comprise; reduced glutathione (GSH), urate, ascorbic acid, vitamin E (alpha-tocopherol), carotenoids (beta carotene), ubiquinone, taurine and hypotaurine, selenium, zinc, coenzyme Q-10, help in sperm protection during freezing (Therond *et al.* 1996; Holt, 2000; Sinclair, 2000) and others like riboflavin, trehalose, alpha-tocopherol and various natural oils like coconut oils to get better post-thaw semen quality (Aisen *et al.* 2000; Iqbal *et al.* 2016). To improve semen quality parameters to get better fertility rates, semen extenders are supplanted with substances like ascorbic acid (Azawi and Hussein, 2013). Ascorbic acid (AA) reduces tocopherol radical and thereby liberating tocopherol to be available for scavenging more molecules of ROS like hydroperoxides and peroxyl (Doba *et al.* 1985).

Vitamin C (ascorbic acid) is secreted in the seminal plasma from vesicular glands (Azawi and Hussein, 2013). Seminal plasma has higher concentrations of vitamin C as compared to blood plasma i.e. seminal plasma has 364 *vs.* 40 µmol/L in blood. Vitamin C is a water-soluble vitamin and is very potent in chain-breaking of ROS with the lipids of sperm plasma membranes thereby giving protection from LPO and also protecting sperms from these ROS (Dandekar *et al.* 2002) and detoxifies 55% of those ROS. It is considered among major non-enzymatic electron trapping antioxidants in semen (Zeitoun and Al-Damegh, 2015) which also protect the sperm genetic integrity by preventing from oxidative damage to DNA (deoxyiribo nucleic acid) (Alvarez and Storey, 2005). Ascorbic acid is a key antioxidant in plasma and inside cells, but it may relate with the plasma membrane by liberating electrons to the α-tocopherol radical and a trans-plasma membrane oxidoreductase movement (Dalvit *et al.* 1998; Sierens *et al.* 2002; Zieve, 2009). Ascorbic acid reduces the oxidative stress of testicular impairment in animals (Acharya *et al.* 2008). Ascorbic acid helps in improving the sperm viability, motility during cryopreservation (Soren *et al.* 2016) by improving the enzymatic antioxidant activity of seminal fluid (Foote, 2002). Vitamin C increases the level of catalases and superoxide dismutase (SOD) which act as important antioxidants against hydroxide ion and superoxide (Hu *et al.* 2010a). Effects of vitamin C as antioxidants have also been explored in recent studies (Kheradmand *et al.* 2006; Attia *et*

al. 2019; Raad *et al.* 2019). The objectives of the study are to compare post-thaw semen quality parameters using two extenders i.e., TCGY supplemented with ascorbic acid and Triladyl®.

MATERIALS AND METHODS

The study was conducted on Sahiwal bulls (*Bos indicus*) all managed under the same nutritional and managemental conditions at Semen Production Unit Qadirabad, District Sahiwal, Punjab, Pakistan.

Semen collection and processing

Five bulls ranging from 4-7 years of age were selected for semen donation. Two ejaculates per day, for three weeks were collected from each bull (six ejaculates from each bull). Each ejaculate was extended using TCGY extender, TCGY supplemented with vitamin C (ascorbic acid) and a commercial extender "Triladyl" (Minitub, Germany). Semen was collected by using an artificial vagina (AV) with inner liner (IMV®) at a temperature of 42-45 ˚C, AV was lubricated with non-spermicidal gel. The area around prepuce was washed and dried before collection. Each bull was given a false mount and a second mount was used to collect semen ejaculate. After 10 minutes, the second ejaculate was obtained without any false mount. Separate AV was used for every ejaculate of the same and different bull. Collected semen was shifted to the water bath immediately after collection at 37 ˚C. A withholding time of 10 minutes was given to each sample so that seminal plasma and sperms get homogenized.

Semen was evaluated grossly for volume, color, consistency and any contamination. Contamination free samples were evaluated by light microscopy (olympus BH2 phase contrast with heated stage at 37 ˚C). Mass activity and individual sperm motility were observed at 100X and 200X, respectively. In individual motility, a drop of 5 microliters (µL) was covered with a pre-warmed glass cover slip and observed at 20X (total magnification 200X), those samples with at least 70% morphologically normal and motile were passed for further test and those below this threshold value were discarded. The thawed samples from three treatment groups were assessed subjectively with phase-contrast microscope. To eradicate any subjective results/error, semen samples were also evaluated using CASA-CEROS II systems (Hamilton-Thorn Inc. USA).

The concentration of sperms was measured by using spectrophotometer (IMV ACCUCELL La'igle, France) with 510 nm wavelength. In this method 40 µl of semen was diluted with 3960 µL microliters of normal saline (NS) in a glass cuvette. Only those samples were accepted with concentration equal to or higher than 500 million sperm/mL of semen. Sperm concentration was kept 30 million spermatozoa per dose (0.5 mL straw).

Extender preparation

The extender for control group was prepared by dissolving 24.20 grams TRIS (24.2 g), citric acid (13.40 g), fructose (10 g) into 730 ml of distilled water (DW). This buffer solution was stirred with a magnet and put on hot plate stirrer to pasteurize at 65 ˚C for 30 minutes and then cooled down gradually to 37 ˚C. This buffer solution was added with 200 mL (20%) hen egg yolk, glycerol (70 mL) 7% and penicillin (1 million IU) and streptomycin (1 g) while stirring continuously. Experimental extender (AA group) consisted of TCGY extender supplemented with 2.1 grams of ascorbic acid with little modification as used by Hu *et al.* (2010b). Ascorbic acid was made part of the buffer solution when TRIS and other constituents were being mixed at 65 ˚C for 30 minutes. The extender used in (TA group) was a commercial product, Triladyl® by Minitube, Germany. The extender is also TRIS based extender which is supplied in 250 mL plastic bottles. The working extender is prepared by adding this 250 mL product into 730 mL of double distilled water and 200 mL of hen egg yolk, as instructed by the manufacturer.

Semen dilution, straw filling, cooling and cryopreservation

Ejaculates were divided into 3 aliquots. The first aliquot was diluted with the TCGY extender (control), 2nd with TCGY supplemented with 2.1 grams of ascorbic acid (AA group) and 3rd with Triladyl® (TA group). French straws (IMV®) with a volume of 0.5 ml were filled with diluted semen and sealed with automatic filling and sealing machine (Cryovet MRSPIC EECC3S) (Baracaldo, 2006). Semen filled straws were cooled to 4 ˚C and equilibrated for three and a half hours. Straws were frozen on static liquid nitrogen (LN_2) vapors 4 cm above from the liquid nitrogen (LN_2) in a wide mouth container (capacity 350 liters) on steel racks. These straws were frozen for 9 minutes. After this straw were directly immersed in LN_2. After 24 hours, one straw from each treated group was thawed at 37 ˚C for 45 seconds. The contents of the straws were emptied into pre-warmed and sterilized glass vials. A drop of 5 µL was put on a glass slide, covered with a cover slip and observed under 200X total magnification for post-thaw sperm motility. The same samples were assessed using Computer Assisted Semen Analyzer (CASA) system CEROSII by Hamilton-Thorn Inc. USA, for kinematic parameters.

Sperm plasma membrane integrity test

Hyposmotic swelling test (HOST) was used to assess sperm plasma membrane integrity. Jeyendran HOST solution was

used (Jeyendran *et al.* 1984). This solution was prepared by dissolving sodium citrate (7.35 g) and fructose (13.5 g) dissolved in 1 liter of distilled water. The solution had the final osmolarity at 155 mosmol/kg. The solution was pasteurized. Those sperms with plasma membranes (PM) intact swelled at their tail with bead formation while those with damaged PM had coiled tails. The test was carried out by mixing 500 µL of HOST solution with 50 µL of diluted thawed semen and incubated at 37 ˚C for 30 minutes. After 30 minutes, a drop of 5 µL was placed on a pre-warmed glass slide and covered with a cover slip and observed at 400X total magnification. The swollen heads or tails with bead formation were counted as +ve HOST while curled/coiled tails were counted as –ve HOST and 250 sperms were counted. Eosin Y test was performed to distinguish between live and dead spermatozoa.

Normal apical ridge

To see the intact acrosome membranes or acrosome processes, formal-saline solution (1%) was used. This solution was made by dissolving 1 mL of formaldehyde (37%) and sodium chloride (2.9 g) into 1000 mL of distilled water and pasteurizing the solution. The test was carried out by incubating 500 µL of a solution with 50 µL of thawed diluted semen at 37 ˚C for 45 minutes together. After this time, a drop of 5 µL was put on a glass slide with a cover slip on it and observed at 400X total magnification. Those sperms with clear demarcation of head boundaries against the background had intact acrosome processes while those without clear demarcation had their acrosomes reacted (Iqbal *et al.* 2016). At least 250 sperms were counted in different microscopic fields.

Statistical analysis

Analysis of variance (ANOVA) was carried out to determine differences among treatments on sperm motility, plasma membrane integrity and normal acrosome morphology and kinematics parameters like average path velocity (VAP), straight-line velocity (VSL), beat cross frequency (BCF) using Tukey's test. The analysis of variance table (ANOVA), 26 by using SPSS software was performed to check the significant differences among the treatment groups at 5% level of significance (SPSS, 2011).

RESULTS AND DISCUSSION

The total motility (%) was the highest in Triladyl® treated samples, (93.6±0.94), followed by 88.53 ± 1.38 in ascorbic acid and 83.33 ± 1.71 in the control TCGY group. The values differed significantly (P<0.05) for Triladyl® in comparison to the other two groups.

The values for progressive motility, although highest in AA treated group followed by TCGY and Triladyl®, did not differ significantly (P>0.05). Plasma membrane integrity and acrosome membrane integrity were preserved significantly better by Triladyl® treated groups in comparison to AA and TCGY group. The means of normal morphology do not differ significantly (P>0.05). Eosin Y test to distinguish between alive and dead spermatozoa shows that AA and Triladyl® provided better sperm protection against cryo-damage and showed significantly improved values than the TCGY group.

The kinematic parameters of three groups show that sperms have faster speed in Triladyl® and ascorbic acid extenders than in the control extender (Table 1). Similarly straight-line velocity values 56.53 ± 2.06 and 59.93 ± 2.03 for AA and Triladyl® are not statistically different but these two groups do different than the control group (P<0.05). The values for BCF are 26.31 ± 0.78, 25.92 ± 0.81 and 28.2 ± 0.35 for the three treatments lie in the same range and no significant difference was observed (P>0.05). Values for straightness shows Triladyl® and AA treatment groups have no significant difference but still have higher values than the control TCGY group.

The values of four semen parameters i.e. VAP, VSL, BCF and straightness are higher in both groups as compared to the control (Table 2). The comparison between the ascorbic acid group and Triladyl group reveals that the Triladyl treated semen doses yielded better results. Length of the average path (µm), length of a curvilinear path (µm) and length of straight-line path (µm) in the ascorbic acid group are significantly high as compared to control and Triladyl groups while linearity is significantly lower in ascorbic acid group only. A similar pattern is observed in other parameters like linearity (%), track speed (µm/s) and wobble % as well.

Sperms are fusogenic, as they have to undergo many vital changes/functions before they acquire the ability to fuse with the ovum and fertilize it. These functions are maturation, motility, energy metabolism to produce vigorous filamentary motions, capacitation and acrosome reaction, to name a few. All of these activities are only possible owing to the fluidity of sperm plasma membrane (PM) which makes possible for the sperms to fuse with PM of the ovum (syngamy) and this fluidity/flexibility owes its existence to PUFA in its lipids profile within the plasma membrane. These higher levels of PUFA and smaller amounts of cytoplasm make the sperms susceptible to attack by various ROS in its journey to reach the ovum. ROS cause oxidation/peroxidation of sperm plasma membrane making sperms PM fragile and rendering them infertile or even kill them (Aitken *et al.* 1996; Griveau and Le Lannou, 1997).

Table 1 Comparison of post thaw semen quality parameters in different treatment groups

Parameters	Treatment groups		
	Control (TCGY)	Ascorbic acid (2.1 g/mL)	Triladyl®
Total motility (%)	83.33±1.71^{a}	88.53±1.38^{b}	93.6±0.94^{c}
Progressive motility (%)	28.71±1.61^{ab}	31.97±1.79^{a}	23.32±2.6^{b}
Plasma membrane integrity (%)	56±0.98^{a}	65.2±0.95^{b}	67.47±0.62^{b}
Acrosomal integrity (%)	63.47±0.89^{a}	76.2±0.67^{b}	74.93±0.61^{b}
Normal morphology (%)	92.41±1.3^{ab}	91.77±1.05^{b}	95.85±0.53^{a}
Eosin Y (%)	56.27±0.89^{a}	65.67±1.02^{b}	68.33±0.72^{b}

TCGY: tris-citric acid-glycerol-yolk.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Table 2 Comparison of Computer Assisted Semen Analyzer (CASA) parameters among different treatment groups

Parameters	Control (TCGY)	Ascorbic acid (2.1 g/mL)	Triladyl®
Average path velocity (µm/s)	68.72±2.2^{a}	77.59±2.7^{b}	77.99±2.99^{b}
Straight line velocity (µm/s)	49.95±1.58^{a}	56.53±2.06^{b}	59.93±2.03^{b}
Beat cross frequency (Hertz)	26.31±0.78^{a}	25.92±0.81^{a}	28.2±0.35^{a}
Straightness (%)	66.83±1.1^{a}	72.9±1.09^{b}	75.78±1.0^{b}
Amplitude of lateral head displacement (µm)	4.74±0.13^{b}	8.47±0.27^{a}	5.00±0.22^{b}
Length of average path (µm)	23.51±0.39^{b}	50.23±1.79^{a}	25.33±2.12^{b}
Length of curvilinear path (µm)	38.65±0.77^{b}	85.01±3.03^{a}	38.89±1.48^{b}
Length of straight line path (µm)	20.42±0.29^{b}	37.28±1.33^{a}	19.39±0.84^{b}
Linearity (%)	54.88±1.19^{a}	47.10±0.95^{b}	53.14±2.06^{b}
Track speed (µm/s)	92.11±1.88^{b}	130.93±5.33^{a}	89.13±3.47^{b}
Wobble (%)	10.40±0.17^{b}	11.40±0.26^{a}	10.72±0.20^{ab}

TCGY: tris-citric acid-glycerol-yolk.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Although there is normally a balance between these ROS and their respective scavengers (antioxidants), this balance is disturbed during the pathological process of ultra-deep freezing of sperms using liquid nitrogen and subsequently thawing them. To make up for this loss, antioxidants are supplemented in the sperm preserving media to keep cryo-damage at the lowest possible level. Ascorbic acid is one such water-soluble chain-breaking antioxidant, which normally occurs in seminal plasma of many mammalian species (Alvarez *et al.* 1987; Aitkin, 1995; Amidi *et al.* 2016).

In the present study results of total motility were significantly better with Triladyl® treated group than the other two. These results are not in line with the results by Amirat *et al.* (2005). They showed Triladyl® treated group had more sperm damages and had values of velocity related parameters lower than Biociphos-Plus and another LDL based extender. May be this difference existed because they compared Triladyl® with egg yolk free extenders, as these extenders might have lesser viscosities than egg yolk based extenders, so that is why Triladyl® treated group produced poorer results in their study. AA treated group successfully preserved plasma and acrosome membranes integrity, and similar results in the study of Hu *et al.* (2010b). Eosin Y test (live/dead spermatozoa %) also produced significantly different results using Triladyl® and AA treated groups (P<0.05) in comparison with the control group. This finding also contradicts the findings by Amirat *et al.* (2005).

In present study, motility of sperms increased after addition of the vitamin C (ascorbic acid) in the TCGY based extender (83.33±1.71% *vs.* 88.53±1.38%) while highest motility (93.6±0.94%) was observed in the Triladyl® group presenting significant differences (P<0.05). Singh *et al.* (2015) revealed that vitamin C supplementation in TCGY based extender showed significantly higher progressive motility (P<0.05) as compared to simple TCGY extender which may be attributed to inhibition of LPO damage. Similar results of improvement in progressive motility by AA supplementation were observed in canine semen (Wittayarat *et al.* 2012), for bull semen (Stolbov and Rimanova, 1983; Raina *et al.* 2002) and ram semen (Azawi and Hussein, 2013). Natural antioxidants increase the motility of spermatozoa thus finally enhancing the fertility potential of the semen (Breininger *et al.* 2005). The significantly higher percentage of live spermatozoa (48.21%) was observed in vitamin C supplemented sample pre and post freezing stages (Singh *et al.* 2015) which is comparable to the results of live spermatozoa (48.40%) (Kumar *et al.* 1994). Sperm motility and viability increase have been observed after supplementing the extender with vitamin C (Wittayarat *et al.* 2012). In the present study acrosomal integrity 63.47 ± 0.89% in TCGY extender, 76.2 ± 0.67% in the AA supplemented TCGY extender and 74.93 ± 0.61% in the Triladyl® based extenders have been observed. The results are in line with previously reported

studies for acrosomal intactness as using vitamin C as a diluent in semen extenders the sperm acrosome intactness has been observed to increase in good quality (66-79%) and poor quality (53-68%) in bovines (Beconi *et al.* 1993) and 89.29% in the buffalo semen (Bhosrekar *et al.* 1994). The intact acrosome 50.68% has been observed in bull semen (Singh *et al.* 2015) which is lower than previously reported 60.70% and 70.70% (Singh, 1994). The effect of locally made semen extender containing LDL from egg yolk sources was better than commercial extender Optidyl® (Amirat *et al.* 2004). In a comparison of extenders and methods, local TRIS egg yolk based extender cryopreservation at room temperature packaging was more effective than commercial Biociphos-Plus® in bovine semen (Thun *et al.* 2002). Further, in comparison to Triladyl® and Optixell® and Tris egg yolk based extender, better post-thaw semen quality parameters and kinematics were observed using Optixell® for buffalo bull semen cryopreservation (Naz *et al.* 2018).

CONCLUSION

It is concluded from the present study that supplementation of ascorbic acid into TCGY extender improves plasma membrane integrity, acrosome status besides improving sperm velocity which can be used as a replacement for commercially available semen extenders, however, Triladyl® can also be used for better post-thaw semen quality.

ACKNOWLEDGEMENT

The author is thankful to Dr. Faiz ul Hassan for his generous efforts in the execution of research. I am especially thankful to all officials and lab staff of Semen Production Unit, Qadirabad, Sahiwal, for providing the opportunity to conduct this study.

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