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Post-Thaw Evaluation of Cryopreserved Bull Semen Extended In Four Different Semen Extenders

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ABSTRACT
BACKGROUND: Artificial insemination represents one of the most applicable technologies in cattle breeding. Semen quality post cryopreservation have been associated with many factors such as impending storage intervals, source of sperm, cryopreservation protocol and the most important is the type of extenders used. **OBJECTIVE:** This study aimed to evaluate the extending capacity of four different types of extenders namely, AndroMed ®, BioXcell ®, Triladyl® and Tris-egg yolk in terms of motility, viability, morphology, DNA integrity, plasma membrane integrity and acrosome integrity of post-thawed bull sperms. Three healthy matured Brangus-Hereford bulls were selected for this study. **MATERIALS AND METHODS:** Twenty four semen samples were collected using an automatic electro-ejaculator and semen quality was analysed using Computer Assisted Semen Analyser (CASA). Eosin-Nigrosin (EN) staining technique was applied to evaluate the morphology, viability and acrosome integrity whilst sperm DNA integrity was evaluated using modified acridine orange stain and hypo-osmotic swelling test was applied to evaluate sperm plasma membrane integrity. **RESULTS:** Amongst the four extenders, AndroMed ® resulted in superior extending capacity to sperm motility, morphology, DNA integrity, plasma membrane integrity and acrosome integrity. BioXcell ® and Tris-egg yolk extenders had the highest capacity to protect sperm viability. Triladyl ® showed lowest extending potential capacity to bull sperm. **CONCLUSION:** From the present study, AndroMed ® can be considered as the best suitable extender for bull sperm cryopreservation. BioXcell®, Triladyl® and Tris–egg yolk based extender can be still applied, but there is a need for more studies in future to improve their extending potential capacity.

INTRODUCTION

Many studies have been conducted after the first successful bull semen cryopreservation since 50 years ago (Gurler *et al*., 2016). Later on, through semen extenders capability for preserving semen, the technique of artificial insemination has been developed and many females were inseminated using single ejaculate (Büyükleblebici *et al*., 2014). Sperm examination in commercial farms is a fast growing technique in many developing and developed countries (Akhter *et al*., 2010). The conventional examination targets at providing information about the nature of sperm which is intended to be used for Artificial Insemination (AI). The result

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of such finding is crucial in determining the success of In Vitro Fertilization (IVF).The procedures carried out in cryopreservation of sperm are very laborious and tend to produce side effects on sperm viability.

The post-thawing sperm quality have been associated with many factors such as impending storage intervals, source of sperm and above all, the type of extenders used in achieving cryopreservation (Celeghini *et al*.,2008). Extenders are used in sperm preservation since they act as source of nutrients for the sperm, prolong its viability, extend the volume, shield against cold shock and other effects of chilling injury (Akhter *et al*., 2010). Low temperature levels are maintained to achieve sperm cryopreservation while using glycerol, a cryoprotectant, has been proven to protect semen (Morrell, 2006).

Egg yolk, a part of many commercial extenders, is a factor that many believed to be conferred as a preservatory cover on cryopreserved sperm, basically due to its low-density lipoprotein which protects the motility of sperm, integrity and fertilization capacity (Bencharif *et al*., 2010). On the other hand, egg yolk, as an animal protein resource, reduces the sperm quality through producing adverse biological risks. It also produces toxins, metabolic end products and cause infection (Kaka *et al*., 2015).Furthermore, time and technical constraints should be managed to produce effective egg yolk-based extenders. On the other hand, there are different kinds of egg yolks available with variable action, due to its variation in their phospholipids content (Aydin and Dogan, 2010).

Phosphatidyl choline, one of the main components of egg yolk phospholipids, play an important role in post-thaw sperm protection and it can be obtained from other resources (Pillet *et al*., 2012). Some alternatives to egg yolk for cryoprotection of sperm during freezing are reported such as soy lecithin, fatty acids such as stearic acid, oleic acid, and palmitic acid (Kumar *et al*., 2015). Aires *et al*. in 2003 has successfully used a commercial soybean-based extender in dairy bulls. Similarly, commercial extenders have been used in rams (Fukui *et al*., 2008) and domestic bucks (Salmani *et al*., 2014). Additionally, equine spermatozoa were protected using commercially available, liposomes-based extenders which are composed of egg yolk phospholipids and other kind of commercially available extenders during cryopreservation (Pillet *et al*., 2012; Leisinger *et al*.,2017) and likewise, bulls sperm was also reported by various studies (Röpke *et al*., 2011;Kaka *et al*., 2015; Khumran *et al*., 2015). Though there are different kinds of products available as commercial extenders, their effectiveness for sperm cryopreservation among cattle is yet a matter of debate.

A lot of information is available in literature indicating conservative potentials of various extenders, but they are not overwhelming (Clemente-Sànchez *et al*., 2014).Therefore, more studies need to be conducted at regular intervals to double check the quality and preservative capacities so as to avoid high cost of commercial extenders (Amirat *et al*., 2010; Rekha *et al*., 2016). The aim of this study is therefore to evaluate which of the four extenders provides the best cryopreservative capacity on bull semen.

MATERIALS AND METHODS

All the chemicals used in this study were purchased from Sigma-Aldrich chemical company/USA, unless chemicals are specified otherwise.

Animals and Study Area:

Three healthy Brangus-Hereford bulls of 4-5 years old weighing about 570±14.5 kg were selected from the university agricultural park farm (2^o 9'18.36" N, 101^o 43'49.61" E) in Universiti Putra Malaysia (UPM), Serdang, Malaysia. They were maintained under uniform management conditions and fed with freshly harvested *Brachiaria decumbens* grass and commercial supplements of palm kernel cake concentrates (2kg/animal/day) containing 15% protein, 22% profat, 17% fiber and 8% moisture. Water was supplied *ad libitum*.

Experimental Design:

Twenty four semen samples were collected from three healthy bulls by electro-ejaculation procedure on a weekly basis which were then transported and freshly evaluated, extended using four different semen extenders, then equilibrated at 4ºC for 2-3 hours. Further the samples were loaded in 0.25 mL French straws, cryopreserved manually by slow freezing using portable racks in a container (25 cm width, 50 cm long, 30 cm height) half-filled with liquid nitrogen, then stored in liquid nitrogen storage. Post-thawed straws were evaluated in two different storage time i.e., one week and two weeks of cryopreservation (-196ºC).

Semen Collection and Transportation:

An electro-ejaculator (Electrojac 6, USA) was used to collect 24 ejaculates i.e., 8 ejaculates from each bull at 4 days interval on a weekly basis of sample collection. The ejaculate was collected in a 15 mL conical glass test tube and immediately stored at 37°C in a box containing warm water (37°C) and then transported within 20-30 minutes to the laboratory of Theriogenology and Cytogenetic (Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine , UPM, Malaysia) where pre-freezing evaluations were documented. In this

study, only semen samples with general motility of more than 70%, concentration of at least 500×10^6/mL and viability of more than 80% were considered (Kaka *et al*., 2015).

Semen Dilution and Preparation of Extenders:

Semen was diluted with each of the four extenders. As shown in Table 1, the first extender is AndroMed® (Minitube, Germany), free from animal protein, was diluted in a ratio of 1:5 using distilled water. The second extender is BioXcell® (IMV, France) which was diluted in a ratio of 1:4 using distilled water. The third extender is Triladyl® (Minitube, Germany) which was diluted in a ratio 1:3 using distilled water along with freshly prepared whole chicken egg yolk of 20% (v/v). The fourth extender is Tris-egg yolk extendercomprising20 % v/v of chicken egg yolk, 2.42 g Tris (base), 1.48 g citric acid, 1.00 g fructose, 6.4 ml glycerol, 25mg gentamicin, 50,000 IU penicillin, and qsp 100 ml of double distilled water (Amirat-Briand *et al*., 2010). The pH of the extenders were adjusted to 6.7. Thereafter, semen samples were extended to adjust the concentration of sperm to 25×10^6 cells in a 0.25 mL straw (IMV, French) sealed by polyvinyl chloride powder and then chilled slowly to a temperature of 4°C for a period of 2-4 hours to attain equilibrium. The packed straws were frozen using vapour freezing method. The straws were later placed on racks and held horizontally 3-4 cm above liquid nitrogen for about 8-10 min. The straws were left to float on the racks for just 3-5 minutes before plunging them in the liquid nitrogen.

Tests and Procedures Used In Post-Thaw Evaluation of Sperms:

Fresh Semen Quality Evaluation:

The samples were evaluated using a Computer Assisted Semen Analyser (CASA, IVOS Hamilton Thorne Biosciences, version 12.2.). Magnification, cell size, contrast and integrity were 1.92, 5 pixels, 40 and 55 respectively. Ten µl of diluted semen was dropped in both the chambers of a pre-warmed glass slide (Hamilton Thorne research 2X-cell, 20 µm), covered with thin cover slide and loaded in CASA for analysis (Khumran *et al*., 2015).

Post-thaw Evaluation of General Motility of Sperms:

Post-thaw evaluation was performed after one week of storage using CASA in which four straws from each extender were evaluated separately. The process was repeated once at 14 days of storage. Four straws of the same extender were randomly selected, thawed in water bath at 37°C for about 30 seconds, wiped dry with tissue paper followed by cutting the sealed end so that the semen from one straw was poured into 5 mL pre-warmed glass test tube. The process was repeated with three straws of the same extender. Each tube was manually shaken to mix the semen well. Then 10 µl of the semen from each of the four tubes was dropped on both the chambers of a pre-warmed CASA slide (Hamilton Thorne research 2X-CEL dual-sided sperm analysis chamber; depth: 20µm), then covered with a cover slip, and immediately loaded in CASA for analysis.

Morphology and Viability of Sperms:

Morphology and viability was evaluated using EN stain, as described by Evans and Maxwell (1987). The stained slide was subsequently air dried and examined under light microscope at 400× microscope at 1000× magnification (Nikon Eclipse 50i, Japan). Sperm cells were considered alive when the head is unstained, while pink–stained heads indicated a dead sperm. Abnormalities of the sperm were also examined using the same slide which was used for viability evaluation. The percentage of normal sperm cells were calculated out of the 200 examined sperm cells (Memon *et al*., 2012).

Acrosome Integrity of Sperms:

Acrosome integrity was evaluated by dropping semen sample into three drops of EN stain. A smear was made and the stained slide was subsequently air dried and examined under a phase-contrast microscope at 1000× (oil) magnification under oil immersion (Yildiz *et. al*., 2000). A sperm cell, with smooth-oval head and a clearly defined cap (acrosome) adjoined to the tail by mid-piece without any visible defect was considered as a normal acrosome (Nagy *et al*., 2013).

DNA Integrity of Sperm:

As described the Tejada *et al*. (1984), the DNA integrity was assessed. Ten µL of post-thawed semen sample was gently smeared on a glass slide and air-dried. The smeared slide was fixed in methanol-glacial acetic acid (1:3 v/v) overnight. The slide was removed from the fixative, air-dried and stained with 0.1% acridine orange staining solution for 5 minutes in a dark room. After staining, the slide was gently rinsed by a stream of distilled water, kept in a wooden slide box for one hour until evaluation. Spermatozoa were evaluated under fluorescent microscope (Zeiss Eurostar, Germany 100×). Sperm with green head fluorescence is considered normal, whilst sperm with red head is considered as denatured DNA. The percentage of denaturated DNA sperms was calculated out of total 100 evaluated sperms.

Australian Journal of Basic and Applied Sciences, 11(5) April 2017, Pages: 80-87

Sperm Plasma Membrane Integrity of Sperms:

Plasma membrane integrity of sperms was evaluated using hypo-osmotic swelling test.The hypo-osmotic swelling stock solution was prepared as described by and Revell and Mrode (1994) i.e., 9 g of fructose with 4.9 g of sodium citrate per liter of distilled water. Plasma membrane integrity was performed by incubating 30 µL of semen with 300 µL of hypo-osmotic solution at 37ºC for 60 min. After incubation, 0.2 ml of the mixture was spread with a cover slip on a warm slide. Two hundred sperms were evaluated (magnification 1000×) with bright-field microscopy. Sperm with swollen or coiled tails were recorded (Buckett *et al*., 1997).

Statistical Analysis:

The data was analysed using SPSS software system (Version 12.0, SPSS Chicago, IL). The sperm quality parameters were analysed using a 2-way factorial analysis of variance. Values were expressed as mean ± standard error of the mean (S.E.M).The level of statistical significance was at $P<0.05$.

Results:

The extender that produced the highest acrosome integrity was AndroMed® with 94% and 93.90% for first and second weeks respectively. The acrosome integrity of sperms which were extended with four extenders was found to be lower for Triladyl with 64.89% and 63.47% for first and second weeks respectively (Table 3).

This was followed by BioXcell® with 90.99% and 89.90% acrosome integrity for first and second weeks respectively. Meanwhile the Tris-egg yolk-based extender showed an acrosome integrity of 90.56% and 88.38% for weeks 1 and 2 respectively (Table 3). The differences among the sperm extenders for acrosome integrity were significant at $P < 0.05$.The highest motility was recorded with AndroMed® (i.e., 76.25% and 77.05% for week 1 and 2). The effects of the four extenders on sperm motility showed lower motility with Triladyl® 60 % and 61% for week 1 and 2 respectively (Table 2). The sperms cryopreserved in Tris-egg yolk had a motility value of 65.58% and 66.08% for weeks 1 and 2 respectively. BioXcell® however showed 63% and 62.23% for weeks 1 and 2 respectively. The differences among the sperm extenders for sperm motility were significant at $P < 0.05$.The highest sperm viability was with BioXcell® which showed 91% and 90.46% for weeks 1 and 2followed by Tris-egg yolk based extender (91.17% and 90.26% for weeks 1 and 2),. Among the extenders, Triladyl® produced lower viability of 89% and 89.29 % for week 1 and 2 respectively (Table 2). AndroMed® had viability of 89.967% and 88.37% for weeks one and two respectively. The differences among the sperm extenders for viability were not significant at $P < 0.05$.

The DNA integrity was highest in AndroMed® with 94.25% and 92.90% at week 1 and week 2 respectively (Table 3). This was followed by BioXcell® which had85.12% and 84.56% for week 1 and week 2 respectively. The lowest sperm DNA integrity observed in both Triladyl® (64.55% and 64.90% for week 1 and week 2 respectively) and also in Tris-egg yolk based (65.34% and 64.09% for weeks 1 and 2 respectively).There were significant differences among the semen extender for DNA integrity of the sperms. ($P < 0.05$). Sperm's normal morphological appearance was found to be highest in AndroMed® with 88.39% and 88.03% for weeks 1 and 2 respectively (Table 2) followed by BioXcell® for weeks 1 and 2 (88.03% and 87.90%) and Triladyl® (with minimal difference for the weeks 1 and 2 (87.38% and 88.38%)). Tris-egg yolk based extender secured the last position with the data for weeks 1 and 2 (88.12% and 87.46%). The differences among the sperm extenders for normal morphology were not significant at $P < 0.05$.

The sperm plasma membrane integrity was significant in AndroMed® with 74.23% and 73.47% for weeks 1 and 2 respectively (Table 3). This was followed by BioXcell® with 68.25% and 67.58% for weeks 1 and 2 respectively. Then followed by Tris-egg yolk based extender with 59.89% and 59.0% for weeks 1 and 2 respectively. The lowest plasma membrane integrity was in Triladyl® with 56.87% and 55.48% for weeks 1 and 2 respectively. There were significant differences ($P < 0.05$) among the semen extenders for sperm plasma membrane integrity.

Discussion:

The present study evaluated certain indicators that determine the fertilizing capacity and quality of cryopreserved sperms. Acrosome integrity, motility, viability, DNA integrity, morphology and plasma membrane integrity were carefully measured. The sperm samples were extended using four different types of semen extenders such as AndroMed®, BioXcell® Triladyl® and Tris-egg yolk-based extender. The parameters were measured in two separate storage times (i.e., week 1 and week 2) in liquid nitrogen (-196 ºC).

The result of the present study showed that the sperm motility was highest in AndroMed® in both weeks. In this study, results of sperm motility concur with previous studies which indicated that the same, AndroMed® had the highest extending potentials in African buffalo (Herold *et al*., 2004). But other studies had defied Triladyl as the one which has the highest extending capacity (Beran *et al*., 2012; Fleisch *et al*., 2017). The findings of our study disagree with the findings of Fleisch *et al*. (2017) which might be due to age of bulls used (18-36 months), final sperm concentration (60×10^6 spermatozoa) per mL, the equilibration time before semen cryopreservation, or automatic freezer used. Our findings are in agreement with that of the study conducted by

Australian Journal of Basic and Applied Sciences, 11(5) April 2017, Pages: 80-87

Akhter *et al*. (2010) but it differs from those observed in water buffalo (Herold *et al*., 2006)might be due to final sperm concentration per mL (80×10^6 spermatozoa / mL) or might be due to the way how semen is collected (Artificial vagina) which reported better total motility in liposome-based extender than in soybean-based AndroMed® and BioXcell® (Kumar *et al*., 2015).Lowest motility was observed with Triladyl extender indicated in both weeks. This findings disagree with the results of Herold *et al*. (2006). This disagreement might be due to the way how sperms are collected (flushed epididymal sperm) or the species of the animal (buffalo) used in that study.

The reports obtained from the study done by Akhter *et al*.(2010) evaluated several sperm extenders and this study inferred that BioXcell® extenders had highest potentials in preserving sperm viability than Tris-egg yolk based extender. However, BioXcell® was not regarded as a good extender for preserving viability of sperm (Celeghini *et al*, 2008).The reasons for this disparity might be attributed to the way how sperms are collected, species and age of animals used (buffalo), suggested sperm concentration stored 50×10^6 motile sperm, size of straws used (0.5 mL) or laboratory techniques used in measuring this parameter. In this study, the normal sperm morphology was within the standards among the four different extenders which emphasizes that the media of all the four different extenders did not affect the morphology of the sperm cells negatively.

The results for acrosome integrity of post-thawed sperm in this study contradicted with the reports of Maxwell (2007) in which he reported that Tris-egg yolk based extender showed highest post-thawed sperm acrosome integrity, plasma membrane activity and DNA integrity than AndroMed®. Furthermore, sperm cryopreservation as process, induces oxidative reaction that leads to inactivity of sperm's plasma membrane and acrosomes fusion (Mostek et.al. 2017). On the other hand, the result of the present study, in terms of acrosome integrity of post-thawed sperms agree with the reports of Akhter *et al*. (2010). However, from literature all the various methods of sperm stimulation had always been used and their applications were undoubtedly a matter of choice and convenience (Galli et. al.2003).

Generally, DNA fragmentation index could be higher in post-thawed semen than in fresh semen, due to high production of hydrogen peroxide as end product during the sperm metabolism (Gurler *et al*., 2016).DNA integrity was highest in AndroMed® than the other extenders used in this study and this could be due to the ability of AndroMed® to reduce the hydrogen peroxide levels during cryopreservation process. While BioXcell® showed DNA integrity lower than AndroMed® during the same environment and during same cryopreservation process. Furthermore, Triladyl® and Tris-egg yolk extender showed the lowest extending capacity through DNA integrity percentage, whilst post-thaw sperm quality was better in ram semen frozen in Triladyl® than Tris-fructose egg yolk (Rekha *et al*., 2016). While study of Clemente-Sànchez *et al*. (2014) in white-tailed deer used different commercial soybean-based extender (BioXcell®) presented similar post-thaw parameters to Triladyl® and Biladyl® 8% extenders, this could explain the species variation related with the kind of extender.

This means that the egg yolk based extenders are weaker in extending capacity to sperm DNA. This findings disagree with the result of Water house *et al*(2010)for which the reason could be that our study evaluated post-thawed sperm DNA integrity while Waterhouse *et al*.(2010) evaluated sperm DNA integrity throughout the incubation time with Triladyl®. There is a significant difference among DNA integrity of the sperm extenders as $P < 0.05$. The sperm plasma membrane integrity was assessed together with DNA integrity of the sperm in the present study. This results agree with the findings of Aitken *et al*. (2009). This was followed by BioXcell® in protecting the DNA of the sperm and this agree with the results of Akhter *et al*. (2010) while our findings showed that Tris-egg yolk and Triladyl® were providing less protection to sperm DNA than in BioXcell® and the reason either could be due to the high concentrations of glutathione in BioXcell® or the lipoprotein in egg yolk resulted in low protection of post-thawed sperm DNA. There is a significant difference among the semen extenders for plasma membrane activity of post-thawed sperm at $P < 0.05$.

Table 1: Preparation of the four different bull semen extenders.

Extender	Dilution factor	Egg yolk
AndroMed®	1: 5	--------
BioXcell®	1:4	--------
Triladyl®	1:3	20%
Tris-egg yolk	2.42 g Tris (base), 1.48 g citric acid, 1.00 g fructose, 6.4 ml glycerol, 25mg gentamicin, 50,000 IU penicillin, and qsp 100 ml bidistilled water	20%

Table 2: Effect of four semen extenders on frozen-thawed sperm motility %, sperm viability % and sperm normal morphology % ± SEM in two different storage time.

Extender	Sperm Motility %		Sperm Viability %		Sperm Morphology %	
	week 1	week 2	week 1	week 2	week 1	week 2
AndroMed®	76.25±1.55[a]	77.05 ±1.09 [a]	89.67 ±1.98 [a]	88.37 ± 1.23[a]	89.39 ± 1.12[a]	88.03 ±1.34[a]
BioXcell®	63 ± 1.75 [b]	62.23± 1.56 [b]	91 ± 2.64 [a]	90.46 ± 2.23 [a]	88.03± 0.56 [a]	87.90± 0.78[a]
Triladyl®	60 ± 2.24 [b]	61 ± 1.69 [b]	89 ± 2.14 [a]	88.29 ± 1.89[a]	87.38 ±0.42 [a]	88.38±0.56 [a]
Tris-egg yolk	65.58±2.48 [b]	66.08 ±2.12 [b]	91.17±2.06 [a]	90.26± 1.90 [a]	88.12 ±0.27 [a]	87.46 ±0.67[a]

Within columns, [a, b] values of different letters are significantly different. ($P \leq 0.05$).

Table 3: Effect of four semen extenders on sperm acrosome integrity %, sperm plasma membrane integrity % and sperm DNA integrity % ± SEM of frozen-thawed spermatozoa in two different storage time.

Extender	Sperm acrosome integrity %		Sperm plasma membrane integrity %		Sperm DNA integrity %	
	week 1	week 2	week 1	week 2	week 1	week 2
AndroMed®	94 ± 1.34 [a]	93.90 ± 1.23 [a]	74.23±1.89 [a]	73.47 ± 1.45 [a]	94.25 ±1.34 [a]	92.90 ± 1.22 [a]
BioXcell®	90.99± 1.2 [a]	89.90 ± 1.34 [a]	68.25±1.76 [b]	67.58± 1.55 [b]	85.12 ±1.88 [b]	84.56± 1.67 [b]
Triladyl®	64.89±1.78 [b]	63.47 ± 1.89 [b]	56.87±2.12 [c]	55.48 ± 1.93 [c]	64.55± 1.99 [c]	64.90 ±1.45 [c]
Tris-egg yolk	90.56 ±1.17 [a]	88.38± 1.24 [a]	59.89± 1.18 [c]	59± 1.23 [c]	65.34 ± 2.12 [c]	64.09 ±1.89 [c]

Within columns, [a, b, c] values of different letters are significantly different. (P≤ 0.05).

Conclusion:

To conclude the present study, AndroMed ® can be considered as the best suitable extender for bull sperm cryopreservation. BioXcell®, Triladyl® and Tris–egg yolk-based extender can still be applied but need more studies in future to improve their extending potential capacity. This present study showed that AndroMed® possess highest extending property compared to other extenders despite the lingering opinion from previous studies that encouraged the application of egg-yolk based extenders for sperm extension. On the other hand, our study confirmed that all the mentioned extenders either commercial (AndroMed ®, BioXcell®, and Triladyl®) or locally produced extenders (Tris-egg yolk based extender), possess good extending capacity and all are still within the accepted value in semen cryopreservation. Storage time of cryopreserved semen samples were not significantly different among all the four extender groups. Finally, it is concluded that the manual freezing of the semen sample still competing the programmable freezing of the loaded straws unless it is a little bit time consuming process.

Future work:

For future work, we might suggest that AI protocol should be repeated using fixed timed artificial insemination with semen produced by different commercial extenders and its effect on the fertility trail and connecting such results with *invitro* embryo production (IVP).

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