JOURNAL OF TROPICAL LIFE SCIENCE
2023, Vol. 13, No. 2, 383 – 392
http://dx.doi.org/10.11594/jtls.13.02.17

Research Article

The Effectiveness of Adding Different Sucrose in Tris-Egg Yolk Diluent on the Sperm Fertility of Sexing Swamp Buffalo (*Bubalus bubalis*)

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Article history:
Submission August 2022
Revised December 2022
Accepted December 2022

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ABSTRACT

Spermatozoa sexing requires a diluent that can protect and provide an optimal environment to maintain the quality of spermatozoa. This study targets to determine the effectiveness of adding sucrose to tris-egg yolk diluent on the sperm quality of the swamp buffaloes produced by sexing with the egg albumin method. Semen was collected once a week for 12 consecutive weeks with an artificial vagina from three male buffaloes at the Indonesian Center for Animal Research and Development (ICARD) in April - June 2021. Semen was separated by sedimentation using 10% and 30% egg albumins. Semen from sexing was divided into four diluent treatments, namely 20% tris-egg yolk (TEY), TEY+0% sucrose, TEY+0.2% sucrose, TEY+0.3% sucrose, and TEY+0.4% sucrose. Parameters measured included: motility, viability, intact plasm membrane, and intact acrosome cap of buffalo sperm. The results showed the motility of X sperm: 43.75-47.5%, Y sperm: 40-45%, the viability of X sperm: 78.25-79.75% and Y sperm: 77.25-79.75%, intact plasm membrane of X sperm: 70.5-71.5% and Y sperm: 70.5-71%, and viable acrosome-intact for X sperm: 79-80% and Y sperm: 78.75-79.5%. The addition of sucrose up to 0.4% into tris-egg yolk diluent was proven can maintain motility, viability, and integrity of cell membrane and acrosome of spermatozoa of water buffalo that was sexed with egg albumin.

Keywords: Acrosome, Intact membrane plasm, Motility, Sexing, Spermatozoa, Sucrose

Introduction

Buffalo is one of the ruminants that become a source of meat, where buffalo contribute 8% of the protein needs of the Indonesian people. The total increase in the buffalo population in Indonesia from 2018 to 2020 was only 1.9% [1]. It was due to the reduced number of males as the effect of the large number of males used on religious holidays and community customs [2]. The use of artificial insemination technology with sperm sexing is one solution to overcome the shortage of males [3].

The sexing process with sedimentation (albumin column) has been carried out on dairy cows [4], Sumba cattle [5], and buffalos [6]. The albumin column is based on the difference in the motility of spermatozoa X and Y as an implication of differences in mass and size, in which Y spermatozoa move faster or have high penetration [7]. One of the weaknesses of buffalo sperm is its sensitivity to changes in temperature and physical treatment due to the specific phospholipid composition [8]. Holt [9] stated that the causes of the low quality of buffalo sperm are the type of diluent, the condition of the spermatozoa, and the cryoprotectant used. The use of carbohydrates in semen diluents, apart from being a source of energy, also functions as a cryoprotectant [7]. Bearden & Fuquay [10] added that tris egg-yolk can maintain sperm viability as egg yolk contains low-density

How to cite:
Yendraliza, Bukhori A, Erwan E, *et al.* (2023) The Effectiveness of Adding Different Sucrose in Tris-Egg Yolk Diluent on The Sperm Fertility of Sexing Swamp Buffalo (*Bubalus bubalis*). Journal of Tropical Life Science 13 (2): 383 – 392. doi: 10.11594/jtls.13.02.17.

protein that can replace damaged and carbohydrates of the disaccharide group are able to protect the plasm membrane of sperm by binding to water molecules in the medium so as to prevent nucleation and the formation of ice crystals when there is a change in temperature [11]. The centrifugation during the sexing process makes sperm require greater energy to survive. One of the possible measures to maintain the quality of sexing buffalo spermatozoa is by adding sugar (carbohydrates) to the diluent solution. Fructose is a monosaccharide carbohydrate commonly added to semen diluents of various livestock as it is easily converted into energy [12]. Sucrose is a disaccharide composed of one molecule each of glucose and fructose. So, it is expected that the addition of sucrose in the semen diluent will produce twice as much energy as glucose and fructose because it has more C, H and O elements. Sucrose has been shown to improve the quality of frozen semen in cattle [13]. Rizal *et al.* [14] showed that the addition of sucrose to the Andromed® diluent could improve the quality of the striped buffalo epididymal spermatozoa after thawing. Moreover, the addition of 0.3% sucrose into tris-egg yolk diluent can maintain the quality of Garut sheep spermatozoa during storage at 5°C [15].

This study aimed to determine the right concentrations of sucrose in tris-egg yolk diluent to maintain the quality of the sexing spermatozoa of buffalo. Improving the quality of spermatozoa after separation is expected to increase the success of pregnancy in various reproductive technologies, such as Artificial Insemination (AI).

Material and Methods

Semen collection

This research has been approved by the ethics committee of the Faculty of Agriculture and Animal Science, State Islamic University of Sultan Syarif Kasim Riau No: KE/KEP-FPP/04/04/2021**.** The semen used in this study came from three bulls swamp buffalo aged between 7 and 10 years which were kept Indonesian Center for Animal Research and Development (ICARD), Ciawi, Bogor, West Java. Buffaloes were kept according to the management at the ICARD and placed in individual cages. They were fed with 45 kg/day/head of elephant grass and 5 kg/day/head of concentrated feed while drinking water was provided ad-libitum.

Semen was collected once a week for 12 consecutive weeks at 8 AM using an artificial vagina (IVM, France) at 42°C. The quality of fresh semen was evaluated. The semen that was further processed was those that had motility of >65%, abnormalities of <15% [8] and intact plasm membrane percentage of >65% [16].

Separation medium preparation

The material used for sexing was egg white (albumin). Albumin was dissolved in tris-buffer solution. 2 mL of 30% albumin was used as the lower fraction, added into a separator tube with a diameter of 1.8 cm and a volume of 10 mL, followed by adding 2 mL of 10% albumin as the upper fraction.

Diluent preparation

A 3.049 g Trisaminomethane, 1.7 g citric acid, 1.25 g fructose, sucrose (Table 1), and antibiotics (penicillin 0.1 g and streptomycin 0.1 g) were put into an Erlenmeyer and homogenized with 80 mL double-distilled water. Before being used, 20% egg yolk was added to the diluent.

Spermatozoa separation and dilution

The evaluated semen was diluted in the tris-

Table 1. Composition of 100 ml tris-egg yolk diluent with different levels of sucrose

Ingredient	Sucrose			
	0%	0.2%	0.3%	0.4%
Tris-aminomethane (g)	3.049	3.049	3.049	3.049
Fructose (g)	1.25	1.25	1.25	1.25
Citric acid (g)	1.7	1.7	1.7	1.7
Egg yolk (mL)	20	20	20	20
Penicillin (g)	0.1	0.1	0.1	0.1
Streptomycin (g)	0.1	0.1	0.1	0.1
Sucrose (g)	-	0.318	0.477	0.636
Distilled water (mL)	80	80	80	80
Total (mL)	100	100	100	100

Source: Modification of ICARD standard for diluent composition

egg yolk (control). Diluted semen (1 mL) was put in a syringe containing the separation medium and left for 45 minutes at room temperature [17]. The lower and upper fractions (2 mL for each) were then mixed with diluent (1 mL) in separated centrifuge tubes. Next, 1 mL of the middle fraction was discarded. Each fraction was centrifuged for 5 minutes at 1500 rpm. The supernatant was discarded to obtain clean spermatozoa precipitate from the separating medium. The precipitate was added with 0.5 ml of diluent and stored in a vessel filled with water at 30-33 C. Spermatozoa were then diluted with a diluent to obtain a concentration of 50-100 million sperm/ml.

Determine the percentage of sperm motility

Semen (20 µL) was dripped on top of the glass slide with a micropipette, covered with a cover glass, and observed in five fields of view under a microscope with 400x magnification and equipped with a heating table (37 °C). Sperm motility was assessed with the following formula:

$$\frac{\sum moving\ sperms}{\sum total\ sperms} \times 100\%$$

Determine the percentage of live spermatozoa

One drop of fresh semen was mixed with one drop of eosin-nigrosin on a slide glass and smeared with cover glass [18]. The smear was observed under a microscope with a magnification of 400x. Spermatozoa that absorb colour mean that the spermatozoa are dead, while those that do not absorb colour are alive. The percentage of live sperm was measured as follow:

$$\frac{\sum live\ sperms}{\sum total\ sperms} \times 100\%$$

Determine the percentage of intact plasm membrane

Assessment of the plasm membrane integrity of spermatozoa was using the Hypoosmotic Swelling Test (HOS-Test). The HOS-Test solution consisted of a mixture of solutions A (1.35 fructose + 50 ml distilled water) and B (0.735 sodium citrate + 50 ml distilled water) (IRIAP, 2021). Semen (20 µL) was added with 80 µL of HOS-Test solution and left for 30 minutes in a water bath (37 C) [19]; [2]. After incubation, 10 µL of semen mixture was observed under a microscope with 400× magnification. Straight tails characterized Spermatozoa with damaged plasm membranes, whereas the intact plasm membranes were noticed by circular or bulging tails. The percentage was then measured as follows:

$$\frac{\sum sperms\ with\ intact\ plasma\ membranes}{\sum total\ sperms} \times 100\%$$

Determine the percentage of intact acrosome

The semen was mixed with formal saline solution (physiological NaCl + 1% formalin) in a ratio of 1:5 into an Eppendorf tube, left to rest for a while, and examined with a phase-contrast microscope using a 400× magnification to count a minimum of 200 spermatozoa [20]. The intact acrosome was marked with black colour on the head of the sperm. The percentage was assessed as follow:

$$\frac{\sum sperms\ with\ intact\ acrosome}{\sum total\ sperms} \times 100\%$$

Data Analysis

The data obtained were analyzed using a randomized block design and regression analysis to examine the effect of adding sucrose on the sexing sperm quality of buffalo. The data was processed using SAS version 9.0.

Results and Discussion

Characteristics of the fresh semen of water buffalo

The characteristics of the fresh semen observed in this study are presented in Table 2. The volume of collected semen in this study was 1.63 ml, in line with Sianturi *et al.* [21], who stated the semen volume of buffalo ranged from 1.44 to 1.76 ml. However, several studies had contradictions to this result. Yendraliza *et al.* [2] found that the ejaculated semen of buffalo has a lower volume (0.9 mL), while Herbowo *et al.* [22] and Ghodasara *et al.* [23] reported higher results of 2.25 mL and 5.11 mL, respectively. The difference in the semen volume is caused by environmental factors, season, age of livestock, and feeds [24]. The motility, viability, intact plasm membrane, and intact acrosome of fresh semen in this study have met the criteria to be further diluted. This is following the standard for fresh semen of Indonesian buffalo, which is 50-70% [25]. The color of the semen obtained was milky to creamy white. Jainudeen & Hafez [26] stated that the semen of buffalo is cream, creamy white, or milky white.

The consistency of semen in this study was

Table 2. Characteristics of fresh buffalo semen

Characteristic	Value
Volume (ml/ejakulation)	1.6 ± 0.15
Color	Milky white-Creamy white
Consistency	Moderate
Odor	Specific smell of semen
Concentration (x 10^6 sperm/mL)	1 360 ± 5.22
Mass movement	++ - +++
Motility (%)	72.5 ± 2.88
Viability (%)	83.75 ± 2.62
Intact plasm membrane (%)	76.75 ± 0.95
Intact acrosome (%)	84.25 ± 1.70

Table 3. Spermatozoa concentraton (10^6 spermatozoa/ml) after separation process (sexing)

Stage	Concentration (million/mL)
Upper fraction (X sperm)	220 ± 2.94
Lower fraction (Y sperm)	87.5 ± 2.5

moderate or rather thick, as Ghodasara *et al.* [23] found in their research. Consistency correlates with sperm concentration [8]. The concentration of buffalo sperm in this study was 360 × 10^6 spermatozoa/mL with a mass movement of ++ - +++. The semen motility observed in this study was higher than that of the Murrah buffalo (66.3%) [27]. The viability and intact plasm membrane of fresh semen of water buffalo differed from the swamp buffalo reported by Eriani *et al.* [28]. Likewise, the acrosome of the water buffalo was higher than that of the Egyptian buffalo [29]. Differences in ejaculatory characteristics from previous research may be influenced by several factors, including animal type, age, health condition, season, collection experience, collection frequency, and ejaculation treatment and management [30]; [31].

Sexing quality of water buffalo semen

The concentration of spermatozoa after separation using albumin was divided into two, that was upper and lower fractions. The higher the albumin concentration in the lower layer, the smaller the spermatozoa concentration (Table 3).

Motility

The average motility of separated spermatozoa decreased compared to the motility of fresh semen. This decrease is due to the spermatozoa passing through the separation process that requires large energy to maintain physiological conditions [31]. The addition of 0.4% sucrose in tris-egg yolk diluent produced the best motility value compared to 0.2% and 0.3% sucrose for both X and Y spermatozoa. However, the motility of X sperm was lower than Y sperm, as the Y sperm in the lower fraction moved further to penetrate the albumin layer and expend more energy. Y spermatozoa have a high penetration power to penetrate solutions, such as egg albumin [32]. Based on SNI [33], the sexing semen from this study is suitable for Artificial Insemination (AI) as it shows minimum spermatozoa motility of 40%.

Based on Figure 1, it can be seen that addition of sucrose increased the sperm motility after the separation (sexing) process in both fractions. Apart from being a source of energy, sucrose in tris-egg yolk diluent is a cryoprotectant for sperm storage. Uchida *et al.* [34] stated that the carbohydrates would bind to water molecules in the diluent to prevent nucleation and the formation of ice crystals. On the other hand, the tris-egg yolk contains low-density lipoprotein (LDL). Farstad [35] mentioned that LDL could adhere to the surface of the sperm plasm membrane to replace lost and damaged phospholipids. Furthermore, Bergeron *et al.* [36] and Akhter *et al.* [37] added that LDL would bind to the protein PDC-109, prevent phospholipid efflux, and produce a protective layer on the surface of the sperm membrane.

The sperm motility after sexing in this study was lower than the sperm motility of Pakistani buffalo using the swim-up method with 68.33% for X sperm and 70.83% for Y sperm [38]. In the same separation method, the motility value of water buffalo sperm was lower than that of Sumba cattle, 63-65% for X sperm and 49-59% for Y sperm [5]. This difference is caused by the type of livestock, method and type of diluent, and the quality of fresh semen [39].

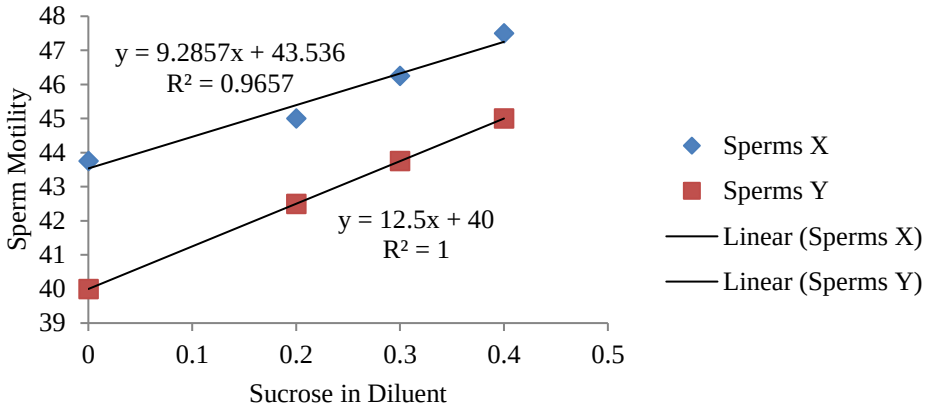

Figure 1. The Motility of Separated (sexing) Spermatozoa

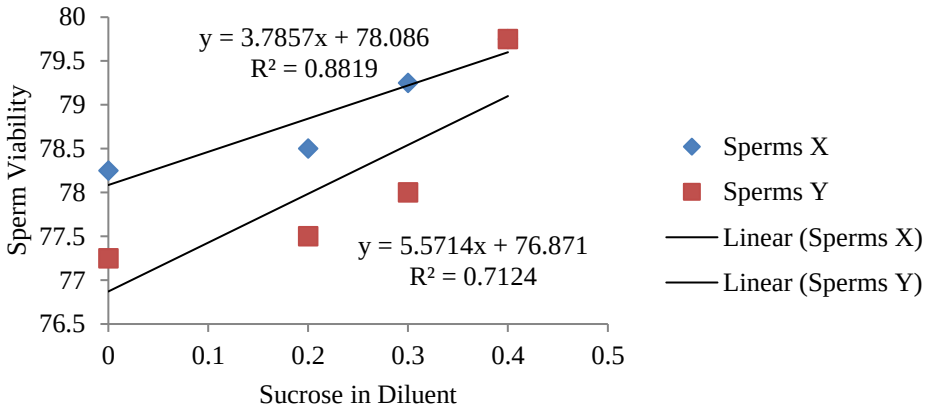

Figure 2. The Viabilty of Separated (sexing) Spermatozoa

Viability

The addition of several concentrations of sucrose into tris-egg yolk diluent showed an increase in the percentage of viability of both X and Y spermatozoa (Figure 2). The presence of tris in the sexing diluent of buffalo sperm can maintain pH, osmotic pressure, electrolyte balance, and the availability of energy sources for sperm [40]. Thus, the viability value of buffalo sperm after separation is categorized as good (Indonesian National Standardization [25]. Fahy [41] suggested that sucrose in sperm diluents can help vitrify solutions, stabilize proteins and membranes. Therefore, the separated sperm viability increased along with the increase in the dose of sucrose. This is reinforced by Qadeer *et al.* [42], that sucrose in diluent will be metabolized through the glycolysis pathway or continued with the Krebs cycle to produce energy in the form of ATP, which is utilized by sperm to move.

This study recorded the viability of X and Y spermatozoa was 78.25 – 79.75 % and 78.75 – 79.5 %, respectively. These results were higher compared to the Nili-Ravi buffalo in Pakistan [43] and Sumba cattle [5]. This difference may be due to the type of animal, the method of separating, and the type of diluent used [44].

Intact plasm membrane

The administration of several concentrations of sucrose in tris-egg yolk had no significant effect (P>0.05) on the percentage value of intact plasm membranes in both X and Y spermatozoa. The viability of separated buffalo sperm at different concentrations of sucrose in egg yolk was categorized as good (70.5%-71.5%) (Figure 3). This is presumably because the carbohydrates contained in the cell envelope are still available. Srivastava *et al.* [45] stated that the cell plasm membrane contains carbohydrates that are bound to lipids (glycolipids) or to proteins (glycoproteins) called the cell envelope or glycocalyx [46]. Manjunath [47]

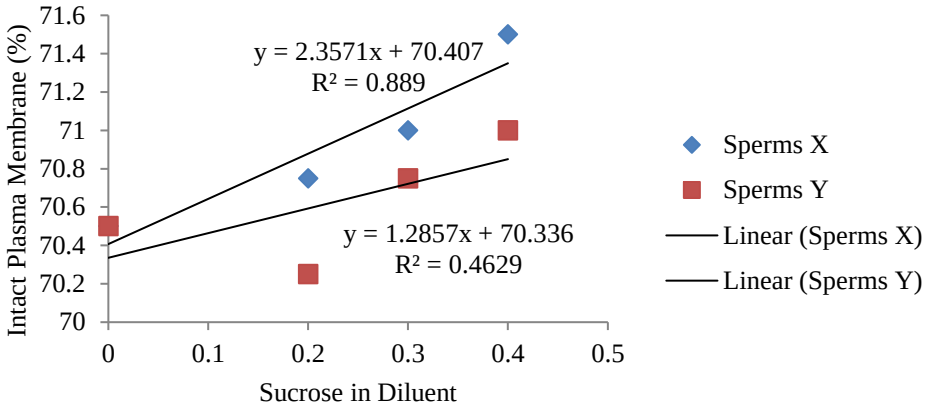

Figure 3. The Intact Plasm Membrane Percentage of Separated Spermatozoa

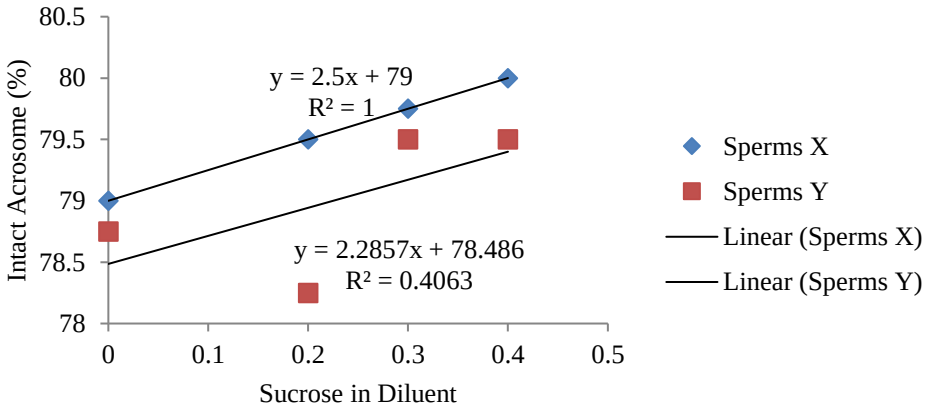

Figure 4. The Intact Acrosome Percentage of Separated Spermatozoa

also mentioned that plasm membrane plays an important role in regulating all processes that occur in the cell. Moreover, sucrose is an effective extracellular cryoprotectant to maintain the integrity of cell membranes [48]. The good integrity of the plasm membrane in this study is reflected by the good motility and viability of the separated spermatozoa, as explained by Amirat *et al.* [49]. Sperm resistance is related to the hydraulic permeability properties of the membrane and the tolerance level and domain arrangement of the sperm membrane [50].

The intact plasm membrane of Y spermatozoa (56.33%) in this study was lower than Nili-Ravi buffalo. However, the X spermatozoa had a similar percentage (70.5 – 71.5%) to the Nili-Ravi buffalo, as reported by Asma-ul-Husna *et al.* [43]. In contrast, the plasm membrane integrity of spermatozoa from Sumba cattle is 63.44 -71.58% for X spermatozoa and 48.43 – 70.64% for Y spermatozoa [5]. With the same separation method, Rasad *et al.* [51] noted a higher integrity percentage for both spermatozoa in goats. The difference in percentage values was due to the different tolerance levels of sperm interacting with diluents and physical treatment during the process. This study showed that the osmotic stress and mitochondrial performance in counteracting free radicals in sperm were still functioning, reflected by the high motility and viability [45].

Intact acrosome

Both spermatozoa from water buffalo were proven to have a quite high percentage of intact acrosome (78.75% -80%) (Figure 4). The high percentages of the intact plasm membrane and acrosome after separation indicate that most spermatozoa have not experienced changes in the acrosome reaction. Because carbohydrates are the main component of the acrosome [47], they can be utilized in the metabolic process of spermatozoa. Furthermore, the head of the spermatozoa has an

acrosome with a double-wall structure located between the plasm membrane of the anterior part of the nucleus. However, the value of the intact acrosome between the X and Y spermatozoa was different. This difference was due to the function of the membrane as a protective acrosome of spermatozoa [44]. However, the administration of sucrose in tris-egg yolk for separating the semen of water buffalo still had an impact on acrosome values. The acrosome has a pivotal role in the fertilization process. The acrosome status determines the success of fertilization to perform the acrosome reaction and oocyte fusion [48]. Awan *et al.* [52] stated that sperm quality as an indicator of AI's success is based not only on the progressive motility of the spermatozoa but also on the integrity of the acrosome, which should surpass 50%. This result is in line with the opinion of Yildiz *et al.* [53], which concluded that the combination of monosaccharides and disaccharides with appropriate concentrations could protect spermatozoa.

However, the results of this study differ from several studies on various types of animals. By adding trehalose and EDTA, the motility of frozen sheep semen and diluted semen became 64% and 52.10%, respectively [47]. The addition of sucrose or trehalose in the diluent significantly increased the motility of spermatozoa in frozen bovine semen [13]. The mixture of tris and sucrose also improved the frozen semen quality of Bali cattle. The differences above are due to differences in the type of animals, diluent, and method used.

Conclusion

Adding sucrose up to 0.4% in tris egg yolk diluent can maintain the values of motility, viability, intact plasm membrane, and acrosome for X sperm and Y sperm of water buffalo produced by sexing with albumin.

Acknowledgment

The author would like to thank the Institute for Research and Community Service of the State Islamic University of Sultan Syarif Kasim Riau, which funded this study under contract number 0245/R/2021.

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