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A comparative study of Sephadex, glass wool and Percoll separation techniques on sperm quality and IVF results for cryopreserved bovine semen

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The aim of this study was to compare the effects of spermatozoa separation techniques on sperm quality and *in-vitro* fertilization (IVF) results for cryopreserved bovine semen. Sephadex, glass wool and Percoll gradient separation techniques were used for sperm separation and sperm motility, morphology and membrane integrity were evaluated before and after separation. Also, cleavage and blastocyst developmental rate were investigated after IVF with sperm recovered by each separation technique. The motility of samples obtained by the three separation techniques were greater compared to the control samples ($p < 0.05$). The percentage of spermatozoa with intact plasma-membrane integrity, identified by 6-carboxyfluoresceindiacetate/ propidium iodide fluorescent staining and the hypo-osmotic swelling test, was highest in the glass wool filtration samples ($p < 0.05$). The cleavage and blastocyst rate of total oocytes produced from glass wool filtration samples were also higher than the control and Sephadex filtration samples ($p < 0.05$), but were not significantly different from Percoll separation samples. However, a significantly greater number of cleaved embryos produced by glass wool filtration developed to blastocyst stage than those produced by Percoll separation ($p < 0.05$). These results indicate that spermatozoa with good quality can be achieved by these three separation techniques and can be used for bovine IVF. In particular, it suggests that glass wool filtration would be the most effective method of the three for improving sperm quality and embryo production for cryopreserved bovine spermatozoa.

Keywords: glass wool, *in-vitro* fertilization (IVF), Percoll, Sephadex

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Introduction

Sperm selection is essential to obtain spermatozoa of good quality and high density from frozen-thawed semen for *in-vitro* fertilization (IVF). Most spermatozoa are damaged during semen freezing and thawing processes. Freezing and thawing procedures are mostly harmful to sperm membranes, since temperature- and osmotically-induced changes occur in the organization, fluidity, permeability, and lipid composition of sperm membranes. Thus the freezing and thawing process produces a low motility percentage and damages membrane structures resulting in a low half-life in the female genital tract and concomitant fertility decay [18]. Furthermore, these dead and abnormal spermatozoa exert toxic [28] and lytic [39] effects on companion cells in semen, and therefore have negative effects on fertility.

Assisted reproductive techniques such as artificial insemination, IVF and intracytoplasmic sperm injection (ICSI) bypass cervical mucus which affords clear advantages for genetic control, disease reduction and economical production of food-producing animals through differential selection of motile spermatozoa and by acting as a physical barrier to nonmotile cells. Therefore, spermatozoa separation techniques capable of acting as this physical barrier are required to remove spermatozoa damaged by the freeze-thaw process in IVF, as selecting spermatozoa with good quality is a major factor in achieving successful fertilization through IVF [22].

There are a number of semen manipulation techniques available for removing undesirable spermatozoa, seminal plasma, cryoprotective agents and other factors. The techniques include the Sephadex column, glass wool filtration, and the Percoll density gradient centrifugation technique. These spermatozoa separation procedures have been characterized with human spermatozoa [4,10,22,33,38,42] and have also been evaluated for use with bovine spermatozoa [1,2,27]. Filtration through a Sephadex

column [1,2,21] and isolation by density gradient centrifugation in Percoll [27,35] have allowed improvements in the quality of bovine semen. In cases of high viscosity [34,46], poor semen quality [23] or cryopreserved ejaculates [8], the glass wool filtration method has proved to be advantageous [12].

However, comparative data concerning the effectiveness of biophysical treatment methods such as Sephadex, glass wool and Percoll in cryopreserved bovine semen has been lacking, although previous experiments have established that various semen manipulation techniques increase the qualitative features of spermatozoa [8,23,27,35]. Also, very few reports are available regarding IVF results of spermatozoa isolated by Sephadex and glass wool filtration despite their excellent ability to improve sperm quality in post-thaw bovine and other species semen [46]. Therefore, the aim of this study was to find the most effective method by comparing the efficacy of sperm separation methods (Sephadex, glass wool and Percoll) on sperm quality, such as motility, morphology and plasma-membrane integrity, and evaluating the effect of these methods on IVF results in frozen-thawed bovine semen.

Materials and Methods

Chemicals and biologicals

All chemical reagents used for this experiment were purchased from Sigma Chemical Company (USA) except for fetal calf serum (FCS), Dulbecco's phosphate-buffered saline and Tissue culture medium 199 (TCM 199), which were from Gibco BRL (USA). Cryopreserved bovine (Korean native cattle; Bos Taurus coreanae) semen in 0.5-mL straws were purchased from the National Agricultural Cooperative Federation (Korea) and the same batch of frozen semen from the same animal was pooled after thawing for experimentation.

Column preparation

Sephadex filtration column: A Tris-glucose-citric acid solution [24 mg/mL Tris (hydroxymethyl) aminomethane, 14 mg/mL citric acid and 8 mg/mL glucose in distilled water, osmotic pressure 325 mOsm/kg, pH 7.0] without glycerol and egg yolk was used to prepare 20% (w/v) slurries of Sephadex G-15. Sephadex was allowed to swell overnight at 5°C as previously described [1]. The filtration column was prepared in a 3-mL disposable plastic syringe. A small amount of glass wool was compressed with the plunger to the bottom of the barrel to prevent loss of Sephadex. Approximately 5 cm of plastic tubing (inner diameter: 1.5 mm) was attached to the tip of the syringe and clamped. One mL of Sephadex G-15 slurry was gently layered over the glass wool and allowed to settle for 3 ~ 4 min. Immediately before semen filtration, the buffer part of slurry was removed by releasing the tubing clamp. The free end of the tubing was inserted in the collection tube at 37°C.

Glass wool filtration column: Glass wool (microfiber code 112; John Manville, USA) filtration was performed as previously described [12] with slight modifications. Briefly, 25 mg of pre-cleaned glass wool micro fiber was gently placed at a depth of 1 cm in the barrel of a 1-mL disposable syringe. The column was vertically suspended and rinsed repeatedly with Brackett and Oliphant (BO) medium [5] including 5 mM caffeine sodium benzoate and 10 μg/mL heparin to remove any loose wool fibers prior to filtration. The rinsed column was inserted in the collection tube at 37°C.

Percoll density gradient column: Percoll density gradient separation was performed as described by Parrish *et al.* [32] with some modifications. A stock of Percoll solution was prepared at a 9 : 1 mixture of Percoll and a ×10 stock of salt solution (2.889 g NaCl, 0.238 g KCl, 0.116 g KH_2PO_4, 0.112 g $CaCl_2$ and 0.163 g Hepes in 50 mL distilled water). The 90% Percoll solution was obtained by diluting a stock of Percoll solution with BO medium. To prepare the 45% Percoll solution, the 90% Percoll solution was mixed at a 1 : 1 ratio with BO medium. In a 15 mL conical tube, 1.5 mL of the 90% Percoll solution was placed, and 1.5 mL of 45% Percoll was smoothly layered over this.

Semen separation procedures

Frozen bovine semen in 0.5-mL straws was thawed in a water bath for 1 min at 37°C and was used for control (not filtered), Sephadex, glass wool and Percoll density gradient separation. One mL of thawed semen was gently layered onto each column. Sephadex and glass wool filtration samples were filtered by placing columns in a water bath at 37°C for 5 ~ 10 min. Percoll density gradient separation was performed by centrifugation at 300 × g for 20 min; the pellet was recovered after aspiration of the supernatant. All recovered semen samples were washed with 6 mL of BO medium by centrifugation at 300 × g for 5 min. After washing, sperm samples were adjusted to 5×10^6/mL in BO medium containing 5 mM caffeine sodium benzoate, 10 μg/mL of heparin, 10 mg/mL of bovine serum albumin (BSA) to evaluate sperm quality and to use as 100 μL-droplets of spermatozoa for IVF.

Evaluation of sperm

For evaluation of progressive motile sperm, 10 μL of diluted semen was placed on a clean microscope slide, and covered with a coverslip. The percentage of progressive motile spermatozoa was determined by observing a minimum of 300 sperm, in at least 6 different fields with a bright field microscope at ×400.

Morphology of spermatozoa [29] was evaluated by DiffQuik staining kit (International Reagents, Japan). A

drop on a glass slide was drawn out as for blood smear, and allowed to air-dry. The slide was placed in each of the three DiffQuik solutions for 5 min each, then rinsed and allowed to dry. At least 200 spermatozoa were evaluated with light microscopy at ×1,000.

Sperm membrane integrity was assessed using a 6-carboxyfluoresceindiacetate/propidium iodide (CFDA/PI) fluorescent staining technique and the hypo-osmotic swelling (HOS) test. Staining media for CFDA/PI stain was prepared within 1 h prior to use, using 20 μL of formaldehyde stock solution (2.5 mg/mL in water), 20 μL of 6-carboxyfluorescein diacetate stock solution (0.5 mg/mL in DMSO) and 20 μL of propidium iodide stock solution (0.5 mg/mL in water) per mL of BO medium. CFDA/PI staining was carried out by incubating 100 μL of semen with 300 μL of staining media at 37°C for 15 min in the dark. A 5-μL aliquot of stained suspension was placed on a slide and covered with a coverslip. Random fields were observed under a fluorescence microscope (×400) and 200 spermatozoa were counted. Staining with CFDA was assessed using a B-2A filter (blue excitation range, with a 450～490 nm excitation filter; Nikon, Japan), while staining with PI was assessed using a G-2A filter (green excitation range, with a 510～560 nm excitation filter; Nikon, Japan). Sperm showing partial or complete red fluorescence (PI staining) were considered membrane-damaged, while sperm showing complete green fluorescence were considered membrane-intact. The HOS test was performed by incubating 30 μL of semen with 300 μL of a 100 mOsm hypoosmotic solution (9 g fructose plus 4.9 g sodium citrate per liter of distilled water) at 37°C for 45 min. After incubation, 200 spermatozoa were evaluated under ×400 with phase contrast microscopy. Sperm with swollen or coiled tails were considered membrane-intact.

In vitro fertilization

IVF was performed with sperm samples prepared by each treatment. First, IVF was performed to compare results of IVF among control, Sephadex and glass wool filtration samples. The method showing the best results among these methods was then compared with the Percoll separation samples.

Bovine (Korean native cattle; *Bos Taurus coreanae*) ovaries were collected from a local abattoir and transported to the laboratory in saline containing antibiotics (100 IU/mL penicillin G and 100 μg/mL streptomycin). Oocytes were aspirated from follicles (2～8 mm diameter) using an 18-gauge needle and cumulus-oocyte complexes (COCs) were selected on the presence of multilayered compact cumulus cells and homogeneous ooplasm. Selected COCs were rinsed in TCM 199 supplemented with 10% FCS. Sets of 20 COCs were matured in 100-μL droplets of maturation medium (TCM 199 containing 10% FCS, 0.5 μg/ mL FSH, 0.5 μg/mL LH and 1 μg/mL β-estradiol) under mineral oil at 38.5°C for 20 to 22 h in an atmosphere of saturated humidity and 5% CO_2. After maturation, COCs were washed with BO medium containing 5 mM caffeine sodium benzoate, 10 μg/mL of heparin, and 10 mg/mL of BSA to partially remove expanded cumulus cells from oocytes. Sets of 20 oocytes were then fertilized with 100-μL droplets of spermatozoa (5×10^6/mL) that had been prepared by the three treatment methods and control. At 5- to 6- h post-fertilization, these sets of 20 presumptive zygotes were washed with TCM 199 containing FCS and cultured in 100-μL droplets of TCM 199 containing 10% FCS at 38.5°C and 5% CO_2. During culture, fertilization and embryo developmental rates were defined by cleavage and blastocyst rates evaluated at 48 h and on day 7 to 9 after fertilization. Blastocyst rates were also reevaluated by calculating blastocyst production of cleaved embryos as well as total oocytes.

Statistical analysis

Statistical analysis of data was performed by SPSS 15.0 software. For data with normal distribution, ANOVA and *t*-test were used, and the Least Significant Difference multiple comparison test was used to calculate the difference between samples in case of showing significant difference in ANOVA. Otherwise, nonparametric Kendall's W test was used in violation of normal distribution. p values < 0.05 were considered statistically significant. All data are presented as mean ± SE.

Results

In order to evaluate and to compare the effectiveness of different spermatozoa treatments, the percentage of motile spermatozoa from cryopreserved bovine semen was determined. From the data presented in Fig. 1, spermatozoa recovered by the different spermatozoa treatments showed a significant increase in the percentage of motility with respect to control samples ($p < 0.05$), but the percentage of motility did not differ among the spermatozoa treatments ($p > 0.05$). The percentage of motility was 45.83 ± 7.35, 64.17 ± 6.51, 65.83 ± 5.98 and 70.83 ± 6.25% for control, Sephadex filtration, glass wool filtration and Percoll separation samples, respectively. The percentage of spermatozoa with normal morphology was not significantly different among all groups (Table 1) and was above 80% in all groups.

The percentage of intact plasma-membrane was identified by CFDA/PI fluorescent staining and hypo-osmotic swelling test (HOST) (Fig. 2). Spermatozoa obtained by glass wool filtration had the highest percentage of intact membrane from the two evaluation methods ($p < 0.05$). In CFDA/PI fluorescent staining, the different spermatozoa treatments significantly increased the percentage of spermatozoa with intact plasma-membrane versus control.

The percentage of spermatozoa with intact plasma-membrane showed greater value in the order written; control (54.75 ± 8.59%), Sephadex filtration (66.93 ± 6.06%), Percoll separation (74.95 ± 4.43%) and glass wool filtration samples (87.07 ± 1.77%) ($p < 0.05$). In HOST, the percentages of intact plasma-membrane were higher in glass wool filtration (75.52 ± 3.96%) and Percoll separation samples (58.38 ± 2.22%) than in control samples (44.97 ± 3.54%) ($p < 0.05$). But Sephadex filtration samples (54.00 ± 5.19%) were not significantly difference with control and Percoll separation samples.

To compare the ability of spermatozoa to fertilize oocytes and oocytes development into blastocysts *in vitro* according to different sperm treatments, cleavage and blastocyst rates were investigated after IVF. First, a comparison of control, Sephadex and glass wool filtration samples is shown in Table 2. The samples recovered by glass wool filtration had higher cleavage and blastocyst rate of total oocytes than control and Sephadex filtration samples ($p < 0.05$). The blastocyst rate of cleaved embryos produced by glass wool filtration samples was higher than that of control samples ($p < 0.05$), but did not differ significantly from that of Sephadex filtration samples ($p > 0.05$). The samples obtained by glass wool filtration produced more blastocysts by producing more cleaved embryos than the other experimental samples, but cleaved embryos development into blastocysts was not statistically significantly different between glass wool and Sephadex filtration samples. The Sephadex filtration did not improve the cleavage and blastocyst rates versus

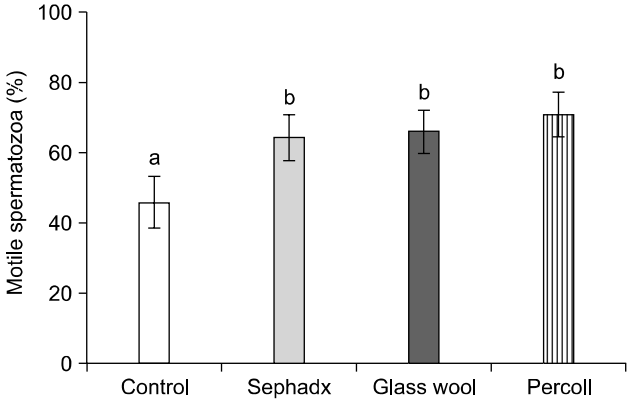

Fig. 1. Sperm motility in treated and control samples. Data are presented as mean ± SE. [a,b]Different superscripts indicate significant differences among treatments ($p < 0.05$, n = 6).

Table 1. Percentage of spermatozoa with normal morphology in treated and control samples

	Separation techniques of spermatozoa			
	Control	Sephadex	Glass wool	Percoll
Normal morphology (%)	88.00 ± 1.51	85.50 ± 2.49	88.83 ± 1.92	88.83 ± 1.52

There were no significant differences across groups. All data are presented as mean ± SE (n = 6).

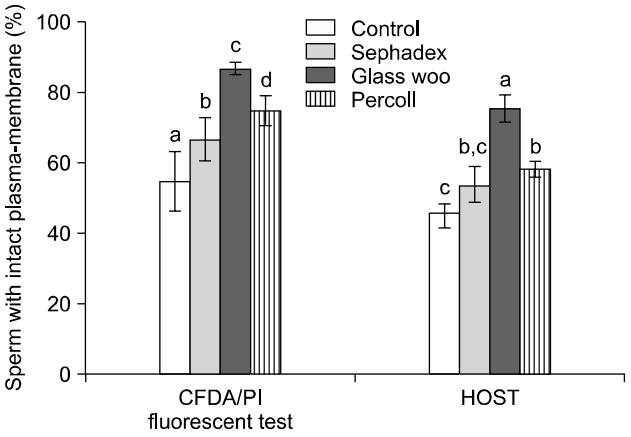

Fig. 2. Sperm plasma-membrane integrity evaluated by carboxyfluoresceindiacetate/propidium iodide (CFDA/PI) fluorescent staining and hypo-osmotic swelling test (HOST) in treated and control samples. Data are presented as mean ± SE. [a,b,c,d]Different superscripts indicate significant differences among treatments within an evaluation method ($p < 0.05$, n = 6).

Table 2. Effect of control, Sephadex and glass wool filtration of spermatozoa on *in-vitro* fertilization (IVF) results

Spermatozoa treatment	Cleavage		Blastocyst		
	Frequency	Cleavage rate (%)	n	Blastocyst rate of total oocytes (%)	Blastocyst rate of cleaved embryos (%)
Control	2,654/4,007	66.09 ± 1.61[a]	587	14.59 ± 0.94[a]	22.28 ± 1.43[a]
Sephadex	2,731/3,971	68.54 ± 1.30[a]	629	15.83 ± 0.87[a]	23.29 ± 1.32[a,b]
Glass wool	2,949/3,989	73.73 ± 1.42[b]	776	19.21 ± 0.76[b]	26.24 ± 1.09[b]

[a,b]Different superscripts within columns indicate significant differences ($p < 0.05$). All data are presented as mean ± SE (n = 28).

Table 3. Effect of glass wool filtration and Percoll separation of spermatozoa on IVF results

Spermatozoa treatment	Cleavage		Blastocyst		
	Frequency	Cleavage rate (%)	n	Blastocyst rate of total oocytes (%)	Blastocyst rate of cleaved embryos (%)
Glass wool	1,653/2,363	70.20 ± 1.78[a]	424	17.75 ± 1.02[a]	25.21 ± 1.24[a]
Percoll	1,680/2,324	73.31 ± 1.76[a]	353	15.22 ± 1.27[a]	20.86 ± 1.69[b]

[a,b]Different superscripts within columns indicate significant differences ($p < 0.05$). All data are presented as mean ± SE (n = 16).

control ($p > 0.05$).

The final experiment compared the effect of glass wool filtration and Percoll separation of spermatozoa on *in vitro* embryo development (Table 3). The cleavage and blastocyst rate of total oocytes was not significantly different between glass wool filtration and Percoll separation samples ($p > 0.05$). But cleaved embryos produced by glass wool samples had a significantly greater development rate to blastocyst stage than cleaved embryos produced by Percoll separation samples ($p < 0.05$).

Discussion

Damaged spermatozoa are removed by different mechanisms among Sephadex, glass wool and Percoll methods. Glass wool is thought to mechanically trap damaged spermatozoa, which are unable to pass the physical barrier of the glass wool [37]. The mechanism by which Sephadex retains dead or damaged spermatozoa is still not well understood. This process is believed to be a complex hydrodynamic phenomenon involving the counter-current orientation of spermatozoa with progressive motility. Motile cells do not approach the limiting layer area surrounding Sephadex spheres, where the flow becomes almost null and they swim counter-current, while dead cells are dragged until they randomly leave the fast flow area and retained when approaching the Sephadex spheres [7]. Furthermore, Sephadex particles appear to provide a physical barrier, forcing immotile/dead spermatozoa to aggregate. Percoll consists of colloidal silica particles coated with polyvinylpyrrolidone that select spermatozoa according to their density, which seems to be related to their maturation stage and their integrity [31]. Spermatozoa with good nuclear morphology are denser and are deposited in the area of greater density [26]. In addition, motile spermatozoa deposit faster than nonmotile cells with the centrifugal force, because of the alignment of their movements with this force [34].

Motility is an essential requirement to achieve oocyte fertilization. Percoll gradient and Sephadex filtration effectively increased the quality in low-motility semen samples; caused by either freeze-thawing or asthenospermia [24,26]. Glass wool filtration significantly improved sperm motility in humans [12]. Our results in bovine semen showed that all treatments improved progressive motility versus control. This indicates that these techniques increased potential fertility of semen samples.

Sperm separation techniques have previously been reported to reduce morphologically abnormal spermatozoa [44]. Effective removal of abnormal spermatozoa from cattle [16] and buffalo [15] semen with Sephadex columns has also been reported. However, in our study, there was no significant difference in the reduction of morphologically abnormal spermatozoa among all experimental groups. There was little change in the percentage of spermatozoa with normal morphology after thawing of cryopreserved bovine semen. Therefore, the effectiveness of treatments cannot be concluded with this criterion because the normal morphology of control samples was already within the normal range ($> 80\%$) in this study.

Sperm outer membrane (plasmalemma) integrity and proper function is essential for sperm metabolism, capacitation, ova binding and acrosome reaction [3,24]. Hence, assessment of plasmalemma characteristics may be useful for predicting the fertilizing ability of sperm. Because both the physical and functional integrity of the sperm plasma-membrane are essential for cell survival [3] and are closely related to fertility, sperm membrane integrity was evaluated by CFDA/PI fluorescent staining and HOST [24]. It has been reported that vital stains such as CFDA/PI fluorescent stain are used to evaluate physical plasmalemma damage, while HOST evaluates plasmalemma biochemical activity as an intact plasmalemma does not ensure that it is functional [9,14,30,47]. The glass wool filtration samples showed the highest values in both evaluation methods but the Sephadex filtration and Percoll separation samples did not show the same results between CFDA/PI and HOST. This may be due to differences among the sperm separation techniques in the removal of spermatozoa damaged in the plasma-membrane of the sperm head (CFDA/PI) and the sperm tail (HOST). These results indicate that glass wool filtration is the best method for recovering spermatozoa with intact head and flagellum plasma-membranes.

Positive correlations have been observed between membrane integrity and fertility. In humans, HOST results were highly correlated with zona-free hamster oocyte penetration rates [6,43]. For boar semen, the proportion of intact sperm identified by CFDA/PI was included in the model that best explained the *in vitro* fertilization rate [14]. The IVF results appeared to be similar to the plasmalemma integrity results in this study. However, these studies could not simply estimate the relationship between sperm membrane integrity and fertility since replicates for evaluation of sperm quality were small in size and interaction between the two was not investigated.

Fertilization rates were not significantly different between glass wool filtration and Percoll separation samples, but the blastocyst rate of fertilized embryos from glass wool samples was significantly higher than that from Percoll sample. In previous studies, glass wool filtration improved chromatin integrity and viability compared to the density gradient centrifugation method [25] and resulted in a significantly higher percentage of normal chromatin-condensed spermatozoa compared with the ejaculate [20]. Glass wool filtration also enhanced embryo quality compared to the density gradient centrifugation method following ICSI [45].

In the context of assisted conception both in animal models and in clinical studies, the degree of DNA aberrations or damage in sperm cells has been linked to the impairment of fertilization and embryo development [11,19,41] and a reduced chance of producing live offspring [17,36,40]. In one report, sperm DNA damage did not impair fertilization of the oocyte or completion of the first 2～3 cleavages, but rather blocked blastocyst formation by inducing apoptosis [13]. Therefore, glass wool filtration might improve embryonic development by recovering spermatozoa with normal DNA in cryopreserved bovine semen compared with other treatment groups. However, further studies are required to determine whether glass wool filtration could remove more DNA-damaged spermatozoa than Percoll separation and have positive effects on developing fertilized embryos into blastocysts.

In conclusion, the biophysical spermatozoa separation methods were effective for removal of nonmotile spermatozoa, and the glass wool filtration was the most efficient among the experimental methods for removing spermatozoa with damaged membranes. Moreover, because glass wool filtration increased the production of cleaved embryos versus Sephadex filtration and had a higher development of cleaved embryos to blastocyst compared to Percoll separation, it could be a promising technique for use in bovine IVF with cryopreserved semen.

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