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Antioxidant effects of bovine serum albumin on kinetics, microscopic and oxidative characters of cryopreserved bull spermatozoa

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Abstract

The aim of this study was to determine the effects of bovine serum albumin (BSA) as an antioxidant on post-thaw characters of bull spermatozoa such as motion variables, viability, plasma membrane integrity, morphology, lipid peroxidation, total antioxidant capacity (TAC), total thiols (TT) and the enzymes activities. Ejaculates were collected from six proven bulls and diluted with a citrate-based extender supplemented with various concentrations of BSA (0, 0.5, 1, 1.5 and 2 g/100 mL). The results showed that the semen extender supplemented with various concentrations of BSA increased ($p < 0.05$) the post-thaw total motility, linearity, lateral head displacement, straight linear velocity, average path velocity, viability, hypo-osmotic swelling test, total normal morphology and normal acrosome morphology. The highest ($p < 0.05$) value for TAC and highest activity of catalyze was achieved by inclusion of 1 g/100 mL BSA and the highest activity of superoxide dismutase was obtained by 1.5 g/100 mL BSA in the semen extender. Total thiols were increased ($p < 0.05$) by supplementation of 1.5 and 2 g/100 mL BSA. No differences ($p > 0.05$) were detected in progressive motility, sperm track straightness, beat cross frequency, curvilinear velocity and malondialdehyde production between the experimental groups. The highest concentration of BSA (2 g/100 mL) in the semen extender reduced ($p < 0.05$) the sperm motion variables. The results indicated that the most effective concentration of BSA is 1 g/100 mL in the semen extender which is associated with an increase in the total motility, antioxidant enzymes activities, TT and TAC.

Additional key words: bull sperm freezing; lipid peroxidation; anti-oxidant activity.

Introduction

Artificial insemination with cryopreserved semen has become one of the most effective methods in animal biotechnology and breeding techniques. Mammalian spermatozoa may damage during semen cryopreservation process (Watson, 2000). It is reported that the cold shock during freeze-thaw process injure mitochondria (Pena *et al*., 2009), plasma and acrosome membranes (Meyers, 2005) of spermatozoa which may lead to reducing the spermatozoa functional integrity and decreasing its fertilizing aptitude (Gillan & Maxwell, 1999).

Free radicals at physiological concentrations are effective in intracellular signaling involved in the regular processes of cell proliferation, differentiation and migration (Piantadosi, 2008). Also, reactive oxygen species (ROS) are important mediators of normal sperm function, such as signal transduction mechanisms that affect fertility (Bennet *et al*., 1987; de Lamirande &

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Abbreviations used: ALH (lateral head displacement); BCF (beat cross frequency); BSA (bovine serum albumin); CAT (catalase); FRAP (ferric reducing antioxidant power); HOST (hypo-osmotic swelling test); LIN (linearity); LPO (lipid peroxidation); MDA (malondialdehyde); PM (progressive motility); ROS (reactive oxygen species); SOD (superoxide dismutase); STR (sperm track straightness); TAC (total antioxidant capacity); TBA (thiobarbituric acid); TM (total motility); TT (total thiol); VAP (average path velocity); VCL (curvilinear velocity); VSL (straight linear velocity).

Gagnon, 1993; Fialkow *et al*., 1994). Production of a high level of ROS by non-viable sperm, molecular oxygen in extenders and even live sperm throughout respiration occur during sperm freeze-thaw process (Bamba & Cran, 1992). Excessive production of ROS may produce peroxides and free radicals which damage the components of sperm (Bilodeau *et al*., 2002). Lipid peroxidation (LPO) also damages the sperm membrane leading to deprivation of motility (Alvarez & Storey, 1982), inactivation of glycolytic enzymes, impairment of the acrosomal membranes (Alvarez & Storey, 1984) and DNA oxidation. Gil-Guzman *et al*. (2001) reported that these events in sperm after a high production of ROS during the freeze-thaw process may render the sperm cell unable to fertilize the oocyte or a non-viable pregnancies my occur.

Supplementation of the antioxidants in the freezing extender may decrease oxidative stress of ROS production (Donoghue & Donoghue, 1997) and may improve sperm quality after thawing. It is shown that BSA as antioxidant protects the sperm of rabbit and men from free radicals (Alvarez & Storey, 1983; Lewis *et al*., 1997). BSA is known to eliminate free radicals generated by oxidative stress and protect membrane integrity of equine and human sperm cells from heat shock during freeze-thaw process of semen (Kreider *et al*., 1985; Klem *et al*., 1986; Lewis *et al*., 1997). To our knowledge, the effect of BSA on oxidative characters including antioxidant enzyme activities, total antioxidant capacitaty (TAC), total thiol (TT) and LPO in cryopreserved bull spermatozoa has not previously been assessed. It is needed more research to establish the effective concentration of BSA supplementation in the bull semen extender. This work was therefore conducted to evaluate the effects of various concentrations of BSA supplementation in freezing extender on sperm motility variables, viability, plasma membrane integrity, morphology, and TAC, LPO, TT antioxidant activities [superoxide dismutase (SOD), catalase (CAT)] in post-thawed bull semen.

Materials and methods

Chemicals and semen sources and preparation

BSA (A9418) and other chemicals used in this study were obtained from Sigma-Aldrich, St. Louis, MO, USA. This trial was performed at the Semen Freezing Laboratory of Animal Breeding Center, located in Tabriz city, NW Iran. Ejaculates were collected from six proven Holstein bulls, 3-6 years old, regularly used for breeding purpose based on their fertility estimation through in vitro and in vivo fertility tests. The bulls were fed concentrate and good quality hay and water was supplied ad libitum. Semen samples were collected twice a week using artificial vagina (45°C). The ejaculates were transferred to the laboratory and submerged in a water bath (34°C), until semen evaluation. The volume of ejaculates was estimated in a conical tube graduated at 0.1 mL intervals. The sperm concentration was determined by means of an Accucell photometer (IMV, L'Aigle, France). The ejaculates were included for evaluation if the following criteria were met: volume, 5-12 mL; sperm concentration of 10^9 sperm mL^{-1}; motility of 70%; and morphology, 10% abnormal cells in the semen sample. The ejaculates were then mixed in a pool, balancing the sperm contribution of each male to remove individual differences in bulls (Gil *et al*., 2003).

The citrate-egg yolk extender (2.9 g dL^{-1} sodium citrate dehydrate, 1000 IU mL^{-1} penicillin, 1000 μg mL^{-1} streptomycin, 25% egg yolk, 7% glycerol, and double distilled water to make a volume of 100 mL) was used for all the ejaculates. BSA was added in the extender to achieve five different final concentrations: 0, 0.5, 1, 1.5 and 2 g/100 mL (control, T1, T2, T3 and T4, respectively). Each pooled ejaculate was split into five equal aliquots and diluted with the BSA supplemented extender, for a final concentration of 10^8 spermatozoa mL^{-1}. Extender had two fractions and BSA added to first fraction.

Semen samples were cooled slowly to 5°C at a liner rate of –0.3°C min^{-1} in a refrigerator. After addition of second fraction, samples equilibrated at 5°C for 80 min. The semen was then packaged in 0.5 mL straws. The straws were sealed via automatic filling and sealing machine (IMV Technologies, MRS 1, France) and they were frozen by a semi-automatic freezer machine (Minitube, Germany) with liquid nitrogen vapor. The straws were cooled according to the standard freezing curve (approximately –15°C min^{-1} from +5 to –150°C). Then, they were transferred to a liquid nitrogen tank and stored. Frozen straws were thawed at 37°C for 30 s in a water bath immediately before using for evaluation.

Semen evaluation

Viability, motility and morphology characters

The viability of spermatozoa in samples was assessed by means of the nigrosin-eosin stain method. The final composition of the stain was: 1.67 g eosin-Y, 10 g nigrosin, and 2.9 g sodium citrate, dissolved in 100 mL distilled water. Sperm suspension smears were prepared by mixing a drop (5 μL) of sperm sample with two drops of stain on a warm slide and spreading the stain with a second slide. Viability was assessed by counting 400 sperm cells in a microscope (Olympus CX21) at 1000X magnification, using immersion oil. Sperm displaying partial or complete purple staining were considered non-viable. Only sperm showing strict exclusion of stain were counted as viable (Balestri *et al*., 2007).

The sperm motility was assessed after thawing the samples for about 5 min at 37°C. A computer-assisted sperm motility analysis (CASA, VideoTesT-Sperm 2.1, Russia) was used to analyze sperm motion characteristics. Semen was diluted 1:9 (v/v) in a citrate-based extender (without egg yolk) and evaluated immediately after dilution. A 4 μL sample of diluted semen was placed onto a pre-warmed microscope slide and covered by a cover slip. Sperm motility characteristics were determined using phase-contrast microscope (Labomed LX400) with a 10x objective at 37°C. The following motility values were recorded: TM (total motility, %), PM (progressive motility, %), VSL (straight linear velocity, μm s^{-1}), VCL (curvilinear velocity, μm s^{-1}), VAP (average path velocity, μm s^{-1}), BCF (beat cross-frequency, Hz), ALH (lateral head displacement, μm), STR (sperm track straightness, %) and LIN (linearity, %). For each evaluation, five microscopic fields were analyzed to include at least 400 cells.

For the evaluation of sperm abnormalities, at least three drops of each sample were added to Eppendorf tubes containing 1 mL of Hancock solution (62.5 mL of 37% formalin, 150 mL of 0.31 M NaCl solution, 150 mL buffer solution and 500 mL double distilled water) (Schafer & Holzmann, 2000). One drop of this mixture was laid on a slide and covered with a cover slip. The percentages of acrosome and total abnormalities were determined by counting a total of 200 spermatozoa under phase-contrast microscopy (1000x magnification, oil immersion).

Assessment of membrane integrity

The hypoosmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails. This was performed by incubating 30 mL of semen with 300 mL of a 100 mOsM hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) at 37°C for 60 min. After incubation, 0.2 mL of the mixture was spread with a cover slip on a warm slide. 400 sperm were examined with 400x magnification with bright-field microscopy. Sperm with swollen or coiled tails were recorded (Buckett *et al*., 1997).

Biochemical assay

The samples were thawed before the biochemical assays. An aliquot (500 μL) of semen from each sample was centrifuged at 800 *g* for 10 min, sperm pellets were separated and washed by resuspending in PBS and recentrifuging (3 times). After last centrifugation, 1 mL of deionized water was added to spermatozoa and they were snap-frozen and stored at –70°C until further analysis (Roca *et al*., 2005).

The ferric reducing/antioxidant power (FRAP) assay was used to measure the total TAC (Benzie & Strain, 1996). FRAP reagent (1 mL) was mixed with 50 mL sample solution. The reaction mixture was then incubated at 37°C for 10 min and absorbance was recorded at 593 nm with a Unico UV/2100 PC spectrophotometer. Results were expressed as equivalent of vitamin C as a potent antioxidant.

The total lipid peroxidation product, as indicated by malondialdehyde (MDA) formation in the sample, was assayed using the thiobarbituric acid (TBA) method (Buege & Aust, 1978). Briefly, 0.5 mL of the sample was added to a 2 mL TCA-TBA solution (0.67% TBA in 20% TCA), vortexed and incubated for 40 min in a boiling water bath. After cooling and centrifuging at 2000 *g* for 10 min, the absorbance of the supernatant was read at 532 nm.

For determination of total thiol groups, samples were mixed with 0.2 M Tris buffer pH 8.2 and 0.1 M 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB). The tubes were shaken and centrifuged at 3000 g. The absorbances of supernatant were read within 5 min at 412 nm (Sedlak & Lindsay, 1968).

The method described by Goth (1991) was used for the determination of CAT activity in the samples.

Sperm samples (0.2 mL) were incubated in 1 mL of hydrogen peroxide (65 mmol mL^{-1}) in 60 mmol L^{-1} phosphate buffer (pH 7.4) for 60 s. The enzymatic reaction was terminated with 1 mL of 32.4 mmol L^{-1} ammonium molybdate, and hydrogen peroxide was measured at 405 nm using a spectrophotometer. One unit of CAT decomposes 1 mmol of hydrogen peroxide per minute, under the indicated conditions.

Total SOD activity was determined by the procedure previously described (Marklund & Marklund, 1974) based on the SOD ability to inhibit the pyrogallol autooxidation. The assay system contained 1 mM EDTA, 50 mM Tris buffer, pH 8.5 and 100 µL of sample in a final volume of 2 mL. The reaction was initiated by the addition of 100 µL of freshly prepared 2.6 mM pyrogallol solution in 10 mM HCl to attain a final concentration of pyrogallol of 0.13 mM in the assay mixture. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation.

Statistical analyses

Statistical analyses of replicated experiment results were used for treatment comparisons and were carried out one-way analysis of variance (ANOVA) using SAS program. Means of treatments were compared for differences using Duncan's Multiple Range Test. All data were expressed as mean ± SE. Differences were considered significant at $p < 0.05$. Five replicates were used in each treatment.

Results

The effects of the various concentration of BSA in the freezing extender on post-thaw motion variables, viability, plasma membrane integrity using HOST analysis, total and acrosome abnormalities in bull spermatozoa are set out in Tables 1 and 2. Addition of BSA to the freezing extender had a beneficial effect on sperm motility. The maximum percentage of TM was achieved in T1 treatment (54.77 ± 4.8%). Percentages of PM (26.71 ± 1.6%) and STR (83.34 ± 1.8%) did not differ ($p > 0.05$) between control and those frozen in T1 to T3, while it decreased ($p < 0.05$) in T4 treatment (12.86 ± 1.5% and 73.01 ± 2.3%, respectively). The percentage of LIN (37.51 ± 2.2%) increased ($p < 0.05$) in T3 compared to the other groups. ALH in T2 (1.02 ± 0.1 µm) was higher ($p < 0.05$) than in control (0.89 ± 0.01 µm) but it decreased ($p < 0.05$) in T4 (0.73 ± 0.03 µm) compared to other treatments. VAP and VSL increased ($p < 0.05$) in T2 (27.40 ± 1.5 µm s^{-1} and 24.81 ± 2.11 µm s^{-1}, respectively) compared to the control (23.49 ± 1.9 µm s^{-1} and 22.57 ± 0.5 µm s^{-1}, respectively), however, they decreased ($p < 0.05$) in T4 (15.59 ± 0.2 µm s^{-1} and 13.67 ± 1.0 µm s^{-1}, respectively). VCL decreased ($p < 0.05$) in T4 (44.58 ± 4.4 µm s^{-1}) compared to other groups. No significant differences ($p > 0.05$) were observed in BCF following supplementation of the freezing medium with BSA after the freeze-thaw process.

Immediately after thawing, the percentage of live sperm in T2 (60.68 ± 6.8%) was higher ($p < 0.05$) than in other groups (Table 2). The percentage of sperm with intact plasma membrane (HOST value) was increased ($p < 0.05$) in T2 (51.17 ± 4.9%) compared to control (41.36 ± 5.6%). Supplementation of the medium with 1 and 1.5 g/100 mL BSA decreased ($p < 0.05$) total abnormality and acrosome abnormality rates in comparison to the control group.

Results of the effects of BSA on TAC, LPO determined with MDA, enzymes activities (CAT and SOD) and TT in thawed bull semen are summarized in Table 3. Addition of 1 g BSA increased ($p < 0.05$) TAC compared to other groups. No significant differences ($p > 0.05$) were observed in the level of MDA following supplementation of the freezing medium with BSA during the freeze-thaw process. The activity of CAT increased ($p < 0.05$) in T1 and T2 treatments. SOD ability to inhibit the pyrogallol autooxidation increased ($p < 0.05$) in T3 compared to other groups. TT increased ($p < 0.05$) in T3 and T4 (298.33 ± 26.2 µmol L^{-1} and 301.56 ± 41.1 µmol L^{-1}; respectively) compared to control (226.03 ± 38.2 µmol L^{-1}) group.

Discussion

The present results showed that BSA may protect microscopic and oxidative characters of cryopreserved bull spermatozoa by increasing enzyme activities and TAC against cryodamage. BSA supplementation (1 g/100 mL) in the semen extender also increased the sperm motion characteristics and viability. These results are consistent with results of Lornage *et al*. (1983) and Uto & Yamahama (1996) who found higher percentages of motile and viable spermatozoa in the groups with BSA supplementation. The axosome and

Table 1. Mean (±SE) percentages of sperm motion variables after freeze-thaw process of bull semen with different concentrations of bovine serum albumin (BSA)

Variables[1]	BSA concentrations (g/100 mL)				
	0 (Control)	0.5 (T1)	1 (T2)	1.5 (T3)	2 (T4)
TM (%)	38.18 ± 0.8^{d}	42.02 ± 2.1^{dc}	54.77 ± 4.8^{a}	48.39 ± 2.1^{b}	42.48 ± 1.5^{c}
PM (%)	26.71 ± 1.6^{ab}	26.01 ± 3.0^{ab}	27.88 ± 2.3^{a}	23.94 ± 2.6^{b}	12.86 ± 1.5^{c}
STR (%)	83.34 ± 1.8^{a}	85.43 ± 2.2^{a}	84.81 ± 2.5^{a}	81.60 ± 5.6^{a}	73.01 ± 2.3^{b}
LIN (%)	29.47 ± 0.7^{c}	34.05 ± 0.7^{b}	33.67 ± 2.8^{b}	37.51 ± 2.2^{a}	26.73 ± 2.8^{c}
ALH (μm)	0.89 ± 0.0^{b}	0.91 ± 0.0^{ab}	1.02 ± 0.1^{a}	0.92 ± 0.1^{ab}	0.73 ± 0.0^{c}
VAP (μm s^{-1})	23.49 ± 1.9^{b}	22.68 ± 1.1^{b}	27.40 ± 1.5^{a}	25.03 ± 1.2^{b}	15.59 ± 0.2^{c}
VSL (μm s^{-1})	22.57 ± 0.5^{b}	23.70 ± 2.1^{ab}	24.81 ± 2.1^{a}	22.99 ± 1.3^{ab}	13.67 ± 1.0^{c}
VCL (μm s^{-1})	57.50 ± 0.7^{a}	59.10 ± 2.5^{a}	62.41 ± 7.5^{a}	56.28 ± 4.3^{a}	44.58 ± 4.4^{b}
BCF (Hz)	8.74 ± 0.2^{a}	8.83 ± 0.1^{a}	8.56 ± 0.1^{a}	8.55 ± 0.2^{a}	8.63 ± 0.2^{a}

[1] TM: total motility; PM: progressive motility; STR: sperm track straightness; LIN: linearity; ALH: lateral head displacement; VAP: average path velocity; VSL: straight linear velocity; VCL: curvilinear velocity; BCF: beat cross frequency. ^{a,b,c,d} Data with different superscripts within rows differ significantly, $p < 0.05$ (ANOVA one way).

Table 2. Mean (±SE) percentages of viability, HOST (hypo-osmotic swelling test), total and acrosome abnormalities after the freeze-thaw process of bull semen with different concentrations of BSA

Characters	BSA concentrations (g/100 mL)				
	0 (Control)	0.5 (T1)	1 (T2)	1.5 (T3)	2 (T4)
Viability (%)	44.39 ± 4.3^{c}	47.91 ± 2.5^{bc}	60.80 ± 6.8^{a}	53.32 ± 4.2^{b}	45.18 ± 3.5^{c}
HOST (%)	41.36 ± 5.6^{b}	44.29 ± 3.5^{b}	51.17 ± 4.9^{a}	47.67 ± 1.5^{ab}	44.12 ± 2.6^{b}
Total abnormalities (%)	15.24 ± 2.7^{ab}	13.33 ± 0.5^{bc}	9.63 ± 1.3^{d}	11.45 ± 0.6^{dc}	16.62 ± 1.8^{a}
Acrosome abnormality (%)	7.40 ± 1.0^{a}	6.14 ± 0.6^{a}	3.91 ± 0.9^{b}	4.64 ± 0.5^{b}	6.50 ± 0.6^{a}

^{a,b,c,d} Data with different superscripts within rows differ significantly, $p < 0.05$ (ANOVA one way).

Table 3. Mean (± SE) oxidative characters after freeze-thaw process of bull semen with different concentrations of BSA

Characters [1]	BSA (g/100 mL)				
	0 (Control)	0.5 (T1)	1 (T2)	1.5 (T3)	2 (T4)
TAC (μM Vit C)	12.57 ± 1.4^{b}	12.60 ± 0.6^{b}	16.43 ± 1.8^{a}	12.45 ± 1.2^{b}	14.06 ± 1.0^{b}
MDA (nmol dL^{-1})	0.94 ± 0.1^{a}	0.76 ± 0.2^{a}	0.83 ± 0.1^{a}	1.18 ± 0.3^{a}	1.17 ± 0.3^{a}
CAT (U mL^{-1})	0.25 ± 0.0^{c}	0.70 ± 0.1^{b}	1.60 ± 0.3^{a}	0.15 ± 0.0^{c}	0.05 ± 0.0^{c}
SOD (% inhibition)	5.09 ± 1.0^{b}	5.03 ± 1.0^{b}	5.12 ± 0.8^{b}	23.00 ± 3.4^{a}	5.09 ± 1.1^{b}
TT (μmol L^{-1})	226.03 ± 38.2^{b}	224.73 ± 37.3^{b}	255.92 ± 16.7^{ab}	298.33 ± 26.2^{a}	301.56 ± 41.1^{a}

[1] TAC: total antioxidant capacity; MDA: malondialdehyde; CAT: catalase; SOD: levels activity of superoxide dismutase. TT: total thiol. ^{a,b,c} Data with different superscripts within rows differ significantly, $p < 0.05$ (ANOVA one way).

associated dense fibers of the middle pieces in sperm are covered by mitochondria that generate energy from intracellular stores of ATP. It is well established that ROS can induce axonemal and mitochondial damage, resulting in the immobilization of sperm (Aitken & Clarkson, 1987; Peris *et al*., 2007).

Destruction of membrane integrity causes a raise in membrane permeability and decreases the ability of sperm to control the intracellular concentrations of ions involved in the sperm motion (Baumber *et al*., 2000). The current results showed that supplementation of the freezing media with BSA increased sperm plasma membrane integrity of bull semen after freezing procedure.

SOD, CAT and glutathione peroxidase are important parts of the antioxidant enzyme defense systems in

sperm which convert superoxide (O_2^-) and peroxide (H_20_2) radicals into O_2 and H_2O, GPX eliminating peroxyl radicals from various peroxides (Irvine, 1996). CAT and SOD also eliminate O_2^- produced by NADPH-oxidase (Alvarez & Storey, 1989; Jeulin *et al*., 1989). The importance of thiol groups for sperm motility, metabolism and survival is well established (Bilodeau *et al*., 2001). Thiol compounds such as cysteine and glutathione have been shown to protect the motility and glycolytic activity of spermatozoa against the inhibitory actions of heavy metal ions (Macleod, 1951; White, 1955) and oxidizing agents (Wales *et al*., 1959). In the present study, the activity of CAT, SOD and TT increased by inclusion of BSA in the freezing medium.

MDA production is greatly used to determine the LPO in a kind of cell types involving sperm (Sikka, 1996). Nair *et al*. (2006) showed that the amount of MDA produced in spermatozoa was negatively correlated to the motility and viability of spermatozoa. However, in this study, addition of BSA did not affect the prevention of MDA formation. The results showed that addition of BSA increases TAC. This may due to the stimulatory effects of BSA on the enzyme activity involved in antioxidant defense.

In the current study, different concentrations of BSA (0.5-2 g/100 mL) were assayed and the most effective concentration of BSA in the bull sperm freezing extender was found to be 1 g/100 mL. Extreme doses of antioxidants in the freezing medium can counteract the oxidative stress and it can also affect the normal sperm functions connected with the ROS. Since limited production of ROS is associated with physiological functions of spermatozoa such as hyperactivation, capacitation and acrosome reaction (Aitken, 1995), the effective concentration of BSA must be used in the freezing extender. In conclusion, supplementation with 1 g/100 mL BSA in the freezing medium has beneficial effects on the motility, viability, normal morphology and plasma membrane integrity in bull spermatozoa after freeze-thaw process. The results suggested that the protection effects of BSA on spermatozoa are associated with an increment in antioxidant enzymes activity, TT and TAC.

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