Global Veterinaria 11 (4): 420-431, 2013
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2013.11.4.7645

Improvement of the Efficacy of Buffalo Oocytes Vitrification

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Abstract: Interest in oocyte cryopreservation has recently increased with the growing importance of *in vitro* embryo production, nuclear transfer and gene banking. Cryopreservation of oocytes collected from slaughtered animals of high genetic value and their subsequent utilization for production of transferable embryos may provide an opportunity to replenish the valuable germplasm lost and lead to genetic improvement of the species. Cryopreservation of oocytes is still an open problem because of their structural and molecular sensitivity to the cooling and freezing process. Vitrification is a common method for cryopreservation of gametes. Although successful oocyte vitrification has been achieved in several animal species, subsequent progress is still limited especially in buffalo. This limitation is due to the alterations which may take place during vitrification process. The goals of this review are to discuss the factors that improve vitrification protocol which can overcome the alterations in buffalo oocytes during vitrification.

Key words: Cryopreservation · Oocytes · Buffalo · Vitrification Improvement

INTRODUCTION

In recent years, there has been increasing interest in the preservation of oocytes from several mammalian species and occasional reports of success have been published in humans and animals. Despite decades of research, oocyte cryopreservation remains a challenge in virtually all species due to the complex structure of the oocyte. Gamete and embryo preservation is proposed as being essential for conserving the genome of all species [1-3]. Although sperm and embryos of many species are readily cryopreserved, oocyte preservation has been less successful. Conventional slow freezing commonly leads to intracellular ice crystallization and cell damage, so the need for an alternative solution is a must. Although vitrification of bovine oocytes is being studied increasingly, there is yet to be a comprehensive understanding of oocyte cryobiology and low fertilization rates need to be resolved before oocyte cryopreservation can be routinely applied. There have been an increasing number of publications regarding vitrification of oocytes as it is a relatively simple and inexpensive method of cryopreserving oocytes. Cryopreservation strategies are based on two main principles: cryoprotectants and cooling-warming rates. Because water is not very viscous, it can only be vitrified by extremely rapid cooling of a small sample or using high concentrations of cryoprotectants [4].

Although successful oocyte cryopreservation has been achieved in several species, subsequent progress is still limited specially in buffalo. Oocyte survival rate after cryopreservation is affected by both morphological and biophysical factors [5]. The main biophysical factors causing cellular disruption during cryopreservation are intracellular ice formation [6] and osmotic injury [7]. These factors can be minimized by adequate cryopreservation techniques such as proper cryoprotectant choice and concentration [8,9], cryoprotectant exposure and removal time [10] and proper cooling and warming rates [11]. The goal of this review is to show the affected factors on the vitrification of oocytes which improve vitrification process.

Type of Cryodevices: Different vitrification protocols have been experimented to cryopreserve *in vivo* and *in vitro* produced embryos or oocytes. These protocols differ in

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many ways, including the type and concentration of the cryoprotectant, number of equilibration steps, time of exposure, number of dilution steps at warming and type of cryopreservation device used [12-15] and it is widely believed that each of these factors can affect the results.

Different instruments have been used for vitrification, such as straws [9], open pulled straws (OPS) [14], electron microscope grids [16], cryoloops [17]. In addition, various holders or tools have been employed such as insemination pipettes, flexipet-denuding pipette, microdrops, solid surface metal blocks, nylon coils or mesh [4, 18].

In the 1980's, the plastic straw became the container of choice for vitrification and storage of embryos [19, 20]. The 0.25 ml standard insemination straws were used almost exclusively for vitrification of oocytes and embryo with a relative cooling speed of 2,500°C/min by direct plunging into LN_2. The introduction of the 0.25 ml plastic insemination straw as a freezing container or acryodevice allowed for easy storage of the bovine embryo. Although the 0.5 ml straw is generally used in semen storage in the USA, embryo transfer practitioners use the smaller size in embryo cryopreservation. The advantages of the straw over 1 ml glass ampoules containers included better exposure of the embryo and medium to the cooling effect of liquid nitrogen and the ease of storing large numbers of straws in nitrogen storage units [21].

As an attempt to reduce cryoinjuries resulted from cryopreservation, Subramaniam *et al.* [22] reported that resting the straws in the air is vital in preventing zona fracture. But, Yunus and Ayhan [23] reported that although straws were rested in the air for 5 s, oocytes were recorded to have broken of zona pellucida and the explanation for this may be due to the occurrence of devitrification especially during thawing brings about critical cooling levels in 0.25 ml straws. Therefore, they concluded that more successful results could be achieved by using containers that obtains higher cooling-warming ratios like open-pulled straw, Nylon bags, electron microscope grids or Cryotop. So conventional straw method for buffalo oocytes vitrification didn't give the result expected but moreover result in some cryoinjuries and so the need for a new device was a must to overcome this problems [24].

In order to facilitate vitrification by higher cooling rates, it is necessary to minimize the volume of the vitrification solution as much as possible by using special carriers [25]. So Cryotop technique is at present one of the most efficient methods for cryopreservation of oocytes [4]. The Cryotop device designed by Kuwayama *et al*. [26] consists of a fine, transparent polypropylene film (0.4 mm wide × 20 mm long × 0.1 mm thick) attached to a plastic handle and equipped with a cover straw hard plastic cover (3 cm long) on top of the Cryotop sheet to protect it during storage in liquid nitrogen containers.

Once the oocytes are placed on the Cryotop, almost the entire loading solution is removed before direct immersion in liquid nitrogen; thus, the final volume is approximately <0.1 µl [27, 28]. The greatest advantage of this method is that an extremely fast cooling rate is achieved and chilling injury is avoided. The extremely small volume also helps to achieve a faster warming rate, thereby avoiding ice crystal formation during warming. A further benefit of the Cryotop method is that the permeable cryoprotectant concentration is reduced to 30%, minimizing potential toxic effects. One of the most successful ultra-rapid vitrification techniques is the Cryotop vitrification (CTV) that has resulted in excellent survival and developmental rates with human and bovine MII phase oocytes [26]. Muenthaisong *et al*. [29] demonstrated that *in vitro* matured Swamp buffalo oocytes vitrified by the CTV method retain the capability to cleave and develop into blastocysts following parthenogenetic activation. Moreover, vitrification using Cryotops improved the cleavage rates of both cow and calf oocytes over those obtained using OPS and led to a greater number of blastocysts [30]. During the past few years, Cryotop technique has rapidly spread in human medicine, producing impressive results in terms of healthy offspring after oocyte and/or blastocyst cryopreservation [31, 32]. Moreover, Cryotops have been successfully used to cryopreserve immature and *in vitro*-matured horse [33], cow [34], pig [35], sheep [36] and buffalo [37] oocytes, rabbit zygotes [38], cow embryos [39,40] and buffalo embryos [41], as well as oocytes from exotic species such as minke whale [42].

Type and Concentration of Cryoprotectant Agents (CPA): Cryoprotectants are defined in a functional manner as compounds that allow a higher degree of cell survival during freezing in their presence than in their absence. In other words they are compounds used to achieve cellular dehydration and avoid intracellular ice crystal formation upon freezing [43]. When mammalian cells suspended in ordinary dilute saline solutions are cooled and frozen, they are inevitably damaged. However, it was accidentally discovered that fowl spermatozoa would survive freezing when suspended in a solution of 10%

glycerol plus albumin [44]. Later, calves were born from artificial insemination with frozen-thawed sperm [45, 46]. These were the first explicit demonstrations that mammalian cells can be successfully frozen with the addition of a supplement to the culture medium. Since those first discoveries, it has been found that many low molecular weight compounds protect cells against freezing damage. These compounds, now referred to as CPAs.

Cryoprotectants Are Divided into Two Types: membrane-permeating (e.g., glycerol, ethylene glycol (EG), dimethyl sulfoxide (DMSO), propanediol) and membrane non-permeating (e.g., sucrose, glucose, Ficoll, proteins, lipoproteins). The membrane permeating cryoprotectants act through several mechanisms: 1) they decrease the freezing point of the solution and prevent oocyte damage from high electrolyte concentrations; 2) they interact with the membrane modifications occurring during cryopreservation process (from a relatively fluid state to a relatively rigid state); and 3) they prevent the exposure of oocytes to high concentration of both intra and extra cellular electrolytes by linking to the electrolytes and they may act as partial substitute to the water [47]. For slow freezing procedures the concentrations of cryoprotectants are limited to 1 to 1.5 M and hence the toxicity of the cryoprotectants is very low. In contrast to vitrification where the concentration can be as high as 8 M. Low molecular weight, glycerol and DMSO are permeating agents. In the past decade EG at a concentration of 1.5 to 1.8 M has emerged as a useful cryoprotectant for cattle embryos [48].

The membrane non-permeating cryoprotectants exert their beneficial effects by increasing the concentration of solutes generating an osmotic gradient across the cell membrane. Adding sugars to the vitrification solution could enhance viscosity of the solution, whereby incubation of cells in this solution before vitrification helps to withdraw more water from the cells and reduce exposure of cells to the toxic effects of the cryoprotectants [49]. Some non-permeating cryoprotectant protects the cells in the immediate post-thaw phase by helping to stabilize the cell membranes. High molecular weight non-permeating polymers or macromolecules have been shown to protect against zona pellucida cracking. The majority of solutions used for oocyte vitrification contain a macromolecular component of fetal calf serum (FCS) or bovine serum albumin (BSA). Other macromolecules used in vitrification solutions include polyethylene glycol, polyvinylpyrrolidone, Ficoll and polyvinyl alcohol [50]. Cryoprotectants influence the ability of buffalo oocytes to survive cryopreservation [8, 51]. Dimethyl sulfoxide, ethylene glycol, 1,2-propanediol, propylene glycol and glycerol have been used in different combinations for vitrification of mammalian oocytes and embryos [21]. Several studies demonstrated that EG would be the ideal cryoprotectant [52] because it penetrates membranes faster than glycerol and is less toxic than other permeable cryoprotectants [16, 53, 54].

The damaging effects of solutes can be lessened by using a mixture of two or more cryoprotectants or by stepwise equilibration (two or more steps) in solutions of intermediate concentrations at room temperature or after cooling to refrigeration temperature. At present, the combined use of two types of cryoprotectants is the standard. It was reported that cryoprotectant mixtures have some advantages over solutions containing only one permeable cryoprotectant [14, 35]. Mahmoud *et al.* [9] found that, the best combination of cryoprotectant was EG + DMSO for vitrification of immature buffalo oocytes using either straw or OPS methods.

Effect of Antioxidant on Vitrification: The formation of reactive oxygen species (ROS) such as superoxide anions (O_2), hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2) is a normal process that occurs in the cell when there is a deviation of electrons to oxygen (O_2) during electron transfer reactions [55]. *In vitro* oocyte or embryo culture results in higher O_2 concentrations than *in vivo* environments, leading to increased ROS levels. ROS such as O_2 are able to diffuse and pass through cell membranes and alter most types of cellular molecules such as lipids, proteins and nucleic acids. This can affect the early development of mouse, hamster and bovine embryos [56]. Living organisms possess natural protective equivalents known as ROS scavengers (anti-oxidants) that counteract the negative effects of ROS. These anti-oxidants include enzymes such as superoxide dismutase, which will eliminate O_2, catalase and selenium-dependent glutathione (GSH) peroxidase, which will transform H_2O_2 into H_2O and O_2, as well as lipid- and water-soluble anti-oxidants such as vitamins C and E and uric acid. However, during *in vitro* oocyte and embryo culture, the levels of anti-oxidants are lower than *in vivo* because the oocytes or embryos are divorced from the donor body and do not benefit from the maternal anti-oxidant protection. The addition of an anti-oxidant to the culture medium,

therefore, may be important for *in vitro* oocyte maturation and embryo development [57].

Various thiol compounds especially cysteine and cysteamine are commonly added to IVM media to support GSH synthesis and to improve the developmental competence of oocytes. The precursor of GSH in oocytes is cysteine [58], which may play an important role as an antioxidant supplement to *in-vitro* culture systems [59]. Low molecular weight compounds such as β-mercaptoethanol and cysteamine enhance cysteine mediated GSH synthesis in bovine embryos [60]. The addition of cysteamine in maturation medium enhanced the GSH synthesis and improved oocyte maturation by protecting the oocytes from oxidative stress and probably by acting on delicate process of cytoplasmic maturation [61, 62]. However, thiol compounds like cysteamine when added to culture medium could have different effects depending on the concentration used, the species and type of oocyte [63, 64].

Buffalo oocytes are likely to be more sensitive to oxidative damage due to lipid content [61]. Moreover, vitrified oocytes were significantly lower glutathione content and higher in H_2O_2 levels. Addition of cysteamine to maturation media improved blastocyst rate in fresh ovine and vitrified bovine oocytes [65]. On the other hand, Oyamada and Fukui [66] found that, cysteamine had no positive effect on nuclear maturation, but improved fertilizability, developmental competence and cryoresistance following vitrification in bovine. In buffalo, Singhal, *et al.* [67] reported that supplementing the maturation medium with IGF-1 + cysteamine improved the production of buffalo embryos significantly *in vitro* culture. No data were recorded on the effect of cysteamine in buffalo vitrified oocytes.

Effect of Meiotic Stages on Vitrification: Numerous studies have been conducted to determine optimal meiotic stages for oocyte vitrification, yet the results are inconclusive. Maturation stages ranging from GV [68], GVBD [69] through MII [70] have been described as the most favorable stage for vitrification. In contrast, a few previous studies indicate that the meiotic stage does not influence the ability of a oocyte to survive after cryopreservation [71, 72].

Otoi *et al.* [73] reported that mature oocytes are more resistant to slow freezing than immature oocytes and subsequently develop to blastocyst stage` *in vitro* at a greater rate than oocytes frozen at earlier stages of maturity. However, when sucrose was added as cryoprotectant, immature oocytes cleaved at a greater rate after cryopreservation/IVF than those frozen at MII. These results indicate that the tolerance of oocytes at different meiotic stages to cryopreservation is affected by the type of cryoprotectant and may be one of the reasons for the differing results. In addition, Lim *et al.* [74] observed that oocyte survival following vitrification by different methods varied according to stage of meiosis.

In buffaloes, El-shahat and Hammam [75], Mahmoud *et al.* [76] and El-nabby [77] found higher proportion of blastocyst in MII than germinal vesicle stage. The poor development in vitrified groups was attributed to the damage in oocytes itself, rather than fertilization failure [70]. In contrast, Barnes *et al.* [69] indicated that oocytes cooled at the germinal vesicle breakdown stage of meiosis cleaved and developed into blastocysts at higher rates than the oocytes cooled at the GV or MII stage. They added that, choosing an intermediate stage, may circumvent some of the problems associated with cryopreservation of GV and MII oocytes.

Effect of Lipid on Vitrification: Previous studies on mammalian oocytes and embryos have led to the conclusion that the amount of intracellular lipids may reflect the embryo quality, developmental potential and cryotolerance [78-80]. The survival rate after cooling or cryopreservation remains very low [81]. As a consequence of lipid physical changes that lead to cryoinjury [50]. Intracellular lipids are stored mainly in the form of lipid droplets (LD) [81]. The presence of the numerous and large LD is also associated with the reduced embryo cryotolerance [82]. Droplets are damaged during cryopreservation into smaller LD [50] with altered morphology [83]. Lipid composition contributes to the embryo quality and chilling sensitivity because different types of lipids play an important role in numerous cellular processes. For example, pig oocytes may use intracellular triglycerides as an energy source [81,84] and ratio of phospholipids to cholesterol influences the physical properties of biological membranes. Different types of phospholipids regulate oocyte maturation, relationship between the phosphatidylcholine and cAMP as well as adenylatecyclase activity [85].

Also, lipids play a role in intracellular signalization and are substrates for different hormones and prostaglandins [86]. Therefore, studies on lipids are vital for the improvement of oocyte and embryo cryopreservation. Until now, stereological analysis of LD volume and gas chromatography gave precise data on the total lipid content in the oocytes and in both non-cultured and cultured embryos up to the blastocyst

stage [87]. Moreover, previous studies found that differences in total lipid content between developmentally matched cultured and non-cultured embryos exist for all pre-implantation stages [81]. Thus the quantification of different types of lipids in embryos, especially in the cultured blastocyst, may serve as an indicator of embryo metabolism associated with its viability and should be performed as the primary effort.

Whatever the mechanism(s), an increase in intracellular lipids impairs the quality of the embryos by increasing their sensitivity to oxidative stress and cryopreservation [88]. Moreover, the sensitivity to chilling and cryotolerance of mammalian oocytes and embryos seems directly correlated to their lipid content [89, 90]. Several methods have already been used to evaluate the lipid content in oocytes and embryos. Fatty acid composition can be analyzed by thin-layer or gas-chromatography [88, 89, 91]. Kit-based assays can be used to measure the different classes of lipids in oocytes or embryos [92]. Abe *et al*. [90]; Rizos, *et al.* [93] used electron or light microscopy after staining with Sudan Black B. A lipid specific fluorescent dye, Nile red, was used for the first time to visualize the lipid droplets and to evaluate the lipid content of single oocyte. The more lipid droplets present in an oocyte, the higher the amount of emitted fluorescent light will be after staining. In order to allow the evaluation of the lipid content in single oocyte embryo, fluorescence was quantified using a photometer connected to a microscope. Nile red fluoresces yellow to orange. After staining with Nile red, neutral lipids, like triglycerides (lipid droplets), fluoresce yellow while polar lipids (phospholipid bilayers) fluoresce in the orange spectrum. Nile red is commonly used to visualize intracellular lipid droplets in all kinds of cells [94].

Large amounts of cytoplasmic lipid droplets serving as energy resources increase sensitivity of oocytes to chilling injuries during cryopreservation [34]. Mechanical removal of lipid droplets [34, 95] or their reduction by chemical agents [96] improved tolerance of oocytes and embryos to cryopreservation. L-carnitine is an enhancer of lipid metabolism in animal cells; it has a role in the transport of fatty acids from the cytosol to the mitochondria to fuel beta-oxidation [97]. Recently, supplementation of embryo culture medium with L-carnitine reduced lipid content in bovine embryos and increased tolerance to freezing [98].

Effect of CPA Exposure Time on Vitrification: The concentration of cryoprotectants required to achieve vitrification is very high which can lead to toxic effects on oocytes [99]. Therefore, the concentration of cryoprotectants and the duration of time for which oocytes are exposed to cryoprotectants are of critical importance. Two approaches have been followed to minimize these toxic effects, (1) the very brief exposure of oocytes to high concentrations of cryoprotectants in the vitrification solution is preceded by equilibration of oocytes in an equilibration solution containing lower concentrations of cryoprotectants [100, 101] and (2) the exposure time may be shortened since the amount of intracellular cryoprotectant(s) required for successful vitrification has been reported to be rather small [102].

The duration of exposure to cryoprotectants depends primarily upon their permeability into the oocytes/ embryos. EG has the highest permeability due to its lowest molecular weight compared with glycerol, DMSO and PG. Mahmoud *et al.* [103] reported that, the morphological examination for the oocytes after exposure showed that the survival rate of the immature buffalo oocytes did not differ among the three exposure time (30 seconds, 1 minutes, 5 minutes) for dimethyl sulfoxide, ethylene glycol and glycerol. In this respect, Wood *et al.* [101] reported a high proportion of mouse and hamster oocytes exposed for 3-5 minutes to 1.5 M DMSO appeared morphologically normal on recovery. In this work, the maturation rate was severely affected in DMSO group followed by EG and glycerol groups at the three exposure times. The maturation rate was lowered at 5minutes exposure than 1 minute and 30 seconds. Martins *et al.* [104] reported that a high concentration (40%) of EG in addition to a long equilibration time (5 or 15 minutes) was detrimental to bovine oocytes maturation. The exposure time is a very important parameter in the selection of cryoprotectants. So the strategy to avoid toxicity of cryoprotectants was to shorten the exposure time. Therefore, optimal exposure time for successful vitrification must be compromised between preventing toxic injury and preventing intracellular ice formation [105].

Effect of Macromolecule Addition: Naturally, cells contain high concentration of protein, which are helpful in vitrification. Higher concentrations of CPAs are needed for extracellular vitrification than for intracellular vitrification. The addition of a polymer with a high molecular weight such as polyvinyl pyrrolidone (PVP), polyethylene glycol (PEG) or Ficoll is sufficient in order to vitrify extracellularly with the same cryoprotective

concentration used intracellularly. Furthermore, the polymers may be able to build a viscous matrix to encapsulate the oocytes/embryos and also prevent crystallization during cooling and warming [106]. Parks and Ruffing [107] observed that addition of PEG resulted in greatly improved viability of oocytes following cryopreservation and vastly reduced the viability seen with vitrification solution alone. The other investigators added Ficoll in the vitrification solution which was thought to stabilize the glass formation and form a protective coating around embryos [48]. Large-molecular-weight polymers are frequently used as extracellular cryoprotectants. Their presence alters the temperature at which the solution vitrifies in relation to variables such as cooling rate and pressure [52]. Even though macromolecules do not penetrate the cell membrane, they can reduce the amount of intracellular cryoprotectants required to achieve vitrification [108], reducing the toxicity of the solution. Vitrification solutions that do not contain large molecular weight synthetic polymers usually contain high concentrations of bovine serum albumin (BSA), or serum, in order to achieve a similar protective effect.

Currently most solutions used for bovine oocyte vitrification contain a macromolecule plus serum or BSA, or simply serum as the macromolecular component. The presence of fetal calf serum in the media for cryopreservation of mouse or human oocytes does not prevent premature release of cortical granules, but prevents the conversion of the zona pellucida proteins to a state known as zona hardening [109]. A direct comparison has not been done with bovine oocytes, but most reports with reasonable results have FCS in both vitrification and warming media [14, 16, 110]. Although FCS is usually added to vitrification solutions for oocytes, such products of biological origin are potential sources of infectious agents [52] and can vary considerably from batch to batch. In cryopreservation process it is easy to transport embryos and gametes worldwide; therefore, extreme precautions must be taken to avoid disease dissemination and the use of animal blood serum is an inherent risk. In addition, *in vitro* culture of embryos in the presence of serum is correlated with many abnormalities. Consequently, numerous research groups seek defined media for all procedures and try to avoid the use of FCS in their formulations [109].

Effect of Cumulus Cells: One factor that could affect oocyte quality following vitrification is the presence or absence of cumulus cells around the oocyte prior to cryopreservation. It has been hypothesized that cumulus cells and glycoproteins slow cryoprotectants penetration that may lead to unequal intracellular distribution of the cryoprotectant and inadequate cell protection [111-103]. It was previously demonstrated that removal of cumulus cells prior to vitrification is advisable for oocyte vitrification in buffalo [37]. On the other hand, when denuded oocytes are vitrified their embryo development is in part affected by the lack of cumulus cells during the process of fertilization, as previously demonstrated for non-vitrified oocytes in this species [114].

The cumulus cell removal prior to *in vitro* maturation or vitrification have shown to have a detrimental effect on oocyte morphology for both immature and mature vitrified equine [115], mouse [116] and bovine [117] oocytes. However, Zhang, *et al.* [118] observed no difference in the survival rate of vitrified mature ovine oocytes with or without cumulus cells. Cumulus cell removal increases the MPF activity and accelerates the transition to metaphase stage and the redistribution of cortical granules [119]. In contrast, Bogliolo, *et al.* [120] reported that immature ovine oocytes vitrified without cumulus cells showed a significantly higher survival and meiotic maturation rate than those with cumulus cells and no differences in spindle and chromatin organization between two groups were observed.

Modina *et al*. [117]; Attanasio *et al.* [121] and Purohit *et al.* [122] showed that vitrified oocytes must be surrounded by cumulus cells in immature mouse oocytes, as a compact layer of cumulus cells blocked the permeation of high toxic cryoprotectant and might help prevent swelling of oocytes during removal of the cryoprotectant. El-nabby [77] in buffalo, Im *et al*. [123] in bovine and Park *et al*. [124] in mouse reported that intact mature oocytes had a higher developmental competence than denuded oocytes. Also, Tharasanit *et al*. [115] reported that cumulus removal from equine oocytes prior to IVM or vitrification resulted in reduced meiotic competence, MII spindle and chromatin quality.

CONCLUSION

Vitrification in buffalo has yet to produce convincing results capable of widespread application. It is critical that researchers achieve more consistent results to further elucidate the species-specific mechanisms influencing poor survivability following vitrification and to establish a "universal" protocol that can be applied for the cryopreservation of oocytes at different developmental

stages which can be used later on the commercial scale for different applications.

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