Improvement of Development of Vitrified Two-Cell Mouse Embryos by Vero Cell Coculture

Mojtaba Rezazadeh Valojerdi,¹⁻³ Mansourah Movahedin,¹ and Ahmad Hosseini²

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Purpose: The purpose of this study was to determine the developmental potential of two-cell mouse embryos resulting from vitrification could increased using monolayer of Vero cells.

Methods: Two-cell mouse embryos were divided into vitrified and nonvitrified groups. Embryos in the vitrified group were frozen with a combination of 10% ethylene glycol, 30% ficoll, and 0.5% M sucrose (EFS10) as cryoprotectants, and thawed rapidly with 0.5 M sucrose. The survived embryos were cultured either with Vero cells monolayer or in T6 medium. Accordingly the embryos of the nonvitrified group were also cultured. The rates of the development in all the groups were daily determined and statistically compared. At the end of the cultivation period, several expanded blastocysts from each group were stained with ethidium bromide and the mean number of the blastomers were counted and statistically compared.

Results: After 4 days of culture, the developmental potential of vitrified-thawed embryos were significantly reduced in Vero cell-free medium, and the mean cell number of embryos reaching the expanded blastocyst stage were also lower than that of nonvitrified embryos. With exception of last day of culture, Vero cell coculture, resulted in a significant increase in the rate of development of vitrified-thawed embryos as well as improved the mean cell number of expanded blastocysts. On the other hand, the mean cell number of expanded blastocysts of nonvitrified group was significantly improved in coculture group. However, the rate of embryo development except for the first day of culture was similar to that of medium alone.

Conclusions: The developmental potential of vitrified-thawed embryos appears to be retarded in conventional medium and Vero cell monolayer is capable to eliminate the postthaw deleterious effect of vitrification during the first 3 days of cultivation, but not for a longer period.

KEY WORDS: Coculture; mouse embryos; Vero cells; vitrification.

INTRODUCTION

Experience gained in the experimental freezing of mammalian embryos, over the last 25 years, has produced numerous modification in the freezing procedure (1). Most of these efforts have been directed

toward improving the survival rate of the frozen-thawed embryo and simplifying the method of freezing (2). This has resulted in the development of several methods that are quick and relatively simple to perform.

Vitrification is an inexpensive and simple method for cryopreservation of mammalian embryos, which was described for the first time by Rall and Fahy (3). These investigators vitrified the 8-cell stage of mouse embryos successfully using a combination of dimethyl sulfoxide (DMSO), acetamide, 1,2 propandiol or propylene glycol, and polyethylene glycol (PEG) as a cryoprotectant solution. Vitrification solidifies

¹ Department of Anatomy, Tarbiat Modarres University, Tehran, Iran.

² Department of Anatomy, Medical School of Shahid Beheshti University, Tehran, Iran.

³ To whom correspondence should be addressed at Royan Institute, Simin St. Asef Cross, Zafaraniyeh, P.O. Box 19395-4644, Tehran, Iran; e-mail: royan@ams.ac.ir.

a liquid by producing an extreme increase in viscosity during cooling. High concentrations of cryoprotectants that are added during the cooling process form amorphous state and devoid of any crystallization at extremely low temperatures. However, at room temperature and at the concentration required for vitrification, these solutions are toxic and should be exposed either at low temperatures or for short periods of time. The cooling rate also should be sufficiently rapid to allow water to pass directly into a glass state without forming ice crystals. Because formation of ice crystals during the freezing procedure may reduce the viability of embryo (4).

Since 1985 until now, there are many reports indicated vitrification is a successful method in the cryopreservation of mammalian embryos. Scheffen *et al.* (5) reported that the high survival rate of mouse embryos after vitrification using a simple vitrification solution containing two permeable cryoprotectants (glycerol and propylene glycol) in which embryos were equilibrated in two step. Complete vitrification of mouse blastocysts with the absence of both intra- and extracellular ice crystals was confirmed using cryoelectron microscopy (6). Kasai *et al.* (7) also described a cryoprotectant solution containing ethylene glycol, ficoll, and sucrose (EFS) and they could retain high survival rate after warming.

Despite many reports on successful vitrification of mammalian embryos, the rate of development of vitrified-thawed embryos for unknown reasons is still lower than that in nonvitrified embryos (4). Loss of membrane integrity (8), impairment of activity of the facilitated glucose transporter (9), reduction of activity of both the Na^+/H^+ antiporter and $\text{HCO}_3^-/\text{Cl}^-$ exchanger (10), structural changes such as high lipid content (11), and poorly developed cell-to-cell contacts (12,13) have been reported in cryopreserved embryos, but these features are probably only some of several things reflecting inappropriate in vitro culture conditions that may play a decisive role.

To increase the developmental rate of cryopreserved embryo, efforts are focused either to vary the cryopreservation techniques (14) or to improve in vitro conditions (15). In the latter, Shamsuddin *et al.* (16) observed the significant of in vitro culture conditions on the postthaw viability of bovine blastocysts. In addition to that, the serum was reported to be not essential for the development of embryos that survived following cryopreservation (17). Both groups sought to improve the survival of in vitro-produced bovine embryos by changing the media in which the embryos were cultured. Bautista *et al.* (18) also showed

that renewing the culture media can significantly help the vitrified mouse zygotes. Other investigators suggested that coculturing of the frozen-thawed embryos with different cells such as bovine cumulus cells (19), bovine oviduct epithelial cells (14), and monolayers of Vero cells (20) can support embryo development. In previous investigation, we have also indicated that Vero cell coculture system had a beneficial effect on the development of frozen-thawed two-cell mouse embryos, using slow technique (21). Now, the purpose of this study was to determine the effectiveness of vitrification for the mouse two-cell embryos, using EFS10, and to find out the improvement of the viability of vitrified-thawed embryos by coculturing with Vero cells. Development and hatching rates were assessed during 96-h cultivation and the total cell number of expanded blastocyst embryos was evaluated at the end of cultivation period.

MATERIALS AND METHODS

Embryos

Six–eight-week-old female NMRI mice were induced to superovulate with an injection of 7.5 IU human menopausal gonadotropin (hMG; Serono) followed by 7.5 IU human chorionic gonadotropin (hCG; Serono), given 48 h apart. Females were mated with males from the same strain and inspected for the presence of the vaginal plug on the following day. Mice with the presence of vaginal plug considered as a pregnant and killed 44–48 h post-hCG by cervical dislocation. Two-cell embryos were flushed from oviduct with T6 medium (NaCl: 4725, KCl: 110, NaH_2PO_4 : 47, MgCl_2 : 47, CaCl_2 : 196, NaHCO_3 : 2100, Na lactated: 2610, Na pyruvate: 30, D glucose: 1000, penicillin G: 63, streptomycin: 50, phenol red: 10 mg/L) supplemented with 5 mg/mL bovine serum albumin (BSA; Sigma). Morphologically normal embryos were washed and pooled in fresh T6 medium before use.

Vitrification Solution and Procedure

Embryos were frozen using the vitrification method described by Kasai *et al.* (7) with some modifications. Briefly, the vitrification solution (EFS10) was prepared, using 30% (w/v) ficoll 70 (average molecular weight 70000), 0.5 M sucrose, and 10% (v/v) ethylene glycol in phosphate buffered saline supplemented with 4 mg/mL BSA. The embryos were transferred to EFS10 at room temperature and held for 2 min. Using a tuberculin syringe, 0.5 mL french straws (A-201, Instruments de medicine veterinaire, France)

were profiled with approximately 100 μL of 0.5 M sucrose solution in phosphate buffered saline, followed by a short column of air and approximately 20 μL of EFS10. Then groups of 15–20 embryos were loaded into each straw and air bubble was added too. The loaded straws were sealed with hematocrite and plunged into liquid nitrogen.

Thawing Solution and Procedure

After 1–2 weeks straws were transferred from liquid nitrogen to room temperature for 10 s, followed by shaking in a 20°C water bath for 20 s. The contents of each straw were then transferred into a petri dish containing 1 mL 0.5 M sucrose in phosphate buffered saline and kept for 5 min. With emptying the contents of the straw into the petri dish, the column of the sucrose solution was mixed with the column of the vitrification solution and diluted ethylene glycol out of the embryos. Thereafter, the embryos were transferred to about 2 mL of fresh phosphate buffered saline, kept at 18°C to rehydrate for another 5 min. All embryos were scored for survival and then transferred to drops of T6 medium with 5 mg/mL BSA with or without a monolayer of Vero cells.

Preparation of Vero Cell Monolayer

Confluent monolayer of Vero cells with 150,000 cells derived from single vial of a frozen stock (Cell Bank at Pasteur institute Tehran, Iran). The cells were thawed 3 days prior to each anticipated coculture study and culture in Dulbecco's modified eagle's medium nutrient mixture F-12 Ham (1:1) (D-8900; Sigma) supplemented with 10% fetal calf serum (FCS; GIBCO) in 50 mL flasks. The cells then were trypsinized with 0.5% trypsin and 0.2 mg/mL EDTA (GIBCO) in phosphate buffered saline. After two successive washes, cell were cultured in drops of 100 μL T6 medium supplemented with 5 mg/mL BSA at a density of 10^6 cells/mL. A drop of the same medium was placed adjacent to the former drop culture and both were overlaid with light paraffin oil (Sigma). Culture dishes were then incubated at 37°C with an atmosphere of 5% CO_2 in air. Twenty-four hours before embryo cultivation, the medium was changed and remained in the same medium until the end of experiment.

Experimental Design

After collection of two-cell embryos, they were divided into two groups. The first group was nonvitrified

and considered as control and the second group was vitrified and stored into liquid nitrogen as described above. Controls, nonvitrified embryos were randomly allocated into the drops of T6 medium with 5 mg/mL BSA (Control 1) and drops of Vero cell monolayer (Control 2). Vitrified embryos after thawing were also randomly allocated into the drops of T6 medium with 5 mg/mL BSA (Exp 1) and drops of Vero cell monolayer (Exp 2). The experiments were replicated 10 and 9 times for nonvitrified and vitrified groups, respectively. Embryos were observed every 24 h for 4 days. The rate of development of the embryos to a higher stage was recorded daily, and a statistical comparison using χ^2 test was done.

Blastocysts Staining

Expanded blastocysts of each group of cultures were randomly selected on Day 4 of cultivation and stained with ethidium bromide. The blastocysts were placed in Acid Tyrod's to remove zona pellucida, washed with phosphate buffered saline and stained with ethidium bromide (EB; Sigma) for 30 min. Embryos were then treated with Triton X-100 (Sigma) for 20 s to remove cytoplasm of the blastomeres and floated into a drop of glycerol on a slide. Nuclei of the blastomeres were counted using a fluorescence microscope with a magnification of 400X. To avoid the problem of superimposing images of cell, the counting was done level by level under microscope by changing the focus with minimum of three levels in each count (Fig. 1). Data were analyzed by one-way analysis of variance.

Fig. 1. A fluorescent photomicrograph of ethidium bromide stained blastocyst used for counting of the blastomeres (400X).

Table I. Embryos Collection and Effect of Vitricification-Thaw Procedure on the Number of Survival Embryos

Number of embryos flushed	1275
Morphologically normal embryos	1213 (95%)
Number of nonvitrified embryos	612
Number of nonvitrified embryos in Control 1	242
Number of nonvitrified embryos in Control 2	370
Number of vitrified embryos	601
Number of embryos survived after thawing	513 (85%)
Number of vitrified embryos in Exp 1	291
Number of vitrified embryos in Exp 2	222

RESULTS

Embryo Collection and the Effect of Vitricification on Embryos Survival (Table I)

One thousand two hundred seventy-five two-cell stage embryos were flushed from the oviducts of superovulated female mice. Embryos were observed carefully for detection of any morphological abnormality. One thousand two hundred thirteen (95%) appeared normal under an inverted microscope and were randomly allocated in nonvitrified ($n = 612$) and vitrified ($n = 601$) groups. Embryos from nonvitrified group were randomly cultured in cell-free medium (Control 1; $n = 242$) and Vero cell coculture (Control 2; $n = 370$).

Embryos from vitrified group were cryopreserved as described under Materials and Methods section (mean number of embryos/straw = 12 ± 2). After thawing 513 out of 601 embryos (85%) were survived and considered as normal. Survived embryos were also randomly cultured in cell-free medium (Exp 1; $n = 291$) and Vero cell coculture (Exp 2; $n = 222$).

Development of Nonvitrified and Vitrified Two-Cell Embryos in Vero Cell-Free Medium Following 96-h Culture (Table II)

During the first 24 h of cultivation of embryos in Vero cell-free medium, for the vitrified group (Exp 1) and the nonvitrified group (Control 1), the rate of cleavage to 4–8 cell stage was nearly identical (53 vs. 55%). Whereas the rate of development to morula in the vitrified group was significantly lower than that of the nonvitrified group (14 vs. 40%, $P < 0.001$).

After 48 h in culture, the rate of development to morula and early blastocyst stage in the vitrified group was significantly lower than that of the nonvitrified group (41 vs. 50%, $P < 0.05$). Subsequently, less embryos in the vitrified group had developed to expanded blastocyst compared to that of the nonvitrified group: 10, 16, and 13% embryos in the vitrified group at Days 2, 3, and 4, respectively. This was significantly lower ($P < 0.001$) than that of the nonvitrified group, in which 32, 55, and 26% reach to this stage of development at Days 2, 3, and 4, respectively.

After 72 h in culture, the rate of hatched blastocyst also increased daily both in vitrified and nonvitrified groups, but this was lower in the vitrified group: 8 and 15% vitrified embryos hatched at Days 3 and 4, respectively, which was significantly lower ($P < 0.001$) than that of the nonvitrified embryos: 18 and 42% embryos at Days 3 and 4 of cultivation, respectively. During cultivation period, more embryos of the vitrified group were degenerated than that of the nonvitrified group: 10, 44, 55, and 72% embryos at Days 1, 2, 3, and 4, respectively. This was significantly higher ($P < 0.001$) than the nonvitrified group, in which 3,

Table II. Development of Vitrified and Nonvitrified Two-Cell Embryos in Coculture with Vero Cell and Vero Cell-Free Medium Following 96-h Cultivation

Treatment	Group	Number	24 h			48 h			72 h			96 h		
			4–8 cell	M	D	M and Eb	Exp. b	D	Exp. b	Hb	D	Exp. b	Hb	D
Nonvitrified	Control 1	242	132	96	6	122	78	33	132	43	58	62	101	77
			(55)	(40)	(3)	(50)	(32)	(14)	(55)	(18)	(24)	(26)	(42)	(32)
	Control 2 ^a	370	69 ^s	264 ^s	7	231 ^s	92 ^s	20 ^s	191	90	51 ^s	78	110 ^s	156 ^s
			(19)	(71)	(2)	(62)	(25)	(5)	(52)	(24)	(14)	(21)	(30)	(42)
Vitrified	Exp 1 ^b	291	153	42 ^s	28 ^s	120 ^s	29 ^s	129 ^s	45 ^s	24 ^s	159 ^s	39 ^s	44 ^s	208 ^s
			(53)	(14)	(10)	(41)	(10)	(44)	(16)	(8)	(54)	(13)	(15)	(72)
	Exp 2 ^c	222	124	45	13	122 ^s	34	57 ^s	53 ^s	30	77 ^s	22	42	159
			(56)	(20)	(6)	(55)	(15)	(26)	(24)	(14)	(35)	(10)	(19)	(72)

Note. Control 1 and Exp 1, embryos cultured in Vero cell-free medium; Control 2 and Exp 2, embryos cultured in Vero cell; M, morula; Eb, early blastocyst; Exp. b, expanded blastocyst; Hb, hatched blastocyst; D, degenerated embryos; value in parentheses are percentages.

^a In this row ^s indicates significance for Control 2 vs. Control 1.

^b In this row ^s indicates significance for Exp 1 vs. Control 1.

^c In this row ^s indicates significance for Exp 2 vs. Exp 1.

Table III. Number of Cells of the Blastocysts Produced in Vero Cell-Free Culture and Vero Cell Coculture System

Culture system	Nonvitrified embryos	Vitrified embryos
Vero cell-free ^a	77.31 ± 12.55 ^b (17)	55 ± 17.79 ^b (15)
Vero cell coculture	99 ± 17.61 (14)	104 ± 2.25 (15)

Note. Values are mean ± SD. Number of embryos examined in parentheses.

^a Means a significant difference between nonvitrified and vitrified embryos ($P < 0.05$).

^b Means a significant difference between Vero cell-free and Vero cell coculture ($P < 0.05$).

14, 24, and 32% embryos degenerated at Days 1, 2, 3, and 4, respectively.

At the end of cultivation period, the mean cell numbers of blastocysts of the vitrified group was also significantly lower than that of the nonvitrified group (55 ± 17.79 vs. 77.31 ± 12.55 , $P < 0.05$), see Table III.

Development of Nonvitrified Two-Cell Embryos in Vero Cell Coculture and Cell-Free Medium Following 96-h Culture (Table II)

During the first 24 h of cultivation, Vero cell coculture system could improve the development of nonvitrified embryos. In coculture group (Control 2) compared with that of the medium alone (Control 1), less embryos ceased at stage of 4–8 cell (19 vs. 55%, $P < 0.001$), while more embryos reached to morula (71 vs. 40%, $P < 0.001$).

After 48 h in culture, the rate of development to morula and early blastocyst stage in coculture group as compared with that of the medium alone was significantly high (62 vs. 50%, $P < 0.05$). But less embryos reach to expanded blastocyst (25 vs. 32%, $P < 0.05$).

After 72 h in culture, the rate of expanded and hatched blastocysts stages for both groups of coculture and medium alone were nearly identical (52 and 24% vs. 55 and 18%, respectively).

After 96 h in culture, although the rate of expanded blastocyst between the two groups was identical (21 vs. 26%), less embryos hatched in coculture group as compared with that of the medium alone (30 vs. 42%, $P < 0.05$). The rate of the embryo degeneration from the first day of cultivation until the end of the third day was low in coculture (2 vs. 3%, 5 vs. 14%, $P < 0.001$; 14 vs. 24%, $P < 0.05$, respectively), but on the last day of culture it contrasted that of the medium alone (42 vs. 32%, $P < 0.05$).

At the end of cultivation period, the mean cell numbers of blastocysts in coculture group was also significantly higher than that of the medium

alone (99 ± 17.61 vs. 77.31 ± 12.55 , $P < 0.05$), see Table III.

Development of Vitrified Two-Cell Embryos in Vero Cell Coculture and Cell-Free Medium Following 96-h Culture (Table II)

During 72 h of cultivation, Vero cell coculture system could improve the development of the vitrified embryos. On the first day, the rate of cleavage to the 4–8 cell stage in the vitrified group coculture with Vero cells (Exp 2) as well as those cultured in Vero cell-free medium (Exp 1) was identical (56 vs. 53%). On the same day, although the rate of the development to morula was high in coculture group, but it was not significant (20 vs. 14%).

After 48 h in culture, the rate of development to morula and early blastocyst stage in coculture group as compared with that of the medium alone was significantly high (55 vs. 41%, $P < 0.05$). The rate of expanded blastocyst was also higher in coculture group, but it was not significant (15 vs. 10%).

After 72 h in culture, the rates of expanded and hatched blastocysts in the coculture group were increased, but in comparison with those of the medium alone, the former was significant (expanded blast: 24 vs. 16%; $P < 0.05$; hatched blast: 14 vs. 8%). These values after 96 h in culture, were identical (expanded blast: 10 vs. 13%; hatched blast: 19 vs. 15%). The rate of embryo degeneration from the first day of cultivation until the end of third day was low in coculture (6 vs. 10%, 26 vs. 44%, $P < 0.001$; 35 vs. 54%, $P < 0.001$, respectively), but on the last day, it suddenly increased in coculture too (72 vs. 72%).

At the end of cultivation period, the mean cell numbers of blastocysts in coculture group was also significantly higher than that of the medium alone (104 ± 2.25 vs. 55 ± 17.79 , $P < 0.05$), see Table III.

DISCUSSION

The present investigation indicated that one step dehydration performed within 2 min at low concentration of ethylene glycol is a suitable, easy, and simple procedure. This method resulted in high survival rate for the vitrification of two-cell mouse embryo, which was in agreement with Bautista *et al.* (18) who vitrified one-cell mouse embryo using one step dehydration and also was in agreement with the other investigators who used different concentrations of ethylene glycol (7), different types of cryoprotectants (22),

different durations for dehydration (4–22), and two-step dehydration (4–14) for the vitrification of mouse embryos. However, despite of high survival rate with low concentration of ethylene glycol, the rate of development of the vitrified-thawed two-cell mouse embryos in conventional medium still is lower than that of the nonvitrified embryos (18). This may be related to the ultrastructural changes of vitrified-thawed embryo that remained during the cultivation (23), or to inappropriate in vitro culture medium resulted in slow efflux of ethylene glycol from the vitrified-thawed embryos (18). Lane *et al.* (10) reported the poor activity of both the Na^+/H^+ antiporter and $\text{HCO}_3^-/\text{Cl}^-$ exchanger in the vitrified-thawed two-cell hamster embryos, and suggested that the addition of Eagle's nonessential amino acids in the culture medium might support the development of the vitrified-thawed embryo. Bautista *et al.* (18) claimed that frequent changing the culture medium could improve the in vitro development rates of vitrified-thawed mouse zygotes to which could assisted the progress of the embryo toward the expanded blastocyst stage. The present investigation also indicated that Vero cell coculture system could improve the rate of development of the vitrified-thawed two-cell mouse embryos until the end of third day of cultivation. But the precise mechanism that promotes development of vitrified-thawed embryos remained unclear. Bongso *et al.* (24) described two possible mechanisms for the effect of feeder cells on the development of mammalian embryos. The first mechanism that is called positive coculture effect where the feeder cell can enhance the embryo development through the secretion of embryo-tropic substances such as insulin-like growth factor (IGF) (25), insulin-like growth factor binding proteins (26), tubal epithelial Proteins 1 and 2 (27) taurine (28) in the culture medium, and also by stimulating the transcription of growth factor receptors on the embryos (29,30). While the other mechanism that is called negative coculture effect where the feeder cell can enhance the embryo development through the removal of deleterious agents from the culture medium (24). The former mechanism does not seem consistent enough to explain our finding. Because our data in the present study showed that Vero cells, except on the first day of cultivation, were unable to improve the rate of development of fresh, nonvitrified embryos, even in the last day of culture the rate of hatched embryos in Vero cells was lower than that of the cell-free medium. This result disagreed with authors who claimed the beneficial effect of Vero cells for improve-

ment of the embryos in bovine (31), mouse (32), monkey (33), and human (34), but confirmed the finding of other investigators who failed to demonstrate a clear beneficial effect of Vero cells on the development of mouse embryo (28).

These contradictory reports may be either related to the mode of the action of Vero cells on the development of embryos from different species to the type of conventional medium in which both embryos and Vero cells are cultured (32), or to inadequate production of embryo-tropic substances such as taurine by Vero cells (28). The other mechanism may be more consistent with the finding of this study where the embryos in both groups of coculture (vitrified and nonvitrified) showed lower degeneration rate during the first 3 days of cultivation; however, this is followed by higher degeneration rate at the fourth day. The possible explanation is that Vero cells during the first period had active secretion of proteolytic enzymes such as chemotrypsin (32), which assisted the embryo to degrade the inhibitory factors, like the chemicals released from cryopreserved embryos. On the other hand, the secretion of this enzyme may be decreased later, which resulted in degeneration of the embryos. The other possible explanation is the consumption of vital chemicals during the growth of Vero cells, where these cells compete with the embryos for survival and resulted in degeneration of the embryos. Two other explanations can also be proposed, the first one is that the high degenerative rate on the fourth day of cultivation could be due to malnutrition or due to the fact that the cells were over populated and reached to the stationary phase. In such a stage, the cells will not only compete with embryos for nutrition, but also halt the secretion embryonic growth factors and even secrete toxic factors to prevent embryo development. Secondly low pH of the culture media can be a reason for the degeneration of the embryo where CO_2 built up in the medium occurred because of the use of microdroplet under paraffin oil.

In addition, the present results also indicated that Vero cells monolayer improve the number of blastomeres in both groups of vitrified and nonvitrified. This finding was in agreement with those authors who cocultured noncryopreserved embryo with different cells monolayer and obtained the same results (35,36). The exact reason for this finding is not clear, but the possible explanation is that Vero cells either secrete mitogenic factors, which is not confirmed yet (37), or reduce the environmental stresses that is harmful for embryo progression.

CONCLUSION

Vero cells coculture system can improve the rate of the development of vitrified-thawed two-cell mouse embryos during the first 3 days of cultivation, but not for a longer period. This culture system also causes an increase of mean cell number of growing blastocysts from vitrified-thawed two-cell embryos.

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