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The Interspecific Effect of Human and Porcine Adipose–derived Stem Cell Conditioned Medium for *in vitro* Culture of Parthenogenetically Activated Porcine Embryos

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Adipose–derived stem cells (ASC) have been known to secrete immunomodulatory and regenerative factors that could influence the *in vitro* culture (IVC) environment for embryo development and exist in conditioned medium (CM) as well. However, the interaction between different species derived embryos and ASC–CM during IVC has never been investigated. In this study, we examined the interspecific effects of ASC–CM obtained from humans (HCM) and pigs (PCM) on IVC of porcine embryos generated by parthenogenetic activation. *In vitro* matured cumulus–oocyte complexes were electrically activated for parthenogenesis and subsequently divided into a control group cultured in porcine zygote medium–5 and treatment groups in which HCM and PCM were supplemented with various concentration. The embryo development competence of each group was evaluated including cleavage rate, blastocyst formation rate and total cell number of porcine parthenotes. As a result, the blastocyst formation rate of 2.5% PCM treated group and the control group was significantly higher than that of 2.5% HCM treated group ($P<0.05$) but there were no differences in the cleavage rate and total cell number of blastocyst among groups. Our study suggested that the application of ASC–CM for embryo IVC system could be more effective within the same species rather than interspecific treatment.

Key words: adipose–derived stem cell, conditioned medium, interspecific effect, *in vitro* culture, porcine embryo

INTRODUCTION

The suitability of animal models for human disease research can be determined based on their physiological function, evolutionary phenotypes (Vodicka *et al.* 2005), organ similarity (Avramopoulos 2009), and size and composition of the genome (Bendixen *et al.* 2010), all of which make pigs ideal biomedical models. Assisted reproductive technology, including *in vitro* maturation (IVM), *in vitro* culture (IVC), *in vitro* fertilization (IVF) and embryo transfer, is essential for production of genetically modified pigs and has been employed for improvement of *in vitro*–derived embryo development from pigs used in research laboratories (Romar *et al.* 2016). Despite the utility of pigs, obtaining *in vitro* developed embryos, particularly embryos with equivalent quality to those developed *in vivo*, is very limited. As the possibility of *in vitro* embryo production is not completely improved, research on embryos IVC systems that can compensate for these shortcomings and enhance the embryo developmental competence needs to be actively conducted (Rodriguez–Osorio *et al.* 2007). IVC of embryos is a critical procedure for development of embryos generated by the contemporary *in vitro* production techniques including IVF, somatic cell nuclear transfer or parthenogenetic activation (PA). Considering that growth factors and cytokines from the oviduct are required for embryo development and implantation, creating the microenvironment during IVC similar to in vivo has implications for the development of high–quality embryos (Bhardwaj *et al.* 2016) and in fact supplementation of embryo IVC medium with various growth factors have shown to reduce apoptosis and degeneration, increase the embryo developmental competence in various animal species (Richter 2008). Consequently, there have been diverse studies on embryo IVC systems involving cell co–culture (Moshkdanian *et al.* 2011), specific factors (Dhali *et al.* 2009) or conditioned medium supplementation (Bhardwaj *et al.* 2016), particularly with mesenchymal stem cells (MSC).

Adipose tissue-derived stem cells (ASC) are a type of MSC that are capable of self–renewal and embryonic lineage differentiation and thus are very valuable in regenerative medicine applications (Pittenger *et al.* 2002). ASC carry distinctive advantages in that they are the most easily obtained and abundant among adult stem cells originating from adipose tissue, a soft tissue that occupies a large part of the body (Tsuji *et al.* 2014), and 1×105 cells can be obtained from 1g of fat tissue without ethical issues or complex surgical procedures (Ra *et al.* 2011). MSC including ASC secrete paracrine factors such as interleukin (IL)–6, IL–10, indoleamine 2,3–dioxygenase, hepatocyte growth factor, transforming growth factor (TGF) β and matrix metalloproteinase, with immunomodulatory and regenerative therapeutic effects on their micro–environment (van Buul *et al.* 2012). It

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was found that these MSC–derived factors exist in cell culture media, so–called stem cell conditioned medium (CM), which can be mass–produced and utilized irrespective of cell donor and recipient compatibility (Pawitan 2014) (Pawitan 2014). In fact, included in ASC–CM are cytokines such as epidermal growth factor, fibroblast growth factor, insulin–like growth factor, granulocyte colony–stimulating factor, granulocyte–macrophage colony–stimulating factor, platelet–derived growth factor, vascular endothelial growth factor and TGFβ (Kim *et al.* 2009). However, it has been revealed that discrepancies exist with regard to the clinical application of MSC because of interspecies variations (Ren *et al.* 2009).

Therefore, we hypothesized that adipose–derived stem cell conditioned medium (ASC–CM), in particular homospecific CM, could support the embryo development during the IVC procedure. The purpose of this study was to investigate the interspecific and homospecific effects of ASC–CM derived from humans and pigs on IVC of porcine parthenotes.

MATERIALS AND METHODS

Ethics approval

Both human and porcine ASCs were provided from R Bio Stem Cell Research Center under GMP conditions and this research was approved by the Life Ethics Committee of the Biostar Stem Cell Technology (RBIO 2015–12–001). All cell donors gave consent to participate in the study and to collect ASC. The details of specific standards are found in the Code of Federal Regulations, Title 21 (21CFR) and Section 610.

Chemicals

All chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated.

Preparation of conditioned medium

With the consent of cell donors, human ASC were isolated from adipose tissue extracted from the lower abdomen through liposuction. The primary culture of cells was performed as previously described (Ra *et al.* 2011). Under the conditions of 37°C and 5% CO2, 3×107 human ASCs were seeded and cultured in T–175 flask (175 cm^2) with RCME (Medium for adipose–derived stem cell culture; R BIO, Seoul, Korea) supplemented with 3.5% newborn calf serum (NCS). On day 3 after cell seeding, the medium was replaced with serum–free basal medium, Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Grand Island, NY, USA). The medium was collected after 24 h culture and centrifuged at 2500 rpm for 5 min. Following addition of serum–free DMEM and repeated supernatant collection 5 times, CM from the first to fifth day were mixed and the final human ASC–CM was recovered through a sterilization and filtration process using a 0.22 um filter. For porcine ASC–CM collection, porcine ASC that were established and cryopreserved previously (Oh *et al.* 2014) were cultured and CM was obtained using the same method as for human ASC as described above.

Oocyte collection and *in vitro* maturation

Porcine ovaries were collected from prepubertal gilts at a local abattoir and transported to the laboratory within 3 h in physiological saline at 32–35°C. Fluids were aspirated from 3 to 6 mm sized ovarian follicles with an 18–gauge needle connected to a 10 ml syringe and kept in a 50 ml conical tube for 5 min at 37°C to allow settlement of cells. The supernatant was discarded and the sediment was washed three times with washing medium containing 9.5 g/l of tissue culture medium–199 (TCM–199; Invitrogen, Carlsbad, CA, USA), 5 mM sodium hydroxide, 2 mM sodium bicarbonate, 0.3% polyvinyl alcohol, 10 mM HEPES and 1% penicillin–streptomycin (Invitrogen). Cumulus–oocyte complexes (COCs) with more than 3 layers of cumulus cells and homogeneous cytoplasm were selected under a stereomicroscope and then washed three times in washing medium. Selected COCs were incubated in IVM medium, TCM–199 medium supplemented with 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 5 μl/ml insulin–transferrin–selenium solution (ITS–A) 100X (Invitrogen), and 10% porcine follicular fluid for IVM at 38.5°C under 5% CO2 in 95% humidified air. The COCs were incubated in IVM medium with hormones, 10 IU/ml equine chorionic gonadotropin (eCG) and 10 IU/ml human chorionic gonadotropin (hCG), for 22 h and then transferred into hormone–free IVM medium for an additional 22 h after washing twice in fresh IVM medium.

Parthenogenetic activation and *in vitro* culture of oocytes

After 42–44 h of IVM, COCs were denuded with 0.1% hyaluronidase by gentle pipetting and washed in Tyrode's albumin lactate pyruvate (TALP) medium. The denuded oocytes were gradually equilibrated in activation medium composed of 0.28 M mannitol, 0.5 mM HEPES, 0.1 mM CaCl2 and 0.1 mM MgSO4. Oocytes with homogeneous cytoplasm were selected and placed in a chamber with two electrodes covered with activation medium and connected with a BTX Electrocell Manipulator ECM 2001 (BTX Inc., San Diego, CA, USA). The oocytes were electrically activated with a single direct current (DC) pulse of 1.5 kV/cm for 60 μsec and then washed three times in porcine zygote medium–5 (PZM–5; Funakoshi Corporation, Tokyo, Japan). The parthenotes were randomly divided and cultured *in vitro* in droplets of PZM with various concentrations of conditioned medium for 7 days in a humidified atmosphere of 5% O2, 5% CO2 and 90% N2.

Evaluation and total cell counts of parthenotes

From day 0, when parthenogenetic activation (PA) was conducted, the cleavage rate of parthenotes was evaluated on day 2 and blastocyst formation rate was assessed on day 7 using a stereomicroscope. The total cell number of blastocysts of each group was counted on

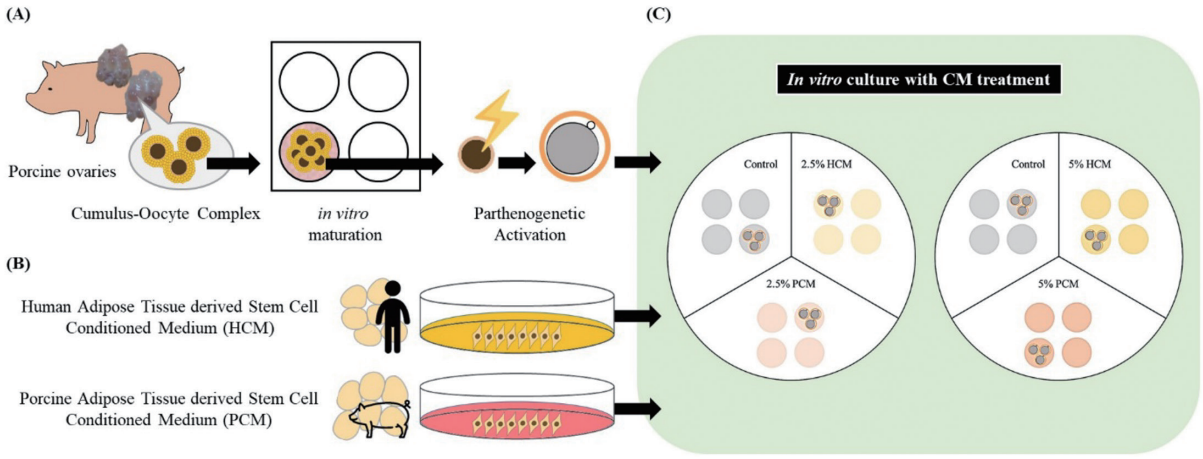

Fig. 1. A schematic illustration of the experimental process and design. (A) Porcine cumulus–oocyte complexes obtained from ovaries were *in vitro* matured and parthenogenetically activated for the experiment. (B) For the preparation of HCM and PCM, adipose–derived stem cells were isolated from human and porcine fat tissue and cultured with serum free medium replacement. (C) *In vitro* culture with HCM and PCM respectively performed to examine the interspecies effect of 2.5% and 5% CM on embryo development competence.

day 7 after staining with 5 ug/ml Hoechst 33342 for 7 min. The stained blastocysts were washed in TALP medium, moved to glycerol drops on a glass slide and covered with a glass coverslip and then observed under a fluorescence microscope (Nikon Corp., Tokyo, Japan).

Experimental design

In total of 3 experiments were conducted with human adipose tissue derived stem cell conditioned medium (HCM) and porcine adipose tissue derived stem cell conditioned medium (PCM) of various concentration treated during parthenotes *in vitro* culture respectively. The same concentration of HCM and PCM were treated for examining the interspecies effect as the final step (Figure. 1).

In experiment 1, 3 kinds of CM concentration were tested with respective control group to determine the optimal concentration for CM treatment and the experimental groups are as follows: 1) control; 100% PZM, 2–1) 2.5% HCM; 97.5% PZM+2.5% HCM, 2–2) 5% HCM; 95% PZM+5% HCM, 2–3) 10% HCM; 90% PZM+10% HCM.

In experiment 2, according to the preliminary result of experiment 1, 2.5% and 5% PCM were treated in parthenotes *in vitro* culture medium and for precise comparison of each concentration, 2.5% and 5% PCM were simultaneously tested with a control group. The experimental groups are as follows: 1) control; 100% PZM, 2) 2.5% PCM; 97.5% PZM+2.5% PCM, 3) 5% PCM; 95% PZM+5% PCM.

Experiment 3 was performed to explore the interspecies effect of CM derived from human and porcine ASC individually. The experimental groups are as follows: 1) control; 100% PZM, 2) 2.5% HCM; 97.5% PZM+2.5% HCM, 3) 2.5% PCM; 97.5% PZM+2.5% PCM and 1) control; 100% PZM, 2) 5% HCM; 95% PZM+5% HCM, 3) 5% PCM; 95% PZM+5% PCM.

Statistical analysis

In experiment 1, the cleavage rate, blastocyst formation rate and total cell number of blastocysts were analyzed by unpaired t test with Welch's correction. In experiment 2 and 3, the analysis factors were equal to experiment A described above and Tukey's multiple comparison test following one–way ANOVA was used. All experiments were repeated at least three times and GraphPad prism software version 5 was used for statistical analysis (GraphPad, San Diego, CA, USA). The values are means±standard error of mean and P–value less than 0.05 among groups were considered as statistically significant difference.

RESULTS

Effect of HCM treatment during IVC on porcine parthenotes development

We evaluated the effects of HCM supplementation with various concentration, 2.5%, 5%, and 10%, on parthenotes during IVC. The rate of porcine parthenotes developed to blastocyst and total cell number of blastocysts were assessed and there was no significant difference between control and 2.5% HCM group. In terms of 5% HCM, blastocyst formation rate of 5% HCM group (20.06±2.75%, $P<0.05$) was significantly lower than that of control (28.78±2.64%, $P<0.05$) but significantly higher total cell number of blastocysts (54.33±6.10, $P<0.05$) was shown in HCM group than that of control (39.64±2.69, $P<0.05$). The blastocyst formation rate of 10% HCM group (9.68±1.55%, $P<0.05$) was https://miro.com/app/board/o9J_lD7mMOU=/significantly decreased compared to control (26.33±3.67%, $P<0.05$) and no significant difference appeared with respect to total cell number of blastocyst (Table. 1).

Effect of PCM treatment during IVC on porcine parthenotes development

Based on the result of human ASC–CM, the effect of 2.5% and 5% PCM were evaluated, excluding the concentration of 10%. There was no significant difference

Table 1. Effect of HCM supplementation during *in vitro* culture on development of porcine parthenotes

Experimental group		No. of embryos	No. of embryos developed to blastocyst (%)	Total cell number of blastocysts
A	Control	107	23 (22.22±5.27)	51.33±6.36
	2.5% HCM	110	16 (14.69±2.27)	44.73±2.95
B	Control	93	27 (28.78±2.64)[a]	39.64±2.69[a]
	5% HCM	95	19 (20.06±2.75)[b]	54.33±6.10[b]
C	Control	90	25 (26.33±3.67)[a]	49.00±8.53
	10% HCM	101	10 (9.68±1.55)[b]	55.17±6.79

Results are presented as the mean ± SEM of an experiment repeated at least 3 times.
Different superscripts indicate statistical differences within experimental groups, P<0.05.
Control: 100% PZM.
n% HCM: treatment group with n% HCM + (100–n)% PZM.

Table 2. Effect of PCM supplementation during *in vitro* culture on development of porcine parthenotes

Group	No. of embryos	No. of embryos developed to (%)		Total cell number of blastocysts
		≥2 cells	Blastocyst	
Control	110	103 (93.64±0.94)	29 (27.42±4.36)	52.00±5.90
2.5% PCM	108	101 (93.75±2.44)	29 (26.88±2.02)	42.75±3.65
5% PCM	108	101 (93.25±1.46)	21 (18.00±3.93)	51.17±6.92

Results are presented as the mean ± SEM of an experiment repeated at least 3 times.
Different superscripts within a column indicate statistical differences, P<0.05.
Control: 100% PZM.
2.5% PCM: 97.5% PZM + 2.5% PCM treatment group.
5% PCM: 95% PZM + 5% PCM treatment group.

Table 3. Effect of 2.5% HCM and PCM supplementation during *in vitro* culture on development of porcine parthenotes

Group	No. of embryos	No. of embryos developed to (%)		Total cell number of blastocysts
		≥2 cells	Blastocyst	
Control	117	106 (93.61±1.21)	23 (27.41±4.35)[a]	48.86±5.76
2.5% HCM	118	108 (93.67±0.96)	12 (10.43±3.68)[b]	45.80±4.10
2.5% PCM	116	109 (96.25±1.25)	21 (23.89±3.99)[a]	44.57±3.65

Results are presented as the mean ± SEM of an experiment repeated at least 3 times.
Different superscripts within a column indicate statistical differences, P<0.05.
Control: 100% PZM.
2.5% HCM: 97.5% PZM + 2.5% HCM treatment group.
2.5% PCM: 97.5% PZM + 2.5% PCM treatment group.

Table 4. Effect of 5% HCM and PCM supplementation during *in vitro* culture on development of porcine parthenotes

Group	No. of embryos	No. of embryos developed to (%)		Total cell number of blastocysts
		≥2 cells	Blastocyst	
Control	100	91 (91.59±1.82)	25 (25.55±5.46)	48.86±5.76
5% HCM	99	92 (92.94±2.32)	14 (14.02±4.75)	50.25±12.04
5% PCM	99	94 (93.45±1.57)	13 (12.67±2.40)	52.71±6.05

Results are presented as the mean ± SEM of an experiment repeated at least 3 times.
Different superscripts within a column indicate statistical differences, P<0.05.
Control: 100% PZM.
5% HCM: 95% PZM + 5% HCM treatment group.
5% PCM: 95% PZM + 5% PCM treatment group.

in cleavage rate, blastocyst formation rate of porcine parthenotes and total cell number of blastocysts between control and treatment groups with 2.5% and 5% PCM (Table. 2)

Comparison of HCM and PCM treatment in equal concentration during IVC on porcine parthenotes development

To determine the interspecific effect of HCM on porcine embryo development, the equal concentration of HCM and PCM were supplemented in IVC medium of respective groups. As shown in Table 3, no significant differences were observed in terms of cleavage rate of porcine parthenotes among control, 2.5% HCM and 2.5% PCM groups (93.61±1.21% vs. 93.67±0.96% vs. 96.25± 1.25%, respectively, p=0.2685). The blastocyst formation rate in 2.5% PCM group (23.89±3.99%) was significantly higher than 2.5% HCM (10.43±3.68%, P<0.05) but similar to the control (27.41±4.35%). With respect to total cell numbers of blastocyst, there was no significant difference among groups (48.86±5.76 vs. 45.80± 4.10 vs. 44.57±3.65, p=0.7703).

Table 4 shows that the cleavage rate of parthenotes was similar among the control, 5% HCM and 5% PCM groups (91.59±1.82% vs. 92.94±2.32% vs. 93.45±1.57%, respectively, p=0.7844). No difference was found in blastocyst formation rates (25.55±5.46% vs. 14.02± 4.75% vs. 12.67±2.40%) and total cell numbers of blastocysts (48.86±5.76 vs. 50.25±12.04 vs. 52.71±6.05) among these groups.

DISCUSSION

In the present study, two concentrations, 2.5% and 5%, were selected for investigating the interspecific effect of ASC–CM on embryo development after PA. Our experiments demonstrated the result of 2.5% CM treatment during porcine parthenotes IVC could be diverged depending on species from which ASC originated, but interestingly it did not pertain to 5% CM treatment.

Contrary to our initial hypothesis, HCM and PCM would not lead to a dramatic improvement in parthenotes development. The media where the control group were cultured consisted of 100% PZM–5, a chemically defined medium developed specifically for porcine IVC with the inorganic and energy substrate concentrations based on porcine oviductal fluid (Yoshioka *et al*. 2002). The medium is supplemented with amino acids including glutamine, hypotaurine, basal medium eagle amino acids and minimum essential medium nonessential amino acids, known to improve the embryo development while present in culture medium (SuzukiYoshioka 2006). Furthermore, polyvinyl alcohol is included in PZM–5, known as available substitute for serum albumin (Yoshioka *et al*. 2002; SuzukiYoshioka 2006; Kim *et al*. 2007), instead of undefined macromolecules derived from animal source. In both HCM and PCM treated groups, a certain amount of the essential components mentioned above became reduced depending on the volume of the CM replaced with the original culture medium. Even though in CM exist various factors secreted from ASC substantially advantageous to embryos, given that all of components with complex combination collaborate together in culture medium, the most adequate condition and concentration of CM for embryo IVC need to be examined without undermining the stability of original medium.

The optimal concentration of CM for treatment varies depending on the type of cell from which CM is derived and the target cell used to confirm its effect. When human embryo conditioned medium was added to the culture medium of human endometrial microvascular endothelial cells, stimulation of cell proliferation was maximized with 5% CM. Other concentrations of CM such as 2.5% and 10% significantly increased tube formation but 20% CM reduced vascular structure growth (Kapiteijn *et al*. 2006)(Kapiteijn *et al*. 2006). In the case of human embryonic stem cell–derived conditioned medium, 10% CM treatment showed a greater bovine embryo *in vitro* development rate than 5% and 15% CM (Kim *et al*. 2011). From dose–response experiments in another study, 50% of human ASC–CM significantly promoted migration of human umbilical vein endothelial cells, fibroblasts and keratinocytes compared to lower (0, 10, 25%) and higher (75, 100%) concentrations (Hu *et al*. 2016). Diverse causes of these differential effects have been speculated from the above studies. A low concentration of CM contains low concentration of cytokines and probably induces low activity in target cells. Nevertheless, a high concentration of CM does not ensure high activity and supports the possibility that both stimulatory and inhibitory factors are present in CM but their effects can change depending on the level and timing of CM treatment (Hu *et al*. 2016).

Studies have been reported regarding the effect of human stem cell secreted factors on porcine embryo development. Co–culture with human endothelial progenitor cells significantly increased pluripotency–related gene expression in blastocysts developed from porcine parthenotes (Lee *et al*. 2018). Human ASC co–cultured porcine embryos showed higher blastocyst development rates and total cell numbers in blastocysts than the control (Nugraha Setyawan *et al*. 2018)(Nugraha Setyawan *et al*. 2018). In contrast, the number of porcine parthenotes that developed into blastocysts was significantly lower than the control when 10% human ASC bioactive material, characterized as culture medium with ASC–secreted cytokines, was treated during culture for 6 days (Park *et al*. 2013). Even though the capability of stem cells would not be reduced by interspecific treatment, species variation needs to be considered. Firstly, phenotypic differences exist between human and porcine MSC populations and cross–reactivity to human MSC has not yet been found in several surface markers of porcine MSC. In addition, differences in lifespan and age of donor species might result in differential regenerative effects and comparison between interspecies donors could become ambiguous (Bach *et al*. 2015)(Bach *et al*. 2015). Most of all, as a result of research on species variation in the mechanisms of MSC–mediated immunosup-

pression, it has been demonstrated that soluble factor-mediated immune regulation of human MSC differs in species–specific mechanisms (Ren *et al*. 2009).

According to the present study, we recommend the homospecific treatment of ASC–CM for porcine embryo culture condition upon consideration of the possibility that interspecific treatment could cause detrimental effects. Further research on the optimal culture condition using CM with serum supplement, concentration validation and the mechanism investigation of MSC–mediated immunosuppression will be conducted.

AUTHOR CONTRIBUTIONS

Kihae RA conducted the experiments, performed statistical analysis, interpreted the data, and drafted the manuscript. Hyun Ju OH coordinated the design of studies, analyzed the data and mainly contributed in editing the manuscript. Eui Hyun KIM conducted the experiments and contributed in editing the manuscript. Nobuhiko YAMAUCHI review and edited the manuscript. Byeong Chun LEE supervised the research. All authors read and approved the final manuscript.

DECLARATION OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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