DOI: 10.1111/rda.12850

ORIGINAL ARTICLE

WILEY Reproduction in Domestic Animals

The nuclear and developmental competence of cumulus–oocyte complexes is enhanced by three-dimensional coculture with conspecific denuded oocytes during in vitro maturation in the domestic cat model

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Contents

The objective of the study was to assess the efficacy of coculture with conspecific cumulus-denuded oocytes (CDOs) during in vitro maturation in a three-dimensional system of barium alginate microcapsules on the in vitro embryo development of domestic cat cumulus–oocyte complexes (COCs). In Experiment I, COCs were cocultured with conspecific CDOs or cultured separately in a 3D system for 24 hr of in vitro maturation, before assessing the meiotic progression. In Experiment II, the in vitro fertilization of COCs and CDOs was carried out with chilled epididymal spermatozoa and the presumptive zygotes were cultured in vitro separately for 7 days in 3D microcapsules before assesment of embryonic development. The results showed that the viability was maintained and that meiosis was resumed in the 3D culture system. The presence of CDOs during in vitro maturation improved the meiotic competence of the COCs, since the proportions of telophase I/metaphase II were higher than that in the groups cultured separately. The enrichment of the maturation system by companion oocytes also enhanced the ability of COCs to develop into embryos, and increased the percentages of morula and blastoycst stages. The COCs cocultured with CDOs developed at higher rates than the COCs cultured separately and the CDOs themselves. The beneficial effects of coculture with conspecific CDOs were presumably due to the paracrine action of some secreted factors that enhanced many molecular patterns related to the complex of cumulus oophorous cells. Further investigations to understand how the 3D microenvironment can influence the features of oocytes and embryos are required.

1 | INTRODUCTION

Over the last decades, many attempts have been made to improve the in vitro outcome of cumulus–oocyte complexes (COCs). In various animal models, the coculture with somatic cells (granulosa, oviductal or embryonic fibroblasts) as companion cells or monolayer, or the culture in isolated oviducts, gave promising results in terms of nuclear and cytoplasmic oocyte maturation (Chigioni, Perego, & Luvoni, 2005; Lee, Quaas, Wright, Toth, & Teixeira, 2011; Luvoni, Chigioni, Allievi, & Macis, 2003). The cleavage, as well as the advanced embryo development, were also enhanced when COCs were matured with companion cells, rather than alone (Hatoya et al., 2006; Jahromi, Mosallanezhad, Matloob, Davari, & Ghobadifar, 2015).

The biological evidence of oocytes as central regulators of follicular cell functions and meiotic resumption prompted their use as companion cells. The production of several growth factors, known as oocyte-secreted factors (OSFs), that act on cumulus cells has been well established (Gilchrist, Ritter, & Armstrong, 2004; Gilchrist &

Thompson, 2007), and their effect in coculture has been proved. Some studies showed that the presence of conspecific cumulus-denuded oocytes (CDOs) positively affected the meiotic maturation in vitro of cocultured bovine, murine and feline COCs and improved their competence to develop into an embryo (Gilchrist, Lane, & Thompson, 2008; Godard, Pukazhenthi, Wildt, & Comizzoli, 2009; Hussein, Thompson, & Gilchrist, 2006).

Coculture is traditionally performed in bi-dimensional (2D) systems, as microdrops of medium in a Petri dish, but recently a wide range of innovative three-dimensional (3D) systems has been developed for in vitro culture. These systems, named scaffolds, are designed to maintain the physiological spatial organization of cells, by preventing their flattening in culture dishes typical of the traditional 2D cultures. The maintenance of cell architecture and communication through gap-junctions between various compartments ensures the structural and functional activity of cultured cells (Desai et al., 2010). The gene expression profiles and the secretion of specific signalling factors turn out to be similar to those observed under in vivo conditions (Cukierman, Pankov, & Yamada, 2002). To create scaffolds for oocyte in vitro culture, various natural biomaterials such as the physiological components of extra-cellular matrix (ECM; collagen and hyaluronic acid) or inert polymers (alginate), have been used. Promising results in terms of oocyte meiosis resumption and achievement of full competence have been obtained in various species (Dorati et al., 2016; Morselli, Canziani, Vigo, & Luvoni, 2016; Morselli, Vigo, & Luvoni, 2014; Pangas, Saudye, Shea, & Woodruff, 2003; Songsasen, Comizzoli, Nagashima, Fujihara, & Wildt, 2012; Zuccotti et al., 2015). The preliminary application of coculture systems in a 3D alginate microenvironment resulted in the resumption of meiosis in domestic cat oocytes, but the in vitro embryo development was not investigated (Morselli et al., 2014, 2016).

This study was developed to check whether coculture with conspecific CDOs during in vitro maturation in a 3D system might improve the in vitro embryo development of cat COCs.

2 | MATERIALS AND METHODS

The study was approved by the Ethical Committee of the University of Milan (December 9th 2014), and all animals were enrolled following written consent by the owner.

2.1 | Chemicals and reagents

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

2.2 | Animals and collection of feline oocytes

Ovaries from domestic cats (n = 58) were harvested at random stages of the oestrous cycle during routine ovariectomy at veterinary clinics. After surgery, the ovaries were immediately placed in a phosphate-buffered saline (PBS) with a mixture of antibiotics (AB) and antimycotics (100 IU/ml of penicillin G sodium, 0.1 mg/ml of streptomycin sulphate and 0.25 μg/ml of amphotericin B) and transported at room temperature (RT) to the laboratory where they were processed.

Ovaries were sliced in PBS and AB with 0.1% (w/v) polyvinyl alcohol to release cumulus–oocyte complexes (COCs). Only immature COCs (n = 617) with darkly pigmented ooplasm completely surrounded by one or more layers of cumulus cells (grade 1; Wood & Wildt, 1997) were selected for the experiments. A subsets of COCs were used to produce the CDOs by removing the cumulus cells with a small bore pipette.

2.3 | In vitro maturation, in vitro fertilization and in vitro culture in 3D system

In vitro maturation was performed for 24 hr in a controlled atmosphere (38.5°C and 5% CO_2 in air) in Quinn's Advantage Protein Plus Blastocyst (SBP, SAGE® In Vitro Fertilization, Trumbull, Connecticut, USA) medium (b-SBP) supplemented with 10 μg/ml FSH, 10 μg/ml LH (National Institutes of Health, Bethesda, MD, USA), 10 ng/ml of epidermal growth factor (EGF), AB and 0.6 mM cysteine (c-SBP).

In vitro fertilization was performed with chilled feline epididymal spermatozoa obtained from isolated testicles after orchiectomy at veterinary clinics. Briefly, the epididymides were isolated from the testicles and epididymal spermatozoa were released by mincing epididymal cauda with a scalpel blade. The sperm suspension was centrifuged (300 g, 5 min) and diluted 1:2 with Tris-egg yolk buffer for chilling at 4°C degree for 24 hr. Before use, a swim-up treatment was performed and concentration and motility were determined in the sperm suspension (Luvoni & Pellizzari, 2000).

Before the in vitro fertilization, the oocytes were washed twice in b-SBP medium supplemented with 5% of foetal calf serum (FCS) and AB (c-SBP2) and transferred into fertilization drops. Immediately prior to insemination, the sperm suspension was diluted in c-SBP2 to a final concentration of 0.75–1 × 10^6 motile spermatozoa/ml, and 10 μl were added to each fertilization drop containing the oocytes.

At 18–24 hr post-insemination, cumulus cells of COCs and unbound spermatozoa were removed from the oocytes using a small bore glass pipette. After washing, the presumptive zygotes were cultured separately in vitro for 7 days in c-SBP2 in 3D system. Fresh culture medium (c-SBP2) was added every other day and the embryonic development was recorded.

For the 3D system, a two-step encapsulation technique in barium alginate (BA) was used as previously described (Conte et al., 1999; Morselli et al., 2016; Vigo et al., 2004). Briefly, the sodium alginate powder (0.5%) was dissolved into sterile water to obtain a melting solution of medium viscosity (3500 cP, centipoise). A saturated solution of $BaCl_2$ was then added to an aliquot of b-SBP medium to obtain the dropping solution of $BaCl_2$ (40 mM) that was dropped at RT with a 25G needle into the melting solution maintained stirred for 30–40 min. The microcapsules were then collected, washed twice in PBS and suspended in the b-SBP medium for immediate use, or maintained until use at 4°C in a Petri dish with PBS (Figure 1).

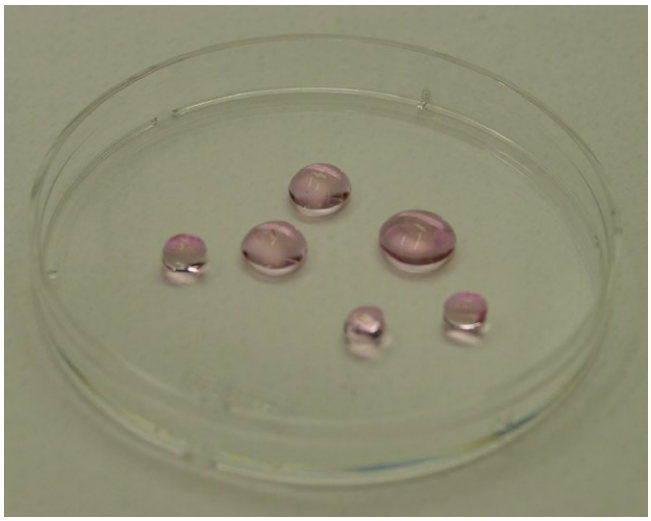


FIGURE 1 Three-dimensional barium alginate microcapsules in a Petri dish

The feline oocytes and embryos were injected into the inner core of the microcapsule by a small bore pipette (Figure 2) and subsequently immersed in the culture medium in a multiwell dish.

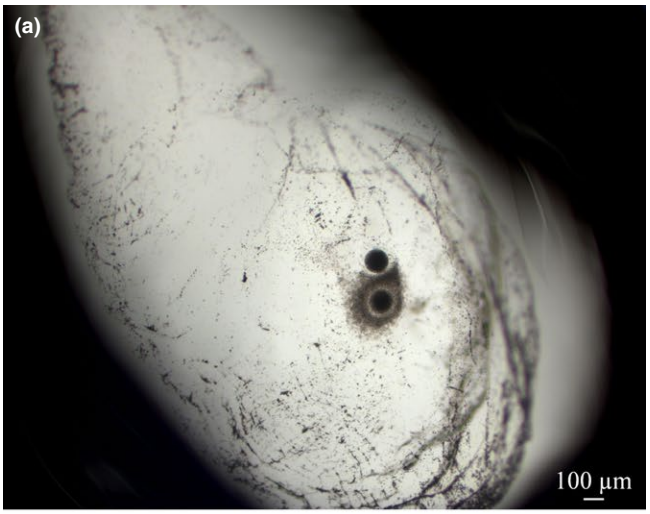
2.4 | Assessment of viability, nuclear maturation and embryonic development

Sequential stainings with fluorescein diacetate/propidium iodide (FDA/PI) for viability assessment and with bis-benzimide (Hoechst 33342) for the evaluation of chromatin configuration were carried out in Experiment I.

Briefly, the oocytes were maintained in the dark in 50 µl of the staining solution (PI: 10 mg/ml; FDA: 5 mg/ml) for 5 min and then evaluated under a fluorescent microscope (Axiovert 100, Zeiss, Arese, Italy). This differential staining allowed the evaluation of viable (bright green fluorescence) or dead cells (red fluorescence). After washing, the COCs (after removal of cumulus cells) and the CDOs were placed on a slide with a minimum amount of medium and then covered by 10 µl of Hoechst solution. After 5 min of incubation in the dark, the Hoechst solution was removed and the oocytes were covered with an antifade reagent (Fluoromount™ Acqueous Mounting Medium). The fixed oocytes were then observed under a fluorescent microscope at 400 × magnification for evaluation of nuclear stages.

The chromatin configurations of the oocytes were classified as follows (Bolamba, Borden-Russ, & Durrant, 1998; Hewitt & England, 1999):

- germinal vesicle (GV): identification of nucleolus and very fine filaments of chromatin;
- germinal vesicle break down-Anaphase I (GVBD–AI): identification of different patterns of chromatin condensation (GVBD) or identification of bivalents (AI);
- telophase I-Metaphase II (TI–MII): identification of two groups of chromosomes moving to opposite ends of the meiotic spindle (TI) or two sets of chromosomes clearly visible (MII);
- degenerated: collapsed nucleus or irregular nuclear conformation.



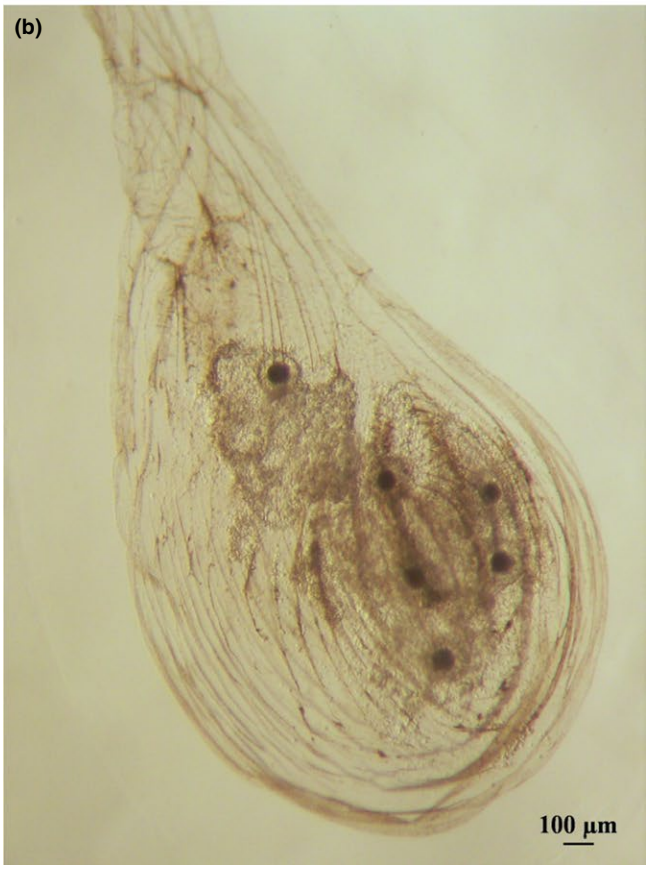


FIGURE 2 (a) Domestic cat cumulus–oocyte complexes (COCs) and conspecific cumulus-denuded oocytes (CDOs) in a 3D barium alginate microcapsule. (b) Domestic cat cumulus–oocyte complexes (COCs) cultured separately in a 3D barium alginate microcapsule

In Experiment II for assessment of embryo development, cleaved embryos, 8-16 cell, morula and blastocyst stages were recorded along 7 days of culture. Then, the embryos were fixed and stained with bis-benzimide (Hoechst 33342) and evaluated under a fluorescent microscope at 400 × magnification.

2.5 | Experimental design

2.5.1 | Experiment I

Fresh feline COCs were matured in vitro in coculture with conspecific CDOs (ratio 1:1) using a commercial medium in a 3D system (barium alginate microcapsules). Control groups of COCs and CDOs were cultured separately under the same 3D conditions. After 24 hr, viability and maturation rates of the various treatment groups were determined (12 replicates).

2.5.2 | Experiment II

Fresh feline COCs were matured in vitro in coculture with conspecific CDOs (ratio 1:1) using a commercial medium in a 3D system, as in Experiment I. After in vitro fertilization with chilled epididymal feline spermatozoa, presumptive zygotes were cultured separately in 3D microcapsules. A control group of COCs was matured in vitro, fertilized and cultured under the same conditions but separately. Embryonic development was recorded over 7 days of in vitro culture (8 replicates).

2.6 | Statistical analysis

Data for viability, maturation and embryonic development rates were analysed by the Chi-square test, and the level of significance was set at $p \leq .05$.

3 | RESULTS

3.1 | Experiment I

In all treatment groups, the 3D barium alginate microcapsules were able to sustain the viability of feline oocytes (range: 93%–97.1%).

The meiotic resumption rate of COCs cocultured with conspecific CDOs and of COCs cultured separately was significantly higher ($p < .01$) than that of CDOs in coculture or CDOs cultured separately (Table 1). Interestingly, the percentage of TI-MII stage obtained with COCs cocultured with CDOs was lower ($p < .01$) than that of COCs cultured separately but was similar ($p > .05$) to that of CDOs cultured separately (Table 1). The proportions of degenerated COCs were similar to those of CDOs ($p > .05$) when in coculture but were lower when cultured separately ($p < .05$; Table 1).

3.2 | Experiment II

The COCs comatured with conspecific CDOs led to higher percentages ($p < .05$) of cleavage, 8–16 cells, morulae and blastocysts, than COCs cultured separately and CDOs (Table 2). Few CDOs were able to reach the 8–16 cell stage and advanced embryo stages (morulae and blastocysts) but in similar proportions ($p > .05$) as COCs cultured separately. The advanced developmental competence (morulae and blastocysts/total number of cleaved embryos) of COCs comatured with CDOs was higher ($p < .05$) than that of COCs cultured separately (Table 2). The few CDOs that developed into an embryo had a competence similar to

TABLE 1 Meiotic progression of feline cumulus–oocyte complexes (COCs) and cumulus-denuded oocytes (CDOs) after in vitro maturation in 3D system (12 replicates)

Groups	Total No. of oocytes	Meiotic resumption (GVBD-MII) No. of oocytes (%)	GVBD-AI No. of oocytes (%)	TI-MII No. of oocytes (%)	Degenerated No. of oocytes (%)
COCs in coculture	72	62 (86.1)[a]	*35 (48.6)[a]*	*27 (37.5)[a]*	4 (5.5)[a,b]
CDOs in coculture	72	29 (40.2)[b]	*20 (27.7)[b]*	*9 (12.5)[b]*	6 (8.3)[b]
COCs cultured separately	70	62 (88.5)[a]	*10 (14.2)[c]*	*52 (74.2)[c]*	1 (1.4)[a]
CDOs cultured separately	72	29 (40.2)[b]	*10 (13.8)[c]*	*19 (26.3)[a]*	7 (9.7)[b]

GVBD, germinal vesicle break down; AI, anaphase I; TI, telophase I; MII, metaphase II.
[a,b,c]Different superscripts indicate significant differences within columns ($p \leq .05$).
3D system: barium alginate microcapsules.

TABLE 2 Developmental competence of cumulus–oocyte complexes (COCs) and cumulus-denuded oocytes (CDOs) cultured in 3D system and fertilized in vitro (8 replicates)

Groups	Total No. of oocytes	Cleavage No. of oocytes (%)	8–16 cells No. of oocytes (%)	Morulae + blastocysts No. of oocytes (%)	Morulae + blastocysts/cleaved No. of oocytes (%)
COCs in coculture	114	58 (50.9)[a]	47 (41.2)[a]	45 (39.5)[a]	*45 (77.6)[a]*
CDOs in coculture	115	17 (14.8)[b]	12 (10.4)[b]	10 (8.7)[b]	*10 (58.8)[a,b]*
COCs cultured separately	102	26 (25.5)[c]	18 (17.6)[b]	14 (13.7)[b]	*14 (53.8)[b]*

[a,b,c]Different superscripts indicate significant differences within columns ($p \leq .05$).
3D system: barium alginate microcapsules.

TABLE 3 Uncleaved and degenerated feline cumulus–oocyte complexes (COCs) and cumulus-denuded oocytes (CDOs) cultured in 3D system (8 replicates)

Groups	Total No. of oocytes	GV/uncleaved No. oocytes (%)	GVBD-AI/uncleaved No. oocytes (%)	TI-MII/uncleaved No. oocytes (%)	Degenerated No. oocytes (%)
COCs in coculture	35	*3 (8.6)*[a]	*14 (40)*[a]	*18 (51.4)*[a]	21 (18.4)[a]
CDOs in coculture	72	*57 (79.2)*[b]	*6 (8.3)*[b]	*9 (12.5)*[b]	26 (22.6)[a,b]
COCs cultured separately	45	*31 (68.9)*[b]	*5 (11.1)*[b]	*9 (20)*[b]	31 (30.4)[b]

GV, germinal vesicle; GVBD, germinal vesicle break down; AI, anaphase I; TI, telophase I; MII, metaphase II.
[a,b,c]Different superscripts indicate significant differences within columns ($p \leq .05$).
3D system: barium alginate microcapsules.

that of the COCs in coculture or cultured separately ($p > .05$; Table 2). Uncleaved oocytes either remained at the GV stage, were arrested during the meiotic progression, failed to be fertilized at the MII stage, or were degenerated (Table 3). The uncleaved COCs in coculture with CDOs resumed meiosis and achieved the full maturational stages (TI-MII) at higher rates than the uncleaved CDOs and COCs cultured separately (51.4% vs. 12.5% and 20%, respectively). In addition, they showed the highest proportions of degeneration and meiotic arrest ($p < .05$).

4 | DISCUSSION

Our data demonstrate that the presence of conspecific CDOs improved the performance of domestic cat COCs cocultured in a 3D microenvironment, promoting the achievement of nuclear and cytoplasmic competence. The positive influence of CDOs during the 24 hr of in vitro maturation resulted in a rate of embryo development higher than that of COCs cultured separately. Specifically, the rate of advanced embryo stages (morulae and blastocysts on the total number of cleaved embryos) obtained in the group of COCs cocultured with CDOs was approximately 78% compared to 54% or 59% in the other groups. The presence of companion cells also decreased the proportions of degenerated and uncleaved COCs.

The benefit provided by coculture with CDOs depends on the inner capacity of oocytes to regulate the functions of somatic cells (granulosa and cumulus oophorus), as already demonstrated in vitro by several authors (Buccione, Vanderhyden, Caron, & Eppig,1990; Vanderhyden, Caron, Buccione, & Eppig, 1990). Paracrine factors secreted by the oocyte themselves, the OSFs, influence positively and regulate the biological functions of the oocyte, such as metabolism, growth and full maturation (Gilchrist et al., 2008; Hussein et al., 2006). Some of them, such as the growth differentiation factor-9 (GDF-9) and the bone morphogenetic factor-15 (BMP-15), also known as cumulus expansion-enabling factors (CEEFs, Dragovic et al., 2007), regulate the differentiation, the activities and the genetic expression of the cumulus cells in various species (Gilchrist et al., 2008; Hussein et al., 2006; Yeo, Gilchrist, Thompson, & Lane, 2008). The cumulus cells are involved in many cellular processes and provide a highly specialized microenvironment for cytoplasmic and nuclear maturation, and further embryo development (Sutton-McDowall, Gilchrist, & Thompson, 2010). In the present study, the improvement of COCs outcomes after maturation with CDOs was presumably related to the OSFs which, by upregulating the COCs cumulus cells molecular functions, gave the oocyte full capability to develop into an embryo.

Our data also show that the 3D barium alginate microcapsules can sustain the viability and the meiotic resumption of domestic cat oocytes, which are prerequisites for further embryo development. To the authors' knowledge, this is the first time that embryo development in a 3D environment has been obtained in this species. Several authors reported that post-hatched and the pre-implantation bovine and porcine embryos cultured in 3D alginate hydrogels or microcapsules developed at higher rates than those cultured in traditional 2D systems (Sargus-Patino , Wright, Plautz, Miles, Vallet, & Pannier, 2014; Yaniz, Santolaria, & Lopez-Gatius, 2002; Zhao et al., 2015). The 3D system mimics more closely the conformation of the biological microenvironment in which the oocytes and the embryos develop in vivo (Kreeger, Deck, Woodruff, & Shea, 2006; Munari et al., 2007; Torre et al., 2006; Zhao et al., 2015). Presumably, coculture associated with the 3D system, that maintains the physiological communications between somatic cells and oocytes, may be of great benefit to enhance the performance of the oocytes in vitro.

In conclusion, the association of companion cells, as CDOs, during in vitro maturation was beneficial to the full competence of the COCs. The presence of CDOs secreting OSFs enhanced the achievement of full maturational and developmental competence of the COCs. The maintenance of oocyte physiological structure in microcapsules deserves further investigations to understand how a 3D microenvironment influences the features of oocytes and embryos. The evaluation of OSFs and the molecular expression of some reference genes, such as the GDF-9 and the BMP-15, in 3D culture systems should be the next step in expanding the knowledge to define enriched culture conditions.

AUTHOR CONTRIBUTIONS

MGM, GCL and PC contributed to the design of the study, to the analysis of the data and to the drafting of the paper. MGM performed the experiments. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

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