J. Mamm. Ova Res. Vol. 13, 1996

Time Sequence of Meiotic Maturation in Cumulus-Enclosed and Denuded Porcine Oocytes Stimulated by Gonadotrophins, Estradiol-17β, Fetal Bovine Serum and Follicular Fluid

—Synergistic Effect of Follicular Fluid with Estradiol-17β or Serum on the Expulsion of First Polar Body—

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Abstract: *This study was conducted to evaluate the effects of gonadotrophins (PMSG, hCG), fetal bovine serum (FBS), estradiol-17β (E2) and porcine follicular fluid (pFF) on the timing of nuclear maturation and the expulsion of first polar body in cumulus-enclosed (CC+) or denuded (CC−) porcine oocytes. CC+ and CC− oocytes were cultured in mTALP-PVA medium supplemented with the above stimulants alone or in combination for 24, 32 and 46 h. PMSG at all doses significantly ($p<0.05$) promoted the meiotic resumption (GVBD) in CC+ oocytes, compared to those in control. When CC+ were stimulated by hCG (15 IU/ml), FBS (15%) and E2 (10 μg) for 32 h, the percentage of GVBD was higher than that of control ($p<0.05$) and for 24 h culture oocytes ($p<0.05$). After 46 h of culture, PMSG, hCG and E2 significantly ($p<0.05$) promoted the expulsion of first polar body (Pb1) in CC+ than CC− oocytes. At the same time, pFF in the presence of E2 or FBS significantly ($p<0.05$) increased the expulsion of Pb1, compared to the stimulation by respective single stimulant. These results suggest that PMSG, hCG and E2 acted through the cumulus cells and stimulated the meiotic maturation; but FBS and pFF directly stimulate, and that pFF in combination with FBS or E2 showed a important role in promoting the expulsion of Pb1.*

Key words: *Porcine oocytes, Meiotic maturation, Hormones, Fetal bovine serum, Follicular fluid.*

Received: July 5, 1995
Accepted: November 28, 1995
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The removal of oocytes from ovarian follicles releases them from inhibitory constraints that originate in the somatic compartment of the follicle and they leads to spontaneous germinal vesicle breakdown in culture. It is also well established that cumulus cells mediate the stimulatory action of a number of ligands in promoting germinal vesicle breakdown in cultured oocytes [6, 10]. The time required to reach complete nuclear maturation *in vitro* varies among immature oocytes [36, 40] and the frequency of chromosomal abnormalities has been found to be high [20]. Oocytes are much more successfully matured *in vivo* than *in vitro* [19], which might suggest that hormonal and follicular factors are required to improve maturation to obtain normal fertilizability and development rate. In the sheep, the steroids and gonadotropins affect maturation, and steroid synthesis inhibitors induce abnormal fertilization following *in vitro* maturation [25]. Maturation conditions, especially those that included the supplementation of maturation medium with gonadotropins and steroids have been observed to have profound effects on the percentage of cumulus-oocyte complexes that complete meiotic maturation *in vitro*.

Gonadotropins as the important factor in the acquisition of competence by the maturing oocytes regulate many events within the follicle cells, including steroid synthesis [7], protein synthesis [18], and the secretion of glycosaminoglycans and hyaluronic acid [8, 12]. The involvement of steroids in reprogramming of mammalian oocyte maturation is controversial [37]. Steroidal

hormones are transported through both cell membrane and cell cytoplasm by diffusion. Binding of steroidal hormone to its receptor starts the synthesis of specific mRNA, which is translocated to the cytoplasm, where it directs synthesis of specific proteins. Most investigators agree that the resumption of meiosis in mammals is not steroid-dependent. On the other hand, some investigators report that distorted steroid patterns during maturation induce chromosomal abnormalities in the later stages of meiosis. However, it is possible that a difference in physiological/hormone environment is responsible for the different results from the *in-vivo* matured oocytes because the pig oocytes used for the *in vitro* maturation are generally collected from hormone-unprimed animals. In addition, removal of the cumulus cells results in dramatic changes in the response of oocytes to culture conditions.

A shortening of the time sequence of germinal vesicle breakdown has already been demonstrated in ovine and porcine oocytes after inhibition of protein synthesis with cycloheximide [27, 17]. It is therefore likely that modulation of both the type of stimulants and its concentration within culture medium plays a vital role in the meiotic response of the oocyte *in vitro*. In the present study, the interaction or synergistic action of cumulus cells with hormone levels involved in the maturation as well as serum and follicular fluid was evaluated.

Materials and Methods

Oocytes Collection and Culture Medium: Pig ovaries were collected from a local slaughterhouse and transported to the laboratory in 0.9% (w/v) NaCl containing antibiotics at 35°C. Only those oocytes completely surrounded by compact cumulus were collected by aspiration of the follicles (2–5 mm in diameter) using a 21 gauge needle and a 5-ml disposable syringe and washed 3 times with maturation medium. The maturation medium consisted of mTALP supplemented with 0.1g polyvinylalcohol/100 ml, 1.0 mM glutamine, 0.2 mM isoleucine, 0.05 mM methionine, 0.1 mM phenylalanine, 0.57 mM cysteine, and 100 μg kanamycin sulfate/ml. The pH of medium was adjusted to pH 7.3. Oocytes were then transferred into the droplets containing 150 μl of mTALP medium supplemented with PMSG (Teikoku Zoki Co., Japan), hCG (Teikoku Zoki Co., Japan), E2 (Sigma), FBS (JRH Biosciences), and pFF, respectively. The culture of oocytes was performed at 38°C in an atmosphere of 5% CO_2 in air for 24, 32, or 46 h. For the preparation of CC− oocytes, cumulus cells were removed from the oocytes by repeating aspiration through a 150- to 200-μm bore pipette. 1) *In vitro* maturation treatments of 5 stimulants were compared to investigate dose responsiveness in mTALP medium. The CC+ oocytes were collected at 24 and 32 h of maturation for the evaluation of meiotic maturation. These times were chosen because germinal vesicle breakdown and resumption of meiosis take place between 24 and 32 hours of maturation. Maximum changes in metabolic coupling also occur during this interval. 2) Treatments with 5 stimulants for *in vitro* maturation were assessed to determine a possible role for these stimulants in enhancing functional performance of oocytes with or without cumulus cells. Cumulus-enclosed and denuded oocytes were incubated for 24, 32 and 46 h. 3) Treatments of oocytes for *in vitro* maturation with each stimulant alone or in combination with pFF were evaluated to assess possible synergistic effects on the expulsion of Pb1 in cumulus-enclosed oocytes.

Evaluation for Nuclear Maturation: The oocytes, mounted on slides and covered by coverslips supported by paraffin-wax pillars, were fixed in acetic-alcohol (1:3) for 24 to 48 hours and then stained with 1% aceto-orcein for microscopic examination (a phase-contrast). The nuclear stage was classified as germinal vesicle, germinal vesicle breakdown (defined by the absence of visible nuclear membrane and nuclear chromatin condensation), Metaphase I, Anaphase I, Telophase I and Metaphase II.

Statistical Analysis: Averages of 4-5 replicate trials for each experiment were examined for statistical differences. Data for the number of oocytes at the germinal vesicle breakdown (culture for 24 or 32 h) and of oocytes with Pb1 (culture for 46 h) were statistically examined using Student's t-test with $p<0.05$ considered significant.

Results

The cumulus-enclosed oocytes stimulated by PMSG at all doses for 24 or 32 h significantly showed the meiotic resumption (GVBD), compared to those in control ($p<0.05$) and did not differ between 24 h and 32 h culture period (Fig. 1). When CC+ oocytes were stimulated by hCG (15 IU/ml) for 32 h, the percentage of GVBD was higher than those of control (72.5 vs 30%, $p<0.05$) and 24 h culture (72.5 vs 48.9%, $p<0.05$; Fig. 1). When they were cultured for 32 h in FBS (15%) supplemented medium, the proportion of oocytes showing the GVBD was greater compared with those of control (59.1 vs 30%, $p<0.05$) and 24 culture period at the same dose (59.1 vs 21.7%, $p<0.05$; Fig. 2). In case of 24 h

culture in 15% pFF supplemented medium, the percentage of GVBD was higher than that of control (39.6 vs 21.4, $p<0.05$) but did not differ from those of 32 h culture period at the same dose (Fig. 2). E2 (10 μg/ml) significantly (55.0 vs 30%, $p<0.05$) promoted the meiotic resumption compared to those of control and at the same dose, the proportion of oocytes cultured for 32 h was higher than for 24 h (55.0 vs 28.3%, $p<0.05$; Fig. 3). After 24 h of culture, PMSG or hCG significantly ($p<0.05$) promoted the meiotic maturation of cumulus-enclosed oocytes than denuded oocytes. However, FBS or pFF stimulately allowed the denuded oocytes than cumulus-enclosed oocytes to initiate the meiotic maturation ($p<0.05$). Also, PMSG or hCG greatly ($p<0.05$) stimulated the meiotic resumption of cumulus-enclosed than denuded oocytes during 32 h of culture period. After 46 h of culture, the expulsion of first polar body highly increased in cumulus-enclosed oocytes than in denuded oocytes stimulated by respective PMSG, hCG or E2 supplemented medium ($p<0.05$; Fig. 4). Follicular fluid in combination with E2 or FBS significantly ($p<0.05$) increased the expulsion of Pb1 compared to the stimulation by E2 or FBS alone in cumulus-enclosed oocytes cultured for 46 h (Fig. 5).

Discussion

The data in the present study demonstrate that the presence of PMSG alone in maturation medium during the first 24 h period was sufficient for the accomplishment of germinal vesicle breakdown (Fig. 1). Successful oocyte maturation *in vitro* has been achieved in several

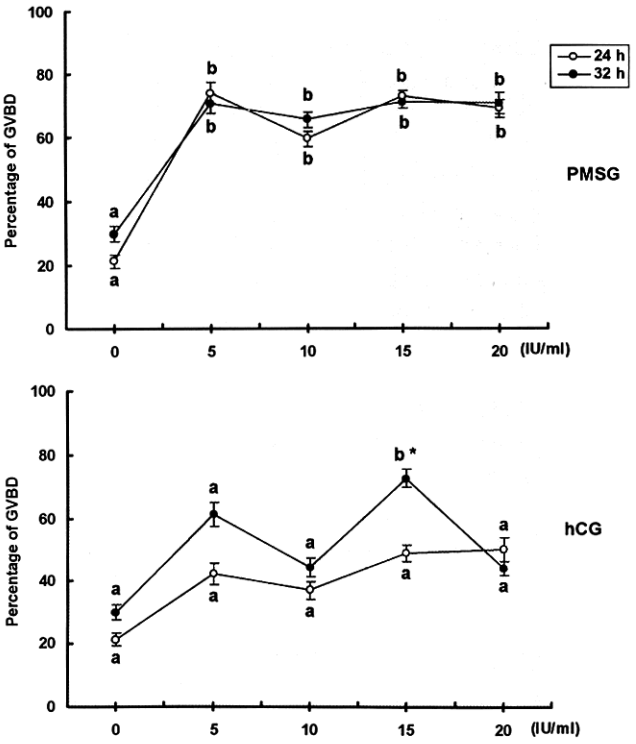

Fig. 1. Effects of PMSG (pregnant mares' serum gonadotrophin) and hCG (human chorionic gonadotrophin) on the meiotic resumption of porcine oocytes. Cumulus-enclosed oocytes were cultured for 24 h or 32 h in the presence of various concentrations of PMSG or hCG and scored for meitoic resumption (% GVBD). GVBD: germinal vesicle breakdown. Each value represents the mean ± S.D. of 5 replicates (10–15 oocytes/replicate). [a,b]Different letters above bars denote a significant difference from respective control ($p<0.05$). *Compared with 24 hours culture group within each dose ($p<0.05$).

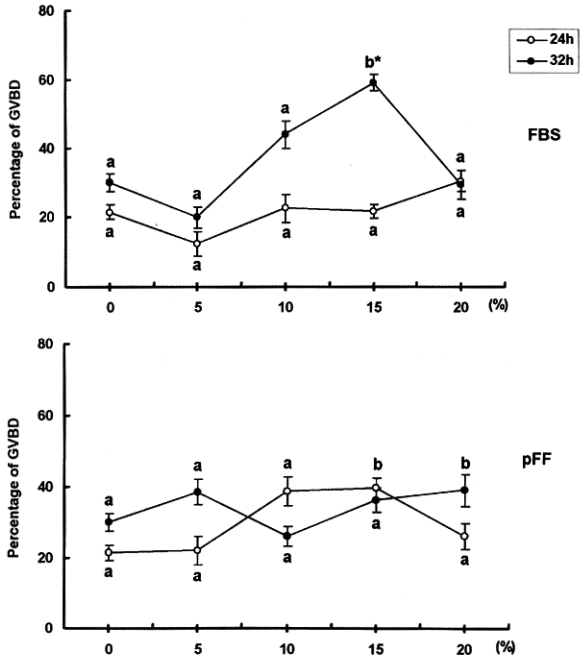

Fig. 2. Effects of FBS (fetal bovine serum) and pFF (pig follicular fluid) on the meiotic resumption of porcine oocytes. Cumulus-enclosed oocytes were cultured for 24 or 32 hours in the presence of various concentrations of FBS or pFF and scored for meitoic resumption (% GVBD). GVBD: germinal vesicle breakdown. Each value represents the mean ± S.D. of 5 replicates (10–15 oocytes/replicate). [a,b]Different letters above bars denote a significant difference from respective control ($p<0.05$). *Compared with 24 hours culture group within each dose ($p<0.05$).

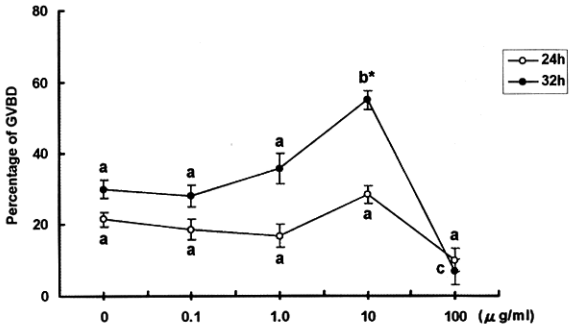

Fig. 3. Effect of estradiol-17β (E2) on the meiotic resumption of porcine oocytes. Cumulus-enclosed oocytes were cultured for 24 or 32 hours in the presence of various concentrations of E2 and scored for GVBD (germinal vesicle breakdown). Each value represents the mean ± S.D. of 5 replicates (10–15 oocytes/replicate). ^{a,b,c}Different letters above bars denote a significant difference from respective control ($p<0.05$). *Compared with 24 hours culture group within each dose ($p<0.05$).

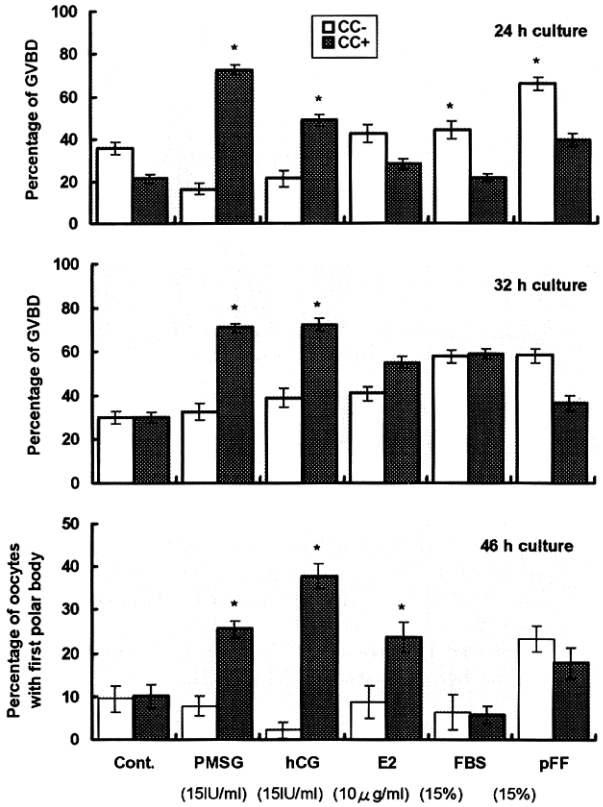

Fig. 4. Effect of cumulus cells stimulated by PMSG, hCG, E2, FBS and pFF on the meiotic resumption and expulsion of first polar body (Pb 1) in porcine oocytes. Cumulus-enclosed (CC+) or -denuded (CC−) oocytes were cultured for 24, 32 and 46 hours in medium containing various stimulants and assessed for GVBD at 24 h and 32 h or Pb 1 expulsion at 46 h of *in vitro* culture. Each value represents the mean ± S.D. of 4 or 5 replicates (10–15 oocytes/replicate). *Compared with that of CC− group within each stimulant ($p<0.05$).

species, but only some of the techniques utilized by the different authors involve the use of gonadotropins. Cow [11] and rabbit [41] oocytes matured in the absence of gonadotropins have, in fact, shown satisfactory developmental competence, although other researches have demonstrated the beneficial effect of hormone supplementation on cow [35], sheep [23] and cat [13] oocytes. Our observation indicates that pig oocytes matured with PMSG, and therefore with an increased intercellular interaction with the cumulus cells, resume meiosis earlier than control oocytes. As far as cumulus oocyte interaction is concerned, it is known that somatic cells are required for oocyte growth and maturation and that this influence is mainly exerted via the gap junctions between cumulus cells and the oocyte. *In vivo*, this intercellular interaction undergoes a progressive reduction starting from the gonadotropin surge to immediately before ovulation. Thus the gonadotropins might play a role in initiating the uncoupling process. Because of the junctional selectivity mentioned earlier, we cannot exclude that a specific inhibitory molecule, produced by somatic cells, becomes excluded by the gap junction pores at certain times, leaving the oocyte free to resume meiosis. In the presence of gonadotropins not only was meiotic resumption anticipated but the percentage of CC+ oocytes extruding the Pb1 was also favorably influenced, compared to the CC− oocytes. It is not clear whether the high proportion of CC− oocytes with hormones or control oocytes that did not extrude the Pb1 after 46 h of culture was simply delayed or meiotically blocked. Judging from our results, PMSG seems to be the gonadotropin which gives better results in terms of meiotic resumption. In our *in vitro* system, hCG could not substitute for PMSG, and its addition to PMSG did not further improve the rate of GVBD. LH or hCG does not reduce intercellular coupling between cumulus cells and oocytes [24], LH increases the uptake of [^{3}H] uridine in cumulus-enclosed oocytes after 26 h of culture [22]. In contrast, FSH promotes the uncoupling of the cumulus cells from the oocytes and stimulates the cumulus expansion significantly [8, 24]. The disruption of cell-cell communication in the cumu-

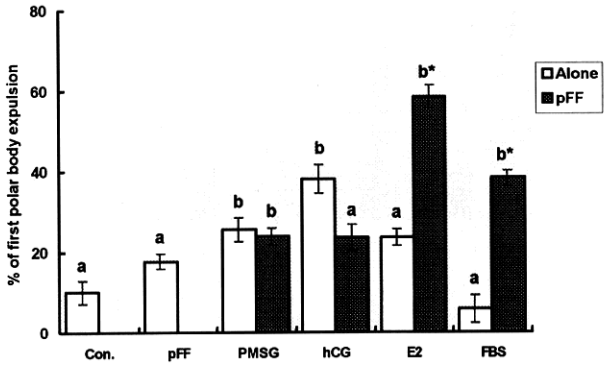

Fig. 5. The synergistic effect of pFF with E2 or FBS on the expulsion of first polar body of porcine oocytes. Cumulus-enclosed oocytes were cultured for 46 hours in medium containing stimulant alone or combination with pFF, and assessed for an expulsion of first polar body. Each value represents the mean ± S.D. of 4 or 5 replicates (10–15 oocytes/replicate). [a,b]Different letters above bars denote a significant difference from control value ($p<0.05$). *Compared with that of single supplemental group in respective stimulant ($p<0.05$).

lus-oocyte complex has been shown in several species to occur after nuclear maturation has started [9, 26, 28]. Delay of meiotic maturation has been reported in *in vitro*-matured porcine oocytes in comparison with *in vivo*-matured oocytes [20]. Cytoplasmic differentiation during *in vitro* maturation of porcine oocytes depends on the maintenance of functional intercellular coupling between follicle cells and the oocytes for at least the first 32 h of maturation [21]. The resumption of meiosis of porcine oocytes and cumulus expansion depend on a transcriptional event as results obtained in other domestic animals. Mammalian immature oocytes are surrounded by several layers of cumulus granulosa cells, which mediate hormonally induced changes within the cumulus-oocyte complexes during oocyte maturation [1, 2].

The observation of polar body emission is a more stringent criterion of normal physiological oocyte maturation than germinal vesicle breakdown. In the present study, the percentage of hCG-treated CC+ oocytes capable of extruding a polar body was nearly 4-folds that observed control oocytes (Fig. 4). However, hCG did not show any effect when added to the culture medium in combination with pFF. Moreover, the ability of pFF in combination with E2 or FBS to extrude the first polar body of CC+ oocytes was observed (Fig. 5). Follicular fluid in antrum contains steroids, glycosaminoglycans, and many metabolites; K+ and Na+ in similar concentrations as in serum. And also follicular fluid contains, only in large follicles, high percentage of estradiol-17β in follicular phase and progesterone at ovulation. From the results of present study, pFF may be related with the stimulation of cumulus-oocyte interaction, resulting in the high proportion of Pb1 expulsion and therefore considered as an indicator of normal nuclear maturation, but further studies are required to determine the part played by the oocyte. Most of the conclusions are drawn from steroid inhibitor experiments and addition of steroids to the culture medium, although the more significance is the importance of steroids, especially estradiol-17β, in the acquisition of competence by the maturing oocyte [23, 25, 39]. The present study using estradiol-17β indicates that it had effect on the Pb1 expulsion through the cumulus cells and furthermore the synergistic action with pFF, but its no effect on the expansion of cumulus cells was observed.

Oocytes denuded of surrounding cumulus cells do not respond to LH, thereby implicating that the cumulus cells mediate the LH effect [1, 42]. *In vivo* and *in vitro*, oocytes in healthy, nonatretic follicles do not resume meiosis until gonadotropin is present. Expansion of the mouse cumulus oophorus *in vitro* was found to be dependent upon regulation of hyaluronic acid synthesis by oocyte [3, 34]. It is also conceivable that the cumulus expansion-enabling factor secreted by pig oocytes is not necessary for the expansion of pig complexes, but plays some other role in the development or function of cumulus cells. In physiological conditions, the cumulus oophorus does not expand before the oocyte resumes meiosis even though gonadotropins and other growth factors may be in follicles at higher concentrations. Recently, several investigators demonstrated in the mouse that mucification, i.e., expansion of cumulus-enclosed oocytes, requires both the endocrine stimulation of gonadotropins and the paracrine action of a soluble factors produced by the oocyte and referred to as cumulus expansion-enabling factor [2, 3, 34, 38]. Most likely, cumulus cells mediate the maturation, fertilization, and development capacity of oocytes. It is known that the somatic compartment is also required for the transmission of certain amino acid, nucleosides, and phospholipid precursors to the oocyte. Apart from their nutrient role, the follicle cells also generate instructional signals which influence the nucleus for direct synthesis of certain structural proteins [5].

It is generally believed that follicular cells surrounding oocyte exert an inhibitory effect on the nuclear maturation of oocyte. However, the recent studies of pig oocyte maturation by either culturing oocytes in medium supplemented with porcine follicular fluid [14, 31]

or co-culturing with follicular cells [15, 21] indicate that the somatic follicular cells play an important role in the cytoplasmic maturation of oocytes. The study of metabolic coupling by Motlik *et al.* [29] indicated that follicular tissue actively controls the degree of intercellular cooperation during the development of oocytes. Maturation media are usually supplemented with proteins, such as fetal calf serum [4, 30], newborn piglet serum [32] and bovine serum albumin [31]. It has been reported that fetal calf serum exerted to inhibit maturation of pig oocytes [33]. However, the number of piglets obtained from *in vitro* fertilization was extremely low. The ability of a mature oocyte or cumulus-oocyte complex to fertilize and develop normally into live offspring requires appropriate dynamics within its microenvironment during the preovulatory, maturational interval. It is considered that irregulatory maturation rate in the porcine oocyts matured *in vitro* may be due to the differences of culture medium and hormones as well as chemical components in medium [16].

In conclusion, the data presented here indicate that only those oocytes surrounded by cumulus cells have sensitivity to hormones to resume meiosis spontaneously and particularly, the addition of PMSG accelerates meiotic progression. Porcine follicular fluid has a synergistic effect with E2 and FBS to allow cumulus-enclosed oocytes to extrude the first polar body.

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