Immunohistochemical Demonstration of Prostaglandin E₂ in Cultured Bovine Embryos

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Abstract. Antiserum to prostaglandin (PG) E₂ and indirect immunofluorescence were used immunohistochemically to demonstrate the presence of PGE₂ in unfertilized and fertilized bovine eggs matured *in vitro*, and embryos from the 2-cell to hatched blastocyst stage developed *in vitro*. Fluorescence showing the presence of PGE₂ was found in the cytoplasm of all the eggs and embryos, though its intensity was weak in unfertilized eggs to morulae, and was strong in blastocysts. No difference in the intensity was observed between unfertilized and fertilized eggs, among embryos at the cleaving stages, and among those at the early, expanded and hatched blastocyst stages.

Key words: Bovine embryo, Prostaglandin E₂, Immunohistochemistry.

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Detection of prostaglandins (PGs) in mammalian embryos was first performed by Dickmann and Spilman in 1975 [1]. They detected, by radioimmunoassay (RIA), PGE-A and PGF in rabbit blastocysts 144 h post coitum (pc), and suggested that the embryos might have the ability to synthesize PGs. PGs have since been detected by RIA in the blastocysts from rabbits [2-4], sows [5], ewes [6, 7], and cows [8]. As for synthesis and release of PGs, there have been positive reports on cultured blastocysts from the animals mentioned above [2, 4, 6, 7, 9-22], and on such from horses [23-25], cynomolgus monkeys [26] and humans [27]. Since both activities have recently been proved to exist in 4-cell human embryos [27] and bovine morulae [22], it is now assumed that PGs must be present in other mammalian embryos at the cleavage stages, aside from the blastocyst stage.

Immunohistochemical detection of PGs has been carried out in mouse, hamster, rat and rabbit

embryos at the stages from unfertilized 1-cell to blastocyst [28, 29], but not yet in bovine embryos. This immunohistochemical investigation, therefore, was designed to examine *in vitro* the presence of PGE₂ in mature unfertilized and fertilized bovine eggs, and embryos from the 2-cell to hatched blastocyst stage.

Materials and Methods

Oocyte collection and maturation in vitro

Bovine ovaries (Japanese Black) were obtained at a slaughterhouse. Immature oocytes covered with cumulus cells were collected by sucking out of visible follicles measuring 2-5 mm in diameter. The cumulus-oocyte complexes were cultured for maturation at 39 C for 22 h in TCM-199 (Gibco Lab., Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (FBS, Nissui Co., Tokyo, Japan) and covered with mineral oil (Squibb and Sons, Inc., Princeton, NJ, U.S.A.) in a

CO₂ incubator (5% CO₂, 95% air).

Sperm capacitation and in vitro fertilization

After having thawed frozen semen obtained from males of the same species, the semen was washed three times in Brackett and Oliphant (BO) medium [30] containing 10 mM caffeine (Sigma, St. Louis, MO, U.S.A.) and 10 µg/ml heparin (Sigma). The sperm suspension was diluted with BO medium containing 10 mM caffeine and 1% bovine serum albumin (BSA, Sigma), so that the final concentration of live spermatozoa was adjusted to 5×10^6 /ml. Cumulus-oocyte complexes after maturation culture were introduced into droplets of the sperm suspension and cultured at 39 C for 6 h under mineral oil in a CO₂ incubator.

In vitro development

After insemination, cumulus-oocyte complexes were washed three times in TCM-199 containing 5% FBS (culture medium) and cultured at 39 C for 48 h in the culture medium, during which the cumulus cells surrounding embryos were removed. The denuded embryos were co-cultured with a monolayer of cumulus cells laid below, at 39 C for 8 days in fresh culture medium with mineral oil on top in a CO₂ incubator. The culture medium was replaced with fresh medium every 48 h.

Indirect immunofluorescence assay

The eggs observed were at the stages of unfertilized and sperm-penetrated (6 h after insemination), and the embryos at the stages of 2-cell (2 days), 4-cell (2–3 days), 8- to 16-cell (3–4 days), morula (5–6 days), early blastocyst (7–8 days), expanded blastocyst (8–9 days) and hatched blastocyst (10 days). These eggs and embryos were washed twice with phosphate buffered saline (PBS, pH 7.4) [31], and the zonae pellucidae were removed in PBS that contained 0.2% pronase (Sigma). Cumulus cells surrounding 1-cell eggs were dissolved beforehand in PBS containing 0.1% hyaluronidase (Sigma). The naked eggs and embryos were washed in PBS and then fixed in PBS containing 10% formaldehyde for 60 min at room temperature. After being rinsed 3 times in PBS for 60 min, they were immersed in PBS containing 0.1% Triton X-100 (Nacalai Tesque, Kyoto, Japan) for 5 min at 20 C and then were placed in PBS. The eggs and embryos were further incubated in the rabbit anti-PGE₂ serum

(Polysciences, Warrington, PA, U.S.A.) for 60 min at room temperature. The anti-PGE₂ serum had been diluted with PBS to 100 times. All these procedures were carried out in a moist chamber. The antiserum to PGE₂ reacts with PGE₂ wholly (100%), 15-keto-PGE₂ (13.2%), PGE₁ (6.5%), 13,14-dihydro-PGE₂ (2.1%), 13,14-dihydro-15-keto-PGE₂ (0.6%) and with the A, B, D or F series of PGs (less than 0.3%). The eggs and embryos were washed again in PBS for 60 min and then incubated in the goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC, Cappel, West Chester, PA, U.S.A.) for 60 min at room temperature. The goat anti-rabbit IgG conjugated with FITC had previously been diluted with PBS to 64 times. The control eggs and embryos were incubated in normal rabbit serum (1:100) before the fluorescein treatment, or in the fluorescein conjugate only. The same procedures of assay were applied 3 times to a group of 15–45 eggs or embryos at every developmental stage. The eggs and embryos thus treated were thoroughly washed in PBS, and were placed a few at a time, on glass slides to be photographed under a reflected-light fluorescing microscope (Nikon Corporation, Tokyo, Japan). Atretic eggs and embryos were eliminated from the observation.

Results

Fluorescence was observed in the cytoplasm of

Fig. 1. Fluorescence showing the presence of PGE₂ in a 2-cell bovine embryo. $\times 300$.

all the eggs both unfertilized and fertilized, and all the embryos at the 2-cell through hatched blasto-

Fig. 2. Fluorescence showing the presence of PGE₂ in an expanded bovine blastocyst. $\times 200$.

Fig. 3. A control expanded bovine blastocyst treated only with the fluorescein-conjugated anti-rabbit IgG. $\times 200$.

cyst stage (Figs. 1, 2). No fluorescence was found in the eggs and embryos incubated in normal rabbit serum or in fluorescein conjugate only (Fig. 3). When positive, the fluorescence was spread evenly throughout the cytoplasm, although its intensity was not even among blastomeres. In blastocysts, there was no difference in the intensity and distribution of fluorescence between inner-cell-mass cells and trophoblast cells (Fig. 2).

The intensity of fluorescence in eggs and embryos at different stages is shown in Table 1. The intensity of fluorescence was weak from unfertilized eggs to morulae, but was strong in blastocysts. No difference in the intensity of fluorescence was found in the eggs between unfertilized and sperm-penetrated groups, among the 4 cleavage groups and among the three blastocyst groups.

Discussion

In bovine embryos, it has been reported that the blastocysts recovered on days 13, 15 and 16 pc contain PGE₂ and PGF₂ α [8], and that the embryos on days 6 through 20 pc metabolize arachidonic acid to PGs, though the metabolites produced from arachidonic acid are different at the developmental stages of embryos; embryos before day 12 pc metabolize it primarily to PGE₂, those on day 13 pc to both PGE₂ and PGF₂ α , and those after day 15 pc to PGI₂ in addition to PGE₂ and PGF₂ α [8, 11, 14, 19, 22]. However, the presence of PGs in 1-cell bovine eggs and in early cleaved embryos has not been examined. The present experiments prove that PGE₂ exists not only in blastocysts but also in 1-cell eggs and cleaved embryos.

Since there is no biochemical evidence for PG synthesis in cleaved embryos, controversy has existed as to whether the PGs were truly produced

Table 1. Intensity of PGE₂ fluorescence in bovine eggs fertilized, and embryos developed *in vitro*

Developmental stages								
1-Cell		2-Cell	4-Cell	8- to 16-Cell	Morula	Blastocyst		
Unfertilized	Fertilized					Early	Expanded	Hatched
+	+	+	+	+	+	++	++	++

The symbol + represents weak fluorescence, and the symbol ++ strong.

by the embryos [17, 28, 29]. Recently, however, it has been reported that 4-cell human embryos [27] and bovine morulae [22] can synthesize PGs from arachidonic acid and release them. Furthermore, PG H synthase, an indispensable enzyme for the production of PGs, has been immuno-histochemically detected in mouse morulae [32]. From such evidence and the findings of the present study, the authors have agreed on the conclusion that the mammalian embryos at the cleavage stage also have the ability to synthesize and release PGs.

It has been suggested that PGs of blastocyst

origin might participate in: intrauterine migration of embryos; blastocyst hatching from zona pellucida; ion transport across trophectoderm; fluid accumulation in the blastocoele; increase of endometrial capillary permeability; blastocystic glucose metabolism [20]; and further steroidogenesis in the embryos of mice, rats, hamsters and rabbits [28, 29]. Our present investigation shows that the amount of PGE₂ in bovine embryos reaches its maximum at the blastocyst stage. This remarkable increase seems to suggest that the PGE₂ must be playing the above mentioned physiological roles in blastocysts.

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