Development of in-vitro-derived bovine embryos cultured in 5% CO_2 in air or in 5% O_2, 5% CO_2 and 90% N_2

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To evaluate the effects of a three gas mixture of 5% O\(_2\), 5% CO\(_2\) and 90% N\(_2\) (OCN) on preimplantation embryo development, bovine in-vitro fertilization (IVF) oocytes were cultured in a defined medium (mBECM) with various supplements either under 5% CO\(_2\) in air or under OCN. When cultured in mBECM alone, embryo development was significantly stimulated in OCN compared to 5% CO\(_2\) in air (experiment 1). In the OCN atmosphere, blastocyst formation was further increased after addition of fetal bovine serum (FBS; 10%) or FBS + cumulus granulosa cells (CGC) to mBECM. The ratio of blastocysts to 8-cell embryos, number of hatched blastocysts and embryo diameter were markedly increased, and zona thickness was decreased after FBS addition. However, development up to the morula stage was fully supported by mBECM alone. There was no significant effect of β-mercaptoethanol (ME; 10 μM) in OCN. In the 5% CO\(_2\) atmosphere, embryo development was significantly (P < 0.05) enhanced after addition of FBS + CGC + ME. In experiment 2, in OCN, FBS added at 60 h post-insemination was effective in stimulating blastocyst formation, but changes in medium volume per oocyte from 13.6 to 1.36 μl had only a marginal effect. In conclusion, OCN gas mixture provides a suitable atmosphere for early embryo growth in vitro and mBECM + FBS is the optimal culture medium under this atmosphere.

Key words: bovine/culture atmosphere/defined medium/embryo/IVF

Introduction

Reproductive physiology during preimplantation development has been widely studied and the results have been utilized for establishing effective embryo culture systems. In most mammalian species, in-vivo-fertilized embryos develop through early cell cycle stages in the lumen of the oviduct (Betteridge, 1995), which maintains lower partial oxygen pressure (pO\(_2\)) than an air atmosphere (Bishop, 1956; Maas et al., 1976). It was reported in previous studies that either use of a low oxygen gas atmosphere (Liu and Foote, 1995) or addition of antioxidants to the culture media (Takahashi et al., 1993; Lim et al., 1996b; Caamano et al., 1996) is effective in supporting bovine embryo development in vitro. The promoting effect of a low oxygen culture environment on embryo development has also been reported for the mouse (Quinn and Harlow, 1978), pig (Wright, 1977), rabbit (Li and Foote, 1993), sheep (Thompson et al., 1990) and human (Noda et al., 1994).

We found that the addition of an antioxidant (Lim and Hansel, 1996), β-mercaptoethanol (ME) (Lim et al., 1996b), fetal bovine serum (FBS) (Lim and Hansel, 1996) and a nitric oxide scavenger, hemoglobin (Hb) (Lim and Hansel, 1998), to the culture medium and the use of cumulus granulosa cells (CGC) (Lim and Hansel, 1996) were major factors in developing optimal culture systems for use with 5% CO\(_2\) in air. However, the actions of these bioactive substances on embryo development in other gas atmospheres that change pO\(_2\) and embryo metabolism (Kosower, 1978; Reed, 1994) have not been evaluated. Consequently, in experiment 1, we examined whether changes in gas atmosphere (5% CO\(_2\) in air versus 5% O\(_2\), 5% CO\(_2\) and 90% N\(_2\) (OCN)) promote development of in-vitro-derived bovine embryos cultured in media with different supplements [FBS, CGC and/or β-mercaptoethanol (ME)]. In experiment 2, the combined effects of FBS and medium volume per oocyte on development were evaluated in the 5% O\(_2\), 5% CO\(_2\) and 90% N\(_2\) (OCN) gas atmosphere. We determined in experiment 2 whether increasing concentrations of embryotrophic factors, by decreasing medium volume per oocyte, would synergize with the promoting effects of FBS shown in experiment 1. A defined medium (modified bovine embryo culture medium; mBECM) supplemented with 19 amino acids and Hb was the basic culture medium for these studies.

Materials and methods

Culture media

The basic medium used for maturation of oocytes was tissue culture medium (TCM)-199 with Earle’s salts buffered with 25 mM HEPES (Gibco BRL, Grand Island, NY, USA). This medium was supplemented with 10% (v/v) heat-inactivated FBS (A-1111; Hyclone Laboratories, Logan, UT, USA), 1 μg/ml oestradiol-17β (NOBL Laboratories, Sioux Center, IA, USA), 3 μg/ml bovine follicle stimulating hormone (FSH; NOBL Laboratories), 6 μg/ml bovine luteinizing hormone (LH; NOBL Laboratories) and 25 μg/ml gentamycin (Sigma Chemical Co., St Louis, MO, USA). The basic medium...
used for treatment of spermatozoa and fertilization of oocytes was a modified Tyrode's medium (Parrish et al., 1988), supplemented with 0.25 mM sodium pyruvate (Gibco BRL, Grand Island, N.Y., USA), 6 mg/ml fatty acid (FA)-free bovine serum albumin (BSA, No. A-10603; Sigma), 15 µg/ml calcium heparin from porcine intestinal mucosa (183 USP/mg; H-8398, Sigma) and 25 µg/ml gentamycin. Culture of embryos was conducted in mBECM (Table I) within a range of 265–290 mOsm, based on a previous report (Lim et al., 1996a). All components for in-vitro fertilization (IVF) and culture of oocytes were purchased from Sigma, except FBS, amino acid solutions (Gibco) and pyruvate (Gibco).

**In-vitro maturation (IVM), IVF and in-vitro culture (IVC) of oocytes**

Bovine cumulus–oocyte complexes used were derived from a commercial source (BOMED Inc., Madison, WI, USA) and shipped to the laboratory in 2 ml of maturation medium in a battery-powered incubator via overnight express mail service. Oocytes matured during transit and arrived within 24 h of exposure to maturation medium pre-equilibrated at 39°C in 5% CO₂ in air. At 22–24 h after exposure to the medium, the oocytes were inseminated *in vitro* with frozen bull semen at a concentration of 2–3×10⁶ spermatozoa/ml as previously described (Lim et al., 1996a). At 18 h post-insemination, oocytes were freed from CGC and were cultured in groups (34–41 oocytes) in either 50 or 500 µl of the designated medium under the paraffin-mineral oil (M-8410, Sigma).

**Assessment of morphology of blastocysts by an image analysis system**

Morphology of bovine blastocysts at 192 h post-insemination was visualized using a ×20 Hoffman objective (Modulation Optics, Greenville, NY, USA) and ×10 ocular lenses on a Nikon Diaphod inverted microscope (Nikon, Tokyo, Japan) equipped with a video camera connected to a video cassette recorder and a colour monitor as previously described (Morgan et al., 1995). Blastocysts were videotaped and morphometric measurements were made from the video image with image analysis software (Image-1 ATΩ; Universal Imaging, Westchester, PA, USA). Four measurements along different axes were taken on each blastocyst for mean embryo diameter (ED) and mean zona thickness of the blastocyst (ZT).

**Inner cell mass (ICM) cell number**

ICM cell numbers of each blastocyst were counted after removing the trophoblasts by immunosurgery as described by Keefer et al. (1994). After videotaping blastocysts at 192 h post-insemination, zonae pellucidae were removed by treatment with a 0.1% protease solution (Type XXV; Sigma). The zona-free blastocysts were washed in mBECM + polyvinyl alcohol (PVA) and then incubated for 10–15 min in anti-eland antiserum (dilution = 1:10), which proved to be effective for bovine blastocysts in a preliminary study (data not shown). The lysed trophoblasts were removed by careful washing in mBECM + BSA (3 mg/ml) with gentle pipetting. The ICM cells were then treated in 0.1% of Hoechst 33342 for 1 h and counted using a fluorescent microscope (Nikon, Tokyo, Japan).

**Experimental design**

In experiment 1, inseminated bovine oocytes (153–158 oocytes/group) were transferred to mBECM containing various supplements and cultured continuously either in 5% CO₂ in air (groups 1 and 2) or in OCN (groups 3–6) until 192 h post-insemination. The culture medium was supplemented as follows: (i) PVA (1 mg/ml), (ii) FBS (10%, v/v) + CGC + ME (10 µM), (iii) PVA, (iv) FBS, (v) FBS + CGC, and (vi) FBS + CGC + ME. Development to the 2-cell, 8-cell, 16-cell, morula, blastocyst (total number of blastocysts, and number of expanded and hatched blastocysts) stages were examined at 48, 60, 120, 144 and 192 h post-insemination respectively. Subsequently, the ratio of blastocysts per 8-cell embryo was calculated. ICM cell numbers, ED and ZT were also evaluated at 192 h post-insemination.

In experiment 2, inseminated oocytes (145–149 oocytes/group) were cultured in mBECM + PVA in OCN. At 60 h post-insemination, 34–38 oocytes were transferred to each of the following systems: (i) 50 µl of mBECM + PVA, (ii) 500 µl of mBECM + PVA, (iii) 50 µl of mBECM + FBS, and (iv) 500 µl of mBECM + FBS. The volume of medium per oocyte was 1.36 and 13.6 µl at 50 and 500 µl of the medium respectively, and oocytes were cultured in the designated volume of the medium under pre-equilibrated paraffin-mineral oil. Neither medium change nor oocyte manipulation was performed, and embryo development to the hatched blastocyst stage was monitored until 192 h post-insemination.

**Statistical analysis**

Each experimental was replicated four times. Inseminated oocytes were randomly allocated to each treatment group. Oocytes developed to the 2-cell, 8-cell, 16-cell, morula, blastocyst, expanded blastocyst and hatched blastocyst stages were individually scored as a '0' (undeveloped). Oocytes that did not develop to the appropriate stages were scored as a '1' (developed). The scores in each stage of development, TE, ZT and mean number of ICM cells were subjected to analysis of variance using the general linear model (PROC-GLM) in the SAS program (Anon, 1990). When significance of the main effects was detected in each experimental parameter, the treatment effects were compared by the least square method.

**Results**

**Development of oocytes to the blastocyst stage (experiment 1)**

A significant ($P < 0.003$) treatment effect was noted in the development to the 2-cell, 16-cell, morula and blastocyst stages and in the ratios of blastocysts per 8-cell embryos (Table II). When cultured in mBECM alone, more ($P < 0.0001$) blastocysts developed in OCN than in 5% CO₂ in air. Furthermore, blastocyst formation from 8-cell embryos was significantly ($P < 0.0001$) stimulated in the OCN compared with the 5% CO₂ gas atmosphere. A similar pattern for treatment effects was noted in development up to the morula stage. Among the groups cultured in OCN, blastocyst formation was promoted by the addition of FBS (37 versus 23%, $P < 0.006$) or FBS + CGC (32 versus 23%, $P < 0.06$). The percentage of blastocysts (37%) and the ratio of blastocysts...
Table II. Development of oocytes after culture in a modified bovine embryo culture medium (mBECM) containing various supplements in different gas atmosphere.

<table>
<thead>
<tr>
<th>Gas atmosphere</th>
<th>Medium supplements</th>
<th>No. of oocytes cultured</th>
<th>No. and (%) of oocytes developed to</th>
<th>Ratio of blastocyst/8-cell embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% CO₂ in air</td>
<td>None</td>
<td>157</td>
<td>129 (82)c</td>
<td>86 (55)</td>
</tr>
<tr>
<td></td>
<td>FBS + CGC + ME</td>
<td>153</td>
<td>124 (81)c</td>
<td>92 (60)</td>
</tr>
<tr>
<td>5% O₂, 5% CO₂, and 90% N₂</td>
<td>FBS</td>
<td>156</td>
<td>122 (78)c</td>
<td>91 (58)</td>
</tr>
<tr>
<td></td>
<td>FBS + CGC</td>
<td>153</td>
<td>117 (76)c</td>
<td>84 (55)</td>
</tr>
<tr>
<td></td>
<td>FBS + CGC + ME</td>
<td>152</td>
<td>111 (73)c</td>
<td>77 (51)</td>
</tr>
</tbody>
</table>

*Percentages of the number of oocytes cultured.
*bTime (h) post-insemination.
*Different superscripts for each parameter were significantly different (P ≤ 0.05).

FBS = fetal bovine serum; CGC = cumulus granulosa cells; ME = β-mercaptoethanol.

Figure 1. Development of blastocysts 192 h post-insemination after culture in a modified bovine embryo culture medium with various supplements and different gas atmosphere. FBS = fetal bovine serum; CGC = cumulus granulosa cells; ME = β-mercaptoethanol. Different letters in each parameter were significantly different (P ≤ 0.05).

per 8-cell embryos (0.63) were highest in mBECM + FBS treatment. Culture in mBECM alone in OCN resulted in the highest percentages of oocytes developing to the morula stage (2-cell, 92 versus 73–82%, P < 0.05; 16-cell, 57 versus 38–46%, P < 0.02; morula, 50 versus 20–40%, P < 0.05). However, transformation of morulae to blastocysts in mBECM alone in OCN was adversely affected and resulted in only 23% in blastocysts and a ratio of blastocysts to 8-cell embryos of only 0.36. No significant effect of ME addition was found when embryos were cultured in mBECM supplemented with FBS + CGC in OCN.

Among the groups cultured in 5% CO₂ in air, more (P < 0.0001) blastocysts were derived from oocytes after addition of FBS + CGC + ME (29%) than after no addition (2%) to mBECM. However, the percentage of blastocysts formed after addition of FBS + CGC + ME in the 5% CO₂ atmosphere was not significantly different from any of the treatments in OCN. The blastocysts per 8-cell embryos ratio after addition of FBS + CGC + ME was nearly identical for 5% CO₂ in air and OCN (0.48 versus 0.49), but a higher ratio (0.63, P < 0.05) was obtained after addition of FBS alone in OCN, compared with those values.

Development of blastocysts to the hatched blastocyst stage (experiment 1)

A significant (P < 0.04) treatment effect was detected in the development of embryos to the hatched blastocyst stage. Regardless of gas atmosphere, no hatched blastocysts were found after culture in mBECM alone (Figure 1). In the 5% CO₂ atmosphere, a greater (P < 0.05) percentage of hatched blastocysts was obtained after addition of FBS + CGC + ME than after no addition. In the OCN gas atmosphere, addition of FBS or FBS + CGC (13–25%) markedly (P < 0.0001) enhanced development. In addition, the proportion of hatched blastocysts after the addition of FBS + CGC + ME in 5% CO₂ in air was not lower than the proportion of hatched blastocysts after any of the other treatments. Although a similar pattern was observed in the proportion of expanded blastocysts, there was no significant effect of treatments on development to the expanded blastocyst stage.

Morphological and cytological analyses of blastocysts (experiment 1)

A significant (P < 0.0001) change in the number of ICM cells/embryo was detected after the experimental treatment.
In-vitro embryo development and gas atmosphere

Figure 2. Number of inner cell mass (ICM) cells after culture in a modified bovine embryo culture medium with various supplements under different gas atmospheres. FBS = fetal bovine serum; CGC = cumulus granulosa cells; ME = ß-mercaptoethanol. Different letters for each parameter were significantly different (P < 0.05).

Table III. Mean embryo diameter (ED) and zona pellucida thickness (ZT) of blastocysts obtained after culture up to 192 h post-insemination in modified bovine embryo culture medium (mBECM) containing various supplements using different gas atmospheres

<table>
<thead>
<tr>
<th>Gas atmosphere</th>
<th>Medium supplements</th>
<th>ED (µm, mean ± SE)</th>
<th>ZT (µm, mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% CO₂ in air</td>
<td>No addition</td>
<td>160.0 ± 21.8bc</td>
<td>16.3 ± 2.9h</td>
</tr>
<tr>
<td></td>
<td>FBS+CGC+ME</td>
<td>179.7 ± 3.4h</td>
<td>12.3 ± 0.4h</td>
</tr>
<tr>
<td></td>
<td>No addition</td>
<td>170.3 ± 3.4h</td>
<td>11.5 ± 0.4h</td>
</tr>
<tr>
<td></td>
<td>FBS</td>
<td>177.7 ± 4.0h</td>
<td>10.6 ± 0.5h</td>
</tr>
<tr>
<td></td>
<td>FBS+CGC</td>
<td>180.5 ± 2.7h</td>
<td>11.1 ± 0.4h</td>
</tr>
<tr>
<td></td>
<td>FBS+CGC+ME</td>
<td>186.2 ± 3.9h</td>
<td>11.3 ± 0.3h</td>
</tr>
</tbody>
</table>

A total of 210 blastocysts was evaluated across treatment groups.

bc Different superscripts for each parameter were significantly different (P < 0.05).

(Figure 2). Among the treatments in OCN, a higher (P < 0.0001) number of ICM cells/embryo was found after no addition (113.1 ± 4.4) or after addition of FBS (107.6 ± 2.8) than after addition of FBS + CGC or FBS + CGC + ME. Mean ICM cell numbers for other treatments ranged from 83.7 to 91.3.

Although there was no significant overall effect of treatment on embryo diameter or zona thickness (Table III), ED was significantly (P < 0.05) lower after culture in mBECM alone (170.3 ± 3.4 µm) than after culture in mBECM supplemented with FBS + CGC or FBS + CGC + ME (180.5 ± 2.7; 186.2 ± 3.9 µm) in OCN. The lowest ED (160.0 ± 21.8 µm) was noted after culture in mBECM alone in the 5% CO₂ atmosphere. ZT was closely and inversely related to ED; the thickest (16.3 ± 2.9 µm) zona was found in the embryos in 5% CO₂ in air without medium supplements and the thinnest (10.6 ± 0.5 µm, P < 0.05) zonae were detected in embryos in the OCN atmosphere in medium containing FBS.

Combined effects of addition of FBS and/or change in the ratio of medium per embryo (experiment 2)

A significant effect of treatments on morula, blastocyst, hatched blastocyst formations, and the ratio of blastocysts per 8-cell embryo (P < 0.001) was found. As shown in Table IV, no significant increase was noted in the proportions of blastocysts and hatched blastocysts after changing the volume of medium per oocyte from 13.69 to 1.36 µl. However, significant effects of FBS addition were found in embryo development to the morula (P < 0.002), blastocyst (P < 0.0001) and hatched blastocyst (P < 0.0001) stages. A decrease in medium volume per oocyte increased (P < 0.03) morula formation (34% versus 22%). No significant interaction between FBS and the medium volume per oocyte was detected. The highest percentage of blastocysts (36%) in this study was obtained when FBS was added in a medium volume of 13.6 µl per oocyte.

Discussion

In our earlier studies, we found that addition of a NO scavenger, Hb, to a co-culture system for IVF bovine embryos increased blastocyst development from ~20 to 30% of oocytes inseminated when the co-culture was conducted in a conventional 5% CO₂ in air. Results of the present experiments suggest that incubation in a three-gas (OCN) atmosphere and co-culture in medium containing Hb may have some advantages over incubation in 5% CO₂ in air. The OCN gas atmosphere yielded more blastocysts than the 5% CO₂ in air atmosphere when no addition was made to the Hb-containing defined medium (Table II).

More than 90% of bovine morulae in the culture system
developed to the blastocyst stage at 192 h post-insemination after addition of FBS to mBECM in the OCN gas atmosphere. Embryo development was also greatly improved after addition of FBS + CGC + ME to mBECM in the 5% CO₂ atmosphere, and the proportion of blastocysts after culture in this system (29%) was not significantly different when compared with any treatment in the OCN gas atmosphere. Nevertheless, a higher percentage of 8-cell embryos developed to the blastocyst stage after incubation in OCN. Blastocysts obtained after addition of FBS in the OCN atmosphere had more ICM cells (Figure 2) and greater total embryo diameters than blastocysts obtained after any of the treatments in the 5% CO₂ in air atmosphere. ZT of blastocysts was decreased after addition of FBS to mBECM in OCN (Table III) and the condition was more favourable for hatching. Thus, the quality of the embryos produced in the OCN gas mixture appears to be improved, especially with addition of FBS.

Compared with other mammalian species, oxygen consumption rates in human oocytes and blastocysts remain at low level (Magnusson et al., 1986). In a study examining trophoblastic tissue of embryos (Rodesch et al., 1992), pO₂ in placental tissue maintained basal level until 10 weeks of gestation period, but a significant increase was detected after 12–13 weeks of gestation. Noda et al. (1994) first reported that preimplantation development and embryo transfer outcome are enhanced by culturing human IVF-derived embryos in Eagle’s medium under the low oxygen gas mixture. Jones et al. (1998) also obtained efficient development to the blastocyst stage by culture of human embryos with Gardner’s G1 and G2 media and the OCN gas atmosphere. However, the promoting effect of the low oxygen gas atmosphere might be dependent on the type of culture system. In the culture system using human tubal fluid medium, use of OCN atmosphere did not significantly promote embryo development compared with 5% CO₂ in air atmosphere (Dumoulin et al., 1995).

Although the OCN gas atmosphere, without any additions, had a remarkable ability to support embryo development to the morula stage (50%) in the defined, Hb-containing medium, only 47% of the morulae obtained in this system developed to the blastocyst stage. The morula to blastocyst transformation was significantly stimulated by the addition of FBS to the culture medium. This promoting effect of FBS was not affected by the time of addition (Tables II and IV), provided it was added to the medium before the 16-cell stage (up to 60 h post-insemination). Comparable results were found by Biggers et al. (1997) who reported that replacement of BSA with PVA severely impaired preimplantation development of mouse embryos. These results strongly suggest that the morula to blastocyst transformation requires additional exogenous factors, found in serum, that specifically stimulate differentiation and proliferation of morulae and blastocysts.

FBS promoted bovine preimplantation embryo development in a Hb-containing medium in this study (Tables II and IV). Nevertheless, caution should be taken in applying this culture protocol to human IVF–embryo transfer programmes. Several reports suggest that human serum albumin (HSA) is preferable to serum for development of human embryos to the blastocyst stage (Ménézo et al., 1984; Khan et al., 1991; Muggleton-Harris et al., 1995; Laverge et al., 1997). A high proportion (40–50%) of pronuclear stage embryos was developed to the blastocyst stage in a culture system using HSA and OCN atmosphere (Jones et al., 1998). Therefore, use of serum-free, HSA-based media, to which putative embryotrophic substances (Harvey and Kaye, 1990; Paria and Dey, 1990; Ryan et al., 1990; Larson et al., 1992; Kane et al., 1997; Gardner and Lane, 1997) are added, might be a good strategy to develop an efficient assisted reproductive technology. In view of the promoting effect of FBS in different media volumes per embryo (Table IV), embryotrophic substances, such as platelet-derived growth factor, transforming growth factor and arachidonic acid (Lim and Hansel, 1996) are likely to be present in FBS.

In previous studies (Takahashi et al., 1993; Caamano et al., 1996; Lim et al., 1996b), addition of ME as an antioxidant to the culture medium enhanced development of bovine embryos to the blastocyst stage in 5% CO₂ in air. Two explanations have been advanced for the action of ME on embryo development. The first is that ME acts only as an antioxidant. The second is that ME has a stimulatory action on various biological reactions, in addition to its action as an antioxidant. The results of the present study support the first hypothesis, that ME exerts its promoting effect solely as an antioxidant, since no further increase was found after addition of ME to the culture medium in OCN. Considering that pO₂ of the OCN gas atmosphere is only one-fourth that of the 5% CO₂ in air atmosphere, the promoting effect of ME is more likely to be due to its action as an antioxidant.
atmosphere, these results suggest that the effectiveness of this gas atmosphere stems from lowered P02.

A decrease in the medium volume per oocyte from 1000 to 1 μl/oocyte was beneficial for preimplantation development in previous studies (Canesco et al., 1992; O’Neill, 1997). Similarly, embryo development to the morula stage in the absence of FBS was significantly (P < 0.02) enhanced by a decrease in the volume of medium (Table IV). These results support the fact that embryos have paracrine function that significantly affects preimplantation development. A positive paracrine role of embryos in preimplantation development has been reported in the mouse (Paria and Dey, 1990; Lane and Gardner, 1992), ovine (Gardner et al., 1994) and bovine (Lim and Hansel, 1996) species.

Nevertheless, a change in the medium volume per oocyte (from 13.6 to 1.36 μl) had only marginal effect on the development of IVF oocytes cultured to the blastocyst stage in the OCN atmosphere in experiment 2. It might appear that the action of embryotrophic substances secreted from the embryo itself is limited to certain stages of pre-hatching development under OCN. However, putative embryotrophic substances secreted from the embryo itself might readily lose their embryotrophic actions under our in-vitro culture conditions and this might result in a reduction of the promoting effect of a decrease in medium volume per embryo ratio. Several embryotrophins of lipophilic nature can lose their biological action when the embryotrophin-containing medium is overlaid by mineral oil (Lane and Gardner, 1992). On the other hand, co-existence of developing embryos with degenerated embryos that can release embryotoxic substances might negatively affect embryo development in a small volume of medium, since paracrine actions among embryos are more critical at a high embryo density than a low embryo density (O’Neill, 1997). In any case, the results of experiment 2 indicate that adjustment of the medium volume/oocyte to ~1.36 μl in the OCN gas atmosphere is not harmful for normal embryo development. Further modification, however, is required for additionally promoting developmental competence in this culture system. Regrouping viable embryos at a certain stage of development, suggested by Jones et al. (1998), might be one of several good alternatives to enhance embryo development.

New approaches to human IVF-embryo transfer are now under evaluation and some newly developed technologies are derived from the methods used in large animal embryo culture. These methods include in-vitro maturation of human oocytes (Cha et al., 1991; Trounson et al., 1994; Russell et al., 1997), culture of embryos up to the blastocyst stage before transfer (Fong et al., 1997; Jones et al., 1998) and co-culture embryos with somatic tissue cells (Wiemer et al., 1989; Menez et al., 1992; Thibodeaux and Godke, 1995; Wiemer et al., 1996). The information contained in this report regarding the effect of the OCN gas on bovine embryo development in a HB-containing medium may also contribute to the development of more effective culture systems for human embryos. A culture system established from the results of these studies may efficiently minimize the frequency of in-vitro manipulations of embryos, such as medium changes and assisted hatching micromanipulations.

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