Impact of Incubator Type on the Yield of in Vitro Produced Bovine Blastocysts

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Avery, B. and T. Greve: Impact of incubator type on the yield of in vitro produced bovine blastocysts. Acta vet. scand. 1992, 33, 341-348. – Because of suboptimal in vitro production of bovine blastocysts a new incubator model (Mini) was tested against the traditional (Heraeus). The difference between their properties seemed only to be the volume of the incubator space. No difference was noted between the CO_2 or the temperature, but the data clearly showed a highly significant increase of the blastocyst rates, 6% versus 51% in the Heraeus and the Mini incubator, respectively, calculated as blastocysts per cleaved embryos. It was concluded that the incubator type or model may be a very important part of the in vitro production of bovine embryos, although we were not able to pin point specific causes for this difference.

in vitro fertilization; CO2; embryo development; blastocyst rate; culture block.

Introduction

The blastocyst rate (blastocysts /cleaved embryos) for in vitro produced bovine embryos is usually reported to be in the range of 20%-35%. In spite of many attempts to improve this rate by variations in the techniques (*Madison et al.* 1992, *Xu et al.* 1992, *Rose & Bavister* 1992, *Pavlok et al.* 1992, *Wiemer et al.* 1991, *Fukui et al.* 1991, *Eyestone et al.* 1991, *Pinyopummintr & Bavister* 1991, *Gordon & Lu* 1990 and many others), and by a better understanding of the optimal conditions for embryo development (reviewed by *Rieger* 1992, *Boone & Shapiro* 1990), it has remained relatively low in many laboratories. Not much has been published about the incubator effect on in vitro development (*Wang et al.* 1992, *Abramczuk JW & Lopata A* 1986), but it has been claimed that smaller incubators might be more efficient (*Boone & Shapiro* 1990, *Bavister* 1987, *Kane* 1987). This article describes how the change to another incubator type dramatically increased the blastocyst rate in a reproducible way to 40%-60%.

Materials and methods

In vitro production of embryos

The final protocol for in vitro production of the bovine embryos was a slightly modified version of the standard methods described by *Madison et al.* (1991, 1992).

Briefly, ovaries were collected from the abattoir and maintained at 33°C in 0.9% sterile saline. From collection of the ovaries and until day 8 post insemination, the temperature was never lower than 33°C. Immature oocytes were aspirated from 2-8 mm antral follicles and cumulus-oocyte complexes of all qualities with unexpanded cumulus cells were selected for in vitro maturation, i.e. grade 1 (surrounded by at least 4-5 layers of cumulus cells), grade 2 (surrounded by 2-3 layers), and grade 3 (partially denuded). Only completely denuded oocytes, or oocy-

tes with expanded cumulus masses scattered in dark clumps in a jelly like matrix, were not selected for in vitro maturation. The oocytes were matured in bicarbonate buffered TCM-199 with Earles salts, supplemented with 10% heat treated estrous cow serum (ECS), 15 I.E./ml Suigonan® Vet (Intervet; one dose contains 400 I.E. PMSG (pregnant mare serum gonadotropin) and 200 I.E. HCG (human chorion gonadotropin)), 0.4 mm L-glutamine, 0.2 mM pyruvate and 50 µg/ml gentamycin. After 24 h maturation the oocytes were inseminated for 20 h in glucose free IVF-TALP (modified Tyrodes solution with 25 mM bicarbonate, 0.2 mM pyruvate, 6 mg/ml bovine serum albumin, 10 µg/ml heparin, 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine and 50 µg/ml gentamycine) with frozen-thawed swim-up treated sperm (2×10^6/ml). (*Parrish et al.* 1986). After fertilization presumptive zygotes were stripped for cumulus cells and cocultured for 8 days with a suspension of bovine oviduct epithelial cells (BOEC) in Menezo-B2 medium (*Menezo,* 1976) supplemented with 10% ECS, 2.5 µg/ml fungizone and additional L-glutamine (0.3 mM) and pyruvate (0.2 mM). Maturation and fertilization took place in 100 µl droplets covered with paraffin oil (10 oocytes/droplet). The oil covered Menezo-B2/BOEC droplets were made 1 day prior to use (40 µl). On day 1 post insemination presumptive zygotes were added, on day 2 post insemination 50 µl additional Menezo-B2 was added (total volume of the droplet 100 µl, 25-40 embryos/droplet, BOEC approximately 1:100 vol/vol). The oviduct cells were prepared from oviducts, collected at the same day as the ovaries.

Evaluation of embryos

For fertilization control a portion of the oocytes/zygotes were fixed (*Xu & Greve*

1988) at 20 h post insemination (day 0 = day of insemination) by the whole mount technique in acetic acid:methanol (1:3), stored at –20°C for 24 h, stained with 1% orcein in 40% acetic acid, and evaluated by interference phase contrast microscopy. Normal fertilization was presumed to have occured when 2 synchronous pronuclei, with or without a second polar body, was observed. Abnormal fertilization included zygotes with 1 pronucleus, > 1 sperm, > 2 pronuclei, or asynchronous development of the pronuclei (*Xu & Greve* 1988).

Evaluation of the embryonic development was based on morphological criterias (*Greve et al.* 1979, *Lindner & Wright* 1983). On day 2 (44 h post insemination) the number of 1-cell, 2-4 cell and 5-8 cell ova was assessed. The cleavage rate was determined as the number of embryos developed to at least the 2 cell stage divided by the total number of ova.

The blastocysts were evaluated on days 7 and 8 post insemination. The blastocyst rate was calculated as the number of day 8 blastocysts divided by the number of cleaved embryos. The number of morulas was not included, since the endpoint was blastocysts.

Variations of the in vitro procedures

Each time a new batch of serum, medium, sperm or gas was introduced, and/or when variations of the technique was tested, it was noted and compared with the outcome of the experiment. New batches were introduced as follows: estrous cow serum (experiments 3, 8, 14, 19, 26, 29); TCM-199 (experiments 5, 14, 18, 20); Menezo-B2 (experiments 14, 23); Bulls (experiments 24 (Ipsen, sperm concentration 1×10^6/ml), 28 (Micro, sperm concentration 1×10^6/ml), 33 (Micro, sperm concentration 2×10^6/ml)); CO_2 supply for the Hereaus incubator (experiments 3, 16, 26). In

experiments 29-30 the estrous cow serum was not heat treated, in experiments 20-30 the room temperature was 28°C-29°C instead of ≥ 33°C, and in experiments 32-39 15 I.E./ml Suigonan Vet® (10 I.E PMSG + 5 I.E. HCG) was added to the maturation medium.

Incubators

Experiments 1-35 took place in a Heraeus CO_2 incubator Type B 5060 EC/CO_2, with inner dimensions of 600×600×588 mm corresponding to a volume of 212 l, and with inner casing of copper. Between the door and the incubator an extra screen consisting of 6 small doors was attached, to ensure that the internal atmosphere was affected as little as possible. The temperature, set at 39°C (39.0°C-39.3°C), was monitored with an electronic thermometer with 2 probes (temperature range -200°C - + 300°C) and continually controlled. Full humidity was supplied by water in the bottom of the incubator. The CO_2 supply was from concentrated CO_2, and

the automatic intake was set at 5%. This level was controlled at least once a week by use of a Fyrite® test Kit (Bacharach), and daily during experiments 34, 35 and 39.

Experiments 36-39 took place in a Mini incubator, (K-System Mini incubator, type G-82, Henning Knudsen Engineering, Klintehøj Vænge 1, DK-3460 Birkerød, Denmark) with inner dimensions of 220×215×85 mm, corresponding to a volume of 4 l. It contained 3 shelves placed with a distance of 27 mm, each shelf being 214×205 mm. The CO_2 was supplied by a ready made gas mixture of 5% CO_2/95% air, which was passed through a flowmeter with regulating valve and flush flow valve. After opening of the incubator, the flush flow valve was used for 10 s for fast recovery of the atmosphere, followed by a minute constant flow of 5% CO_2/95% air. The incubator was heated by a circulating thermostat water bath, with electronic temperature control. The temperature was set at 39°C (39.0°C-39.3°C) and full humidity was
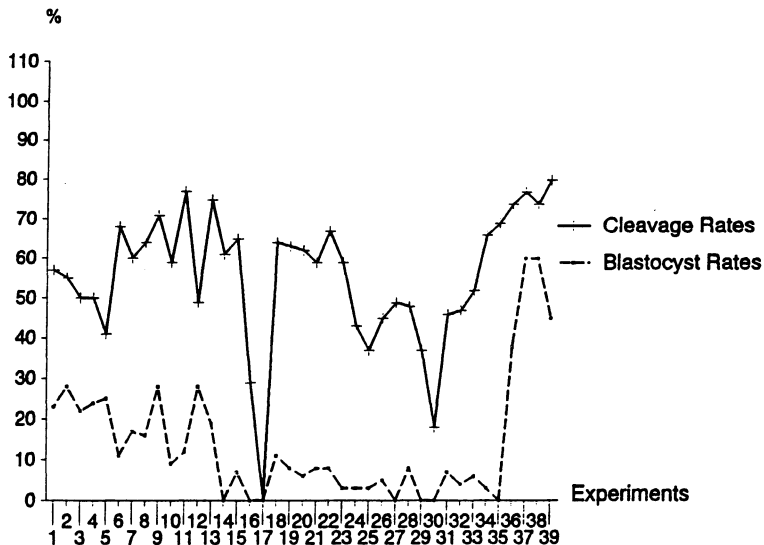

Figure 1.Cleavage- and blastocyst rates for in vitro produced bovine embryos from experiments 1-39.

supplied by water in the bottom of the incubator.

Comparison of incubators

In 2 experiments the oocytes were allocated randomly between the 2 incubators for maturation, fertilization and culture, followed by comparison of fertilization rates, cleavage rates and blastocyst rates (experiments 34 and 35 in the Heraeus were equal to experiments 36 and 37 in the Mini incubator).

In experiment 39 the embryos were allocated for culture in the Heraeus and the Mini incubators after beeing matured and fertilized in the Mini incubator.

Statistics

The statistics were calculated by one way analysis of variance (ANOVA), for testing of whether or not significant differences among the mean values could be stated. The level of significance was $P < 0.05$ (GraphPad IN-STAT).

Results

In the first period (experiments 1-13) the in vitro technique functioned satisfactorily with a fertilization rate of 69%±10%, a cleavage rate of 61%±11% and a blastocyst rate of 20%±7% (Fig. 1, Tables 1-3). This was followed by a long period, in which it was

Table 1. Fertilization rates (% ±SD) for bovine in vitro produced embryos assessed at 20 h post insemination.

Experiment	Replicates	No. ova	Fertilization	
			Total	Normal
1-13	9	121	69±10	56±9
14-35	14	315	69±18	59±14
36-39	2	35	81±8	75±7

Fertilization was not significantly different within columns.

Table 2. Cleavage rates (% ±SD) for bovine in vitro produced embryos assessed at 44 h post insemination.

Experiment	No. ova	Cleaved/ total	5-8 cell/ total	5-8 cell/ cleaved
1-13	1555	61±11[a]	33±6[d]	55±10[g]
14-35	2989	50±17[b]	14±13[e]	25±21[h]
36-39	223	76±2[c]	44±7[f]	58±11[g]

Values with different superscripts within columns differ: (a,b,c p < 0.0001; a,b p < 0.05; a,c p < 0.01; b,c p < 0.006; d,e,f; d,e, p < 0.0001; d,f p 0.0085; e,f p 0.0002; g,h p < 0.0001; g,g p 0.62).

Table 3. Blastocyst rates (% ±SD) for bovine in vitro produced embryos assessed at day 8 post insemination.

Experiment	No. ova	BL/ total	BL/cleaved	BL/5-8 cell
1-13	1555	12±4[a]	20±7[d]	37±14[h]
14-35	2989	3±3[b]	6±8[e]	21±30[h]
36-39	223	39±8[c]	51±10[f]	90±20[i]

Values with different superscripts within columns differ: (a,b,c; b,c; a,c; a,b p < 0.0001; d,e,f; d,e; d,f; e,f p < 0.0001; h,i p < 0.0005; h,h p 0.1).

impossible to overcome the 8-16 cell block (experiments 14-35), resulting in a blastocyst rate of 6%±8%. The mean fertilization rates (69%±18%) and cleavage rates (50%±17%) were in general not so affected (Tables 1-3), although in some of the experiments they were 0% (Fig. 1).

Many attemps were tried to improve the results, but none of them sufficient to overcome the 8-16 cell block.

The change from 1 bull (Ivanhoe, experiments 1-23) to 2 other bulls (Ipsen, experiments 24-27 and Micro, experiments 28-32), temporarily resulted in lower cleavage rates

(18%-49%), but an increase of the sperm concentration from 1×10^6/ml to 2×10^6/ml improved the cleavage rates to 52%-69% (Micro, experiments 33-35, Fig. 1).

In experiment 32 15 I.E/ml (PMSG 10 I.E./ml + HCG 5 I.E/ml) Suigonan Vet® was added to the maturation medium. This resulted in an extreme cumulus expansion, but it did not seem to improve neither the cleavage- nor the blastocyst rate (47%, 4%). However, Suigonan Vet® was added to the maturation medium in all subsequent experiments.

Experiments 34 and 35 (Heraeus incubator) were identical with experiments 36 and 37 (Mini incubator). The cleavage rates were 66% (60/91)-69% (54/78) and 74% (39/53) - 77% (50/65) in the Heraeus and Mini incubators, respectively. The corresponding blastocyst rates were 3% (2/60) - 0% (0/54) and 38% (15/39) - 60% (30/50), respectively (Fig. 1).

In experiment 39 some of the embryos, matured and fertilized in the Mini incubator, were cultured in the Heraeus incubator. The culture dish was covered with aluminum foil, and the incubator was not opened from day 2 to day 6. The cleavage and blastocysts rates were 73% (19/26) and 0% (0/19), compared with 79% (58/73) and 45% (26/58) in the Mini incubator.

The results showed, that the blastocyst yield in the Mini incubator was almost identical with the number of 5-8 cell embryos at day 2 (44 h post insemination). Of 94 5-8 cell embryos (experiments 36-39), 85 (90%) developed to blastocysts (Table 3), which meant that the outcome of an experiment could be predicted already at day 2 post insemination. The 85 day 8 blastocysts were distributed as follows: blastocysts 4 (5%), expanding + expanded blastocysts 63 (74%) and hatching + hatched blastocysts 18 (21%).

Discussion

The chance of succes in an vitro system is multifactorial, depending of the optimal quality of each of the procedures and components. This study showed, that the impact of the incubation system should not be underestimated, since it seemed to play a major role. Our main problem was to overcome the 8-16 cell in vitro culture block, at which stage the embryonic gene expression (maternalzygotic transition) is switched on in the cow (*Camous et al.* 1986, *Barnes & Eyestone* 1990, *Telford et al.* 1990, *Antalikova & Fulka* 1990, *Eyestone & First* 1991).

The low blastocyst rates were observed for the first time in experiment 14. Since all medias and sera came from new batches, it was natural to suspect the commercial medias to be the cause for the sudden drop, and in fact the TCM-199 used in experiments 14-17 resulted in progressively lower cleavage rates (65%-29%-0%). It is well known that medias can degrade fairly rapidly and become cytotoxic, and that differences between batches are often seen because of that (*Nielsen & Bertheussen* 1990). This cytotoxcicity is however more obvious in serum-free medias, since the proteins can bind tocix products.

It did not occur to us to suspect the Heraeus incubator for being the main cause for the difficulties, since it had provided us with blastocysts before. The temperature and CO_2 concentration was extremely stable at steady state. However, the incubator door was opened and closed numerous times during the days, and the incubator was used by several people. This of course disturbed both the atmosphere and the temperature, and complete recovery of the environment was slow. In several of the experiments the incubator door was not opened from late day 2 to early day 6 post insemination, which coincides with the period when the embryos were

supposed to pass the 8-16 cell in vitro block, but in spite of that, no improvment of the results was observed.

Another explanation for the low blastocyst rate could be the presence of toxic ingredients in the CO_2, such as CO, released into the incubator with the gas. However, the CO_2 was of medical grade, and as such considered to be of high quality, and no correlation could be shown between the results and change of CO_2 supply.

The Heraeus incubator had an inner casing of copper, and water in the bottom. This copper serves an anti-bacterial and anti-fungus function, and in fact we never saw fungus on the copper or in the waterbath. The question was if the copper or copper compounds could affect the atmosphere, and hence the development of the embryos? From a chemical point of view this should be possible, since some of the copper in the water should be present in the oxidized form. The water would then contain a certain low concentration of copper ions, which would be in equilibrium with hydrated copper ions in the atmosphere. It is however not easy to explain how the hydrated copper ions could penetrate to the medium through the protecting layer of paraffin oil. A thin layer of oxidized copper was also found on the sides of the incubator. Small particles of oxidized copper might be "blown off" to the atmosphere, fall into the oil, and disturb the development of the embryos. Superoxide radicals and hydrogenperoxide are produced in mammalian cells during aerobic metabolism, and copper ions in conjunction with hydrogenperoxide can result in damage to DNA (*Aruoma et al.* 1991, *Dizdaroglu et al.* 1991, *Sagripanti & Kraemer* 1989). The block stage has been described as particularly sensitive to the adverse effect of oxygen radicals (reviewed by *Rieger* 1992). Since embryonic develop-

ment was mainly affected at this stage, it could be explained by an augmented deleterious effect by copper and oxygen radicals.

Another difference between the incubators was the size. The Mini incubator was only 220×215×85 mm, and the distance between the shelves 27 mm. Apart from protecting the eggs from light it also maintained a very stable temperature and CO_2 environment, meaning that after opening of the Mini incubator the CO_2 atmosphere would be reestablished at 5% almost instantaneously because of the flush valve, and no fall in temperature would be expected either, because of the heat preserving capacity of the solid steel shelves. How the size per se could affect the blastocyst rate is however difficult to visualize.

A simpler and more direct explanation might be the presence of toxic substances in the laboratory air. The air supply to the Mini incubator was bottled air mixed with CO_2, whereas the air supply to the Heraeus incubator was plain air from the laboratory. This air was filtered in the clean bench where all particles ≥ 0.3 µm, but no gasses, were removed. It would then be easy to imagine the presence of noxic fumes in the laboratory air from organic solvents, detergents, acetic acid etc.

This study showed firstly that the incubator type per se may influence the outcome of in vitro production of bovine embryos, and secondly that it is possible to achieve a very high average blastocyst rate in the Mini incubator. We do not know exactly why one incubator was better than the other, but suspect it to be due to a toxic effect of the copper. It is however important to remember that, compared with in vivo conditions, we deal with empirically designed suboptimal culture systems. The 8-16 cell block is an in vitro culture artifact that can be used as a good indicator for the quality of a culture system.

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Sammenfatning

Inkubatorens betydning for udbyttet af in vitro producerede kvæg blastocyster.

Kvæg blastocyster fremstillet ved reagensglasbefrugtning blev sammenlignet efter dyrkning i to forskellige kuldioxid inkubatorer. Bortset fra rumfanget var de to inkubatorer i princippet ens (CO_2, temperatur, luftfugtighed), men udbyttet af blastocyster, beregnet som blastocyster pr totalt antal dyrkede æg, var 3% versus 39% i henholdsvis den store og den lille inkubator (p <0.0001). Indtil 8-16 celle stadiet udvikledes æggene stort set ens. I den store inkubator gik æggenes udvikling i stå ved 8-16 celle blok stadiet, mens æggene i den lille inkubator fortsatte til blastocyst stadiet. Årsagen til denne forskel kendes ikke, men kan måske skyldes en toksisk effekt af kobber, som beklæder den store inkubators indre overflader.