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EFFECT OF OIL ON EMBRYONIC DEVELOPMENT *IN VITRO* AND ON EMBRYO SURVIVAL AFTER ONE-STEP VITRIFICATION IN CATTLE

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Abstract

The effect of two types of oil, routinely used to overlay *in vitro* embryo culture media, on bovine *in vitro* embryonic development and on their subsequent survival after one-step vitrification was investigated. In Experiment 1, silicone oil batch A was compared to paraffin oil. Embryonic development at the eight-cell, compact morula and blastocyst stage was significantly impaired by silicone oil as compared to paraffin oil ($P<0.0001$). None of the blastocysts which were produced under silicone oil survived the vitrification procedure while 59% of the blastocysts survived when they were cultured under paraffin oil both before vitrification and after warming. In Experiment 2, another batch of silicone oil was compared to paraffin oil. No effect of the type of oil on the development of fertilized cells into the eight-cell and compact morula stage embryos could be demonstrated. However, the blastocyst formation rate was significantly lower with silicone oil than with paraffin oil. The survival of vitrified blastocysts after warming was also significantly improved when silicone oil batch A was replaced by batch B (0% and 41%, respectively), although it was still lower when compared with the survival of blastocysts developed under paraffin oil (53%) ($P<0.05$). In Experiment 3, the relationship between the age at which blastocysts were attained and their post-warming survival was studied. Blastocysts were vitrified at different times after fertilization. The survival rate after warming and culture was significantly higher for those embryos that reached the blastocyst stage early. It is concluded that the embryo production rate *in vitro* and the ability of embryos to survive cryopreservation is affected by the type of oil used to overlay the *in vitro* culture medium. Moreover, the age at which the blastocyst is attained is an important factor affecting survival after vitrification.

Keywords: Age of embryo; Bovine; Embryo; Oil toxicity; Vitrification

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Introduction

Irrespective of major improvements in the technique of *in vitro* maturation, fertilization and culture of bovine oocytes, cryopreservation methods developed for bovine embryos produced *in vivo* have proved to be unsatisfactory for bovine embryos derived *in vitro*. This is possibly due to structural differences between these two types of embryos [6, 7, 13, 15]. Recent investigations have revealed that the sequence of factors involved in the embryo production procedure *in vitro* affect the freezing sensitivity of the embryos [18]. One of these factors, which has not yet been investigated and may affect the cryopreservation of embryos produced *in vitro*, is the type of oil used to overlay the *in vitro* culture media. In this paper, the result of an investigation into the effects of silicone oil and paraffin oil on the development of bovine embryos produced *in vitro* and on their survival rate after one-step vitrification are presented.

Materials and Methods

In Vitro Embryo Production

The *in vitro* oocyte maturation (IVM), fertilization (IVF) and embryo culture (IVC) procedures which were used have been described previously [16]. Bovine ovaries were collected in an abattoir. Follicles (2 to 8 mm) were punctured to collect cumulus-oocyte complexes. Oocytes surrounded by a complete cumulus oophorus were matured in TCM199 bicarbonate buffered medium (Cat No. 041-01150 H, Gibco, UK) supplemented with 20% heat inactivated estrous cow serum (ECS), 0.2 mM sodium pyruvate (Cat No. P-3662, Sigma), 0.4 mM glutamine (Cat No. G-6392, Sigma), and 50 µg/ml gentamycin sulphate (Cat No. 043-05710, Gibco) at 39°C in 5% CO_2 in air with maximum humidity for 24 to 26 hours. Groups of approximately 100 oocytes were cultured in 500 µl of the maturation medium without oil overlay.

IVF was performed with frozen-thawed sperm which was subjected to a discontinuous percoll gradient to separate motile from nonmotile spermatozoa. The IVF medium consisted of Tyrode's solution (TALP) [2], and was supplemented with 6 mg/ml BSA (Cat No. A-7030, Sigma), 20 µM D-Penicillamine (Cat No. P-4875, Sigma), 10 µM Hypotaurine (Cat No. H-1384, Sigma), 1 µM epinephrine (Cat No. E-7386, Sigma), and 25 µg/ml heparin (Cat No. H-8514, Sigma). Sperm concentration was adjusted to 1×10^6 spermatozoa/ml. Sperm-oocyte co-incubation lasted for 20 to 24 hours. Fertilization of each 100 matured oocytes was performed in 500 µl of the medium without oil overlay. Subsequently, the cumulus cells were mechanically removed, and groups of 25 fertilized zygotes were cultured in 50 µl droplets of culture media which consisted of Ménézo-B2 medium (Cat No. 01001, INRA, France) supplemented with 10% ECS and 2.5 µg/ml fungizone (Cat No. 043-05290, Gibco). Each droplet was further supplemented with 2 µl bovine oviduct epithelial cells (BOEC) suspension. To prepare BOEC, oviducts were collected from the slaughter house and were dissected free from connective tissue. Oviductal epithelium was removed by scraping the lumen with a scalpel. Epithelial cells were washed three times with TALP medium and cultured overnight in TCM199 medium. One day later, the cells were washed again and 2 µl BOEC (~20 oviduct vesicles) were put in each 50 µl droplet of Ménézo-B2 medium. The droplets were prepared in a 4-well multi-dish plate (Nunc, Denmark) and were covered with oil. All cultures were performed at 39°C in 5% CO_2 in air with maximum humidity.

Vitrification Procedure

The vitrification solution (EFS) previously described by Kasai *et al.* [8] was also used for the present experiments. It consisted of 40% v/v ethylene glycole (Cat No. E-9129, Sigma), 18% w/v ficoll (Ficoll 70, average MW: 73300, Cat No. 09894, Pharmacia, Uppsala), and 10.26% w/v sucrose. EFS solution was prepared in a holding medium (E. T. Freezing medium, Cat No. 04101100 H, Gibco) which is a modified PBS supplemented with sodium pyruvate (0.33 mM), glucose (5.6 mM), BSA (0.4%), and kanamycine sulphate (0.025 mg/ ml). Groups of 3 to 4 blastocysts were transferred into sterile Petri dishes each containing 1.2 ml of EFS. A 0.25 ml French straw was used for the loading of each group of embryos. First, about 4 cm length of the straw was filled with 0.5 M sucrose solution followed by ~1 cm air bubble. Then ~0.5 cm EFS, ~0.5 cm air bubble, ~2 cm EFS together with the embryos, ~0.5 cm air bubble, ~0.5 cm EFS and ~0.5 cm air bubble were aspirated into the straw, respectively. The remaining part of the straw was filled with 0.5 M sucrose solution and the straw was immersed in liquid nitrogen and stored until warming.

After warming of the straws in a water bath at room temperature (20±2°C) for 10 seconds, the content of each straw was emptied into a well of a sterile 4-well multi-dish plate containing 0.7 ml 0.25 M sucrose solution in holding medium. It took approximately 1 minute to find the embryos (back in the well) and to transfer them to a second well which also contained 0.7 ml of 0.25 M sucrose solution. After 4 minutes, they were transferred to a well containing 0.7 ml of holding medium.

Experiment 1

The purpose of this experiment was to study the effect of paraffin oil and silicone oil batch A on: 1) embryonic development and 2) *in vitro* embryonic survival after vitrification. After IVF, the cumulus cells of the oocytes

were mechanically removed and each replicate was randomly divided into two groups. Group 1 was cultured in medium droplets overlaid with 0.8 ml of paraffin oil (Cat No. 7160, Merck, Germany) and group 2 was cultured under 0.8 ml of silicone oil batch A (Cat No. 14,615-3, Batch No. 37113, Aldrich, Germany).

All embryos were evaluated on day 3 after IVF to determine the number of cleaved and eight-cell embryos. On day 6 after IVF, all embryos at the morula stage were transferred to one well. On the basis of the classification method described by Mahmoudzadeh *et al.* [10], embryos without any obvious defects, with a cell mass pushed to one side, with blastocoel occupying more than half of the perivitelline space and with trophoblastic layer lying adjacent to the zona pellucida were regarded as grade 1 blastocysts. Starting at 144 hours post IVF, grade 1 blastocysts were removed for vitrification at 8 hour intervals. The remaining embryos in the plates were further evaluated on day 9 after IVF to record the total number of embryos which had developed into the compact morula and the blastocyst stage.

The vitrified-warmed blastocysts from group 1 and 2 were randomly subdivided into two groups. Each group was cultured in a 50 µl droplet of culture medium (Ménézo-B2+BOEC) which was covered with 0.8 ml of either silicone oil or paraffin oil. The droplets were prepared in a 4-well multi-dish plate and kept in an incubator at 39°C, in 5% CO_2 in air and maximum humidity. Post-warming survival was defined as the percentage of vitrified embryos that expanded or hatched during 72 hours of culture.

Experiment 2

Another batch (B) of silicone oil (Cat No. 14,615-3, Batch No. 67777, Aldrich, England) was compared with paraffin oil. The assessment of embryo development and survival after vitrification was the same as in Experiment 1.

Experiment 3

Culture drops were examined at 8 hour intervals (144, 152, 160 and 168 hours after IVF). At these times, all embryos which attained the blastocyst stage were removed and vitrified. The percentage of blastocyst formation at different times during the culture period and their subsequent survival after vitrification were recorded.

Statistical Analysis

The analysis of multiway tables of frequencies was performed according to a hierarchical loglinear procedure [14] with the software program SPSS Release 5.0. The significance of a difference between two proportions was checked with a Chi-square test. If not explicitly mentioned, a significance level of $P<0.05$ was used.

Results

In all experiments, an added variance in the development rate of the embryos and in their subsequent

Table 1. *In vitro* development of IVM/IVF cattle oocytes in culture medium overlaid with paraffin oil or silicone oil (batch A)

Replicate	Type of oil	Number of oocytes	Number of cleaved cells (%) at day 3	Number of eight-cells (%) at day 3	Number of compact morulae (%) at day 6	Number of blastocysts (%) at day 9
1	Paraffin	289	244 (84)	192 (66)	112 (39)	102 (35)
	Silicone	295	231 (78)	181 (61)	86 (29)	66 (22)
2*	Paraffin	292	251 (86)	188 (61)	107 (37)	91 (31)
	Silicone	205	140 (68)	91 (44)	38 (19)	29 (14)
3	Paraffin	309	218 (71)	136 (44)	70 (23)	64 (21)
	Silicone	310	221 (71)	143 (46)	5 (2)	5 (2)
4*	Paraffin	470	333 (71)	227 (48)	122 (26)	118 (25)
	Silicone	378	259 (69)	140 (37)	72 (19)	68 (18)
Total	Paraffin	1360	1046 (77)	743 (55)^a	411 (30)^c	375 (28)^e
	Silicone	1188	851 (72)	555 (47)^b	201 (17)^d	168 (14)^f

*: Differences in the number of oocytes between paraffin oil and silicone oil are caused by an accidental loss of oocytes during the experiment

$^{a-f}$: Values in columns with different superscripts are significantly different ($P< 0.0001$)

survival rate after vitrification was observed among the replicates (P<0.05). This was taken into consideration when evaluating the effects of paraffin and silicone oil.

Experiment 1

In 4 replicates, 2548 *in vitro* matured and fertilized oocytes were randomly cultured in media overlaid with either paraffin or silicone oil batch A. On average, a significantly higher fraction of presumed zygotes cultured under paraffin oil developed into the eight-cell, compact morula and blastocyst stage (55%, 30% and 28%, respectively) as compared to those cultured under silicone oil batch A (47%, 17% and 14%, respectively) (P<0.0001) (Table1). This effect could be either less or more pronounced for individual replicates (P<0.01).

After vitrification, none of the132 grade 1 blastocysts produced under silicone oil survived, irrespective of the type of oil overlaying the post-warming culture medium (Table 2). In contrast, 50% of the embryos cultured under paraffin oil survived the vitrification procedure (P<0.0001) (Table 2). Furthermore, blastocysts produced under paraffin oil showed a higher survival rate after post-warming culture under paraffin oil than under silicone oil (59% and 40%, respectively) (*P*<0.01). No effect of the oil on the average hatching rate could be demonstrated, some replicates exhibited a higher, while others a lower, hatching rate following post-warming culture under silicone oil.

Experiment 2

In 4 replicates, 2538 *in vitro* matured and fertilized oocytes were randomly cultured in media overlaid with either paraffin oil or silicone oil batch B. The developmental rate of presumed zygotes to the eight-cell stage was not affected by the type of oil covering the culture medium. However, the total developmental rate of the presumed zygotes into compact morulae and blastocysts was higher in the medium overlaid with paraffin oil than with the silicone oil batch B (P<0.01 and P<0.05, respectively) (Table 3).

After vitrification, the average survival rate of blastocysts cultured under paraffin oil was higher than that of blastocysts cultured under silicone oil batch B (53% and 41%, respectively) (P<0.05). This was mainly due to the lower survival rate of vitrified embryos that were cultured under silicone oil and post-warming cultured under paraffin oil (Table 4).

The overall hatching rate of the surviving blastocysts cultured under paraffin oil (39/100=39%) or silicone oil (17/54=31%) was not significantly different. Also, the post-warming type of oil did not significantly affect the hatching rate (Table 4).

Table 2. Survival rate of cattle embryos cultured under different types of oil (IVC-1), vitrified, warmed and recultured under different types of oil (IVC-2)

Replicate	Number of surviving blastocysts/ Number of vitrified blastocysts (Number of hatched blastocysts after vitrification)			
	IVC-1 under silicone oil (Batch A)		IVC- 1 under paraffin oil	
	IVC-2 under silicone oil	IVC-2 under paraffin oil	IVC- 2 under silicone oil	IVC- 2 under paraffin oil
1	0/25 (0)	0/26 (0)	14/31 (0)	23/32 (6)
2	0/17 (0)	0/18 (0)	13/34 (2)	23/39 (8)
3	0/2 (0)	0/2 (0)	8/17 (4)	13/17 (3)
4	0/20 (0)	0/22 (0)	8/26 (3)	13/34 (3)
Total number of surviving embryos (%)	0/64 (0%)[a]	0/68 (0%)[a]	43/108 (40%)[b]	72/122 (59%)[c]
Total number of hatched embryos (%)	0 (0%)[d]	0 (0%)[d]	9/43 (23%)[e]	20/72 (28%)[e]

[a-c]: Values in rows with different superscripts are significantly different (P<0.01)

Table 3. *In vitro* development of IVM/IVF cattle oocytes in culture medium overlaid with paraffin oil or silicone oil (batch B)

Replicate	Type of oil	Number of oocytes	Number of cleaved cells (%) at day 3	Number of eight-cells (%) at day 3	Number of compact morulae (%) at day 6	Number of blastocysts (%) at day 9
1	Paraffin	308	218 (71)	136 (44)	58 (19)	52 (17)
	Silicone	285	203 (71)	117 (41)	45 (16)	40 (14)
2	Paraffin	339	232 (68)	144 (42)	85 (25)	79 (23)
	Silicone	355	242 (68)	149 (42)	60 (17)	59 (17)
3	Paraffin	157	120 (76)	72 (46)	39 (25)	37 (24)
	Silicone	170	119 (70)	74 (44)	23 (14)	22 (13)
4	Paraffin	477	291 (61)	166 (35)	85 (18)	79 (17)
	Silicone	447	271 (61)	168 (38)	82 (18)	77 (17)
Total	Paraffin	1281	861 (67)	518 (40)	267 (21)[a]	247 (19)[c]
	Silicone	1257	835 (66)	508 (40)	210 (17)[b]	198 (16)[d]

[a-d]: Values in columns with different superscripts are significantly different ([a,b] $P< 0.01$, [c,d] $P<0.05$)

Table 4. Survival rate of cattle embryos cultured under different types of oil (IVC-1), vitrified, warmed and recultured under different types of oil (IVC-2)

Replicate	Number of surviving blastocysts/ Number of vitrified blastocysts (Number of hatched blastocysts after vitrification)			
	IVC-1 under silicone oil (Batch B)		IVC- 1 under paraffin oil	
	IVC-2 under silicone oil	IVC-2 under paraffin oil	IVC- 2 under silicone oil	IVC- 2 under paraffin oil
1	2/10 (1)	4/10 (0)	12/19 (5)	17/25 (6)
2	11/20 (4)	5/21 (0)	11/26 (3)	14/26 (7)
3	5/9 (3)	3/10 (0)	9/15 (4)	6/14 (1)
4	13/25 (5)	11/26 (4)	14/32 (6)	18/32 (7)
Total number of surviving embryos (%)	31/64 (48%)[a]	23/67 (34%)[b]	46/92 (50%)[a]	55/97 (57%)[a]
Total number of hatched embryos (%)	13/31 (42%)	4/23 (17%)	18/46 (39%)	21/55 (38%)

[a-c]: Values in rows with different superscripts are significantly different ($P<0.01$)

Experiment 3

From the 8 replicates (2641 oocytes) performed in experiments 1 and 2 under paraffin oil, 622 blastocysts (24%) were produced during 9 days of culture (day of fertilization=day 0). Sixty-seven percent of these blastocysts developed between 144 and 168 hours after fertilization while the remaining embryos (33%) attained the blastocyst stage after 168 hours post fertilization. The same trend of blastocyst formation was observed for the embryos cultured under silicone oil batch B (Table 5).

The survival rate of the blastocysts after one-step vitrification and post-warming culture under paraffin oil was highly dependent on the age at which the blastocysts were attained ($P<0.001$). Compared to the highest survival rate (93%) of the blastocysts which were vitrified after 144 hours post fertilization, a decreasing trend was already noted for those vitrified after 152 and 160 hours post fertilization (63% and 63%, respectively), whereas the survival rate decreased sharply to 38% ($P<0.001$) for the blastocysts which attained this stage 168 hours after fertilization (Table 5). No significant difference in the hatching rate of the blastocysts which were selected at different points of time after fertilization could be demonstrated.

Discussion

The detrimental effect of silicone oil on embryonic development and in particular on embryo survival after cryopreservation is obvious from the results of this report. Blastocyst formation rate in culture medium overlaid with silicone oil batch A was significantly lower than in the medium covered with paraffin oil. However, the percentage of the zygotes developing into blastocysts under silicone oil batch A is still within the acceptable range of *in vitro* embryo production [3, 20]. Moreover, blastocysts produced in oviduct co-culture medium under silicone oil have previously been transferred in large numbers and were capable of producing an initial pregnancy rate of 56% and a calving rate of 33% [17]. Morphological distinction of the blastocysts produced under silicone oil and paraffin oil is almost impossible except for the fact that the embryos which have developed under silicone oil frequently exhibit a darker appearance under the microscope. This difference is more pronounced in the post-warming culture medium after vitrification. It is possible that the lower quality of blastocysts produced under silicone oil may have been responsible for the rather low calving rate which was obtained in the earlier study [17], although it must be stressed that despite a somewhat darker appearance, the embryos seemed morphologically normal.

Evidence of oil toxicity can be observed by rapid sperm death [1] or by membrane lysis of embryonic cells and failure of embryonic development [Van Soom *et al.*, unpublished observation]. The inherent toxic effect of silicone oil, which was already visible in a lower blastocyst formation, appeared to be detrimental when the blastocysts were cryopreserved. None of the blastocysts developed under silicone oil batch A could tolerate the vitrification procedure after 1 minute exposure to EFS solution, while

Table 5. Relationship between the age of cattle embryos produced under paraffin oil or silicone oil (batch B) and their survival after one-step vitrification

		Age of blastocysts (hours post fertilization)				
	Type of oil	144	152	160	168	≥168 to 216
Blastocyst formation rate (%)	Paraffin	27/622 (4)	79/622 (13)	201/622 (32)	112/622 (18)	203/622 (33)
	Silicone*	8/210 (4)	11/210 (5)	70/210 (33)	42/210 (20)	79/210 (38)
Number survived/ vitrified (%)	Paraffin**	13/14 (93)[a]	26/41 (63)[a]	65/104 (63)[a]	23/60 (38)[b]	
	Silicone***	2/4 (50)	2/5 (40)	15/34 (44)	12/21 (57)	
Number hatched/ survived (%)	Paraffin**	6/13 (46)	9/26 (35)	9/65 (29)	6/23 (26)	
	Silicone***	1/2 (50)	0/2 (0)	7/15 (46)	5/12 (42)	

[a-b]: Values in rows with different superscripts are significantly different ($P<0.001$)
*: Data are pooled of 4 replicates (Table 4, IVC-1 under silicone oil)
**: Data are pooled of 8 replicates (Tables 2 & 4, IVC-1 & IVC-2 under paraffin oil)
***: Data are pooled of 4 replicates (Table 4, IVC-1 & IVC-2 under silicone oil)

59% of the blastocysts cultured under paraffin oil re-expanded after 72 hours of post-warming culture under paraffin oil (Table 2). Ultrastructural changes possibly occur in the developing embryos during the culture period under silicone oil thus resulting in a very low viability of the cryopreserved embryos.

Unexpectedly, embryo development and survival after one-step vitrification was much better when the embryos were cultured under another batch of silicone oil (batch B). The type of oil did not interfere with the development of fertilized oocytes to eight-cell stage. However, silicone oil batch B reduced the developmental capacity of the presumed zygotes into compact morulae and blastocysts (Table 3). Also a high percentage of blastocysts cultured under silicone oil survived vitrification (41%), but this was still significantly lower than the survival rate of the embryos produced under paraffin oil (53%).

Factor(s) responsible for the extreme sensitivity to cryopreservation of the embryos produced under silicone oil are not yet known. The interaction of the oil covering the drops of medium with some substances present in the medium, like oestradiol and progesterone, has already been reported [11, 19]. However, the substances which diffused out of the drops of medium into the oil and the degree of diffusion were similar for both paraffin and silicone oil [11]. Differences observed in this report between the two batches of silicone oil illustrate the variability in the preparations of different batches of silicone oil. Blastocyst formation rate and resistance to vitrification were highly improved when batch A of silicone oil was replaced with batch B, yet this was still significantly lower than the survival rate of blastocysts developed under paraffin oil, suggesting the toxic potential of silicone oil for *in vitro* embryo production. Further investigations have to be performed to understand the mechanism of oil toxicity.

Another parameter of *in vitro* embryo production which was studied in this report is the relationship between the age at which blastocysts are attained and their sensitivity to cryopreservation. As demonstrated in Table 5, embryos which had developed earlier into blastocysts exhibited a significantly higher survival rate after vitrification. Similar results were observed following conventional methods of freezing [4]. Developmental stage-dependent cryopreservability of *in vitro* produced embryos has already been reported [5, 12, 20]. Embryos at more advanced stages of development showed better resistance to cryopreservation. Our results together with the results of our previous experiments [9] show that both the age of the *in vitro* produced embryos and their stage of development at freezing affect the result of cryopreservation. Embryos which develop early and are selected at the expanded blastocyst stage have the best chance of surviving vitrification.

More than 30% of the *in vitro* produced embryos developed into blastocysts later than day 7 after fertilization. These embryos are non-cryopreservable for they exhibit a very low survival rate after vitrification [9]. It is necessary to investigate the potential of these embryos for further development following fresh transfers into recipients. The priority for fresh transfer or cryopreservation of different groups of *in vitro* produced embryos must be defined more exactly.

In conclusion, failure of the embryos which were cultured under silicone oil batch A to resist the vitrification procedure demonstrates the significant importance of every single step followed during *in vitro* embryo production. Successful cryopreservation of *in vitro* produced embryos requires a careful reconsideration of routine techniques used for *in vitro* embryo production.

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