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Evaluation of a small volume oil-free in vitro production system for bovine embryos

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Abstract

Background:

Embryos are usually produced in culture systems with an oil overlay, which conveys protection against the evaporation of water and microbial contamination. The oil can also release toxic substances and absorb essential components, such as hormones, which adversely affect the quality of the oocytes and the development of embryos in vitro.

Objective:

The aim of this study was to validate an oil-free bovine in vitro production (IVP) system.

Method:

Cumulus–oocyte complexes collected from abattoir-derived ovaries were matured, fertilized and cultured employing a standard system. The quantity of medium in both groups (with and without an oil overlay) and throughout all stages of IVP was maintained at a volume of 100 μl. The oil group was covered with paraffin oil. The maturation stage of oocytes was assessed using fluorescence staining after 24 hr and developmental stages of embryos were evaluated on day 8. The expanded day 8 blastocysts were assessed by live–dead staining.

Results:

Oocytes matured in the absence of an oil overlay had significantly higher maturation rates when compared against matured oocytes in medium with an oil overlay. Steroid concentration is higher in medium after maturation without oil cover. The developmental rate was significantly higher after culture without oil overlay. The total cell number and the live–dead ratio was not significantly different. The osmolality did not differ between both groups during maturation and slightly decreased during culture without oil.

Conclusion:

Based on the current study, bovine oil-free IVP systems can be suggested as an alternative to oil-covered medium.

KEYWORDS

bovine, in vitro production, oil overlay

1 | INTRODUCTION

Currently, in cattle breeding, in vitro production (IVP) of embryos is one of the most important biotechnologies. According to the data provided by the International Embryo Technology Society, the number of transferred bovine embryos produced worldwide rose from around 139,000 in 2000 to over 740,000 in 2018. Beside the significance of cattle as a source of high-quality food, it is a relevant animal model in research, including its similar reproductive physiology to humans. The embryos of both species (human and cattle) are similar with respect to the microtubule organization during fertilization, metabolic requirements and interaction with used culture conditions as well. Moreover, the use of the bovine model is important in the light of the ethical and legal restrictions in human embryo work (Anderiesz et al., 2000; Menezo & Herubel, 2002; Navara et al., 1995; Neuber & Powers, 2000; Wrenzycki et al., 2005).

As the first reported live bovine offspring was produced totally in vitro (Fukuda et al., 1990), this method has been further refined into a routine procedure. It does, however, still not fully mimic the in vivo process, resulting in reduced quality of gametes and embryos, as well as a limited output with only 30%–40% developing blastocysts (Rizos et al., 2002; Sutton et al., 2003). Changes in the in vitro environment in which the oocytes and embryos are cultured can have a significant effect on this (Lonergan et al., 2003; Wrenzycki, 2016). The most commonly used system is a microdrop culture method, where drops of medium are covered with an oil overlay, because of the simple assessment of cumulus–oocytes complexes (COCs) and embryos (Tae et al., 2006). Furthermore, protection from bacterial contamination, absorption of lipophilic toxic compounds, avoidance from evaporation of the medium, maintenance of osmotic pressure and pH value (Martinez et al., 2018) are usually quoted as beneficial impacts of the drop system. Three different types of oil are used for covering the drops, silicone oil, mineral oil and paraffin oil. Silicone oil is an organic polymerized siloxane, usually polydimethylsiloxane, compared with mineral oil which is a heavy hydrocarbon. Mineral and paraffin oil are basically the same product, even paraffin oil is higher purified and contains more saturated hydrocarbons as reviewed by Morbeck (2012). However, it is known that an overlay of oil is also able to negatively influence IVP (Otsuki et al., 2009; Van Soom et al., 2001). If gametes and embryos are cultured without oil overlay to avoid these undesirable effects, large amounts of media have to be used in order to elude an increase in osmolality (Gasperin et al., 2010). Thus, particular compounds derived from gametes or embryos can be diluted (Canseco et al., 1992; Hoelker et al., 2009). Depending on the individual background, it has to be decided which are the more acceptable effects on the gametes or embryos.

In cases where only limited samples are available, such as from individual animals, cloned or gene edited embryos or from primates, and the negative effects of oil overlay should be avoided, special attention needs to be taken to ensure that the media remain present.

Therefore, the aim of the study was to evaluate an oil-free IVP system with small volumes of medium and the bovine was used as an appropriate model for outlined cases above.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Groups of 20 COCs (abattoir-derived ovaries) were matured (IVM), fertilized (IVF) and cultured (IVC) in vitro as explained in paragraph 2.2.

The quantity of medium in both groups (total IVP with and without an oil overlay) and throughout all stages of IVP was carried out at a volume of 100 µl. The oil group was covered with 75 µl paraffin oil (IVF Bioscience, Falmouth, UK). As a complement, medium (100 µl) was covered with oil (75 µl) in single parts of IVP (A: IVM without oil + IVF/IVC with oil; B: IVM/IVF without oil + IVC with oil; C: IVM/IVF with oil + IVC without oil; D: IVM with oil + IVF/IVC without oil). Each run was performed in duplicate and experiments were performed over several weeks to avoid an effect due to the week. Four wells of a microtitre strips (Thermo Fisher Scientific Germany) with flat bottom were used as culture dish and placed in a petri dish surrounded with approximately 3 ml distilled water (Figure S1). The maturation stage of oocytes was assessed using fluorescence staining (Hoechst bisbenzimide 33342, Sigma-Aldrich, Germany) after 24 hr of maturation. In addition, IVM medium was analysed via radioimmunoassay after 0 hr (control) and 24 hr (with and without COCs) to measure 17 β-oestradiol (E2) and progesterone (P4) concentrations. Pools of each media type were generated from each duplicate. The developmental stage was assessed on day 8 (total IVP with or without an oil overlay/single steps of IVP covered with oil). The quality of expanded day 8 blastocysts (total IVP with or without an oil overlay) was determined by live–dead staining (total cell number as well as ratio of live and dead cells).

2.2 | Collection of cumulus–oocyte complexes and in vitro production

The in vitro production of embryos was performed using the procedure outlined by Abele et al. (2014).

Abattoir-derived ovaries of healthy cows were transported to the laboratory in thermal tanks filled with 30°C phosphate buffered saline (Abele et al., 2014). The COCs were obtained via the slicing method categorized and then selected as previously described (Blaschka et al., 2019). The selected COCs, according to morphological criteria, were transferred in groups of 20–100 µl maturation medium with or without oil overlay. The COCs matured for 24 hr at 39°C and 5% CO2 in a humidified atmosphere.

Groups of matured COCs were transferred and fertilized in Fert-TALP medium supplemented with hypotaurine, epinephrine, heparin and BSA. Thawed semen was used. The COCs and semen (1 x 10^6 spermatozoa ml-1) were co-incubated for 19 hr with identical gas and temperature conditions as used for maturation.

After fertilization, presumptive zygotes were gently denuded. Afterward, they were cultured in synthetic oviduct media with amino acids (SOFaa) supplemented with BSA (FAF) for 8 days at 39°C under O_2-reduced conditions (5% O_2 and 5% CO_2).

The development rates of the embryos were determined morphologically on day 8 of cultivation (percentage of oocytes, which had developed to blastocysts) was recorded.

2.3 | Maturation rate of oocytes

The evaluation of nuclear maturation success was realized using Hoechst bisbenzimide 33342 (0.004%; Sigma-Aldrich, Germany) staining as previously described (Blaschka et al., 2019). Oocytes were classified as matured, when they had reached the metaphase II stage as characterized by the metaphase plate and the occurrence of the first polar body. Oocytes in metaphase I stage presenting chromosomes at the metaphase plate of the first meiotic division, these were considered as immature.

2.4 | Live–dead staining

In addition, a live/dead staining was performed with expanded blastocysts on day 8 according to Stinshoff et al. (2011). Ethidium homodimer (Invitrogen, USA) and Hoechst bisbenzimide 33342 were used at concentrations of 0.01% and 0.004%, respectively, to differentiate between live and dead cells. Hoechst is able to pass intact cell membranes and stains this blue. Ethidium homodimer, in contrast, is just capable to pass injured membranes to stain the cells red. Two to three expanded blastocysts (n = 2–3/group) were stained per pass. The assessment of the stained blastocysts was achieved using a fluorescence microscope Eclipse Ci (Nikon, Düsseldorf, Germany). Image software NIS-Elements D version 5.02. (Nikon, Düsseldorf, Germany) was used to accomplish the cell counting.

2.5 | Analysis of medium

Concentrations of P4 and E2 in IVM medium were determined in duplicates using established radioimmunological methods after extraction of the samples with organic solvents. The measurement of P4 followed the procedure outlined by Hoffmann et al. (1973) as previously described (Klein et al., 2003). Samples (200 µl) were extracted twice with 2 ml hexane. The antiserum applied was generated against 4-pregnene-11a-ol-3,20-dione-hemisucciate-BSA. Intra- and inter-assay coefficients of variation were 8.8 and 8.9% respectively. The limit of quantification was 0.5 ng/ml.

Measurements of E2 were performed as previously described (Klein et al., 2003). Medium samples (200 µl) were extracted twice with 2.5 ml toluene. The antiserum was directed against oestradiol-17b-6-carboxymethyloxim-BSA. The limit of quantification was 12.5 pg/ml. Intra- and inter-assay coefficients were 7.1% and 17.6% respectively. At least, 10 replicates were analysed.

In addition, the osmolality of IVM and IVC medium was analysed in 50 µl of medium using a cryoscopic osmometer (Osmomat 3000basic, gonotec, Berlin, Germany). For each measurement, technical repeats (three times) were performed.

2.6 | Statistical analyses

Statistical analyses were performed with R (version 3.5.1; R Development Core Team, 2018). All data were tested for normal distribution using a Shapiro-Wilk Test. Maturation and development rates were analysed using a binomial test because these are percentages. Data obtained from the live–dead staining were analysed using a t test. For assessment of the hormone concentration and osmolality, the non-parametric Wilcoxon test was used because of non-normal distribution. A p-value ≤0.05 was considered statistically significant.

3 | RESULTS

Numerical values are outlined in mean and standard deviation (*SD*).

3.1 | Maturation rate of oocytes and development assessment of embryos

3.1.1 | Total IVP with or without an oil overlay

Oocytes matured in the absence of an oil overlay had significantly ($p \leq 0.05$) higher maturation rates (71.5 ± 6.8%, stained oocytes n = 200) when compared against matured oocytes in medium with an oil overlay (60.2 ± 9.3%, stained oocytes n = 207).

The developmental rate on day 8 was significantly higher ($p \leq 0.05$) after culture without oil overlay (without oil: 38.4 ± 14.8%, used presumptive zygotes n = 581; with oil: 33.5 ± 12.6%, used presumptive zygotes n = 586). Both are depicted in Figure 1.

3.1.2 | Single steps of IVP covered with oil

On day 8, the development rate was not significantly changed for embryos developed in IVP medium covered with oil in single parts of the procedure (A: 28.0 ± 8.9%, n = 279; B: 27.8 ± 10.3%, n = 295; C: 23.0 ± 12.3, n = 276; D: 26.6 ± 13.6%, n = 300; n referred to as used presumptive zygotes).

3.2 | Steroid concentrations in IVM medium

After the maturation period, the E2 concentration was found to be lower in medium with an oil overlay. The mean concentrations were

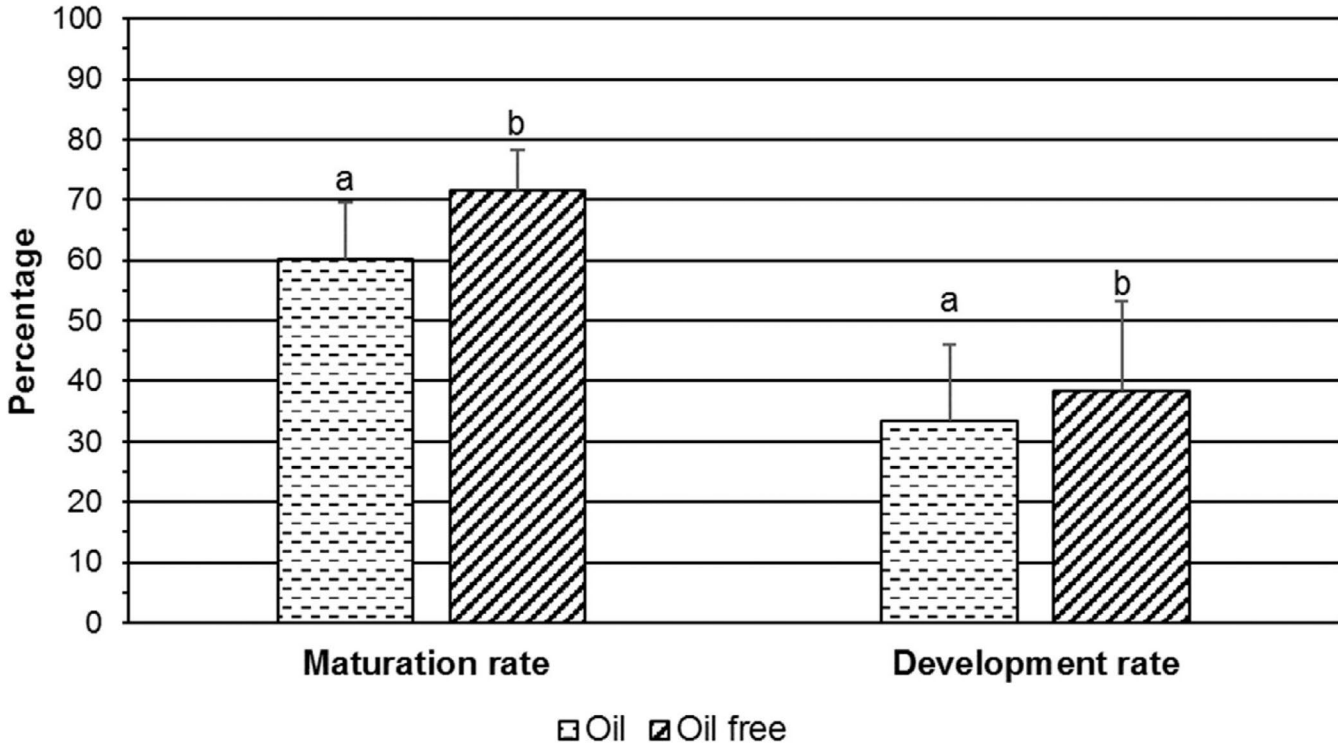

FIGURE 1 Maturation rate of COCs after 24 hr and development rate on day 8 with or without an oil overlay in the entire IVP (mean ± *SD*; a:b $p \leq 0.05$)

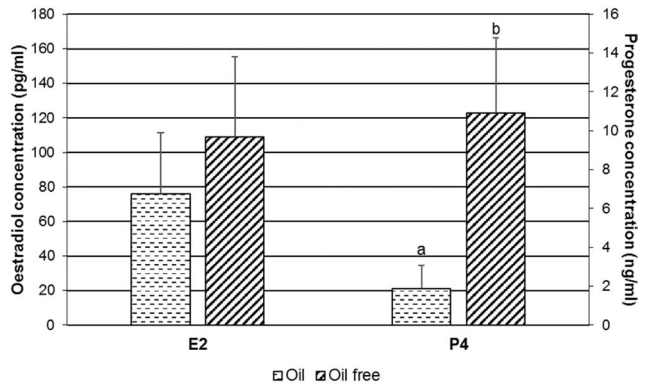

FIGURE 2 Steroid concentration in IVM medium after 24 hr of maturation (E2: 17ß-oestradiol, $p = 0.099$; P4: progesterone, a:b $p \leq 0.05$; mean ± *SD*)

76.0 ± 35.4 pg/ml and 109.0 ± 46.4 pg/ml ($n = 10$) in media with or without oil covering respectively. Numerical values appear to be different, but due to the high variability of the data within the test groups, statistical significance was not achieved (median: with oil: 65.6 pg/ml; without oil: 106.2 pg/ml).

The P4 concentration was significantly lower in medium covered with oil (1.9 ± 1.2 ng/ml) compared with medium without overlay (10.9 ± 3.9 ng/ml; $p \leq 0.05$; $n = 11$). The results are shown in Figure 2.

In medium analysed before incubation (0 hr) and after 24 hr of maturation without COCs, E2 and P4 concentrations were under the limit of quantification (E2: 12.5 pg/ml; P4: 0.5 ng/ml).

3.3 | Live–dead staining

The total cell number and the live–dead ratio were not significantly different (total cell number: without oil: 129.0 ± 30.1, with oil: 119.0 ± 30.0; live–dead ratio: without oil: 22.6 ± 19.7, with oil: 18.0 ± 8.0) as depicted in Figure 3. Descriptive data of the assessment after covering only single steps of IVP with oil (group A-D) are depicted in Table S1.

3.4 | Osmolality

The osmolality of IVM medium did not differ between both groups (with and without oil) and the control before maturation. After 8 days of culture, the osmolality was significantly lower in medium without an oil overlay ($p \leq 0.05$) compared with the medium with overlay and the control (0 hr). The results are shown in Table 1.

4 | DISCUSSION

Currently, the application of oil overlays is common practice in IVP systems because it has advantages in terms of work flow and the assessment of gametes and embryos. It can, however, take up lipophilic steroid hormones, which might negatively affect IVP outcome.

Steroids, especially E2 and P4 have a crucial role such as for follicular development in physiological oestrus cycles (Forde et al., 2011).

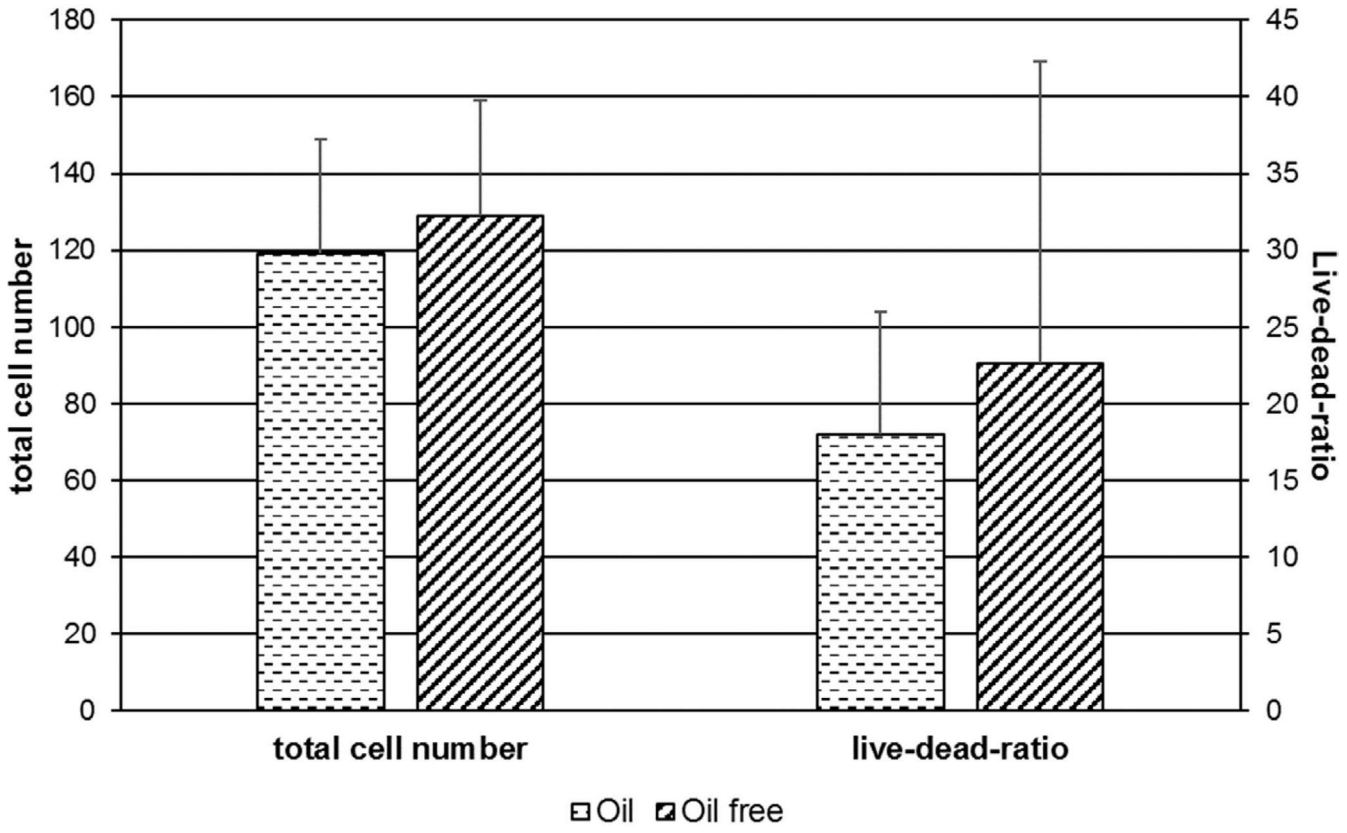

FIGURE 3 Total cell number and live–dead ratio of expanded blastocysts after IVP with or without oil respectively (with oil: $n = 25$; without oil: $n = 26$; mean ± *SD*; $p > 0.05$)

TABLE 1 Osmolality of IVP media (without COCs) with and without an oil overlay (mean ± *SD*)

	Osmolality (mOsm/kg H_2O)	Replication (*n*)		Osmolality (mOsm/kg H_2O)	Replication (*n*)
0 hr (before incubation)	287.8 ± 3.1	4	0 hr (before incubation)	275.2 ± 2.6[a]	5
24 hr IVM with oil overlay	289.7 ± 2.8	6	8 days IVC with oil overlay	275.5 ± 2.7[a]	5
24 hr IVM without oil overlay	288.6 ± 2.7	6	8 days IVC without oil overlay	270.3 ± 2.3[b]	5

Note: IVC, in vitro culture; IVM, in vitro maturation; a:b $p \leq 0.05$.

During the final maturation, a switch from E2 to P4 dominance takes place in the follicle (Dieleman et al., 1983). Mingoti et al. (2002) demonstrated that cumulus cells of COCs are able to secret E2 and P4 into the medium when they are matured in a medium enriched with bovine serum albumin (BSA) and gonadotropins. A pattern similar to that observed in vivo has, however, not been demonstrated and the role of these steroids during IVM remains controversially discussed (Blaschka et al., 2019).

Improving effects of E2 on the resumption of meiosis, increased maturation rates in bovine IVM and enhanced fertilization as well as cleavage rates in human IVM has been reported (Fukui et al., 1982; Tesarik & Mendoza, 1995). A potential impact on nuclear and cytoplasmic maturation (reduced chromosome aberration, stimulated cytoplasmic maturation) has been suggested for P4 (Fair & Lonergan, 2012; Sirotkin, 1992).

In the present study, an obvious reduction in E2 and P4 concentration in medium covered with oil was demonstrated, which is likely due to absorption by the oil overlay. This clearly confirms previous results from IVM with pig COCs (Shimada et al., 2002). Clemente et al. (2009) also showed a reduction in P4 in medium used for bovine embryo culture covered with oil, but with no effect of the P4 concentration on the blastocyst development or quality, even if exogenous P4 was supplemented. However, during maturation, Xu et al. (1988) showed a decreased E2, but not P4 concentration. The kind of oil used for covering has an effect on the steroid concentration as well (Miller & Pursel, 1987). It has been shown that silicon oil takes up seven times more E2 than paraffin oil. In the present study, paraffin oil dedicated to IVP was used. Reinsberg et al. (2004) also reported a different absorption of E2 versus P4 and an effect of the oil/medium ratio in a human system. They suggested a lower

lipophilicity of E2 compared with P4, which might also explain the higher absorbed concentrations of P4 than of E2 in the current study (Figure 2).

Moreover, a delayed nuclear maturation of oocytes in vitro in a system with oil overlay has been reported for pig (Shimada et al., 2002) and a delayed meiosis progression after in vitro follicle culture in mouse oocytes (Segers et al., 2008). In the present study, a significantly reduced maturation rate has been found in the presence of an oil cover.

One of the core factors during in vitro production of embryos is the osmolality. An increasing osmolality has an adverse effect on oocyte maturation and fertilization (Lim et al., 1994), high concentration of sodium chloride in IVF medium can increase the occurrence of polyspermy (Roh et al., 2002) as well as affecting embryo development (Liu & Foote, 1996). A beneficial effect of the oil overlay is maintaining the osmotic pressure. However, Swain (2018) determined a significantly increased osmolality after 6 days of culture in medium covered with different types of oil (mineral and paraffin). To beware an oil overlay and avoid evaporation of medium, large volumes of medium will be usually used. Conversely, with large volumes, important compounds such as paracrine and autocrine factors for embryo interaction can be diluted (Canseco et al., 1992; Martinez et al., 2018; Nagao et al., 2008). Gasperin et al. (2010) showed that an increase in the osmolality in 400 μl medium can be avoided by adding water in the central hole of a four well plate. In an IVP system used for pigs, it could be clearly demonstrated that osmolality can be stabilized if the medium is surrounded by water. But in contrast, no differences could be determined for the maturation or development rate in both systems (with or without oil; (Martinez et al., 2015)). In the present study, the osmolality remained constant during the maturation phase and slightly decreased during culture. However, the osmolality is still in the acceptable range of the used medium (culture medium 270–280 mOsm; Holm et al., 1999).

In the present study, maturation and development rates were higher in the system without an oil overlay, and no negative effect on the quality of the embryos (live–dead ratio, total cell number) was observed in a small volume system. Van Soom et al. (2001) determined a considerable decrease in the development rate (up to 12%), if the bovine IVP system was not covered with oil, with no further detailed suggestions. For pigs no difference in development rate (with oil: 34.2 ± 10.7% vs. with oil: 34.6 ± 16.4%) can be detected, if the osmolality is not changed during culture (Martinez et al., 2015). The total cell number of blastocyst can be affected by their environment (in vivo vs. in vitro (Ushijima et al., 2008); different culture systems (Mucci et al., 2006; Nedambale et al., 2004)). However, if single parts of the IVP system in the present study were covered with oil, the development rates and quality did not change. This could suggest that an oil-free maturation is not the key factor.

The presence or absence of oil per se did not alter the output of an IVP system. If oil is used, however, the type of oil (Martinez et al., 2018; Van Soom et al., 2001) and a potential treatment of the used oil (Tae et al., 2006) have an impact on embryo development and quality. Also, the batch of the oil, especially for silicon oil ((Van Soom et al., 2001); own observations) and the storage (light and temperature; (Otsuki et al., 2009)), affect the results. In contrast, Labied et al. (2019) could not determine an effect of the tested oils on the rate of pregnancy per fresh transfer, live birth rate per fresh transfer as well as miscarriage rate after IVF in women. However, within oil-covered system, components can be shifted from one medium drop to another, which has to be kept in mind, especially in a research context (Miller & Pursel, 1987). Furthermore, the used oil can contain toxic compounds able to transfer to the medium as reviewed by Martinez et al. (2018). Added chelating agents (EDTA or BSA) as well as haemoglobin (nitric oxide scavenger) and co-culture systems are partially able to resolve toxic effects (Lee et al., 2004; Tae et al., 2006; Van Soom et al., 2001). On the other hand, oil may absorb embryotoxic substances (Miller et al., 1994).

5 | CONCLUSION

An oil overlay on medium used for IVP is usually done because of its protective effects. However, oil covering is able to influence the outcome in a significant way. It is still an undefined product with different composition and purity. In the current study, it has been shown that an oil overlay is dispensable during the whole procedure even with small volumes of medium, with a positive effect on maturation and development rate as well as the steroid concentration in maturation medium. A negative effect on the embryo quality could not be demonstrated. Nevertheless, there is need for investigations to assess whether the presence or absence of an oil overlay has an impact on gene expression patterns.

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CONFLICT OF INTEREST

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all authors.

AUTHOR CONTRIBUTION

Carina Blaschka: Conceptualization; Formal analysis; Project administration; Writing-original draft. **Sophie Diers:** Investigation; Writing-review & editing. **Mariya Aravina:** Formal analysis; Investigation. **Swantje Geisler:** Formal analysis. **Gerhard Schuler:** Investigation; Validation; Writing-review & editing. **Jens Tetens:** Formal analysis; Resources; Supervision; Writing-review & editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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