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Embryo production study, week 4, 2022.

Project:

Effect of bovine spermatozoa preparation on embryonic development *in vitro*

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Overview

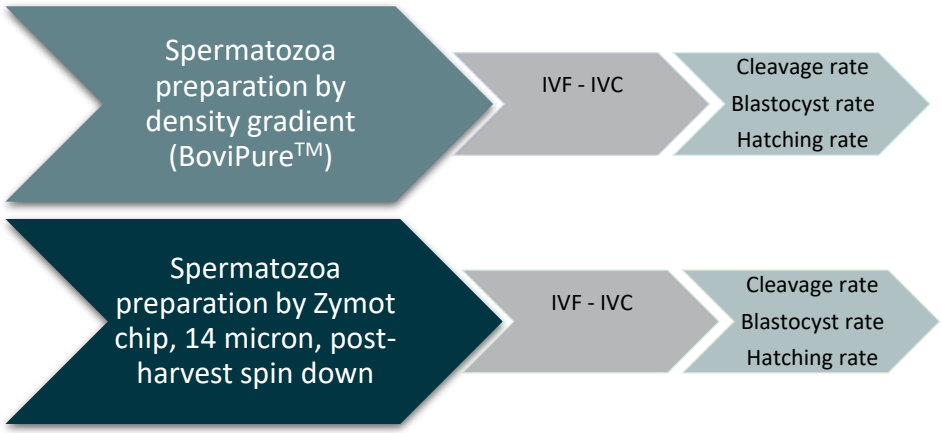
1. Purpose of the study:

1.1. The purpose of this study was to investigate the effect of spermatozoa preparation on bovine embryonic development *in vitro.* It was performed by comparing the effect of density gradient centrifugation and Zymot fertility chip preparation of spermatozoa on the blastocyst rates after *in vitro* fertilization.

1.2. Study setup: Two parallel groups of bovine IVF, where the only difference was the preparation of the spermatozoa.

Group 1: Spermatozoa were prepared by density gradient centrifugation (BoviPure™, Nidacon International AB, Sweden)

Group 2: Spermatozoa were prepared by a Zymot fertility chip, prototype, 14 micron pores, followed by a post-harvest spin down step.



2. Materials and Methods

2.1. Collection of cumulus-oocyte complexes (COC) and in vitro maturation (IVM)

All media were from IVF Bioscience, United Kingdom, unless otherwise stated.
Bovine ovaries were collected at a local abattoir (Danish Crown, Holsted) and transported to the laboratory in physiological saline (0.9%) at 33°C within 3 h after slaughtering.

OBS: Due to Covid-19 restrictions, we were not allowed to enter the slaughterhouse to pick up the ovaries, as we usually do, but we had to wait until the ovaries reached the end of the slaughterhouse. This led to an uncontrolled cooling down of the ovaries and a significantly prolonged time, before the oocytes were aspirated, on Monday 24th January.

Cumulus-oocyte complexes (COCs) were aspirated from 2 to 150 mm diameter follicles using 18G needles attached to a vacuum pump. The oocytes were washed three times in Wash and one time in IVM medium, mixed, and randomly distributed into groups of oocytes going into each well. *In vitro* maturation was performed with BO-IVM medium, and oocytes were incubated in groups of 30-45 oocytes in one well with 500 µl BO-IVM medium at 38.8°C and 6% CO_2 in atmospheric air for 21-24 hours.

2.2. Spermatozoa preparation

Frozen semen from two bulls (Noble and Logan) of known *in vivo* fertility was used for this study. Six straws were thawed and mixed in a non-toxic 2 ml Eppendorf Tube. From this semen mixture 1 ml was mixed with 1 ml BO-SEMENPREP. 850 µl of this suspension were loaded on the Zymot chip (14 micron prototype) inlet with a MEA tested 1 ml syringe. Hereafter, approximately 750 µl of warm media (BO-SEMENPREP) were added onto the membrane surface and the outlet port was primed with media. The chip was then incubated in a humid incubator at 38 °C for 30 minutes. After 30 minutes, 500 µl sperm-containing media were collected from the outlet and added to one tube and centrifuged at 300 *g* for 5 minutes and the supernatant was removed down to the 100 µl line. The pellet was resuspended, and the concentration was determined with a Makler counting chamber.

Density gradient centrifugation was performed according to the manufacturer's instructions (Nidacon International Ab, Göthenburg, Sweden). At room temperature, in a 10 mL centrifuge tube, 500 µl of 80 % BoviPure® was placed in the bottom of the tube and carefully, 500 µl of the 40% Bovipure® were added on top. 250 µl of the semen mixture was carefully added on top and the tube was centrifuged for 15 minutes at 300 *g*. Hereafter, everything but the pellet, was aspirated and removed and the pellet was aspirated and moved to a new tube with 1 ml of BoviWash (Nidacon). The pellet was resuspended, and then centrifuged for 5 minutes at 300 *g*. The supernatant was removed, and the pellet resuspended in approximately 100 µl and the concentration determined with a Makler counting chamber.

2.3. In vitro fertilization (IVF) and culture (IVC)

The COCs were evaluated for cumulus expansion and viscoelasticity and then washed in BO-IVF medium. The group of COCs were placed in a well with 400 µl BO-IVF medium under oil. From each IVM-plate, 2 wells of oocytes were moved into a DGC plate and 2 wells were moved into a Zymot plate, to randomize the oocytes from each IVM plate. The sperm suspension was then added at

approximately 50 µl to obtain a final volume of 500 µl and a concentration of $1*10^6$ spz/ml. COCs and spermatozoa were co-incubated at 38.8 °C and 6 % CO_2 in atmospheric air for 16-20 h. Fertilized oocytes were denuded by vortexing in Wash medium at full speed for 2 minutes. Then they were washed three times in Wash medium and one time in BO-IVC medium. Fertilized oocytes were then cultured in BO-IVC medium at 38.8°C in 6% CO_2, 5% O_2 in 89% N_2.
On the 2nd day of culture (48 hours after insemination) we registered the number of cleaved embryos, on the 7th day the number of blastocysts, and on the 9th day the number of hatched blastocysts.

2.4. Statistical analysis

Initial calculations were performed in Excel and all statistical analysis were performed in GraphPad Prism.

Cleavage rate was defined as: $\frac{n_{Total\ Cleaved\ Zygotes}}{n_{Total\ COCs}} * 100\%$

Blastocyst rate was defined as: $\frac{n_{Total\ Blastocysts}}{n_{Total\ COCs}} * 100\%$

Hatched blastocyst rate was defined as:

$$\frac{n_{Total\ Hatched\ Blastocysts}}{n_{Total\ COCs}} * 100\%$$

The Kinetics score was defined as:

$$\frac{n_{Bl} * 1 + n_{XBl} * 2 + n_{HBl} * 3}{n_{Total\ Blastocysts}}$$

3. Results and discussion

This study was performed with two days of oocyte collection:
Monday 24th January and Tuesday 25th January.
An identical study setup, with two study groups (DGC vs. Zymot) were run in parallel on both days.

3.1.Study day 1: 24th January 2022

On the first day of oocyte harvesting (24th January), we collected 621 oocytes. On this day we had significant challenges on the slaughterhouse with uncontrolled low temperatures and prolonged time before oocyte aspiration, due to Covid-19 restrictions, preventing us from picking up the ovaries in the usual manner.

During maturation all oocytes were pooled in one group. On day 2, the day of insemination, the oocytes were randomly assigned to one of the two groups. Each plate of *in vitro* matured oocytes was split randomly into either group 1 (DGC) or group 2 (Zymot). The oocytes were inseminated with either DGC prepared spermatozoa or Zymot chip prepared spermatozoa.

After 16-20 hours of co-incubation with spermatozoa, the oocytes/presumptive zygotes were washed, denuded, and placed in culture medium. At 48 hours after the onset of insemination (after approximately 28 hours in culture medium) the oocytes/zygotes were evaluated for cleavage. We found a significantly lower cleavage rate (total cleaved zygotes/total oocytes) in the Zymot group (56 %) compared to the DGC group (69 %). The cleavage rate for both groups was slightly lower than expected for IVF studies run during optimal conditions.

On day 7 of culture (after insemination), the number of total blastocysts were counted (blastocysts, expanded blastocysts and hatched blastocysts), and the blastocyst rate was calculated (total number of blastocysts/total number of oocytes). We found no significant difference in the blastocyst rate between the two groups (24% vs. 21%, ns). The blastocyst rates were slightly lower than expected for IVF studies run during optimal conditions.

On day 9 of culture (after insemination), we evaluated the hatching rate (total number of hatched blastocysts/total number of oocytes). We found no significant difference in the hatched blastocyst rate on day 9 (17% vs. 18%, ns). We further calculated a kinetics score, to evaluate the embryo development kinetics. The two groups showed similar developmental kinetics (Kinetics score 1.47 vs 1.69, ns).

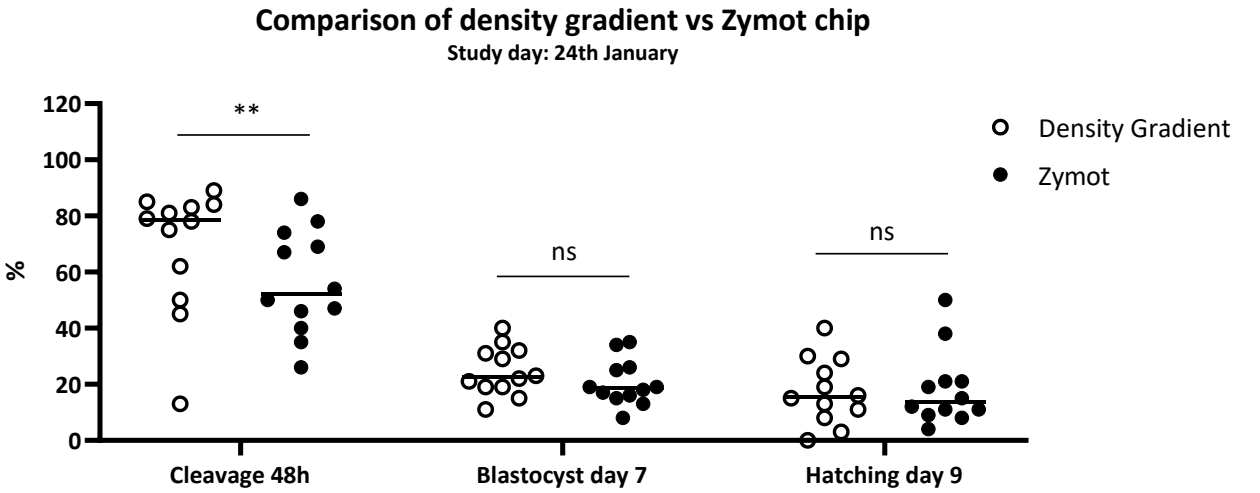


Figure 1. Bovine IVF was performed with either density gradient (DGC) or Zymot prepared spermatozoa, and the embryo outcome was evaluated by three parameters: Cleavage rate (the percentage of cleaved zygotes 48h after the start of insemination), Blastocyst rate day 7 (percentage of blastocysts; including blastocysts, expanded blastocysts and hatched blastocysts) and on day 9 the hatched blastocyst rate (percentage of hatched blastocysts relative to the total number of oocytes). Each dot represents the result from one well in a 4-well petri dish with 13-34 oocytes per well.

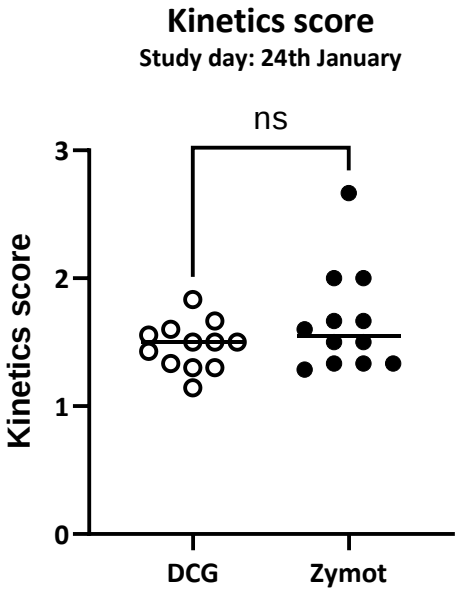


Figure 2. On day 7 all the blastocysts were scored as a blastocyst (score 1), expanded blastocyst (score 2) or hatched blastocyst (score 3), and a kinetics score was calculated (please see the equation under Materials and Methods). Each dot represents the result of one well with 13-34 oocytes per well.

Study day 24th January	Cleavage 48h	Blastocyst day 7	Hatching day 9	Kinetics score
Density gradient	69 % (222/322)	24 % (78/322)	17 % (55/322)	1.47
Zymot	56 % (168/299)	21 % (62/299)	18 % (54/299)	1.69

3.2.Study day 2: 25th January 2022

On the second day of oocyte harvesting (25th January), we collected 297 oocytes.

During maturation all oocytes were pooled in one group. On day 2, the day of insemination, the oocytes were randomly assigned to one of the two groups. Each plate of *in vitro* matured oocytes was split randomly into either group 1 (DGC) or group 2 (Zymot). The oocytes were inseminated with either DGC prepared spermatozoa or Zymot chip prepared spermatozoa.

After 16-20 hours of co-incubation with spermatozoa, the oocytes/presumptive zygotes were washed, denuded, and moved to culture medium. At 48 hours after the onset of insemination (after approximately 28 hours in culture medium) the oocytes/zygotes were evaluated for cleavage. We found no significant difference in cleavage rate (total cleaved zygotes/total oocytes) between the two groups (DGC 82 % vs Zymot 89 %, ns). The cleavage rate was satisfactory in both groups indicating a good level of fertilization.

On day 7 of culture (after insemination), the number of total blastocysts were counted (blastocysts, expanded blastocysts and hatched blastocysts) and the blastocyst rate was calculated (total number of blastocysts/total number of oocytes). We found a significantly higher blastocyst rate in the Zymot group compared to the DGC group (49% vs 34%, $p < 0.01$).

On day 9 of culture (after insemination), we evaluated the hatching rate (total number of hatched blastocysts/total number of oocytes). We found a significantly higher hatched blastocyst rate in the Zymot group compared to the DGC group (35% vs 22%, $p < 0.05$).

We further calculated a kinetics score, to evaluate the embryo development kinetics. The two groups showed similar developmental kinetics (Kinetics score 1.77 vs 1.84, ns).

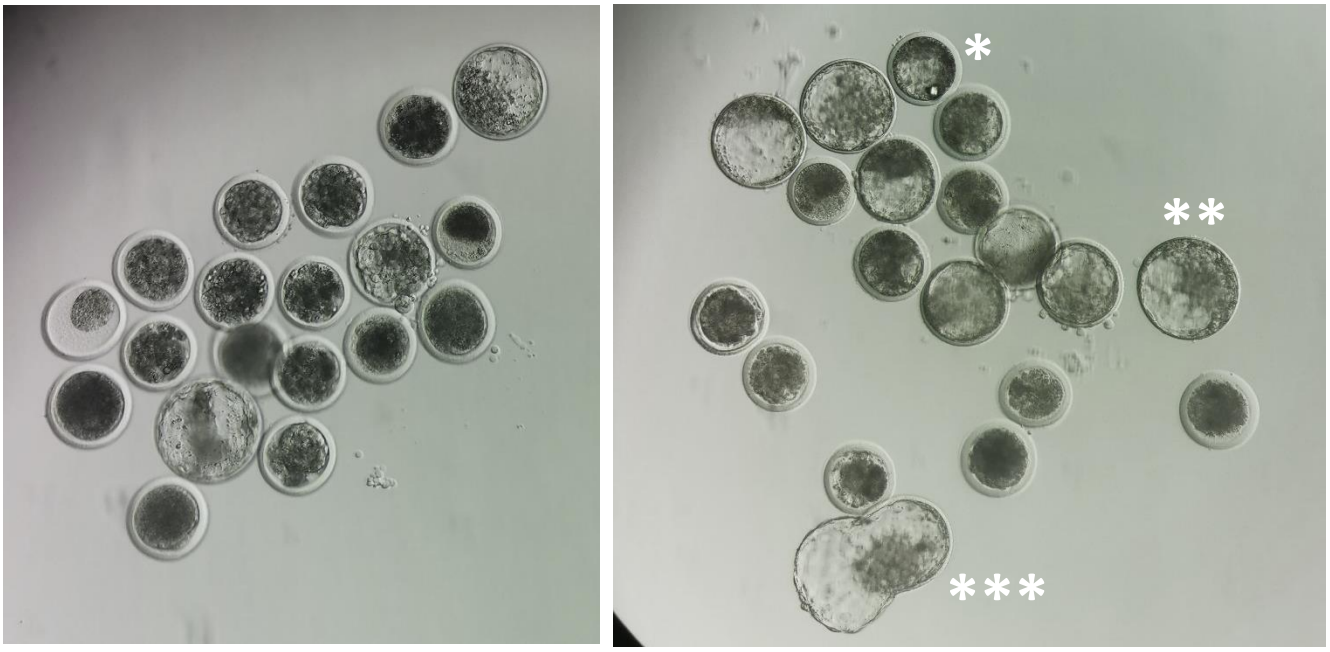


*Figure 3. Representative pictures of one well from the DGC group (to the left) and the Zymot group (to the right) at day 7, during the recording of blastocysts. Examples of the different types of blastocysts are marked with asterisks: *Blastocyst, **Expanded Blastocyst and ***Hatched blastocyst.*

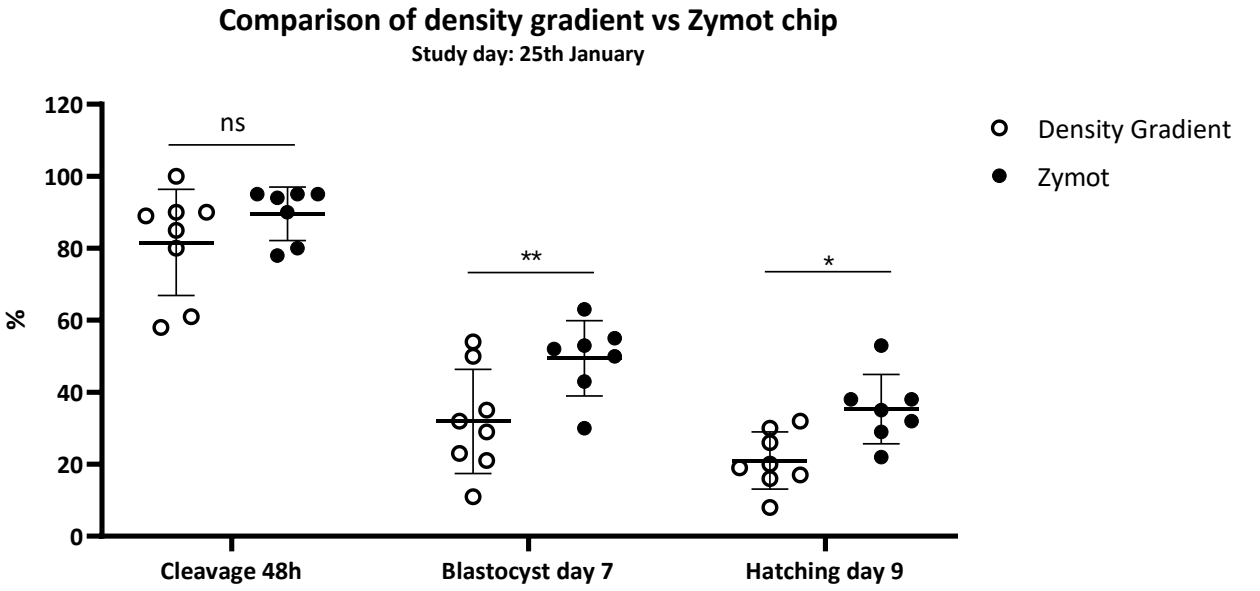


Figure 4. Bovine IVF was performed with either DGC or Zymot prepared spermatozoa, and the embryo outcome was evaluated by three parameters: Cleavage rate, Blastocyst rate day 7, and Hatched blastocyst rate day 9. Each dot represents the result from one well in a 4-well petri dish with 13-34 oocytes per well.

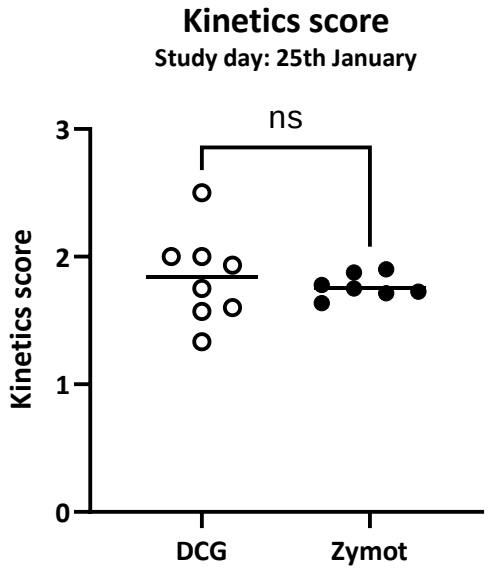


Figure 5. On day 7 all the blastocysts were scored as a blastocyst (score 1), expanded blastocyst (score 2) or hatched blastocyst (score 3) and a kinetics score was calculated (please see the equation under Materials and Methods). Each dot represents the result of one well with 13-34 oocytes per well.

Study day 25th January	Cleavage 48h	Blastocyst day 7	Hatching day 9	Kinetics score
Density gradient	82 % (130/158)	34 % (53/158)	22 % (35/158)	1.77
Zymot	89 % (124/139)	49 % (68/139)	35 % (48/139)	1.84

4. Conclusions

From study day 1, 24th January, it was clear that suboptimal study conditions (Covid-19 restrictions at the slaughterhouse, preventing us from collecting the ovaries in our normal way) affected the rates in the study, leading to lower-than-normal cleavage- and blastocyst rates. The results from this study day, revealed no difference between the two groups, in terms of blastocyst rates, and showed that under sub optimal conditions, DGC and Zymot prepared spermatozoa perform equally well in bovine *in vitro* embryo production.

For the second study day, 25th January, we optimized the collection of ovaries under Covid-19 restrictions and even with a more limited number of oocytes (n=297), Zymot prepared spermatozoa induced a significantly higher blastocyst rate at day 7 (49% vs. 34%, $p < 0.01$) and a significantly higher percentage of hatched blastocysts on day 9 (35% vs 22%, $p < 0.05$) compared to density gradient prepared spermatozoa. The results from study day 2 indicate that the Bovine Zymot chip, could be considered as a better alternative to density gradient preparation of spermatozoa for bovine *in vitro* embryo production.

However, due to the discrepancy between the two study days, we would recommend repeating the study to confirm the findings from study day 2 and obtain sufficient scientific evidence to recommend the bovine Zymot chip as a better alternative to density gradient preparation of the spermatozoa.

5. Abbreviations:

- Bl: Blastocysts
- COCs: Cumulus-oocyte complexes
- DGC: Density gradient centrifugation
- HBl: Hatched blastocysts
- IVC: *In vitro* culture
- IVF: *In vitro* fertilization
- IVM: *In vitro* maturation
- XBl: Expanded blastocysts
- Spz: Spermatozoa