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Influence of Protein Source in Culture Media for IVM, IVF and Embryo Culture on the Development of Domestic Cat Oocytes

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1 Introduction

The underlying causes of species extinction in the animal and plant kingdoms are numerous and can no longer be explained by natural evolution alone. In fact, the current extinction rate is about 1000 times the likely background rate of extinction, i.e. that without the influence of human action (Pimm et al., 2014). The causes are directly or indirectly related to the human activity, such as poaching, habitat loss due to agriculture and urban development, resource depletion, pesticides and waste in soils and waters, in addition to climate change.

For many species, captive breeding efforts offer the most likely short-term approach of ensuring their survival, such as in the case of most wild feline species which are threatened with extinction in their native ecosystems. In the last decades assisted reproduction techniques (ART), *in vitro* production (IVP) of embryos, as well as cryopreservation of gametes and embryos have become an important part of wildlife research and conservation. Understanding reproductive traits and strategies can help in particular in supporting breeding programmes in zoos as well as maintaining genetic variability in wildlife populations.

In the case of endangered feline species, the *in vitro* fertilisation (IVF) of the domestic cat can be studied, because the domestic cat represents a reliable and widely used model organism due to its similarities. Here the continuous improvement of *in vitro* culture conditions is essential.

In fact, *in vitro* culture conditions during fertilisation and early embryogenesis in felines do have an impact on developmental competence of embryos, especially from morula to blastocyst stage (Roth et al., 1994). But also *in vitro* maturation (IVM) and its circumstances can have a great influence on embryo quality, as it is one of the most important requirements for ART in endangered species. Zoo animals, which become mainly available to research when they have to be castrated or euthanized for health reasons or surplus (Fernandez-Gonzalez et al., 2015), often provide immature oocytes in varying amounts and quality levels (Johnston et al., 1991). Because inappropriate IVM culture conditions can quickly become the main cause of poor embryo survival rates, and because the full fertilisation potential of the

oocytes should be exploited (Zahmel et al., 2017), *in vitro* culture of the IVM should be included in testing, in contrast to Roth et al., 1994.

At our laboratory we have been using an IVM medium that worked well for our purposes regarding ART in felines. This so-called SAGE Art-1529 is actually a ready-to-use embryo culture medium for human medicine and is distributed for the U.S. fertility market. Among selected non-essential and essential amino acids, taurine, sodium citrate, vitamins, insulin, lactate and gentamicin it contains 5 mg/ml human plasma protein fraction, i.e. a sterile solution of proteins composed mainly of albumin and globulin derived from human plasma.

SAGE Art-1529 is not commercially available in Europe and therefore we are looking for a replacement for our laboratory. As an alternative for the European market the company that manufactures SAGE Art-1529 has launched the embryo culture medium SAGE Art-1029, whose composition is protein-free and for which protein supplementation is needed.

Albumin is a universally added protein to most human *in vitro* cultures as it has a wide range of physiological roles. Since it is the most ample macromolecule in the human oviduct (Leese, 1988) and mimicking its *in vivo* environment is expected to support the development of embryos to the blastocyst stage (Lane & Gardner, 2007), albumin is a recommended supplementation for media. Bovine Serum Albumin (BSA) is a common addition to culture media used in the model of the domestic cat and other felines for IVM and IVF before switching to fetal calf serum (FCS) in the embryo culture (Fernandez-Gonzalez et al., 2015; Karja et al., 2002). Herrick et al., while seeking for a feline optimized culture medium, even used BSA until the third day after fertilisation in embryo culture and only then switched to FCS (Herrick et al., 2007). However, commercially available media from the human ART market, such as SAGE Art-1529, and complex protein supplements for serum-free media are actually preferable as they help to investigate and standardize *in vitro* cultures between laboratories (Nestle et al., 2012). In addition, more complex rather than simple protein molecules in supplement media may have a beneficial effect by mimicking *in vivo* conditions more closely (Meintjes et al., 2009). Still, it is yet controversial whether a complex protein composition is preferred over a simple one, especially in human ART. Regulatory agencies have been increasingly encouraging laboratories to simplify the human embryo culture

protein supplements since proteins obtained from donors have the potential to transmit diseases to patients undergoing assisted conception treatment, such as Creutzfeldt-Jakob disease (Kemmann, 1998).

In the domestic cat model FCS, which is treated as having an almost universal application, is already commonly used as a supplement for embryo cultures where it boosts blastocyst production and quality. However, studies showed that the development of more than 50 % of domestic cat oocytes arrested during the diplotene stage of Prophase I when cultured in the presence of FCS (Johnston et al., 1993; Wood et al., 1995) and it is not clear what the causes of this negative impact are. In the domestic cat, FCS is therefore not used in IVM and IVF, although it is later used widely in embryo culture. However, since FCS not only raises ethical questions but is also variable in quality from being an animal product the question arises whether FCS can be replaced by a protein supplement in the embryo culture of the protein-free medium too.

In this study, the complex protein supplement Serum Substitute Supplement (SSS) was used. It contains 60 mg/ml protein with 84 % albumin from human serum (HSA) and 16 % α and β globulins and was used in a 1:10 dilution in our medium to adjust the protein levels to SAGE Art-1529.

The aim of this study was to compare SAGE Art-1529 to the protein-free SAGE Art-1029 supplemented with SSS and to find out whether SAGE Art-1029 can be a suitable alternative for working with the domestic cat model. The different media and supplements were used for IVM as well as for embryo culture. It was further investigated whether an embryo culture composed from SAGE Art-1029 supplemented with SSS produced comparable results to the same embryo culture supplemented with FCS.

2 Material and methods

All chemical reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless stated otherwise and were of the highest purity available.

2.1 Sample collection

All organic samples, such as ovaries and testicles, were provided by the Berlin Animal Shelter during the period from March to June 2021. These samples accrued as animal organ waste during routine castrations of stray cats, and have been provided to the Leibniz Institute for Zoo and Wildlife Research (IZW) for over ten years. The castrations were carried out by veterinarians of the Berlin Animal Shelter for the purpose of containing the stray cat population and were neither carried out primarily for the IZW nor arranged by the IZW.

Organ and tissue waste, which was produced during processing of the ovaries and testicles in the laboratory, was stored in freezers at -20 °C and disposed via the pathology department.

2.1.1 Preparation of ovaries

The domestic cat ovaries were obtained by ovariectomy at the Berlin Animal Shelter on the same day. The transport of the ovaries took place in pairs in sample tubes in Minimum Essential Medium Eagle HEPES Modification supplemented with 0.3 % bovine serum albumin and 1 % antibiotic antimycotic solution 100×. After arrival in the laboratory, the samples were stored in the refrigerator at 5–8 °C until further use, but ideally processed immediately, as long transport and storage times reduce the quality of the oocytes.

The preparation was carried out in the laboratory under the laminar flow cabinet. Ovaries were removed from surrounding tissue on an absorbent paper cloth and their cycle stage was recorded. Cycle stages included inactive (no follicles visible), follicular (follicle size over 2 mm on at least one ovary), luteal (one or more corpora lutea visible), and intermediate (follicle size 1–2 mm). They were then transferred to a 60 mm petri dish (Sarstedt, Nümbrecht, Germany) with Dulbecco's Phosphate Buffered Saline supplemented with

1 g/l polyvinyl alcohol (PBS/PVA) at room temperature so that the ovaries were just covered. If the ovaries were heavily bled, 40 u/ml of heparin was added to prevent cells from sticking together. To retrieve the oocytes, the ovaries were incised over their entire surface with a scalpel to open the follicles, allowing oocytes to fall into the medium.

2.1.2 Oocyte collection

The further steps were carried out on a warming plate at 38.5 °C under the stereomicroscope. With the help of a mouth pipette, the oocytes could now be selected and transferred, whereby the surrounding cumulus cells were not to be removed through the glass capillary.

These cumulus-oocyte complexes (COCs) were selected using the classification according to Wood and Wildt (Wood & Wildt, 1997). Class I and II COCs, i.e. with uniform dark cytoplasm and at least three rows of tightly compacted cumulus cells (Figure 1 a, b), were selected and transferred to a Nunc™ IVF four-well dish (Thermo Fisher Scientific, Schwerte, Germany). This is recommended, as the pH can be kept stable longer in a four-well dish under oil. Each well contained 400 µl of wash medium (WM, Medium 199 with Earle´s salts, supplemented with 3 mg/ml BSA, 1.4 mg/ml HEPES, 0.6 mg/ml sodium lactate, 0.25 mg/ml sodium pyruvate, 0.15 mg/ml L-glutamine, 0.1 mg/ml cysteine and 0.055 mg/ml gentamicin), which was overlaid with 400 µl of Mineraloil for IVF light (Reproline, Rheinbach, Germany; light oil) and preheated for at least 4 hrs in the incubator at 38.5 °C and 5 % CO_2 to adjust to pH and oxygen concentration. After this first selection, COCs were again sorted according to quality, washed and transferred with as few other cells as possible, for instance erythrocytes, to another well with WM.

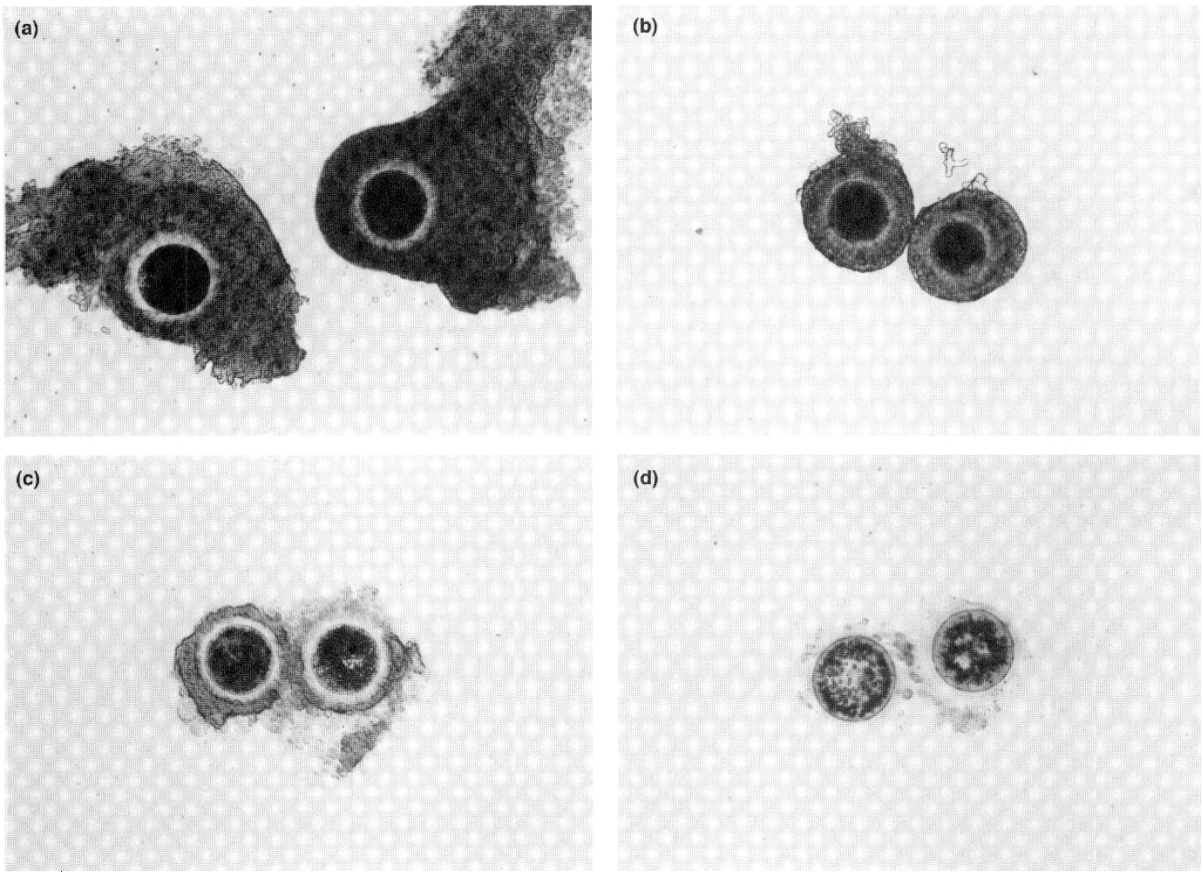

Figure 1 Classification of various cumulus-oocyte complexes (COCs) according to Wood and Wildt. (a) Grade I, excellent. (b) Grade II, good. (c) Grade III, fair. (d) Grade IV, poor. (Wood & Wildt, 1997)

2.1.3 Preparation of testicles

The domestic cat testicles were samples obtained by castration at the Berlin Animal Shelter. Transport of the testicles took place in pairs in sample tubes without any medium. After arrival in the laboratory, the samples were stored in the refrigerator at 5–8 °C until further use.

The preparation was carried out in the andrology laboratory where vas deferens together with head, body and tail of epididymis were carefully removed from the testis and surrounding tissue on an absorbent paper cloth. To avoid too many erythrocytes in the sample later on, it was particularly important to also remove the artery of vas deferens. Vas deferens and epididymis were then transferred to a 35 mm petri dish (Sarstedt, Nümbrecht, Germany) with 500–700 µl WM at 38.5 °C, depending on their size.

2.1.4 Sperm collection and quantification

In the petri dish with WM, they were now cut as small as possible with scissors so that the sperm could swim out into the medium. WM with sperm was removed with a pipette and filtered through a 30 µl CellTrics® cell strainer (Sysmex, Norderstedt, Germany) into a 1.5 ml Eppendorf tube on a heat block at 38.5 °C to remove small pieces of tissue. 500 µl WM were used to rinse the filter. The sperm sample was then centrifuged at 500 × *g* for 5 min.

After centrifugation the supernatant was removed and the sperm pellet was resuspended in approximately 100 µl WM. Again, if the sperm pellet was very small, it was resuspended in only 80 µl WM, if it was very large, it could be resuspended in up to 200 µl WM.

Sperm motility could be estimated on a glass slide under the microscope at an adequate dilution with WM. For the sperm count, an adequate dilution with aqua destillata (a. dest.) was filled into a counting chamber (Thoma/Neubauer classic), counted and calculated. Final results of sperm count were indicated in million spermatozoa per millilitre and, when considering the motility rate, in million motile spermatozoa per millilitre.

2.1.5 Thawing of frozen sperm

If fresh testicle samples were not available or the processed samples contained too few motile sperm, it was possible to thaw sperm that had been supplied earlier by the Berlin Animal Shelter and had been frozen in vials for these purposes following a cryopreservation protocol that is used as standard in the andrology laboratory (Klaus et al., 2016). Vials contained 300 µl sperm suspension with differing quantity and motility of domestic cat spermatozoa.

For the thawing process, frozen sperm were removed from the nitrogen and thawed by swirling a single vial for 90 seconds in a circulating water bath (LAUDA, Lauda-Königshofen, Germany) at 38 °C. The sperm cell solution was diluted dropwise 1+1 with WM and then centrifuged at 500 × *g* for 5 min to remove cryo-protectants, as described in this study's protocol for sperm evaluation and preparation for fertilisation *in vitro* (Fernandez-Gonzalez et al., 2019).

The further steps after centrifugation were performed as described in chapter 2.1.4 Sperm collection and quantification. If a total amount of 1.5×10^6 motile spermatozoa per millilitre was exceeded after motility assessment and sperm count, samples were considered suitable for IVF according to Fernandez-Gonzalez et al (Fernandez-Gonzalez et al., 2019).

2.2 Comparison of culture media SAGE Art-1529 and SAGE Art-1029

The Quinn's Advantage™ Protein Plus Blastocyst Medium (SAGE Art-1529) and Quinn's Advantage™ Sequential Media Blastocyst Medium (SAGE Art-1029) without protein were both purchased from CooperSurgical (Trumbull, USA). SAGE Art-1529 was kindly donated by Pei-Chih Lee, Smithsonian Conservation Biology Institute's (SCBI) Center for Species Survival, Washington, D.C., USA, as it is not for sale in Europe. A direct comparison of the culture media SAGE Art-1529 and SAGE Art-1029 took place in two groups, each containing six batches of oocytes each; group A (SAGE Art-1529) as a control group and group B (SAGE Art-1029) supplemented with 10 % Serum Substitute Supplement (FUJIFILM Irvine Scientific, Tilburg, Netherlands; SSS) in IVM or 5 % FCS in embryo culture.

Table 1 Experimental design for the comparison of culture media SAGE Art-1529 (group A) and SAGE Art-1029 (group B) in IVM, IVF and embryo culture.

		Group A	Group B
(i)	**IVM**	SAGE Art-1529	SAGE Art-1029 + 10 % SSS
(ii)	**IVF**	WM	WM
(iii)	**Embryo culture**	SAGE Art-1529	SAGE Art-1029 + 5 % FCS

2.2.1 *In vitro* maturation

The selected COCs were cultured in two groups at 38.5 °C and 5 % CO_2 in an incubator for 24 hrs (Figure 2). Maturation took place in 400 µl maturation medium in a four-well dish under 400 µl light oil each. The two maturation media were composed of the respective SAGE medium (Table 1 i) and both supplemented with 0.5 IU/ml luteinizing hormone (LH)

and 0.2 IU/ml humane follicle-stimulating hormone (“Bravelle”, Ferring Pharmaceuticals, Saint-Prex, Switzerland; hFSH). At some point, as there was no longer a source of hFSH for our laboratory, it was replaced by 0.2 IU/ml porcine follicle stimulating hormone (Active Bioscience, Hamburg, Germany; pFSH), which was already tested to be an alternative for this purpose.

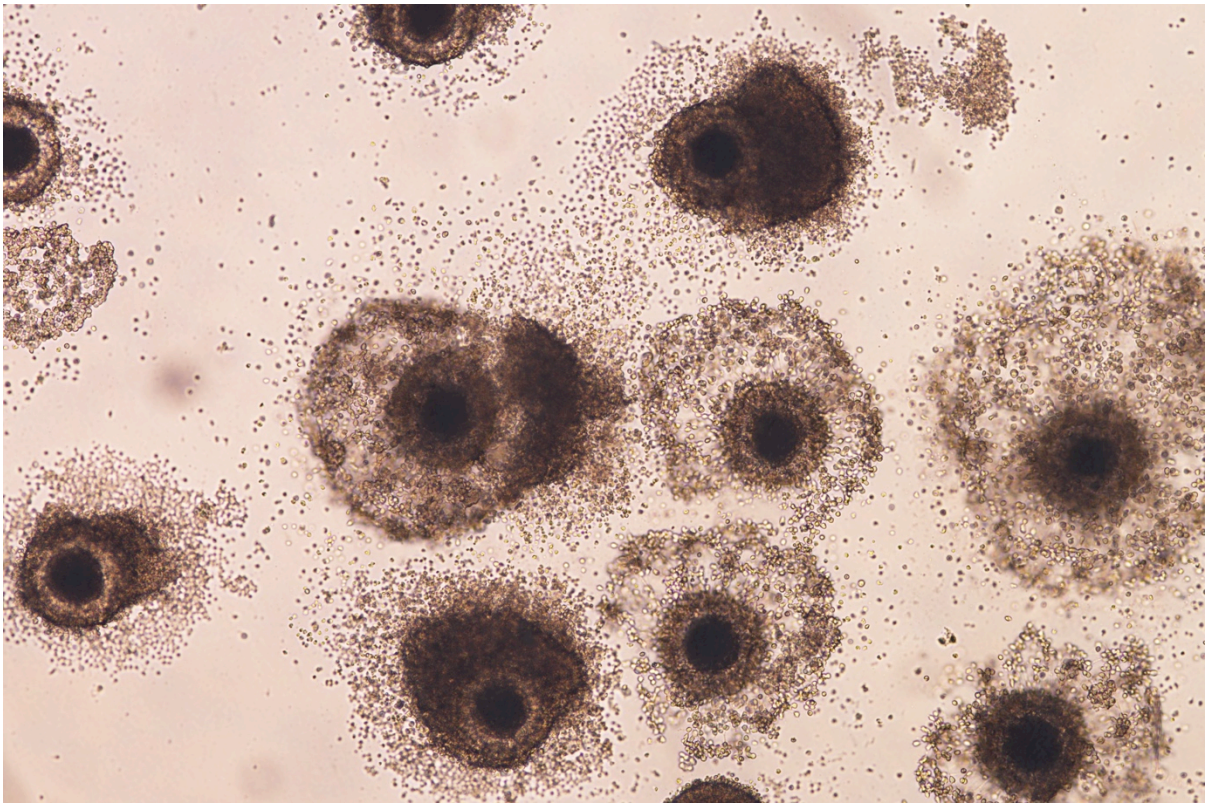

Figure 2 Class I and II ocytes 20 hrs after maturation. Batch *LB270421A*.

2.2.2 *In vitro* fertilisation

IVF was performed for each group in a four-well dish in 350 µl IVF medium with 50 µl sperm solution under 400 µl light oil at 38.5 °C and 5 % CO_2 for 16–20 hrs. IVF medium consisted of WM (Table 1 ii) supplemented with 87 ng/ml heparin, since there was no previous experience of SAGE media in IVF in feline oocytes. The sperm dilution consisted of fresh or frozen sperm diluted with WM with a final sperm concentration of 1×10^6 motile spermatozoa/ml in the IVF well. This means that regardless of whether the sperm were fresh or frozen, the number of motile sperm was the same in each well. Still, it

was possible that frozen sperm performed worse, but since both groups were always tested against each other in each batch, this factor influenced, if so, both groups to the same extent and was therefore not further addressed later on.

After 16–20 hrs of incubation, oocytes were stripped of their cumulus cells under the microscope on the warming plate with the help of a mouth pipette and glass capillary, and then washed in WM before being transferred to embryo culture.

2.2.3 Embryo culture

After IVF, the oocytes were transferred in groups to the already prepared embryo culture dishes and incubated for up to 192 hrs (8 days) at 38.5 °C, 5 % CO_2 and 5 O_2.

For monitoring embryo development in the Primo Vision time-lapse system, Micro well group culture dishes (9 well, Vitrolife, Landshut, Germany; Primo Vision dish) were prepared with a 30 µl drop of embryo culture medium under 3 ml of SAGE oil for tissue culture (CooperSurgical, Måløv, Denmark). Each Primo Vision dish held nine oocytes in lowered wells. As our laboratory had a capacity of only three Primo Vision systems, it occurred that the embryo culture of some experiments could not be monitored by Primo Vision microscopes. As an alternative, these were incubated outside the Primo Vision system and in regular embryo culture dishes for cost reasons. These Nunc™ IVF petri dishes (60 mm, non-treated, Thermo Fisher Scientific, Schwerte, Germany) could hold up to 16 drops of embryo culture medium à 20 µl under 5 ml of SAGE Oil, each drop containing one zygote (single-drop culture).

For the embryo culture medium of group A SAGE Art-1529 was used, whilst the embryo culture medium of group B was composed of SAGE Art-1029 supplemented with 5 % FCS (Table 1 iii).

2.3 Comparison of embryo culture protein supplements FCS and SSS

For the comparison of the culture media supplements FCS and SSS, group C was introduced and compared to group B, with each group consisting of six batches of oocytes each.

IVF and IVM for group B and C (Table 2 i, ii) were performed identically as described before for group B in chapter 2.2.1 and 2.2.2.

Table 2 Experimental design for the comparison of the two different embryo culture protein supplements FCS (group B) and SSS (group C) for the use of SAGE Art-1029 in IVM, IVF and embryo culture.

		Group B	Group C
(i)	**IVM**	SAGE Art-1029 + 10 % SSS	SAGE Art-1029 + 10 % SSS
(ii)	**IVF**	WM	WM
(iii)	**Embryo culture**	SAGE Art-1029 + 5 % FCS	SAGE Art-1029 + 10 % SSS

2.3.1 Embryo culture

Embryo culture was performed as described before for group B in chapter 2.2.3, whilst for group C, instead of using an embryo culture medium composed of SAGE Art-1029 supplemented with 5 % FCS, an embryo culture medium composed of SAGE Art-1029 supplemented with 10 % SSS was used (Table 2 iii).

2.4 Propidium iodide staining and assessment of nuclear stage of uncleaved oocytes

Oocytes that did not divide within 32 hrs after fertilisation were transferred on a glass slide and left for air-drying on a heat block for at least 2 hrs. The oocytes were then fixed in 96 % ethanol at 5–8 °C over night.

For staining, 1 mg/ml propidium iodide (Invitrogen, Darmstadt, Germany; PI) diluted 1:100 in PBS was used with a few drops on the glass slide so that all fixed oocytes were covered. The oocytes were then evaluated under the fluorescence microscope ECLIPSE Ti2-U (Nikon Europe, Düsseldorf, Germany) for signs of maturation in their nuclear stage (Figure 3), such as stained chromatin structures and an extruded polar body that indicate metaphase II (MII).

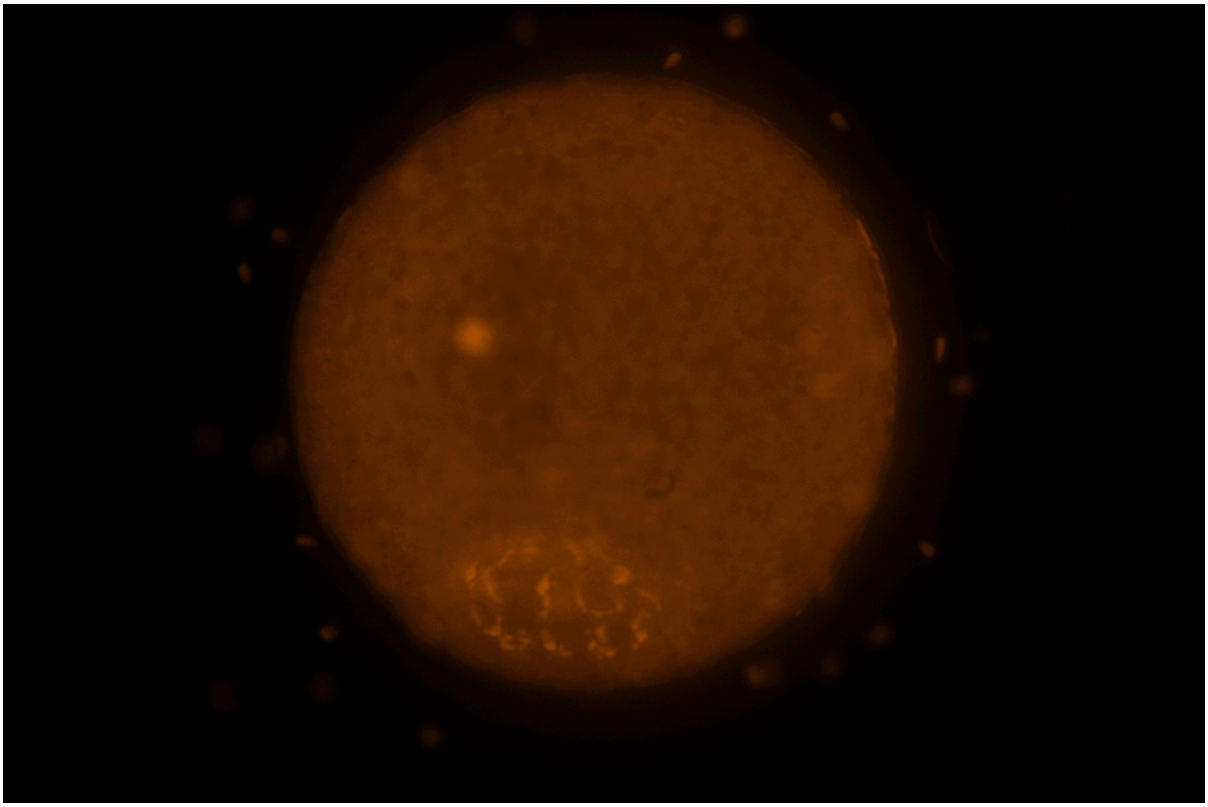

Figure 3 Uncleaved oocyte *LB190421B1* after PI staining showing stained chromatin structures (bottom) that indicate germinal vesicle breakdown (GVBD), thus not being matured.

2.5 Tracking and classification of embryos

Embryos were tracked and evaluated over the course of up to 192 hrs (8 days). Those embryos that degenerated during this period were removed from embryo culture. Those that developed into blastocysts after 144 hrs after fertilisation were submitted to the department's biobank for freezing.

Embryos that were not monitored by the Primo Vision time-lapse system were examined under the microscope every 24 hrs if possible. Of those embryos that were monitored by the Primo Vision time-lapse system, in addition, a snapshot was taken and stored by the Primo Vision microscopes every 30 min.

Early embryos were evaluated in terms of division and quality. Cleavage stages were determined by the number of cells of an embryo at a certain point in time. Furthermore, quality of cleavage was determined by the uniformity of division and the quantity of debris discharged, using a modified classification system according to Moriwaki et al., which was originally developed for human embryo assessment (Moriwaki et al., 2004). For uniformity of division, it was examined whether the divided cells had differences in size and how homogeneous the cytoplasm was. Embryos were then given a score from *A* to *C*; from *A* very symmetrical and homogeneous cells to *C* very large differences in the size of the cells and e.g. vacuoles in the cytoplasm, with in-between scores marked with $^{+}$ and $^{-}$. A score of *1* to *3* was given to assess fragmentation and ejection of cell content; from *1* no fragmentation to *3* much fragmentation and plenty of cell content that was discharged, marked with in-between scores of $^{+}$ and $^{-}$ again. Blastocysts were not scored, as existing classification systems are meant for the assessment of human blastocysts, which differ immensely regarding the morphology. Since feline blastocysts consist of significantly more cells that are much expanded and since there are hardly any morphological differences recognisable amongst them, scoring was omitted.

2.6 Primo Vision evaluation of attended time until cleavage

By using the Primo Vision Analyser software, which creates time-lapse videos of the embryos, their developmental status could be marked and compared at different stages. Values (in hours and minutes) were recorded for the moments of division to 4-cell, 8-cell, early morula and first expanding blastocyst stage for further statistical analysis.

A Primo Vision evaluation of the 2-cell stages had to be omitted here, as many of the 2-cell divisions occurred before the embryo dish was placed in the Primo Vision microscope and

therefore their exact time of cleavage could not be recorded. Values for the division into the 9–16-cell stage cannot be recorded either, as it cannot be clearly differentiated visually in the Primo Vision time-lapse video. This is only possible again as soon as the number of 16 cells of an embryo is exceeded, recognisable by the typical arrangement of the cells and cell size, and the embryo is thus classified as an early morula.

2.7 Statistical analysis

Maturation rate, cleavage rate, morula rate, and blastocyst rate of each group were recorded and then statistically analysed with Fisher's exact test for the comparison of groups A and B, as well as groups B and C.

Later on, the values recorded in Primo Vision for the different moments of embryo division were statistically analysed by using an unpaired two-tailed *t*-test for the comparisons of groups A and B, as well as groups B and C.

A significance threshold (alpha level) of 0.05 was set for both statistical hypothesis tests, with $p \leq .05$ being statistically significant.

3 Results

3.1 Comparison of culture media SAGE Art-1529 and SAGE Art-1029

In group A, where SAGE Art-1529 was used as culture medium, from a total of 53 oocytes, 36 oocytes matured. Of the matured oocytes, 22 divided, while 31 of the 53 oocytes remained undivided. 16 of the 22 divided oocytes developed to morula stage, of which 9 developed to blastocyst stage. In group B, where SAGE Art-1029 was used as culture medium, from a total of 53 oocytes, 39 oocytes matured. Of the matured oocytes, 27 divided, while 26 of the 53 oocytes remained undivided. 22 of the 27 divided oocytes developed to morula stage, of which 15 developed to blastocyst stage. (Table 3)

Table 3 Comparison of group A and group B: Oocyte maturation rates and cleavage rates relative to total number of oocytes. Morula rates and blastocyst rates related to number of cleaved oocytes.

	Total no. of oocytes	No. of matured oocytes	No. of cleaved oocytes	No. of embryos reaching morula stage	No. of embryos reaching blastocyst stage
A	53	37 (69.8 %)	22 (41.5 %)	16 (72.7 %)	9 (40.9 %)
B	53	39 (73.6 %)	27 (50.9 %)	22 (81.5 %)	15 (55.6 %)

Each group contained six batches.

No significant differences were found comparing the *in vitro* cultures of group A and B, regarding cleavage rate, maturation rate, morula rate, and blastocyst rate (Table 4).

Table 4 Fisher's exact test comparing groups A and B regarding maturation rate, cleavage rate, morula rate and blastocyst rate.

	p-value
Maturation rate	0.83
Cleavage rate	0.44
Morula rate	0.51
Blastocyst rate	0.39

*$p \leq .05$

3.1.1 Comparison of attended time of embryonic development

A comparison of groups A and B, regarding the periods of time until a certain stage of embryonic division (Table 5, Figure 4), showed no significant difference for the development until the 4-cell stage, as well as in the early morula stage and the blastocyst stage. However the statistical analysis showed a significant difference for the two groups in the 8-cell stage, with SAGE Art-1029 being used in the group taking longer for the embryos to divide into eight cells.

Table 5 Unpaired two-tailed *t*-test comparing groups A and B regarding attended time until division at 4-cell stage, 8-cell stage, early morula stage and first expanded blastocyst stage.

	p-value
4-cell stage	0.116
8-cell stage	0.011*
early morula stage	0.730
first expanded blastocyst stage	0.320

*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$

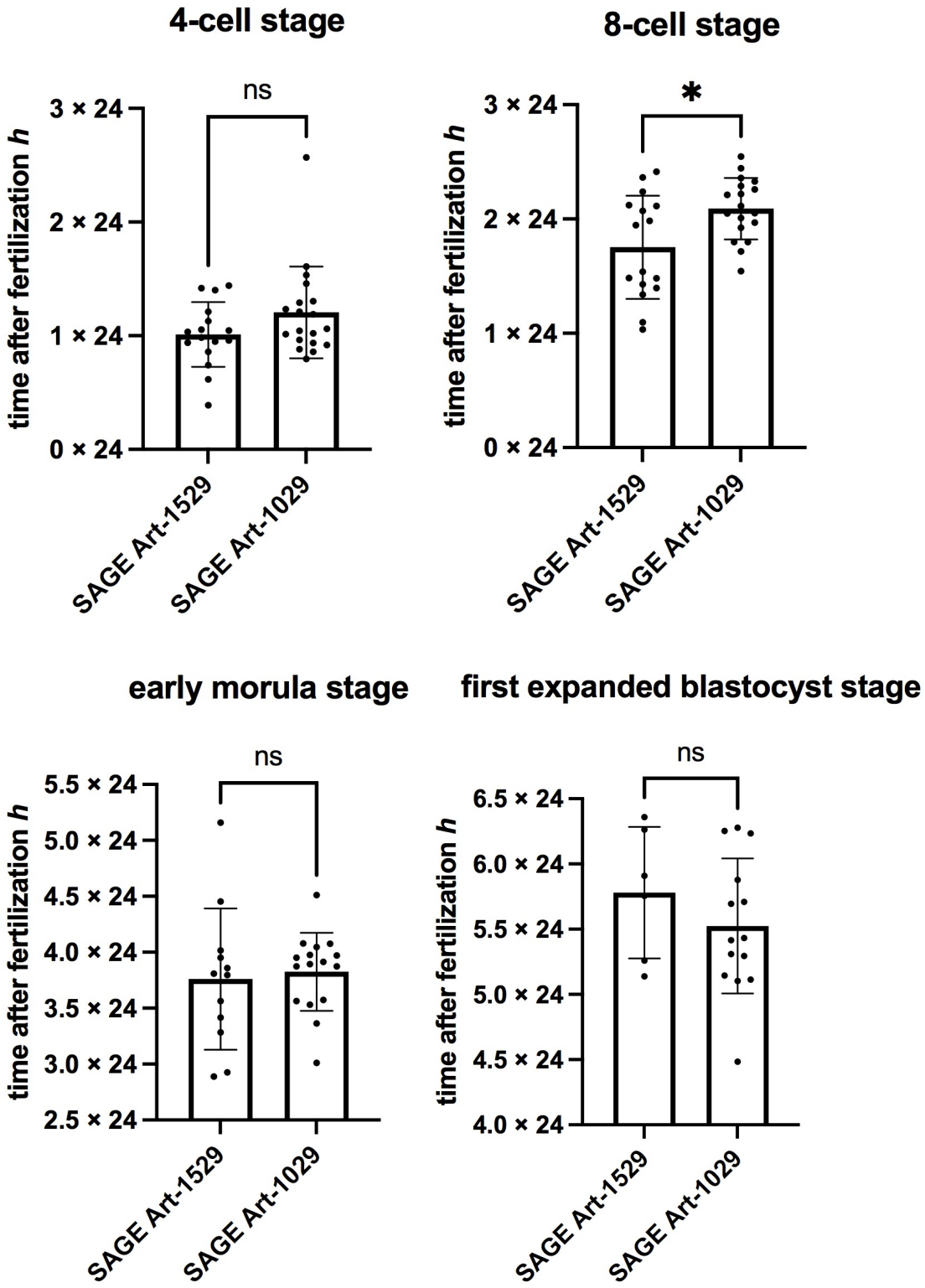

Figure 4 Statistical representation of SAGE Art-1529 compared to SAGE Art-1029 regarding attended time until division at 4-cell stage, 8-cell stage, early morula stage and first expanded blastocyst stage. *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ns = non-significant

3.2 Comparison of embryo culture supplements FCS and SSS

In group B, where FCS was used as an embryo culture supplement, from a total of 50 oocytes, 33 oocytes matured. Of the matured oocytes, 32 divided, while 18 of the 50 oocytes remained undivided. 27 of the 32 divided oocytes developed to morula stage, of which 22 developed to blastocyst stage. In group C, where SSS was used as an embryo culture supplement, from a total of 51 oocytes, 40 oocytes matured. Of the matured oocytes, 39 divided, while 12 of the 51 oocytes remained undivided. 28 of the 39 divided oocytes developed to morula stage, of which 19 developed to blastocyst stage. (Table 6)

Table 6 Comparison of group B and group C: Oocyte maturation rates and cleavage rates relative to total number of oocytes. Morula rates and blastocyst rates related to number of cleaved oocytes.

	Total no. of oocytes	No. of matured oocytes	No. of cleaved oocytes	No. of embryos reaching morula stage	No. of embryos reaching blastocyst stage
B	50	33 (66 %)	32 (64 %)	27 (84.4 %)	22 (68.8 %)
C	51	40 (78.4 %)	39 (76.5 %)	28 (71.8 %)	19 (48.7 %)

Each group contained six batches.

No significant differences were found comparing the embryo cultures of group B and C, regarding cleavage rate, maturation rate, morula rate, and blastocyst rate (Table 7).

Table 7 Fisher's exact test comparing groups B and C regarding maturation rate, cleavage rate, morula rate and blastocyst rate.

	p-value
Maturation rate	0.19
Cleavage rate	0.20
Morula rate	0.26
Blastocyst rate	0.10

*$p \leq .05$

3.2.1 Comparison of attended time of embryonic development

A comparison of groups B and C, regarding the periods of time until a certain stage of embryonic division (Table 8, Figure 5), showed no significant difference for the development until the 4-cell stage. However, there were significant differences between the two groups in the 8-cell stage as well as in the early morula stage, with group C, where the protein supplement SSS was used, developing slower. In turn, there was no significant difference between the two groups in the blastocyst stage.

Table 8 Unpaired two-tailed *t*-test comparing groups B and C regarding attended time until division at 4-cell stage, 8-cell stage, early morula stage and first expanded blastocyst stage.

	p-value
4-cell stage	0.061
8-cell stage	0.002**
early morula stage	0.042*
first expanded blastocyst stage	0.552

*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$

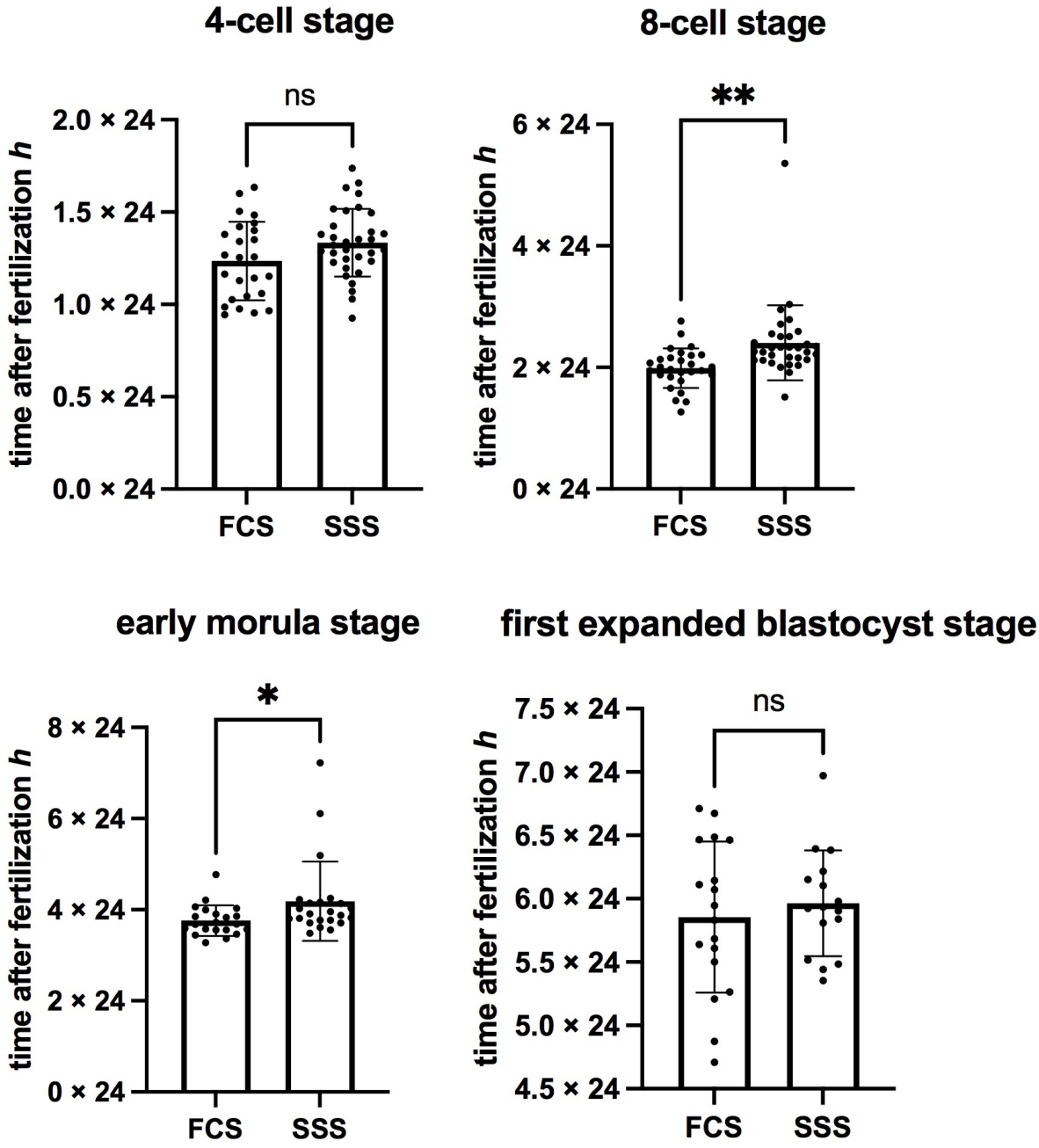

Figure 5 Statistical representation of FCS compared to SSS regarding attended time until division at 4-cell stage, 8-cell stage, early morula stage and first expanded blastocyst stage. *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ns = non-significant

4 Discussion

This study demonstrates whether SAGE Art-1029 supplemented with SSS can be an alternative to the human IVF embryo culture medium SAGE Art-1529, which is also well established for the IVF-system of the domestic cat and where first experiences with exotic felines have also been established (Crosier et al., 2020; Morselli et al., 2017). SAGE Art-1529 is, however, not commercially available in Europe. As this study was conducted from March to July, i.e. during the reproductive season of the domestic cat, comparable results cannot be expected at any other time of the year and are difficult to replicate, as the developmental competence of oocytes will be impaired during the non-reproductive season.

Since the results in Table 4 show no significant differences between groups A and B with regard to maturation rate and division rate of oocytes as well as morula rate and blastocyst rate, the initial question can be answered, being that SAGE Art-1029, which is protein-free, may well be an alternative for SAGE Art-1529 in *in vitro* cultures of the domestic cat when 10 % SSS is added. A more detailed evaluation of the attended time until a certain cleavage in Primo Vision in Table 5 shows that there are also no significant differences between the two groups in the development to 4-cell embryos, early morulae and blastocysts. Thus, the protein-free SAGE Art-1029 does not have a slowing effect on embryo development at these cell stages, a factor that is often assumed to be a sign of poor embryo quality because any delay would be a deviation from development *in vivo* and therefore not optimal (Roth et al., 1994). The only exception is the development to the 8-cell stage where the development of the embryos from group B seems to be significantly slower. Whether this is due to the composition of SAGE Art-1029 and the protein substitute SSS is biologically irrelevant here as this experiment refers to the overall outcome of blastocysts. Even though there might be individual variations, they have no effect on blastocyst development. In the overall outcome the protein-free SAGE medium with a protein supplement does not perform worse than the original SAGE medium.

For the experiment comparing a classical approach with FCS in embryo culture to the new approach with SSS in embryo culture, there seemed to be a trend towards better embryo quality with the addition of FCS. However, this trend was not sustainable in terms of morula

rate and blastocyst rate, as no significant differences were found (Table 7). A non-significant difference of blastocyst rate suggests that the modified classification of early embryonic stages according to Moriwaki et al., 2004, which gives scores from *A* to *C* and *1* to *3*, cannot give any solid indication about the further development of an embryo in the domestic cat. In fact, this classification is an exclusively subjective evaluation of a momentary observation and has its origins in the routine assessment of embryos in human medicine in order to find out which embryo offers the highest growth potential and the highest probability of pregnancy. It has a high potential for error since its parameters cannot be measured exactly and different angles of view of the embryos may show different images under the microscope that can be interpreted differently. Therefore, in this present study on the domestic cat, this classification was only used as a guideline to determine whether an embryo was developing in time, however, no decisions were taken on this basis.

The maturation rate and the division rate did not show any significant differences in this comparison either. This was to be expected, as the culture media only differed after transfer to the embryo culture dishes. During IVM and IVF, the culture media for group B and group C were identical and therefore could not have produced any significant differences.

Embryo assessment supported by time-lapse systems such as Primo Vision is used widely in human ART, but still very little in animals. First studies on embryos from productive livestock reveal the new possibilities offered by the timing of cleavage divisions (Fryc et al., 2021), which were also investigated here. The evaluation of the attended time until a certain cleavage in Table 8 shows that the divisions up to the 8-cell stage display highly significant differences and the divisions up to the morula stage display significant differences, whereby the embryos of group B, which had FCS added in the culture medium, developed faster. Rather unexpected here is, however, that the divisions up to the 4-cell stage and blastocyst stage of groups B and C in turn showed no significant differences. One possibility to explain these different significance levels is that SSS actually only has a slowing effect on the embryos in the ranges of 8-cell divisions and morula divisions. However, it is also possible that the sample size of the experiment, especially in terms of the number of blastocysts, was too low to provide a reliable result. The sample size of 100 oocytes per group originally set by the study leader could unfortunately not be achieved here, due to unexpected low sample

availability. In the future, however, a study with a corresponding sample size on the effects of FCS and SSS in embryo culture in the domestic cat model should provide more reliable results for interpretation.

Generally, the use of FCS poses a scientific problem because FCS is not only undefined, but also varies from lot to lot and thus may interfere with the outcome of experiments. Many of the substances of FCS have not been identified yet, and of those that have been identified, some effects on cell cultures remain unclear (Jochems et al., 2002). Even though this cocktail of hormones and growth factors can help embryos to grow extraordinarily fast, it should still be considered that optimal cell growth does not necessarily coincide with proper cellular function (Gardner, 1998).

Studies have shown that complex protein supplements such as SSS can highly influence embryo quality, and even though these products do have lot-to-lot variability too (Morbeck et al., 2014), they are principally still more defined by being artificial serum replacements. When 10 % SSS was added to media that was already pre-supplemented with 5 mg/ml of HSA it resulted in an overall increase in implantation and live birth rates in humans (Meintjes et al., 2009). Therefore, it would be interesting to test in a large-scale experiment to what extent the speed of embryo development at certain division rates effects not only the blastocyst rate but also the success of embryo transfers in domestic cats in order to be able to make predictions for each embryo early on, and how SSS influences this outcome.

For the application tested here as a supplement for SAGE Art-1029 in the IVF system of the domestic cat not only during IVM, but also during embryo culture, SSS surely seems to be a viable alternative to the former use of SAGE Art-1529 and of FCS in embryo culture in our laboratory.

5 Summary

Assisted reproduction techniques and cryopreservation of gametes have become an important part of wildlife research and conservation. By understanding reproductive traits and strategies, breeding programs in zoos can be supported and genetic variability in wildlife populations can be maintained. In order to understand basic aspects of assisted reproduction of endangered feline species, *in vitro* fertilisation of the domestic cat can be studied, where the continuous improvement of *in vitro* culture conditions is essential. The aim of this work was to find an alternative to the human culture medium SAGE Art-1529, which has been successfully applied in the IVF-culture of domestic cats but is not commercially available in Europe. In another experiment, the Serum Substitute Supplement (SSS) was compared to the previously used fetal calf serum (FCS) as a protein source in embryo culture. Division rates and development speed of the embryos were recorded with the time-lapse system Primo Vision.

SAGE Art-1029 supplemented with SSS was found to have no significant differences to SAGE Art-1529 regarding maturation rate, cleavage rate, morula rate and blastocyst rate. The timing of cleavage division showed no significant differences at the 4-cell stage, morula stage and blastocyst stage, however embryos in SAGE Art-1029 developed significantly slower to 8-cell stage. Though there were individual differences during embryo growth, there were no differences in the overall outcome regarding blastocyst rate, which therefore does not make SAGE Art-1029 perform worse overall, but makes it a viable alternative to SAGE Art-1529.

SSS in embryo culture was found to have no significant differences to FCS regarding maturation rate, cleavage rate, morula rate and blastocyst rate. The timing of cleavage division showed no significant differences at the 4-cell stage and blastocyst stage, however embryos in SSS developed significantly slower to 8-cell stage and morula stage. Further studies could analyse whether the speed of embryo development at certain division rates effects not only the blastocyst rate but also the success of embryo transfers in domestic cats and whether predictions could be made for each embryo at an early embryonic stage in the future, and how SSS affects this outcome. For the application tested here, SSS seems to be an alternative to FCS in embryo culture in our laboratory.

6 Zusammenfassung

Assistierte Reproduktionstechniken und die Kryokonservierung von Gameten sind ein wichtiger Bestandteil der Wildtierforschung und -erhaltung geworden, um Zuchtprogramme in Zoos zu unterstützen und die genetische Vielfalt von Wildtierpopulationen zu erhalten. Um die grundlegenden Aspekte der assistierten Reproduktion von gefährdeten Katzenarten zu verstehen, kann die *In-vitro*-Fertilisation der Hauskatze untersucht werden, wobei eine kontinuierliche Verbesserung der *In-vitro*-Kulturbedingungen von entscheidender Bedeutung ist. Ziel dieser Arbeit war es, eine Alternative zu dem bei der Hauskatze bewährten, aber in Europa nicht kommerziell vertriebenen humanen Kulturmedium SAGE Art-1529 zu finden. In einem weiteren Experiment wurde das Serum Substitute Supplement (SSS) gegen das bisher verwendetet fetale Kälberserum (FCS) als Proteinquelle in der Embryonenkultur verglichen. Teilungsraten sowie Entwicklungsgeschwindigkeit der Embryonen wurden mit dem Zeitraffersystem Primo Vision erfasst.

SAGE Art-1029, angereichert mit SSS, zeigte keine signifikanten Unterschiede zu SAGE Art-1529 hinsichtlich der Reifungsrate, Spaltungsrate, Morularate und Blastozystenrate. Die Embryonalteilungen zeigte keine signifikanten Unterschiede im 4-Zell-Stadium, Morulastadium und Blastozystenstadium, außer im 8-Zell-Stadium. Obwohl es individuelle Unterschiede während des Embryowachstums gab, zeigten sich keine Unterschiede im Gesamtergebnis hinsichtlich der Blastozystenrate, was SAGE Art-1029 daher insgesamt nicht schlechter abschneiden ließ, sondern es zu einer Alternative zu SAGE Art-1529 macht.

SSS in der Embryokultur zeigte keine signifikanten Unterschiede zu FCS hinsichtlich der Reifungsrate, Spaltungsrate, Morularate und Blastozystenrate. Die Embryonalteilungen zeigte keine signifikanten Unterschiede im 4-Zell-Stadium und im Blastozystenstadium, jedoch entwickelten sich die Embryonen in SSS signifikant langsamer bis zum 8-Zell-Stadium und zum Morulastadium. In weiteren Studien könnte untersucht werden, ob sich die Geschwindigkeit der Embryonalentwicklung zusätzlich auf Embryonentransfers auswirkt und ob in Zukunft Vorhersagen für jeden Embryo zu einem frühen Zeitpunkt gemacht werden können, und wie SSS dieses Ergebnis beeinflusst. Für die hier getestete Anwendung scheint SSS eine Alternative zu FCS in der Embryokultur in unserem Labor zu sein.

7 List of abbreviations

Terms regarding nuclear stages and cleavage stages:

nc	not cleaved
GV	germinal vesicle
GVBD	germinal vesicle breakdown
MI	metaphase I
MII	metaphase II
PB	polar body
PN	pro nucleus
2c	2-cell stage
3c	3-cell stage
4c	4-cell stage
5–8c	5–8-cell stage
8c	8-cell stage
9–16c	9–16-cell stage
early Mo	early morula
Mo	morula
Mo comp	compacting morula
early Bla	early blastocyst
Bla	blastocyst
Bla exp	expanded blastocyst
Bla hatched	hatched blastocyst
n.a.	cannot be assessed
deg	degenerated

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9 List of tables and figures

9.1 Tables

9.2 Figures

10 Supplement

Table S 1. A vs. B: Raw data of oocyte tracking and PI staining (SAGE Art-1529)

ID oocyte	d0	d1	d2	d3	d4	d5	d6	d7	d8	PI staining	matured
LB150321A1			4-6c	5-8c		Mo (C2)			Bla		✓
LB150321A2			nc							3c pathogenetic	
LB150321A3			4-6c	9-16c		Mo (C3) comp			Bla		✓
LB150321A4			nc							MI	
LB150321A5			4c	5-8c		Mo (C3)			Bla deg		✓
LB150321A6			nc							MI	
LB150321A7			nc							MII	✓
LB150321A8			deg	deg		deg					
LB150321A9			2c	5-8c		5-8c (C3)			deg		✓
LB150321A10			nc							gone missing	
LB120421A1		nc	8c (B1)	9-16c (B1)			early Mo (C1)	Mo	Mo deg		✓
LB120421A2		nc	4-6c (C2)	9-16c (C1)							✓
LB120421A3		nc	nc	nc PB							✓
LB120421A4		nc	nc PB	nc							✓
LB120421A5		2c	8c (B2)	9-16c (A1)			early Mo (C1)	Bla	Bla hatched		✓
LB190421A1		nc	nc PB							n.a.	✓
LB190421A2		nc deg	deg							deg	
LB190421A3		nc	nc							MI	
LB190421A4		nc	nc							MII	✓
LB190421A5		n.a.	5-8c	9-16c ($B2^+$)			Mo (B2)	Mo (B2)	Mo (B2)		✓
LB190421A6		2-4c (B2)	9-16c (B2)	9-16c (B2)			9-16c (C3)	deg			✓
LB190421A7		2c (A1)	5-8c (A2)	9-16c (B2)			early Mo (B2)	early Mo (B2)	Mo deg		✓
LB190421A8		nc	nc							MII	✓
LB270421A1		nc PB	nc							MII	✓
LB270421A2		4c (B2)	8c (B2)			Mo (C3)	early Bla	Bla			✓
LB270421A3		nc	nc							GV	
LB270421A4		nc	4-6c (B2)			n.a.	Bla exp	Bla			✓
LB270421A5		2c (B1)	8c (B1)			Mo (B2)	Mo (B2)	Bla			✓
LB280421A1		nc			nc						
LB280421A2		nc			nc						
LB280421A3		nc PB			nc PB						✓
LB280421A4		nc PB			nc						✓
LB280421A5		nc			nc						
LB280421A6		nc			nc						
LB280421A7		nc			early Mo (C2)	Mo (C2)	Mo (C2)	Mo deg			✓
LB280421A8		nc			5-8c (C3)	deg					✓
LB030521A1			nc							n.a.	
LB030521A2			nc PB							MII	✓
LB030521A3			nc							MII	✓
LB030521A4			nc PB							2 PN	✓
LB030521A5			4c (B2)	9-16c (B2)			deg				✓
LB030521A6			4c (C2)	9-16c (C2)			9-16c deg	9-16c deg			✓
LB030521A7			4-6c (C1)	9-16c (B2)			early Mo	early Mo			✓
LB030521A8			4c (B2)	9-16c (B2)			Mo deg ?	Mo deg			✓
LB030521A9			nc PB							MII	✓
LB030521A10			8c (B2)	9-16c (A2)			Mo (B2)	Mo (B2)	Mo deg		✓
LB030521A11			nc PB							n.a.	✓
LB030521A12			nc							MI	
LB030521A13			nc PB							MII	✓
LB030521A14			nc deg	nc deg						GVBD	
LB030521A15			4-6c (C2)	9-16c (B2)			early Bla	Bla			✓
LB030521A16			2-4c (C3)	5-8c (C3)			Mo (C3)	Bla			✓
LB030521A17			nc							GVBD	

Table S 2. A vs. B: Raw data of oocyte tracking and PI staining (SAGE Art-1029)

ID oocyte	d0	d1	d2	d3	d4	d5	d6	d7	d8	PI staining	matured
LB150321B1			nc							GVBD	
LB150321B2			4-6c	4-6c		Mo (B2)			Bla		✓
LB150321B3			5-8c	9-16c		9-16c (C2)			deg		✓
LB150321B4			nc							MII	✓
LB150321B5			nc							MI	
LB150321B6			nc							GVBD	
LB150321B7			5-8c	9-16c		Mo (B2) comp			Mo deg		✓
LB150321B8			nc							GV	
LB150321B9			nc							MII	✓
LB150321B10			nc							MII	✓
LB150321B11			2c	5-8c		early Mo (B2)			gone missing		✓
LB150321B12			nc							MI	
LB120421B1		nc	nc PB	nc							✓
LB120421B2		2c	4c deg ?	5-8c deg			5-8c deg				✓
LB120421B3		2c	4-6c (C3)	n.a.			Mo	Mo	Mo deg		✓
LB120421B4		nc	nc PB	nc							✓
LB120421B5		nc	nc	nc							
LB120421B6		n.a.	4-6c (C3)	9-16c (C3)							✓
LB120421B7		nc	nc	nc							
LB120421B8		2c	4c (B2)	9-16c (B2)			Bla	Bla	Bla		✓
LB190421B1		nc	nc							GVBD	
LB190421B2		nc PB	nc PB							MII	✓
LB190421B3		2c (B2)	5-8c (B2)	9-16c (A1)			Bla	Bla			✓
LB190421B4		nc PB	2c (C¯2)	5-8c (C3)			5-8c (C3)	5-8c (C3)			✓
LB190421B5		2-4c (B2)	5-8c (B2)	9-16c (C2)			Bla	Bla			✓
LB190421B6		2c (C2)	5-8c (B2)	9-16c (B2)			Bla	Bla			✓
LB190421B7		2-4c (A2)	5-8c (B2¯)	9-16c (B2)			Bla	Bla			✓
LB190421B8		nc deg	deg							deg	
LB190421B9		2c (A1)	5-8c (B2)	9-16c (B1)			Bla	Bla			✓
LB190421B10		nc	nc PB							2c	✓
LB190421B11		2c (B3)	5-8c (B2)	9-16c (B¯2)			Bla	Bla			✓
LB190421B12		2c (C2)	5-8c (B2)	9-16c (B¯2)			Mo (B2)	Bla			✓
LB270421B1		2c (B1)	6c (A1)			early Mo (B1)	Mo (B2)	Mo (B2)			✓
LB270421B2		2c (B2)	4c (B2)			n.a.	early Bla	Bla			✓
LB270421B3		n.a.	6c (B2)			Mo (B2)	Bla exp	Bla hatched			✓
LB270421B4		nc	nc							MI	
LB270421B5		nc	nc PB							MII	✓
LB280421B1		2c (A1)			Mo (C2)	Mo (C2)	deg				✓
LB280421B2		nc			nc PB						✓
LB280421B3		2c (B1)			9-16c (C2)	early Mo (C2)	Mo (C3)	Mo deg	Mo deg		✓
LB280421B4		nc			nc PB						✓
LB280421B5		nc			nc						
LB280421B6		nc			nc						
LB280421B7		nc PB			nc PB						✓
LB030521B1			4c (B2)	8c (B2)			Mo (B2)	Mo (B2)	deg		✓
LB030521B2			nc							MI	
LB030521B3			nc PB							MII	✓
LB030521B4			4-6c (A1)	9-16c (A1)			early Bla	Bla			✓
LB030521B5			nc							n.a.	
LB030521B6			5-8c (B2)	9-16c (B2)			Mo comp	Bla			✓
LB030521B7			4-6c (B2)	9-16c (B3)			n.a.	Bla			✓
LB030521B8			4c (B2)	5-8c (B2)			5-8c (B2)	5-8c (B2)	5-8c (B2)		✓
LB030521B9			4c (B2)	5-8c (B3)			Bla	Bla			✓

Table S 3 B vs. C: Raw data of oocyte tracking and PI staining (SAGE Art-1029 embryo culture containing FCS)

ID oocyte	d0	d1	d2	d3	d4	d5	d6	d7	d8	PI staining	matured
LB100521B1		4c (B2)					Mo (C2)	Bla			✓
LB100521B2		2-4c (B1)					early Bla	Bla			✓
LB100521B3		2c (B1)					early Bla	Bla			✓
LB100521B4		4c (B1)					Mo (B2)	Bla			✓
LB100521B5		2c (B2)					Mo (C2)	Mo (C2)	Bla		✓
LB100521B6		nc deg					deg				
LB100521B7		4c (A1)					Mo (B2)	Mo (B2)	Mo (B2)		✓
LB100521B8		nc deg					nc				
LB100521B9		2c (C2)					Mo (B2)	Bla			✓
LB190521B1		4c (B2)				Mo (B2)	Bla				✓
LB190521B2		deg				deg					
LB190521B3		2-4c (B2)				Mo (A2)	Mo comp	Mo comp			✓
LB190521B4		2c (B3)				Mo (C2)	Mo comp	early Bla			✓
LB190521B5		2c (C1)				Mo (C2)	Mo (C3) comp	early Bla			✓
LB190521B6		nc				nc					
LB190521B7		2c (A2)				Mo (B2)	Mo	Bla			✓
LB020621B1		2c			9-16c (C2)	9-16c (C2)	9-16c (C2)	9-16c (C2)	9-16c deg		✓
LB020621B2		nc PB			nc						✓
LB020621B3		2c (C2)			Mo (A2)	n.a.	Bla				✓
LB020621B4		4c (B2)			early Mo (B2)	early Mo (B2)	Mo (B2)	Mo deg	Mo deg		✓
LB020621B5		nc deg			deg						
LB020621B6		nc deg			deg						
LB020621B7		2c (A1)			Mo (A1)	Mo comp	Mo comp	Bla			✓
LB020621B8		nc			nc						
LB070621B1		2c (A1)	8c (A1)	9-16c (A2)			Mo (B2)	early Bla			✓
LB070621B2		3c (A1)	5-8c (B2)	9-16c (B2)			Bla	Bla			✓
LB070621B3		nc PB	4c (B3)	8c (B2)			Mo comp	Bla			✓
LB070621B4		nc	5-8c (B2)	9-16c (B2)			Mo (B2) comp	Bla			✓
LB140621B1			4c (B2)	9-16c (B2)			Mo (B2)	early Bla			✓
LB140621B2			nc	nc			n.a.	deg			
LB140621B3			5-8c (C2)	9-16c (C3)			9-16c (C3)	9-16c (C3)			✓
LB140621B4			5-8c (B1)	8c (B2)			Bla	Bla			✓
LB140621B5			5-8c (B2)	9-16c (B2)			Mo deg	deg			✓
LB140621B6			nc	nc			nc	deg			
LB140621B7			4c (B2)	8c (B2)			Bla	Bla			✓
LB140621B8			4c (B2)	9-16c (B2)			Bla	Bla			✓
LB130721B1		nc deg	nc deg							deg	
LB130721B2		nc	nc PB							n.a.	✓
LB130721B3		nc	3c (B2)			9-16c (C3)	9-16c deg	deg	deg		✓
LB130721B4		2c (B2)	4c (C2)			8c (B2)	8c (C2)	8c (C2)	deg		✓
LB130721B5		nc	3c (B3)			Mo (B2)	Mo (C2)	Bla	Bla		✓
LB130721B6		2c (B1)	5-8c (B2)			Mo (C3)	Bla	Bla	Bla hatched		✓
LB130721B7		nc deg	nc deg							deg	
LB130721B8		nc deg	nc deg							deg	
LB130721B9		nc deg	nc deg							deg	
LB130721B10		2c (A1)	4c (A1)			early Mo (B2)	Mo deg	Mo deg	deg		✓
LB130721B11		nc	4c (B1)			9-16c (B2)	9-16c deg	9-16c deg	deg		✓
LB130721B12		nc deg	nc deg							deg	
LB130721B13		nc	nc							GVBD	
LB130721B14		nc	nc							GVBD	

Table S 4 B vs. C: Raw data of oocyte tracking and PI staining (SAGE Art-1029 embryo culture containing SSS)

ID oocyte	d0	d1	d2	d3	d4	d5	d6	d7	d8	PI staining	matured
LB100521C1		2c (B1)					Mo (A2)	Mo (A2)	MoA2		✓
LB100521C2		nc					nc				
LB100521C3		2c (B2)					n.a.	Bla			✓
LB100521C4		2c (C2)					Mo (C3)	Mo (C3)	Mo deg		✓
LB100521C5		nc PB					9-16c (C3)	9-16c deg	9-16c deg		✓
LB100521C6		2-4c (C2)					Bla	Bla			✓
LB100521C7		nc PB					Mo (B2)	Bla			✓
LB100521C8		2c (B1)					early Bla	Bla			✓
LB100521C9		2c (A1)					early Bla	Bla			✓
LB100521C10		2c (A2)					9-16c (C3)	Mo (C3)	MoC3		✓
LB100521C11		2c (A3)					9-16c (B2)	9-16c (B2)	9-16c (B3)		✓
LB100521C12		nc deg					nc deg				
LB100521C13		nc					n.a.	8c deg	8c deg		✓
LB100521C14		2-4c (C2)					Mo (B3)	Bla			✓
LB100521C15		2c (B1)					8c (B3)	9-16c (B3)	Mo (B2)		✓
LB100521C16		2c (A1)					Mo deg	deg			✓
LB100521C17		nc PB					nc				✓
LB100521C18		2-4c (A1)					early Bla	Bla			✓
LB190521C1		2c (B2)				Mo (B3)	Bla				✓
LB190521C2		2c (C1)				n.a.	n.a.	deg		5-8c deg	✓
LB190521C3		4c (C3)				Mo (B2)	n.a.	Bla			✓
LB190521C4		2c (C1)				Mo	Mo	Bla			✓
LB190521C5		2c (B1)				Mo (B2)	Mo (C3)	Mo deg			✓
LB190521C6		nc				nc					
LB190521C7		deg				deg					
LB020621C1		nc PB			9-16c (B2)	9-16c (B2)	9-16c (B2)	9-16c deg	9-16c deg		✓
LB020621C2		2c (B2)			9-16c (C2)	9-16c deg	9-16c deg	deg			✓
LB020621C3		2c (B1)			Mo (B2)	Mo comp	Mo comp	Bla			✓
LB020621C4		2c (B2)			early Mo (C2)	Mo (B2)	early Bla				✓
LB020621C5		nc deg			nc						
LB020621C6		2-4c (B1)			Mo (A2)	Mo comp	Mo comp	Bla			✓
LB020621C7		nc			nc						
LB070621C1		2c (B2)	3c (C3)	4c (C3)			Mo deg	deg			✓
LB070621C2		2c (A2)	8c (A2)	9-16c (A2)			early Bla	Bla			✓
LB070621C3		2c (B1)	4c (C2)	9-16c (C2)			Bla	Bla			✓
LB070621C4		nc	4c (B2)	5-8c (B2)			Mo (C3)	Bla			✓
LB140621C1			4c (B2)	9-16c (B2)			Bla	Bla			✓
LB140621C2			4c (C3)	8c (C3)			Mo (C2)	Mo comp			✓
LB140621C3			2-4c (A1)	n.a.			n.a.	deg			✓
LB140621C4			5-8c (B1)	5-8c (C2)			5-8c (C3)	deg			✓
LB140621C5			5-8c (B3)	8c (C2)			Mo (C2)	Bla			✓
LB140621C6			9-16c (B1)	9-16c (B2)			Bla	Bla			✓
LB140621C7			nc	nc			deg	deg			
LB130721C1		nc deg	nc deg							deg	
LB130721C2		2c	4c			4c	9-16c deg	deg	deg		✓
LB130721C3		nc	nc							deg	
LB130721C4		nc PB	nc							2-4c	✓
LB130721C5		nc	nc							GVBD	
LB130721C6		nc	3c (C2)			Mo (C2)	Mo (C2)	Mo deg	deg		✓
LB130721C7		2c	4c (B2)			9-16c (B2)	Mo (C3)	Mo deg	deg		✓
LB130721C8		nc deg	nc deg							deg	

Table S 5. A vs. B: Amount of time (in hours:minutes) until embryonic cleavage for each embryo in Primo Vision (SAGE Art-1529)

ID oocyte	**2c stage**	**4c stage**	**8c stage**	**early morula**	**expanded blastocyst**
LB120421A1	08:18	09:18	24:48	123:49	
LB120421A2	16:48	17:48	36:54		
LB120421A5	06:48	14:48	26:18	92:36	123:19
LB190421A5	21:02	23:02	35:32	70:14	
LB190421A6		22:32	33:32		
LB190421A7		34:02	47:38	94:50	
LB270421A2		22:47	34:17	91:05	150:18
LB270421A4		24:48	57:54	82:00	126:13
LB270421A5		25:18	50:54	85:30	141:49
LB030521A5		27:06	49:43		
LB030521A6	32:36	33:36	46:43		
LB030521A7	22:06	34:36	50:43	106:55	
LB030521A8	18:36	29:06	56:43	96:25	
LB030521A10		20:37	32:07	69:20	
LB030521A15		25:07	53:44	91:26	138:08
LB030521A16	20:07	23:37	35:37	78:50	152:38
mean	**18:17**	**24:15**	**42:04**	**90:15**	**138:44**

Table S 6. A vs. B: Amount of time (in hours:minutes) until embryonic cleavage for each embryo in Primo Vision (SAGE Art-1029)

ID oocyte	**2c stage**	**4c stage**	**8c stage**	**early morula**	**expanded blastocyst**
LB120421B2		22:28			
LB120421B3		25:28	56:35	95:17	
LB120421B6			53:05		
LB120421B8		30:58	61:05	108:17	130:23
LB190421B3		25:02	49:09	84:45	129:57
LB190421B4	28:02	61:39			
LB190421B5			46:09	72:15	141:03
LB190421B6		35:02	58:39	97:51	127:27
LB190421B7		22:02	43:09	80:45	122:27
LB190421B9		24:32	37:02	94:51	137:03
LB190421B11		28:33	41:10	97:52	123:28
LB190421B12		29:03	43:10	85:46	150:04
LB270421B1		31:19	55:55	85:31	
LB270421B2		24:19	n.b.	n.b.	107:37
LB270421B3	26:49	36:49	54:55	97:07	122:43
LB030521B1	19:08	29:38	53:14	93:57	
LB030521B4		23:08	48:14	92:57	136:39
LB030521B6		20:38	49:14	92:57	149:39
LB030521B7		21:08	50:44	93:27	150:39
LB030521B8		19:08	47:14		
LB030521B9		38:38	54:14	95:27	127:03
mean	**24:39**	**28:55**	**50:09**	**91:48**	**132:35**

Table S 7 B vs. C: Amount of time (in hours:minutes) until embryonic cleavage for each embryo in Primo Vision (SAGE Art-1029 embryo culture containing FCS)

ID oocyte	**2c stage**	**4c stage**	**8c stage**	**early morula**	**expanded blastocyst**
LB100521B1		23:10	39:46	85:22	145:41
LB100521B2		22:40	46:16	97:29	134:35
LB100521B3		30:10	46:46	92:29	146:41
LB100521B4		23:40	44:16	91:59	142:41
LB100521B5		32:10	53:46	100:59	155:41
LB100521B7			49:16	n.a.	
LB100521B9		27:40	52:46	n.a.	155:11
LB190521B1			30:26	78:38	126:21
LB190521B3		23:26	46:02	93:44	
LB190521B4		30:26	53:02	88:14	161:03
LB190521B5		25:26	45:02	85:38	139:57
LB190521B7		27:56	34:26	82:38	147:27
LB020621B1	27:54	38:24	55:30		
LB020621B3		32:24	45:00	86:06	135:19
LB020621B4		22:54	34:54	83:06	
LB020621B7		27:24	37:54	96:42	136:25
LB140621B1			46:41	92:23	160:12
LB140621B3	26:35	33:05	42:41		
LB140621B4		34:05	48:11	85:47	116:59
LB140621B5		25:04	48:11	80:47	
LB140621B7		34:35	51:11	85:17	112:59
LB140621B8		27:05	50:41	88:23	124:59
LB130721B3	25:07	35:37	51:44		
LB130721B4	21:07	39:14	61:14		
LB130721B5	25:37	33:37	49:44	95:56	155:08
LB130721B6		24:37	47:14	89:26	132:02
LB130721B10		30:07	56:14	114:32	
LB130721B11	28:37	36:07	66:20		
mean	**25:49**	**29:38**	**47:41**	**90:15**	**140:31**

Table S 8 B vs. C: Amount of time (in hours:minutes) until embryonic cleavage for each embryo in Primo Vision (SAGE Art-1029 embryo culture containing SSS)

ID oocyte	**2c stage**	**4c stage**	**8c stage**	**early morula**	**expanded blastocyst**
LB100521C1		28:41	54:17	99:59	
LB100521C3		34:11	57:47	92:59	149:12
LB100521C4		25:41	50:47	90:29	167:18
LB100521C5	24:11	31:41	72:53		
LB100521C6		24:41	55:47	90:29	131:36
LB100521C7	24:11	33:11	55:47	96:29	
LB100521C8		30:41	49:47	85:23	141:42
LB100521C9		36:11	66:53	99:29	142:12
LB100521C10		32:42	50:48	146:43	
LB100521C11		39:48	70:54		
LB100521C13	25:12	30:12	56:18		
LB100521C14		26:42	52:48	89:00	153:13
LB100521C15		30:42	128:37	173:19	
LB100521C16		27:42	61:18	89:00	
LB100521C18		22:12	48:48	n.a.	130:37
LB190521C1	22:57	30:57	46:03	83:39	132:22
LB190521C2		29:27	52:03		
LB190521C3			48:03	91:45	146:28
LB190521C4		32:27	49:03	n.a.	143:28
LB190521C5	20:27	32:27	51:03	86:39	
LB020621C1	25:55	38:25	65:07		
LB020621C2		33:25	56:01		
LB020621C3		36:25	54:01	96:43	153:26
LB020621C4	27:55	35:55	56:01	99:13	139:26
LB020621C6		29:55	54:01	91:13	142:26
LB140621C1		32:06	57:12	94:54	128:30
LB140621C2	20:35	41:42	62:12	93:54	
LB140621C3		33:06			
LB140621C4	19:05	28:06			
LB140621C5	20:05	39:12	36:18	101:24	147:36
LB140621C6		31:06	53:12	91:24	140:06
LB130721C2	21:27	31:07	60:14		
LB130721C6	20:37	29:37	60:14	101:56	
LB130721C7	23:07	36:37	51:44	124:32	
mean	**22:44**	**32:01**	**57:41**	**100:27**	**143:06**

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