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In Vitro Embryo Production in Ruminants

Edited by

Ignacio Contreras and Sandra Soto

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About the Editors

Ignacio Contreras-Solís

Ignacio Contreras, was appointed to a tenure track position and assistant professor position at Veterinary Faculty of Central University of Venezuela (1999–2004). After achieving a Ph.D. in veterinary science in 2008, was appointed to a full professor position 2008–2012 as associated professor at the same institute. He has carried out postdoctoral research activities in Spain and Italy. His research is particularly focused on reproductive biotechnology in animal science sector using small ruminants' species.

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Preface to "In Vitro Embryo Production in Ruminants"

In vitro fertilization (IVF) has been a useful tool for accelerating the genetic progress in ruminants. Despite the efforts to improve the IVF outcome in terms of embryo yield, pregnancy rates and life births, there are still many challenges to overcome. Further optimization of this technology will potentially lead to its widespread application in ruminant breeding programs. This Special Issue highlights state-of-the-art advances on IVF technologies -including review and research articles- applied to small and large ruminants. The scope of this issue includes topics such as: novel approaches to protect the oocyte from heat stress during maturation, effects of environment/season on IVF outcomes in goat, technologies to optimize stored semen for IVF and a comparative review about IVF in Dairy Cattle and Mediterranean Water Buffalo. We hope this Special Issue will contribute to enlarge our current knowledge about factors that affect IVF in ruminant species and methods to enhance its success.

Ignacio Contreras-Solís and Sandra Soto-Heras
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Article

Establishment of a Wisent (*Bison bonasus*) Germplasm Bank

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Simple Summary: The wisent (European bison) is a protected species. For this reason, we undertook the use of biotechnologies—such as in vitro maturation of oocytes, in vitro fertilization of matured oocytes, in vitro culture of embryos, and embryo vitrification—to establish a wisent embryo bank. The competencies of the vitrified embryos were tested by transferring the warming embryos to cattle (interspecies embryo transfer). The pregnancy was confirmed biochemically and using USG, and although the fetuses were resorbed, the embryos' competence for development was demonstrated. The results of these studies open the way for the cryoconservation of wisent germplasm.

Abstract: The wisent, or European bison (*Bison bonasus*), belongs to the same family (Bovidae) as the American bison and domestic cattle. The wisent is the largest mammal in Europe, and is called the "Forest Emperor". The wisent is listed as "Vulnerable" on the IUCN Red List, and is protected by international law. Achievements in reproductive biotechnology have opened new possibilities for the cryoconservation of the wisent germplasm. Therefore, this research aimed to improve a strategy for the protection and preservation of the European bison through the creation of a wisent germplasm bank, based on the following procedures: isolation and in vitro maturation (IVM) of oocytes, in vitro fertilization (IVF) of matured oocytes, in vitro embryo culture (IVC), and embryo cryopreservation. Wisent ovaries were isolated from females outside the reproductive season, and eliminated from breeding for reasons other than infertility. Cumulus–oocyte complexes (COCs) were isolated from follicles greater than 2 mm in diameter and matured for 24 h and 30 h. After IVM, COCs were fertilized in vitro with wisent sperm. The obtained wisent zygotes, based on oocytes matured for 24 h and 30 h, were cultured for 216 h. Embryos at the morula and early blastocyst stages were vitrified and then warmed and transferred to interspecies recipients (*Bos taurus*). USG and biochemical tests were used to monitor pregnancies. This study obtained embryos in the morula and early blastocyst stages only after oocytes were fertilized and matured for 30 h. On average, per oocyte donor, 12.33 $\pm$ 0.5 COCs were isolated, and only 9.33 $\pm$ 0.61 COCs were qualified for in vitro maturation (75.68%), while 9.16 $\pm$ 0.48 COCs were matured (84.32%). On average, per donor, 5.5 $\pm$ 0.34 embryos were cleaved (59.96%) after 48 h post-fertilization (hpf), and 3.33 $\pm$ 0.21 achieved the eight-cell stage (36.52%) after 96 hpf, while 1 $\pm$ 0.21 morula and early blastocyst stages (10.71%) were achieved after 216 hpf. A total of six embryos (one morula and five early blastocysts) were obtained and vitrified; after warming, five of them were interspecies transferred to cattle (*Bos taurus*). On day 41 after fertilization, 3 out of 5 pregnancies were detected based on USG, P4, and PAG tests. However, no pregnancy was observed on day 86 after fertilization, indicating embryo resorption.

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This study shows that obtaining wisent embryos in vitro, and subsequent cryopreservation to create a wisent embryo bank, can be applied and implemented for the wisent protection program.

Keywords: wisent; European bison; oocytes; sperm; embryos; embryo transfer; germplasm bank; threatened species

1. Introduction

The wisent, or European bison (*Bison bonasus*), belongs to the same genus (*Bison*) as the American bison (*Bison bison*). Wisent can be found in the forests of Europe, and the American bison includes two subspecies: the plains American bison (*Bison bison bison*), which lives on prairies, and the wood American bison (*Bison bison athabascae*), which lives in forests. European and American bison are essential for cultural and biological reasons, and play a role as an umbrella species [1–3]. Currently, the European bison population in Poland is 2300, and in Europe there are 9100 individuals [4]. The main threat to the European bison is that it is a small, fragmented population with a low level of genetic diversity, which can reduce survival and fertility [5]. This increases the risks due to disease (e.g., tuberculosis, brucellosis, blindness), which can eliminate individuals or even entire herds [6–9].

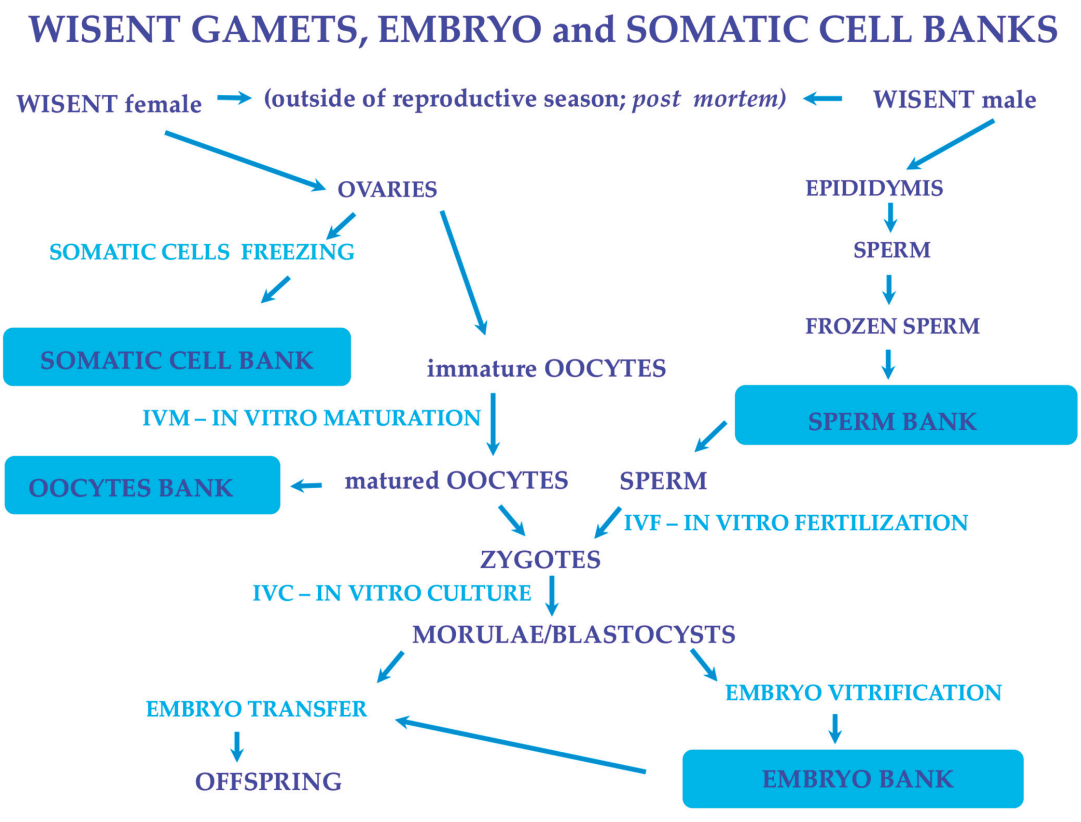
The wisent is a protected species, and is listed on the IUCN Red List as "Near Threatened". Until 2020, the European bison (*Bison bonasus*) was classified as "Vulnerable" [10,11].

Such an outstanding improvement is owed to the in situ and ex situ protection programs that have been implemented in Poland for many years [12–14]. The activities of this program have been supported by cryopreservation of germplasm, i.e., oocytes, spermatozoa, embryos, somatic cells, and ovarian tissues. The germplasm can be used for breeding, conservation, or research purposes. This is possible thanks to achievements in reproductive biotechnology, including procedures such as in vitro embryo production (i.e., in vitro maturation of oocytes, in vitro fertilization of matured oocytes, and in vitro culture of embryos), cryopreservation, embryo transfer, and cell and tissue cultures [15–38]. The germplasm can be cryoconserved through a slow freeze, but vitrification or ultrarapid freezing is now more commonly used. Among these techniques, vitrification deserves special attention, and is widely used by infertility clinics and institutions involved in the in vitro production of domestic and endangered animal embryos [17,18,20–22].

Cryopreservation of germplasm is commonly used for many species, including the American bison [23,28]. Additionally, some of these biotechniques have been implemented to protect wisents [16,32,33,36,37]. Our team has conducted this study since 2014 [39]. In 2017, we obtained the first in vitro wisent embryo [40]. In 2018, the team of Riedl et al. published an abstract about obtaining in vitro wisent embryos [41].

These successes prompted us to continue this study and focus on establishing a wisent germplasm bank, which is a multistage project defining a strategy for protecting the wisent (Scheme 1).

The aims of the first step of this project were as follows: (1) to determine the conditions for oocyte maturation, fertilization, and early development of embryos in vitro; (2) assessment of the possibility of vitrification the embryos obtained in vitro; and (3) confirmation of the developmental potential of the embryos obtained in vitro by transferring them to a recipient.



Scheme 1. The cryopreservation of wisent germplasm: gametes, embryos, and somatic cells. Generally, wisent germplasm is collected postmortem from females and males culled out of the reproductive season (from October to March). Both ovaries and epididymis can be collected, and somatic cells from ovaries can be isolated, frozen, and deposited in a somatic cell bank. Sperm are isolated from the epididymis, frozen, and deposited in a sperm bank. Additionally, immature oocytes are recovered from isolated ovaries for in vitro maturation (IVM). Some of the matured oocytes are vitrified and deposited in an oocyte bank. At the same time, most of the matured oocytes are fertilized in vitro (IVF) using thawed sperm taken from the sperm bank. In the next step, the embryos received due to fertilization are cultured in vitro (IVC) until they achieve the morulae and blastocyst stages. Next, embryos are vitrified and banked in the embryo bank to transfer them to recipients and introduce offspring into the herds.

2. Materials and Methods

This study was conducted during 2015–2021. All experiments and procedures were performed in compliance with the Polish Animal Welfare regulations, and approved by the Local Ethics Commission for Animal Experiments of Warsaw University of Life Sciences. In vitro production procedures were used according to the procedures commonly used in our laboratory [29,30].

2.1. Ovaries Isolation

In this study, 10 female wisents, 3–11 years old, from different herds in Poland, were culled out of the reproductive season (October–March) for reasons other than infertility and were used.

Ovaries from each donor were collected approximately 30 min after death, and were placed in DPBS containing 0.2 mg/mL streptomycin and 250 IU/mL penicillin at 30 °C, and transported in a thermos to the mobile laboratory within less than 30 min. Immature COCs were aspirated from ovarian follicles with a diameter of 2–6 mm and washed twice in the following medium: TCM 199 HEPES without $NaHCO_3$, supplemented with 10% FBS (vol/vol) and 50 μg/mL gentamicin, pH 7.4. Each oocyte donor was handled individually.

2.2. In Vitro Maturation of Oocytes

As described by Cervantes et al. [25], before and after in vitro maturation, COCs were classified as compacted (i.e., at least one complete layer of granulosa cells tightly surrounding the oocytes), expanded (i.e., cumulus cells expanded or partially dissociated), denuded (i.e., oocytes without cumulus cells), or degenerated (i.e., with pyknotic granulosa cells and vacuolated ooplasm). Only compacted, expanded, and denuded COCs qualified for in vitro maturation. COCs from 4 donors were matured for 24 h, and COCs from 6 COC donors were matured for 30 h. Qualified COCs started maturation during transport to the laboratory in a thermos, in a modified maturation medium of TCM 199 HEPES without $NaHCO_3$, supplemented with 10% FBS (vol/vol), 0.02 IU NIH-pFSH/mL, 1 μg/mL β-estradiol, 0.2 mM sodium pyruvate, 0.2 mg/mL streptomycin, and 250 IU/mL penicillin (pH 7.4), at 38.5 °C.

After maturation, COCs were analyzed as described by Cervantes et al. [25], and only expanded, compacted, and denuded COCs qualified for in vitro fertilization.

2.3. In Vitro Fertilization of Matured Oocytes

Only qualified COCs (i.e., expanded, compacted, and denuded) were fertilized in vitro. Sperm were previously isolated from the epididymis of a wisent bull culled out the reproductive season, and subsequently frozen in straws, as described by Kozdrowski et al. [33].

Sperm were thawed at 37 °C, centrifuged (200× *g*) for 10 min, and washed in 2 mL of Sp-TALP containing 6 mg/mL BSA fraction V and 50 μg/mL gentamicin, pH 7.4. Sperm were capacitated using the swim-up method in 1 mL of TL stock solution for sperm capacitation (MINITUBE, Delevan, CA, USA), with 6 mg/mL bovine serum albumin fraction V (BSA V) and 50 μg/mL gentamicin, pH 7.4. In vitro fertilization was performed in TL stock solution for fertilization (MINITUBE, Delevan, CA, USA), supplemented with 6 mg/mL bovine serum albumin–fatty acid fraction (BSA FAF), 0.2 mM sodium pyruvate, PHE (20 μM penicillamine, 10 μM hypotaurine, 1 μM epinephrine), 2 μg/mL heparin, and 50 μg/mL gentamicin, pH 7.4. Sperm were individually incubated with matured COCs for each donor, at a final concentration of 1×10^5 sperm/oocyte, for 18 h at 38.5 °C under 5% CO^2 in humidified air.

2.4. In Vitro Culture of Embryos

After 18 h post-fertilization (hpf), putative zygotes were denuded of cumulus cells by pipetting and washed twice in SOF stock solution (MINITUBE, Delevan, CA, USA) supplemented with 10% FBS (vol/vol) and 50 μg/mL gentamicin. Then, wisent zygotes were co-cultured with Vero cells (ATCC, Rockville, MD, USA) for 8–9 days. Vero cells at a concentration of 2×10^3 were placed in 40 μL of SOF stock solution (MINITUBE, Delevan, CA, USA) supplemented with 10% FBS (vol/vol) and 50 μg/mL gentamicin, and overlaid with mineral oil. The wisent zygotes were individually co-cultured with Vero cells at 38.5 °C under 5% CO_2 in humidified air until 216 h post-fertilization (hpf). At the same time, the medium was partially replaced with fresh medium at the same concentrations after 48, 96, and 144 hpf. After 168 hpf, the medium was partially replaced again, but was supplemented with 3 mg/mL BSA V instead of FBS. Embryo development was evaluated after 48, 92, and 216 hpf. Based on morphology, embryos were classified as G1, G2, or G3, based on the IETS manual [26,42].

2.5. Vitrification

Embryos at the morula and early blastocyst stages were vitrified using the BOVIPRO *Vit-Kit*™ (MINITUBE, Delevan, CA, USA), according to the manufacturer's instructions, and deposited in an embryo bank. Qualified embryos for vitrification were placed in a holding medium. Each of the embryos was transferred from the holding medium to 100 µL of equilibration medium A for 5 min, and then into 100 µL of equilibration medium B for 5 min. During the second exposure, the straw was preloaded with a 90 µL column of DT medium, followed by ~1.5 cm of air. Next, the embryo was transferred to a 30 µL drop of vitrification medium and immediately loaded into the straw, followed by ~1.5 cm of air, and then another 90 µL of DT medium. The straw was then heat-sealed and placed into an empty, pre-chilled 10 mm goblet held in liquid nitrogen (LN2) vapor (−150 to −180 °C). After 1 min of vapor exposure, the goblet was lowered into liquid nitrogen (−196 °C) and the straw was submerged. The total exposure time to the vitrification media was <1 min. The straws were stored in LN2 (−196 °C).

2.6. Interspecies Embryo Transfer

Initially, 20 Polish black-and-white Holstein-Friesian heifers were prepared for interspecies embryo transfer. All heifers were housed in free-stall barns, fed a total mixed ration twice daily according to nutritional requirements, and had free access to water and salt licks. Two months before transfer, they were given premixed VITAMIX KW Fertility® Polmass, Bydgoszcz, Poland) with A, D3, and E (6000 mg), as well as the recommended dose of beta-carotene (2000 mg) with organic selenium and biotin to improve breeding rates [43].

Estrus cycles of the recipients were synchronized by intramuscular (IM) injections of a prostaglandin F2α (PGF) analogue, administered twice at 11-day intervals. All recipients were synchronized using D-cloprostenol at a dosage of 0.15 mg/heifer (Dalmazin®, Fatro S.p.A., Ozzano Emilia (BO), Italy).

After the second injection of prostaglandins, all heifers were checked for signs of estrus three times per day. Heifers seen in standing estrus qualified as recipients (day 0). A day before embryo transfer, the evaluation of the corpus luteum (CL) was performed by palpations per rectum, according to Spell 2001. Only heifers with prominent CL were used for embryo transfer on day 9 (n = 5).

On day 9, five wisent embryos were warmed using the BOVIPRO *Vit-Kit*™ according to the manufacturer's instructions (MINITUBE, Delevan, CA, USA). Straw with vitrified embryo was held in the air for 10 s before submersion in water at 20–24 °C for 10 s. Immediately after initial warming, the straw was held at the sealed end and flicked 3–5 times, dislodging the air pockets to mix the contents. The embryo was allowed to rehydrate via straw dilution for up to 1 min before the sealed end of the straw was cut and its contents emptied into a Petri dish. Embryos were recovered and washed using four small drops (~50 µL) of holding medium. A single warmed wisent embryo was transferred from embryo transfer medium—BioLife Transfer Medium (Agtech Inc., Kansas City, MO, USA) to the heifer uterine horn, ipsilaterally, the ovary displaying a prominent corpus luteum.

2.7. Monitoring Pregnancy

Recipients were monitored daily for heat behavior. Pregnancy diagnosis was made at 41 and 86 days after in vitro fertilization (dpf) by ultrasonography using an HS-2200V (Honda Electronics Co., Ltd., Toyohashi, Japan) with an intrarectal 7.5 MHz linear array transducer.

Additionally, 43 days after in vitro fertilization, progesterone (P4) and pregnancy-associated glycoprotein (PAG) serum levels were also tested to confirm pregnancy. Blood samples were collected from all recipients via puncture of a coccygeal vessel (v. caudalis mediana). The sera were separated and sent on dry ice to laboratory (LABOKLIN GMBH & CO. KG LABOR FÜR KLINISCHE DIAGNOSTIK, Bad Kissingen, Germany) for diagnosis of P4 and PAG levels.

2.8. Statistical Analysis

The qualified immature and matured COC rates were expressed as a ratio of the number of useable COCs to the number of recovered COCs. The rates of embryos at the 2-cell, 8-cell, morula, and blastocyst stages were expressed as a ratio of these individual stages to the number of qualified COCs for in vitro fertilization.

Statistical analyses were performed using Statgraphics Centurion (Statgraphics Technologies, Inc., The Plains, VA, USA). The effect of the time of in vitro maturation (24 h vs. 30 h) on COCs' maturation and embryo development was analyzed using the chi-squared test. Differences at $p < 0.05$ were considered significant, and $p < 0.01$ was considered highly significant.

3. Results

Results of the in vitro maturation of oocytes and the development of embryos, depending on the time of oocyte maturation (24 or 30 h), are presented in Figure 1.

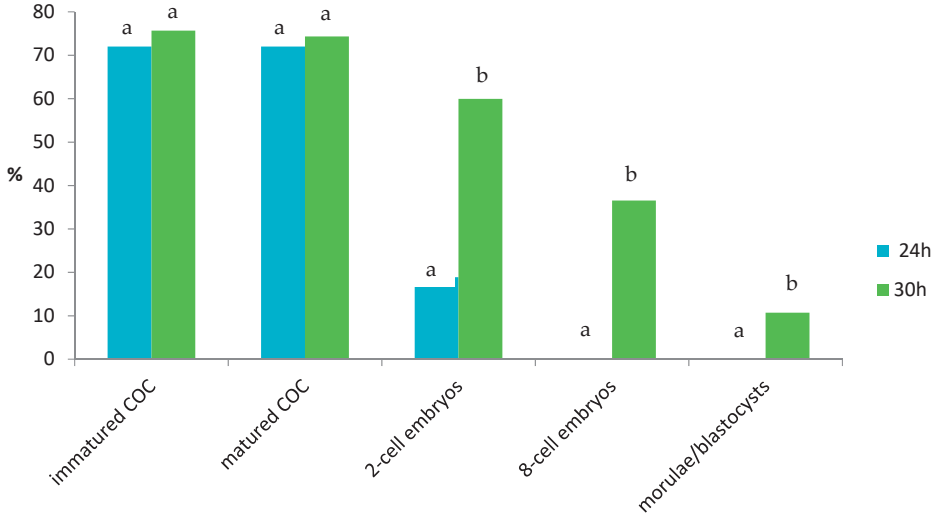


Figure 1. Analysis of percentages of in vitro maturation of oocytes and development of embryos, depending on the time of oocyte maturation (24 or 30 h). COC—cumulus-oocyte complex. Bars with different letters denote statistical differences at $p < 0.01$.

There were no differences between the times of in vitro maturation (24 h vs. 30 h) in terms of the percentage of matured oocytes. Significant differences were found in the percentage of embryos at the 2-cell, 8-cell, morula, and blastocyst stages ($p < 0.01$). Bars with different letters denote statistical differences at $p < 0.01$.

Oocyte maturation for only 24 h reduced the percentage of 2-cell embryos and arrested further embryonic development. In addition, none of the embryos reached the morula or early blastocyst stages, in contrast to embryos arising from oocytes that were matured for longer (30 h), which reached the morula and early blastocyst stages (10.71 ± 2.14).

Results of the in vitro maturation of oocytes and embryo development per oocyte donor, depending on the time of oocyte maturation (24 or 30 h), are presented in Table 1.

Table 1. Analysis of the in vitro maturation of oocytes and development of embryos per oocyte donor, depending on the time of oocyte maturation (24 or 30 h).

Parameters	24 h IVM	30 h IVM
Number of donors	4	6
Number of COCs isolated/per donor	12.50 ± 0.95	12.33 ± 0.5
Immature COCs' quality/per donor:		
Compacted	6.75 ± 1.25	7.2 ± 1.32
Expanded	1 ± 0.82	0.7 ± 0.81
Denuded	1.25 ± 0.95	1.5 ± 0.54
Degenerated	3.5 ± 0.57	3 ± 0.89
Qualified oocytes for IVM/per donor	9 ± 0.4 (72%)	9.33 ± 0.61 (75.68%)
Matured COCs' quality/per donor:		
Compacted	5.75 ± 0.95 [a]	1 ± 1.21 [b]
Expanded	2 ± 1.15 [a]	6.7 ± 1.21 [b]
Denuded	1.25 ± 0.95	1.5 ± 0.54
Degenerated	0	0.16 ± 0.41
Qualified COsC for IVF [1]	9 ± 0.4 (72%)	9.16 ± 0.48 (74.32%)
Embryo development/per donor:		
2-cell	1.25 ± 0.25 (18.88%) [a]	5.5 ± 0.34 (59.96%) [b]
8-cell	0 [a]	3.33 ± 0.21 (36.52%) [b]
M/B *	0 [a]	1 ± 0.26 (10.71%) [b]
Vitrified embryos/per donor	0 [a]	0.83 ± 0 (9.09%) [b]

[1] Data are presented as means ± SEM (%).[a,b] Within rows, values with different superscripts are different $p < 0.01$. * Morulae/blastocysts. IVM—in vitro maturation, IVF—in vitro fertilization.

There were no differences between the times of in vitro maturation (24 h vs. 30 h) in terms of the percentage of matured oocytes (Figure 1). However, the quality of oocytes after oocyte maturation for 24 h was lower than that of those matured for 30 h (expended COCs 2 vs. 6.7—Table 1). Moreover, after 48 hpf, the development of embryos, which arose based on oocytes matured for 24 h, was lower after 24 h of oocyte maturation compared to 30 h ($p < 0.01$). The further development of these embryos was stopped. Only embryos that arose based on oocytes matured for 30 h developed until the morula or early blastocyst stages. Interestingly, one transferable embryo was obtained from each oocyte donor.

Although one embryo was obtained from each donor (six embryos in total—one morula/Grade 2; three early blastocysts/Grade 1; one early blastocyst/Grade 2; and one blastocyst/Grade 2), only five were vitrified, for technical reasons; however, all of these embryos were transferred to cattle (*Bos taurus*)—the interspecies recipients.

Figure 2 shows a wisent (European bison) immature oocyte, early blastocyst, and compact morula after warming.

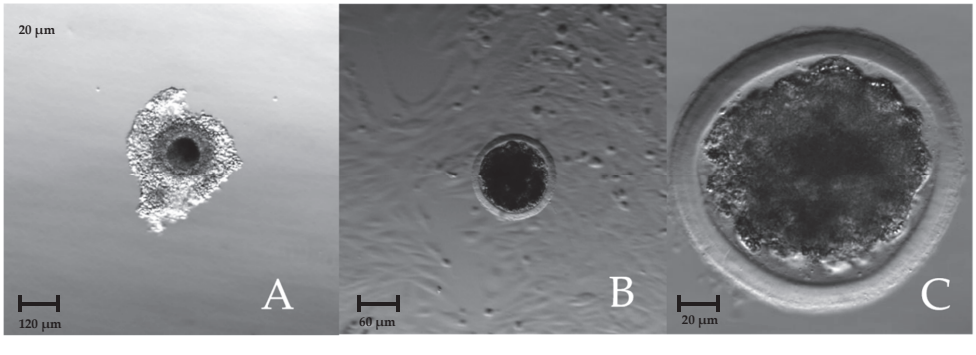


Figure 2. (**A**) Immature wisent oocyte showing the yin–yang conformation; (**B**) wisent early blastocyst 216 hpf; (**C**) wisent compact morula after warming.

The results of wisent embryo (*Bison bonasus*) interspecies transfer to cattle (*Bos taurus*) are presented in Table 2.

Table 2. Analysis of wisent embryo (*Bison bonasus*) transfer to cattle (*Bos taurus*)—interspecies transfer.

Recipient Number	Day after Estrus	Stage of Embryo at 9 dpf */Grade	Ovary/Corpus Luteum	Estrus after ET	Pregnant by USG (41 dpf ***)	Progesterone (ng/mL) (Pregnant 1–10 ng/mL) (43 dpf)	PAG (Pregnant > 0.3) (43 dpf)	Pregnant by USG (86 dpf)
1	9	WB/3	L3	present	negative	0.6	0.035	negative
2	8.5	M/2	L3	present	negative	0.5	0.02	negative
3	9	WB/1	R2	absent	positive	9.9	0.31	negative
4	9.25	WB/1	L2	absent	positive	6.5	0.32	negative
5	9	WB/1	L2	absent	positive	6.5	3.06	negative

* Grade based on IETS [43]; * M: morula, WB: early blastocyst; ** L: left, R: right; *** dpf: days post-fertilization; PAG—pregnancy-associated glycoprotein.

After the transfer, heifers were observed for the next estrus, and two of the heifers showed signs of heat. At 41 days post-fertilization (dpf), the first USG examination was performed, which revealed signs of pregnancy (i.e., enlarged horns and fluid in the uterine horns) in three heifers with the best-quality embryos transferred. The result of the USG examination was confirmed biochemically, based on progesterone (P4) and PAG tests. At 86 dpf, a subsequent USG examination was repeated, and revealed signs of embryo resorption (uterine horns returned to the state before the transfer).

4. Discussion

This study shows that it is possible to obtain wisent embryos in vitro, based on oocyte maturation, fertilization of matured oocytes, and embryo culture. Moreover, we found that embryos at the morula and early blastocyst stages can maintain their competence after vitrification.

4.1. In Vitro Maturation of Wisent Oocytes

Many factors determine the success of these biotechniques, which are crucial for access to wisent gametes. This is due to the small population of the European bison, which is also dispersed over many herds [5,16]. Moreover, gametes can only be isolated outside of the breeding season (October–March) from individuals that have been eliminated from the herd due to health reasons. In addition, gametes are isolated postmortem, which can negatively affect germplasm quality [40].

In this study, the efficiency of isolated COCs per donor ($12.33 \pm 0.5/12.50 \pm 0.95$) was similar to that for American bison [23–28]; however, analysis of COCs indicated that not all of them could qualify for maturation, which was associated with signs of degeneration (Table 1).

The main factors determining nuclear and cytoplasmic competencies for in vitro maturation may be the season, hormonal stimulation, and maturation time. In our 2018 study, we found that extending the oocyte maturation time from 24 h to 30 h impacted the development of embryos [40]. However, it is worth noting that the team of Riedl et al. [41] also conducted a similar study, and only reported that, from 2 wisents, 203 oocytes were recovered (around 102/per donor), of which 169 (83%) matured and 18 reached the morula/blastocyst stages (10.65%/per matured oocytes and 8.87% per isolated oocyte).

Much more knowledge is provided by studies on the American bison [23–28]. Krishnakumar et al. showed that immature oocytes isolated outside the breeding season and matured for 22 h have a reduced ability to fertilize, unlike oocytes isolated during the season [27]. Additionally, exciting research was conducted by Benham et al. [23], indicating that oocytes matured for 24 h reached maturity for fertilization. Thundathil et al. showed that oocytes isolated in spring—mostly from pregnant cows—and matured for 24 h were characterized by high maturity and fertilization [28]. Oocytes were also collected from hormonally stimulated donors in the breeding season. Hence, the competencies of oocytes

for fertilization and embryo development were much greater, based on the work by Cervantes et al. [25,26]. The stimulation and the extension of the maturation time from 24 to 30 h—and even up to 34 h—affect oocyte maturity [26].

In our research, we also observed that oocytes cultured for 24 h mature nuclearly, which means that they achieve metaphase II. However, as confirmed by this research, they do not have sufficient fertilization and embryonic development competence, because only 18.88% of embryos reach the two-cell stage, and none reach the eight-cell stage (Table 1). This means that the embryos do not pass through a block in their in vitro development, resulting from taking control of the embryonic genome over further development [29,30].

Contrary to 24 h COC maturation, after 30 h, COCs were characterized by statistically significant dispersed cumulus (Table 1), which was a prognostic factor for oocytes' acquisition of competencies.

An interesting observation is that, in wisents, the ooplasm is darker and unevenly distributed, which causes the yin–yang conformation effect (Figure 2A) already described in American bison [24]. Presumably, like in American bison, such a conformation does not affect the maturation of oocytes and their ability to fertilize.

4.2. In Vitro Fertilization of Matured Wisent Oocytes

It is worth noting that wisent sperm were also isolated outside of the reproductive season, and their ability to fertilize was tested on hybrids [44]. Furthermore, similar hybrids were also created by other researchers [35].

There seemed to be no difference in fertilization kinetics between wisent and cattle, because zygotes were formed at similar times.

4.3. In Vitro Culture of Wisent Embryos

In our study, embryos achieved the morula and blastocyst stages based on oocytes that matured for 30 h. On the other hand, embryos developing on oocytes matured for only 24 h only reached the two-cell stage (Figure 1). The embryos obtained based on oocytes that matured for longer (30 h) achieved the morula or early blastocyst stages, although the percentage was lower 10.71 ± 2.43% (Figure 1), but this was similar to our first report (16%) [40]. Additionally, a similar result was reported by Riedl et al., where a total of 10.65% blastocysts was achieved [41]. In American bison, the percentage of blastocysts depends on the authors; in terms of wood bison blastocysts, at day 7—27% and day 8—18.4% [26], as well as day 8—8.4% [23]. These results are also similar to ours, but other teams have achieved a higher percentage of blastocysts—45.9% (after 34 h of IVM) [24]—and morulae (32.31 ± 11.78) and blastocysts (16.42 ± 9.49) [27].

Indeed, wisent embryos' development rate is slower than that of American bison, because the early blastocyst stage of the wisent embryos was reached in 8.5–9 days (Table 1, Figure 2B). In contrast, American bison embryos reached the blastocyst stage at the same rate as cattle embryos [31].

In our study, the extension of the culture time was not justified, as the embryos were intended for vitrification.

4.4. Vitrification of Wisent Embryos

Our earlier observations indicated that embryos in the morula and early blastocyst stages should be vitrified [44]. Embryos were vitrified mainly in the early blastocyst stage, i.e., before the blastocyst cavity was entirely formed. However, vitrification did not adversely affect the development of the embryos, as all of them showed normal morphology after warming (Figure 2C). Our results are similar to those of other studies [21,22].

4.5. Wisent Embryo Transfer to Cattle Recipients

In our study, interspecies embryo transfer was performed, and in vitro embryos were transferred to cattle (*Bos taurus*), because access to recipients was extremely limited. Additionally, Riedl et al. [41] carried out interspecies transfer of wisent embryos to *Bos*

taurus. In American bison, Thundathil transferred wood bison embryos to the same species' uterus, with successful offspring delivery in 2007 [27]. Recently, Benham et al. achieved plain bison offspring [23].

The wisent is related to *Bos taurus* and American bison, and crossbred wisent/domestic cattle and wisent/American bison are observed in nature. Hence, it was justified to check the developmental competencies of the embryos by transferring them to another species, i.e., *Bos taurus*. The interspecies transfer of embryos has been performed between many species, such as rat–mouse, donkey–horse, goat–sheep, and wild cat–domestic cat and –tiger, but only donkey–horse and wild cat embryos develop to full term [45].

Wisent embryos showed correct morphology before transfer to recipients, and developmental potential after the transfer (Table 2), which was confirmed by positive USG results at 41 dpf. This was also indicated by the levels of PAG and P4 in maternal blood. PAG is synthesized in ruminants' trophectoderm and, after 28 days of pregnancy, can be detected in maternal blood [46]. Progesterone is a steroid hormone mainly produced by the corpus luteum, and this hormone can be detected after 28 days of pregnancy [47].

The levels of both markers indicated biochemical pregnancies in three out of five ET cases (Table 2). However, later on, based on USG, resorption took place (day 86 post-fertilization). Riedl (2018) also observed resorption after the transfer of wisent embryos to *Bos taurus* recipients [41].

Probable causes of resorption after interspecies embryo transfer have already been described by Widayati et al. [45]. These studies suggest that fetus resorption may have an immunological aspect, resulting from rejection by *Bos taurus* of a xenograft, such as a wisent fetus. There may also be an inappropriate interaction between the wisent trophoblast and *Bos taurus* endometrium, as shown by the study by Tachi and Tachi [48]. Early resorptions may also result from nutritional or support system abnormalities in vitro, becoming apparent later in embryonic development. Since we found biochemical pregnancies, it is possible that while there could be an interaction between the chorion and the endometrium, the placenta was not formed [45].

Wisent embryos could be transferred to *Bos taurus* 8.5–9 dpf at the earliest, because they only reached the stage of morulae or early blastocysts. For this reason, recipients were also between 8 and 9 days of heat. In cattle, the embryos in these stages are most often transferred to recipients on the 7th day. However, it has been shown that it is possible to successfully transfer embryos asynchronously in cattle—that is, 7-day-old or 9-day-old embryos to 9-day-old recipients [49]. Despite fetal resorption, the transfer of wisent embryos to *Bos taurus* recipients confirmed that the wisent embryos maintain developmental potential after warming.

5. Conclusions

In this study, the procedures of in vitro maturation of immature oocytes, in vitro fertilization of matured oocytes, and in vitro culture of embryos resulted in one wisent embryo/per donor, which could also be successfully vitrified. Therefore, these procedures allowed for the establishment of a wisent embryo bank, which can be commonly implemented to preserve and protect this species. Therefore, despite the low efficiency of this biotechnology, this is a very promising path for protecting and maintaining the genetic variability of this threatened species.

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Review

In Vitro Production of Embryos from Prepubertal Holstein Cattle and Mediterranean Water Buffalo: Problems, Progress and Potential

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Simple Summary: In vitro embryo production using oocytes from prepubertal cattle and buffalo collected by laparoscopy can be used to produce embryos from genetically superior females. Following transfer of these embryos into adult recipient animals, multiple offspring can be produced from these elite animals in a very short timeframe, long before they reach sexual maturity, thereby reducing the generation interval and accelerating genetic gain. This review article summarizes recent advances in this technology, outlines the current limitations, and suggests possible avenues to further improve this emerging biotechnology.

Abstract: Laparoscopic ovum pick-up (LOPU) coupled with in vitro embryo production (IVEP) in prepubertal cattle and buffalo accelerates genetic gain. This article reviews LOPU-IVEP technology in prepubertal Holstein Cattle and Mediterranean Water Buffalo. The recent expansion of genomic-assisted selection has renewed interest and demand for prepubertal LOPU-IVEP schemes; however, low blastocyst development rates has constrained its widespread implementation. Here, we present an overview of the current state of the technology, limitations that persist and suggest possible solutions to improve its efficiency, with a focus on gonadotropin stimulations strategies to prime oocytes prior to follicular aspiration, and IVEP procedures promoting growth factor metabolism and limiting oxidative and endoplasmic reticulum stress.

Keywords: Holstein; Mediterranean Water Buffalo; in vitro embryo production; laparoscopic ovum pickup; accelerated genetic gain; prepubertal; embryo development

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1. Introduction

In vitro embryo production (IVEP) and embryo transfer (ET) technologies have had a momentous impact on livestock production, with their use growing substantially in recent years. Despite barely being used on a commercial scale as recently as the late 1990s, IVEP has increased at an average annual rate of 12%, according to data provided by the International Embryo Transfer Society [1,2]. Moreover, it has been applied in most important livestock species, as reviewed in previous publications, e.g., cattle [2], buffalo [3], camelids [4], swine [5], goat and sheep [6,7], and cervids [8]. In cattle, where IVEP is broadly used, the majority of embryos transferred worldwide have been produced in vitro since 2016 [1]. Although no single factor can be attributed as the sole cause of this major milestone, improved media composition, the introduction of sexed semen, faster turnover compared to conventional multiple ovulation embryo transfer (MOET), and the ability to use semen from multiple bulls on oocytes from a single donor at the same time are all believed to be contributing factors [9].

Another key factor that explains IVEP expansion is the refinement of technologies to enable safe and practical collection of oocytes from live females. In large adult animals, most oocytes used for commercial embryo production are collected via ultrasound-guided trans-

vaginal ovum pickup (OPU). However, in species that are too small for oocyte collection via OPU (e.g., sheep, goat, deer), a laparoscopic ovum pick-up (LOPU) procedure was developed in the early 90s [10]. Since then, it has been refined and adapted for use in a wide range of both domestic and wild species [10–18]. The LOPU approach has several advantages over OPU, including that the ovary is viewed directly with a depth of field, rather than on a two-dimensional sonogram, enabling superficial follicles to be aspirated accurately without risking injury to the ovarian stroma [19]. This minimizes ovarian trauma, and hence the risk of sequels including tissue adhesions. As such, LOPU can be repeated on a regular basis while minimizing long-term reproductive concerns [11,20].

Of particular interest is the application of LOPU to conduct IVEP in very young animals. LOPU allows the recovery of oocytes from animals as young as two months of age, long before they are sexually mature or large enough for ultrasound guided OPU. Subsequently, IVEP allows for these oocytes to be fertilized in vitro to produce blastocysts, which are then transferred into adult recipient females, as shown in Figure 1. Using this approach, multiple offspring from the donor animal can be born before it reaches sexual maturity. Using LOPU-IVEP, it is now possible to exploit the large ovarian pool of oocytes present at young ages to rapidly proliferate genetically superior, valuable, or endangered animals [19,20]. It also provides a faster mechanism for the proliferation of animal lineages of particularly valuable genotypes [21]. Additionally, from a more basic-science perspective, prepubertal animals are also excellent negative models for the acquisition of developmental competence, leading to a better understanding of infertility and the development of new fertility treatments [22].

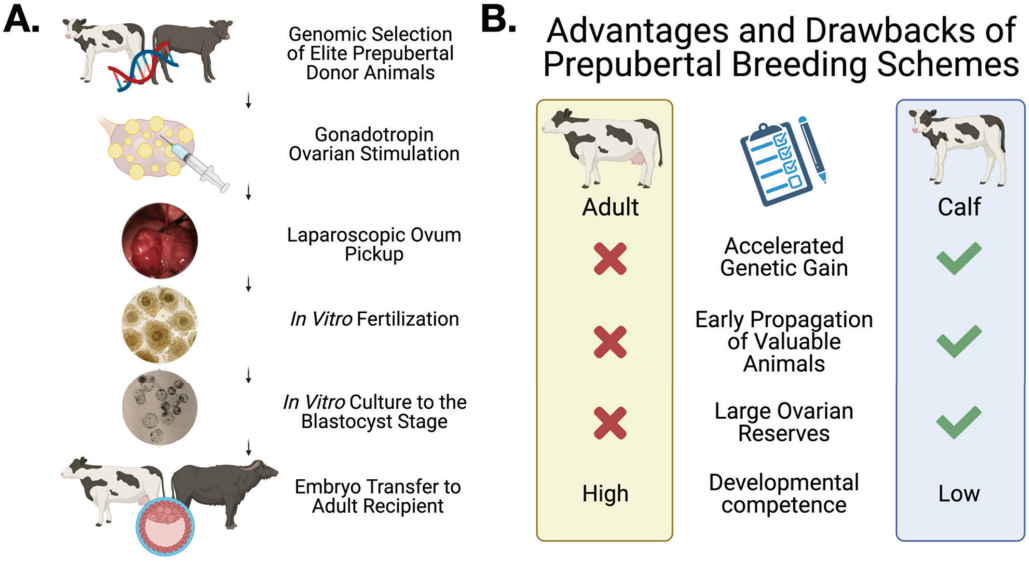

Figure 1. Overview of LOPU-IVEP in Prepubertal Buffalo and Cattle. (**A**): Flow chart showing the typical steps involved in prepubertal LOPU-IVEP programs. (**B**): Comparison between adult and prepubertal breeding schemes, showing the advantages and drawbacks of each. Figure created with BioRender.com, accessed on 30 July 2021.

There are two main reasons for the interest in using prepubertal animals as oocyte donors. First, the ovarian pool of available oocytes is vast; prepubertal animals consistently yield large numbers of cumulus-oocyte complexes (COCs) compared to their adult counterparts [19,23]. Second, early propagation of elite animals results in shorter intervals between generations, thereby increasing the rate of genetic gain [24] and enabling faster access to the latest genetic lineages. However, multiple studies have consistently shown that, although large number of COCs can be recovered, poor embryo development rates

result in few blastocysts from prepubertal-derived oocytes in many domestic livestock species including cattle [25,26], buffalo [27] goat [28,29], sheep [30,31], and pig [32,33]. Although differences in oocyte competence vary among species, in cattle, prepubertal oocytes typically yield a 10–15% blastocyst rate compared to ~30% using oocytes from adult animals [23]. While the exact reasons for the impaired competence are unknown and are most likely a combination of multiple factors, various differences have been noted such as smaller oocyte size, incomplete cytoplasmic maturation, variations in gene expression, and alterations in protein synthesis and metabolism [26,34,35].

This review will focus on prepubertal reproductive technologies, sometimes dubbed 'juvenile in vitro embryo transfer' (JIVET), in Holstein–Friesian cattle (*Bos taurus taurus*) and Mediterranean water buffalo (*Bubalus bubalis*). Together, these species serve as complementary animal models to investigate prepubertal oocyte competence and improve prepubertal reproductive technologies since Holsteins mature relatively quickly while water buffalo mature much more slowly. In normal breeding practices using artificial insemination, Holstein heifers typically give birth to their first calf around two years of age, while, on average, water buffalo heifers are not expected to calf until around three years of age. As such, the goals of this review are to outline the current state of the technology, identify research gaps and suggest possible future avenues of research.

2. Increasing the Rate of Genetic Gain by Shortening Generation Intervals

Selective breeding, or artificial selection, is the practice where individuals are bred based on specific merits in order to proliferate a desirable trait. Broadly speaking, exceptional animals are bred to produce superior offspring. Recently, genomics has revolutionized selective breeding strategies and reliable single-nucleotide polymorphisms for various traits have been identified in both cattle and water buffalo [36]. The rate at which these genetic gains (the difference in genetic value between parent and offspring) take place is inversely correlated with the generation interval [37]. Therefore, it is beneficial to breed the best animals at the youngest age possible in order to maximize the rate of genetic gain. Using buffalo as an example, if a calf undergoes LOPU/IVEP/ET at two months of age, offspring would be born at around the time the donor animal is one year old, effectively decreasing the generation interval by up to two years.

3. History of LOPU-IVEP in Prepubertal Calves

It was identified early on that using prepubertal animals in breeding programs would lead to dramatic increases in the rate of genetic gain. However, most early attempts at using MOET in prepubertal cattle predominantly failed. Some of the first attempts in the early 1970s noted that, when embryos sourced from prepubertal animals were placed in culture, development arrested before reaching the morula stage [38–40]. It should be noted that IVEP technology was still in its infancy at that time. Nevertheless, these pioneering studies showed that prepubertal animals could respond to exogenous gonadotropin stimulation [41]. Although animals responded well to follicle-stimulating hormone (FSH) treatment, they did not ovulate reliably in response to injections of pituitary extracts high in luteinizing hormone (LH) [41,42], resulting in low recovery rates and poor embryonic development [38,39]. Based on those observations, assumptions were made that the prepubertal reproductive tract was detrimental and not conducive to normal fertilization and early embryo development [38,41]. Ultimately, this resulted in MOET strategies being abandoned and attention instead turned to LOPU-IVEP.

Substantial research was done in the 1990s to develop reliable LOPU and IVEP techniques for prepubertal animals in several species. Studies during this period showed that the LOPU component was largely successful, but IVEP was not. Following LOPU, multiple authors reported high oocyte yields in young animals, often more than what is typically recovered from adult animals [19,23,43]. Following IVEP, the oocytes from young animals resulted in blastocyst development rates that were consistently lower than rates in mature animals [25,44–47]. For example, Revel and associates found similar fertilization

and cleavage rates between oocytes from three-month-old heifer calves and adult cattle, but prepubertal oocytes failed to produce similar blastocyst rates [23]. These poor results, combined with the inability to identify genetically superior animals at such young ages at that time, led to the loss of interest in prepubertal LOPU-IVEP research projects for around 20 years.

Since these studies in the 1990s, significant advancements in marker-assisted selection, genomics, and IVEP have renewed interest and demonstrated the potential to circumvent many of the initial shortfalls. Genomic marker selection in particular is having a huge impact on the dairy industry where the production phenotype can be accurately predicted as soon as the animal is born through screening of single-nucleotide polymorphisms [48,49]. Along with the progress in genome selection, significant improvements in IVEP practices have been accomplished in recent years, resulting in the ability to produce high quality embryos in vitro, comparable to their in vivo derived counterparts [9]. Recent innovations such as sequential media compositions and advanced low-oxygen tension incubators have enabled production of embryos in vitro possessing cryotolerance capabilities similar to embryos produced in vivo [2,50]. As genomic selection and modern IVEP technology become more and more cost-effective in the future, their application and use are expected to continue growing.

Despite significant progress in recent years, problems with prepubertal IVEP technologies still exist. As evidenced from studies in different species, prepubertal oocytes have a reduced developmental competence compared to adult oocytes, with fewer IVEP embryos reaching the blastocyst stage, as observed in bovine [23], buffalo [27], ovine [30], caprine [51], and swine [52]. It has been shown that calves respond well to FSH stimulation and produce many follicles, often producing more than cows [19,23,43]. Consequently, prepubertal donors typically produce more COCs and 2-cell stage embryos than adult donors. Blastocysts derived from prepubertal oocytes are competent to support full-term development and normal offspring have been produced in multiple species, including buffalo [7,27] and cattle [23,53,54]. Hence, the primary challenge remains the improvement of oocyte competence to enable higher embryo development rates to the blastocyst stage. In this regard, learning how to prime and prepare prepubertal oocytes, both in vivo inside the follicle and in vitro during maturation and culture, seems the most logical and promising path to consolidate LOPU-IVEP uses in prepubertal breeding schemes, as shown in Figure 2.

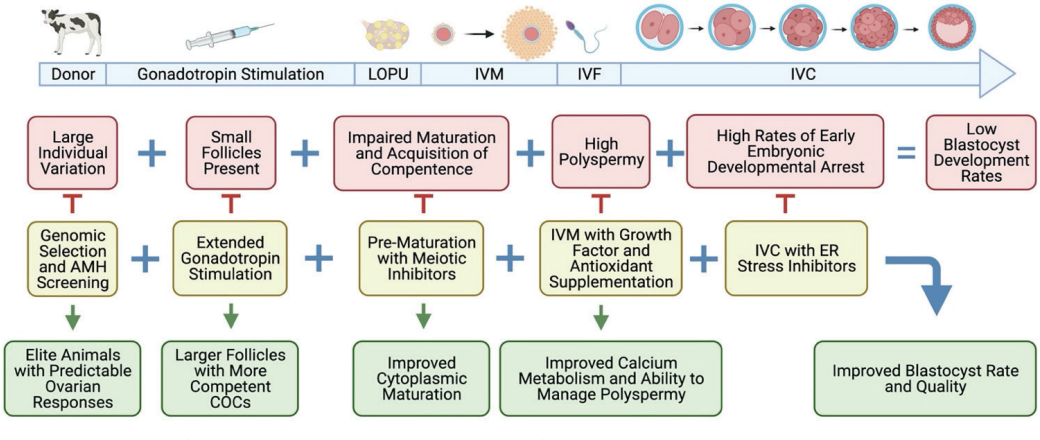

Figure 2. Overview of LOPU-IVEP in prepubertal cattle and buffalo, showing potential targeted approaches to address the main problems holding the technology back. Figure created with BioRender.com, accessed on 30 July 2021.

4. Understanding Developmental Competence of Oocytes

One of the greatest challenges in overcoming the impaired developmental competence of prepubertal oocytes is that the underlying reasons are not fully understood, and the cause is most likely a combination of multiple factors. For example, the hypothalamic-pituitary-ovarian axis in prepubertal animals is immature, which could lead to defective signalling and steroidogenesis in ovarian follicles. In turn, an improper follicular microenvironment could affect metabolism within the oocyte itself or the crosstalk between the oocyte and granulosa cells, ultimately resulting in oocytes unable to reach full developmental competence.

4.1. The Hypothalamic–Pituitary–Ovarian (HPO) Axis

The HPO axis is essential for the management of the oestrous cycle and, consequently, fertility. Kisspeptins (*Kp*) are a family of neuropeptides in the hypothalamus, which were discovered in 2003 to operate upstream of gonadotropin-releasing hormone (GnRH) signalling [55]. GnRH neurons express the receptor for kisspeptin, GPR54, and consequently have been implicated in many critical roles including timing the onset of puberty, secretion of gonadotropins, transmission of the negative and positive feedback loops, and generation of the LH surge [56,57].

This upstream hypothalamus signalling is believed to be the last component of the HPO axis to mature in juvenile heifers, and is the limiting factor determining the HPO functionality prior to puberty [58]. Specifically, the number of *Kp*-positive cells in the arcuate nucleus and pre-optic area are believed to be responsible for the negative and positive feedback loops, respectively, and have been shown to increase during prepubertal development in the ewe [59]. Downstream, in the pituitary, GnRH receptors do not change with age, and secrete gonadotropins in response to GnRH at a very young age [60,61]. In the ovary, the relative mRNA abundance of FSH receptor in granulosa cells is significantly lower in prepubertal Holsteins compared to adult cows, possibly explaining the smaller average follicle size in prepubertal animals, and consequently the reduced developmental competence of oocytes [62].

4.2. Follicular Microenvironment

The lower developmental potential of calf oocytes may be due to environmental deficiencies in vivo prior to retrieval [23,63]. Hence, a clear understanding of the follicle and its follicular fluid is important. Calf follicular fluid contains approximately half the LH concentration compared to cow follicular fluid (2.0 ± 0.2 ng/mL vs. 4.0 ± 0.3 ng/mL) [64]. This is in accordance with the plasma concentration of LH, which is also lower in younger animals [65]. Although changes in LH concentration may have no direct impact on the oocyte itself due to a lack of LH receptors, it would affect steroidogenesis and androgen production in granulosa and theca cells [66]. A disruption in estrogen production would affect the transcription of genes regulated by estrogen response elements. Alternately, impaired androgen metabolism could also affect fertility, as androgen-receptor knock-out mice are sub-fertile [67]. In a similar manner to LH, calf follicular fluid has also been shown to contain approximately half the estradiol content compared to adults (6.3 ± 2.1 ng/mL vs. 12.7 ± 5.5 ng/mL) [64]. Collectively, it can be speculated that these differences in the follicular micro-environment may negatively impact the acquisition of developmental competence, and may partially explain the low IVEP outcomes observed in calves [63]. This further emphasizes the importance of suitable gonadotropin stimulation regimes to emulate a follicular microenvironment that will promote oocyte competence prior to LOPU.

4.3. Oocyte and Granulosa Cell Crosstalk

Oocyte competence is dependent on intercellular communication within the ovarian follicle during follicular growth and development, and is regulated by endocrine, paracrine, and autocrine factors [68]. While direct inter-cellular connections are mediated via gap

junctions and transzonal projections (TZPs) [69], indirect intercellular communication can occur through extracellular vesicles (EVs) secreted into the follicular fluid [70]. Collectively, these pathways facilitate bi-directional communication, signaling and transport of molecules between the oocyte, granulosa, and theca cells [70,71].

Developmental competence increases gradually and sequentially as oocytes increase in size due to transcriptional activity during follicular and oocyte growth [72,73]. This is vital as oocytes from prepubertal animals are smaller and have a thinner zona pellucida than those from adults, despite originating from follicles of the same size [74]. For example, calf oocytes have a mean diameter of 118.04 ± 1.15 µm compared to a mean diameter of 122.83 ± 0.74 µm for mature cows [24,74]. Since a small variation in diameter represents a larger variation in volume, small variations in diameter may have important impacts on developmental competence. As such, the capacity of bovine oocytes to mature to metaphase II during IVM is positively correlated with their diameter [75]. Aside from diameter, several cytoplasmic differences have also been observed between oocytes from prepubertal and adult animals. For example, oocytes from adult cows have more lipid droplets in their cytoplasm compared to those from heifers, both before and after IVM [63]. Other differences include incomplete cytoplasmic maturation, altered gene expression and protein synthesis, as well as defective metabolism in oocytes from young animals [26,34,35].

More recently, the intimate relationship between the oocyte and cumulus cells has been investigated to better define the role of TZPs [76,77]. Although more research needs to be done to determine how the physiology, distribution and retraction of TZPs impacts IVEP outcomes in both prepubertal and adult oocytes, TZPs are known to facilitate communication and the transport of essential molecules between granulosa cells and the oocyte [76,78]. Despite differences observed in the organization of TZPs in COCs from lambs compared to adult ewes, the impact on embryo development remains unclear [79].

In addition to intercellular communication via TZPs, the roles of EVs on intra-follicular cell communication has also become of particular interest [70]. EVs are small lipid bilayer particles secreted by cells into the extracellular space, which then diffuse and act on secondary target cells, transporting various molecules including proteins, lipids, messenger RNA (mRNA), and microRNA (miRNA) [80,81]. Since the initial discovery of EVs in equine follicular fluid in 2012 [82], they have since been described in bovine [83] and porcine follicular fluid [84,85] and were shown to play multiple roles inside the follicle, including granulosa cell proliferation and cumulus expansion [86,87]. Notably, studies have found variability in EV and miRNA profiles when comparing follicular fluid from follicles of different sizes and young vs. old animals [82,86–88]. For example, da Silveira found significant differences in the number and profiles of miRNAs present when comparing follicular fluid from young (3–13 y.o.) and old (>20 y.o.) mares [82,89]. Others have found similar results when comparing younger (<31 y.o.) and older (> 38 y.o.) women [90]. How these findings may translate into prepubertal vs. adult cattle and buffalo remains unknown. However, it has been shown that supplementation with EVs in vitro was able to increase blastocyst rates in cattle to 37%, compared to 26% using IVM with EV-free fetal calf serum [91]. Thus, it is possible that supplementation with adult EVs in prepubertal IVEP programs may help improve oocyte competence.

5. Hormonal Stimulation

Due to the impaired HPO axis in prepubertal animals, an efficient hormonal stimulation protocol is critical to provide the COCs with a conducive intra-follicular milieu prior to LOPU. Previous work in our laboratory showed that FSH stimulation in prepubertal calves was able to mimic a functional HPO axis by increasing mRNA expression of FSH receptor (FSHR) and cytochrome P450 family 19 subfamily A member 1 (CYP19A1), while decreasing levels of steroidogenic acute regulatory protein (StAR) and hydroxy-δ-5-steroid dehydrogenase, 3β-and steroid δ-isomerase 1 (HSD3B1) in calf granulosa cells [62]. The molecular changes that occur during follicular and oocyte growth involving molecules synthesized within the oocyte or imported from granulosa cells are critical for the acquisition of

an oocyte's developmental competence and support the theory that "the history of the follicle determines the future of its oocyte" [92]. In support of this, several studies have shown a positive correlation between the follicular diameter and developmental competence of the oocyte in many species, including sheep [93], goat [94,95], cattle [35,72,92], buffalo [96], and pig [97,98]. For example, in adult cattle, oocytes from follicles 2–6 mm in diameter produced an average blastocyst rate of 34.3%, while oocytes from follicles > 6 mm in diameter produced an average blastocyst rate of 65.9% [72]. A similar pattern was observed in adult buffalo, with oocytes originating from follicles < 3 mm in size resulting in a blastocyst rate of 2.4 ± 1.5% while oocytes originating from follicles > 8 mm in diameter resulted in a blastocyst rate of 16.9 ± 1.7% [99]. This same trend was observed in prepubertal animals, with blastocyst rate per oocyte increasing from 6.8% to 13.8%, comparing oocytes from small (<5 mm) and large (≥5 mm) follicles in Holstein calves [100].

In prepubertal animals, LOPU-IVEP has been performed following hormonal stimulation protocols that were adapted from those used for adult animals. The goals of gonadotropin stimulation are not only to increase the size of follicles, and consequently oocyte competence, but also to increase the number of follicles suitable for aspiration [21]. Follicle stimulating protocols have consisted of multiple injections of FSH, single injections of compounds with a longer half-life such as equine chorionic gonadotropin (eCG), or a combination of both FSH and eCG [101–103]. Due to its short metabolic half-life, FSH is typically re-administered every 12 h for 3–4 days. Studies in the 1990s found that calves had a significantly better follicular response when subjected to multiple injections rather than a single injection of a large dose of FSH [102,103]. However, combining a single FSH injection with one of eCG resulted in a similar ovarian response to multiple FSH injections, suggesting a single dose of FSH is able to recruit but not sustain development of a follicle cohort [102,103]. These data seem to be supported by the fact that combining a single injection of FSH with a low dose of eCG can result in a similar ovarian response to multiple-injection regimes, with the FSH bolus able to recruit a follicle cohort, and the eCG able to sustain continued development [45]. It could be possible that eCG aids in follicle development from its inherent LH activity, which could act synergistically with FSH [103,104]. When comparing the interval between FSH, with and without eCG, we found that FSH injections every 8 h starting 72 h before LOPU, until a single dose of 400 IU of eCG 36 h prior to LOPU, yielded better blastocyst rates compared to FSH injections every 12 h without eCG (17.5 ± 8% vs. 8.9 ± 5%) [100].

6. LOPU and COC Quality

As the LOPU procedure is essentially the same for all ruminants and has been described in detail in other manuscripts [7,10,53], this review will not focus on the technical aspects of the procedure itself. However, it is worth highlighting that LOPU has been shown to be extremely safe and can be repeated on a regular basis. For example, LOPU has been repeated ~10 times in goats [20], and we repeated the procedure every two weeks in prepubertal Holsteins and buffalo between 6 and 9 times over a 3–4 month period [105]. Following this, none of the animals had reproductive problems later in life, as they were used to produce more embryos by trans-vaginal OPU and had normal fertility following artificial insemination. In our experience with prepubertal calves and buffalo, oocyte recovery rate (the proportion of follicles from which COCs were recovered) following LOPU is usually very good. Indeed, the average recovery rate was 77.1 ± 27% in Holstein calves (n = 109 LOPUs) [53], and 84.3 ± 29.3% in buffalo calves (n = 56 LOPUs, unpublished). Concerning COC quality, 87.4 ± 19% were deemed usable including 67% grade 1 and 20.4% grade 2 [53]. In addition, we observed that the gonadotropin stimulation regime used affected COC quality, with a longer stimulation protocol (≥72 h) resulting in a viability rate of 95.3% ± 18%, compared to 85.4% ± 22% for a shorter protocol (36–42 h) [53].

7. Individual Variation

In adult cows, the ovarian response upon gonadotropin stimulation is widely variable among animals [106]. The same variation was observed in calves [21,106], with research in our laboratory revealing similar results in both Holsteins [100] and buffalo [105] calves as shown in Table 1. The large individual variation is problematic in selecting the best calves to be used in a prepubertal LOPU-IVEP scheme, which may be mitigated by determining the serum concentrations of anti-Müllerian hormone (AMH) given its correlation with an individual animal's response following gonadotropin stimulation observed in adult cattle and buffalo [107–109]. Although more work needs to be done to confirm this remains true in prepubertal buffalo calves, data suggest that AMH concentration remains a credible marker for LOPU-IVEP performance in prepubertal *Bos taurus* and *indicus* calves [110]. This is particularly useful since the follicular population is difficult to assess using ultrasound at such a young age.

Table 1. Individual variation of usable COCs recovered from calves over six LOPU sessions.

Species	Number of Animals	Number of COCs Recovered			
		Total	Mean ± SD All Calves (Total Per Calf)	Mean ± SD Bottom Calf (Total)	Mean ± SD Top Calf (Total)
Holstein	11	1393	22.2 ± 14 (126.6)	12.7 ± 4 (72)	38.2 ± 11 (229)
Buffalo	8	774	16.2 ± 9 (81)	10.1 ± 3 (50)	26.6 ± 6 (130)

SD = standard deviation. Data adapted from [100,105].

Seasonality

In the specific case of buffalo, another factor potentially contributing to variation in results is season. Buffalo are sensitive to long photoperiods, with reproductive efficiency improving in the autumn and winter as daylight decreases, similar to sheep & goat [111–114]. Season has been reported to influence the age at puberty [115]. Moreover, in adult Mediterranean buffalo undergoing repeated OPU, embryo yield improved significantly in the autumn [116], but there are yet no studies on the impact of season on prepubertal oocyte quality. Additionally, heat stress is well-researched and known to impact the estrous cycle, follicular development, oocyte quality and embryonic development rates in ruminants [117–119].

8. In Vitro Embryo Production

Following LOPU, oocytes undergo in vitro maturation, fertilization, and culture. Although variations exist in cattle and buffalo, these usually last for 22 h, 18 h, and 7 days, respectively. Most protocols have followed media compositions and procedures consistent with those used for adult animals with minimal derivations [23,101,120]. As such, commercially available media can be used. However, prepubertal oocytes may benefit from specially tailored IVEP protocols supplemented with various factors, which is discussed below.

8.1. Oocyte In Vitro Maturation (IVM)

Although in vivo maturation was the norm for many years, and the first Holstein calf born in the world from IVF was a product of in vivo maturation [121], in vitro maturation has yielded more reliable and consistent results in recent years. The objectives of IVM are both nuclear and cytoplasmic maturation. Nuclear maturation is the transition from germinal vesicle (prophase I) to metaphase II, while cytoplasmic maturation allows morphological, functional and biochemical changes to take place in the cytoplasm.

Multiple studies have shown that, although prepubertal oocytes are able to complete nuclear maturation, their ability to manage cytoplasmic maturation is more ambiguous. For nuclear maturation, it has been shown that oocytes can undergo germinal vesicle breakdown and successfully arrest at metaphase II [43,46,47,103]. It has been suggested that this process may be delayed in lamb oocytes compared to ewes [79]. However, our findings with oocytes collected from Holstein [53,100] and buffalo (unpublished) calves revealed that ~80% were able to mature to the metaphase II stage and successfully extrude the first polar body after 24 h of IVM. In terms of cytoplasmic maturation, electron microscopy studies have shown that organization of the oocyte organelles, such as the number and distribution of cortical granules as well as the population of mitochondria, are different in prepubertal compared to adult oocytes [47,79,122]. Damiani and colleagues compared cortical granule migration in calf and cow oocytes and found that cortical granules did not migrate as efficiently in calf oocytes as only 19% (17/90) of calf oocytes exhibited migration compared to 71% (83/117) in cow oocytes. This may impact normal fertilization and the initiation of the block to polyspermy, since 81% (73/90) of calf oocytes still possessed clusters of cortical granules following IVM [47]. Furthermore, cortical granule migration was delayed in 70% (19/27) of calf oocytes compared to 28% (7/25) in cow oocytes [47]. In addition to cortical granule migration, other cytoplasmic differences have been noted, including the distribution of mitochondria and lipid droplets [47]. These cytoplasmic deficiencies may be associated with the impaired competence of prepubertal oocytes. In support of this, it has been shown that transferring the nuclei of adult oocytes into enucleated calf oocytes resulted in similarly low development rates to those observed in control calf oocytes [123].

8.2. In Vitro Fertilization (IVF)

The ability of calf oocytes to properly manage fertilization, oocyte activation and the block to polyspermy appears to be impaired. Research in the 1990s showed that, although fertilization rates (as measured by sperm penetration) were the same between prepubertal and adult donors, there was a significantly higher rate of abnormal fertilization in prepubertal (16%) than adult (7%) oocytes [24,47]. Work in our laboratory provided additional evidence that polyspermy is a significant problem for IVF in calf oocytes. Working with Holstein calf oocytes and using the industry standard concentration of 1 million motile sperm/mL, polyspermy rates were over 40% [53]. However, when the sperm concentration was reduced to 500,000 motile sperm/mL, the incidence of polyspermy decreased to 19.7% [53]. In addition, the normal fertilization rate, as evidenced by the presence of two polar bodies and two pronuclei, increased from 59.4% to 69.7% [53]. Interestingly, we observed a steady decrease in polyspermy rates with age, declining from 45.5% in animals < 100 days old, to 12.8% in animals >130 days old [53]. We also observed similar results working with buffalo calves, with age and semen dose affecting polyspermy rates [105].

8.3. Embryo In Vitro Culture (IVC) and Transfer

Following fertilization, cell division appears to be delayed, with a low proportion of calf-derived embryos reaching the 4 and 8-cell stages of development at standardized time points [35,120]. In addition, embryo development to the blastocyst stage is significantly lower than what is achieved with adult Holsteins and buffalo oocytes [22,23,27,35,120]. In our experience with Holstein calves, cleavage rates varied between 60–70% and blastocyst rates were around 20%. However, both embryo yield and quality were significantly affected by the gonadotropin stimulation protocol and age of the calves [53]. In buffalo, this is potentially compounded by the fact that both the oocyte donor and sire used during IVF have a large influence on IVEP outcome, with only around 10% of males suitable for IVF [27,124]. Despite limited information in the published literature on the timing and causes of embryonic development arrest, it has been shown in 6–8-month-old heifers that 67% (40/60) of cleaved embryos that failed to reach the blastocyst stage arrested between the 2 and 8-cell stage, which was significantly higher than the 18% (5/28) observed in embryos from adult animals [120]. This suggests that prepubertal oocytes are unable to

transition from oocyte to embryo and properly regulate embryonic genome activation, as the stage of developmental arrest coincides at around this time [125].

Recently, the possible impact of ARTs on the embryonic epigenome has garnered attention, with studies suggesting offspring produced by IVEP may be at higher risk of various disease [126,127]. For example, large offspring syndrome has been associated with epigenomic differences in imprinted genes [128,129]. Furthermore, the extent of cellular reprogramming and epigenetic inheritance of both parental methylomes on the embryo is currently being investigated [130]. Whether prepubertal LOPU-IVEP programs may affect epigenetic inheritance is unclear, however. Evidence in bulls suggest that the age of the sire influences the transcriptome and epigenome of blastocysts produced by IVF [131]. In females, transcriptomic comparison of blastocysts produced from the same heifers between 8–14 months old revealed that genes related to mitochondrial function were impacted in younger heifers [132]. How these differences may affect future embryo development of offspring is unknown.

Despite the lower development to the blastocyst stage, prepubertal embryos can reach this stage in a similar timeframe and have normal characteristics including a visible inner cell mass [120]. In terms of cell numbers, as an indicator of embryo quality, there were no differences in the trophectoderm-inner cell mass ratio between hatched and unhatched blastocysts from cows and 6–8-month-old heifers [120]. However, the total cell count in day 8 blastocysts was slightly lower but not statistically different between embryos of heifers (89 $\pm$ 20) and adult cows (100 $\pm$ 30) [120]. Additionally, heifer-derived and cow-derived blastocysts seem to have similar lipid metabolism, with day 8 blastocysts containing comparable triglyceride concentrations [120].

The ultimate and essential test for blastocyst quality is the ability to establish pregnancy and result in healthy offspring following embryo transfer. Pregnancies and live births with full-term offspring following LOPU-IVEP and embryo transfer have been reported by multiple authors using calf-derived oocytes in both Holsteins [23,43,44,53,101] and buffalo [7,27]. Although earlier studies have suggested lower rates of establishing pregnancy with prepubertal-sourced embryos, our findings revealed more encouraging results. Indeed, we obtained a 62% (13/21) pregnancy rate after transferring LOPU-IVEP blastocysts from Holstein calf oocytes. Of the 13 confirmed pregnancies, 4 were interrupted for experimental reasons and 100% of the 9 that were allowed to continue carried their pregnancy to term [53]. In buffalo, of 10 embryo transfers, 3 became pregnant, all of which delivered healthy calves [7,105]. Other authors reported similar results in prepubertal buffalo by confirming 3 pregnancies and delivery of healthy calves after the transfer of 8 IVEP embryos [133,134]. With the knowledge that these prepubertal LOPU-IVEP-ET schemes do work, animal breeding companies are now starting to offer these programs on a commercial basis. However, further research is needed to improve and ensure the long-term financial viability of these programs going forward.

8.4. Embryo Cryopreservation

In addition to yielding similar rates of embryos and pregnancies following transfer, another goal is for prepubertal-derived embryos to have cryotolerance similar to that of adult-derived embryos. It is well documented that in vivo produced embryos are more cryotolerant than their in vitro produced counterparts [135–137]. As such, embryo quality plays a major role in post-thaw survivability, with the cytoplasmic lipid content, i.e., the number and size of lipid droplets, shown to affect cryotolerance significantly, with more lipids being detrimental [135]. This presents a unique challenge for buffalo embryos, as they have high levels of lipids [3]. To address this problem, L-carnitine supplementation in vitro has been shown to aid in the lipid metabolism, as well as providing antioxidant protection, which improved post-thaw survivability in both Holsteins [138,139] and Buffalo [140,141]. However, this strategy remains to be tested in prepubertal-derived embryos.

9. Future Perspectives: What Can We Do Better?

With the knowledge that prepubertal LOPU-IVEP technologies do work, as evidenced by healthy calves born following embryo transfer, the current challenge is improving efficiency. As such, attention should focus on conditions both in vivo, before LOPU, and in vitro, following LOPU. In vivo approaches should include innovative gonadotropin stimulation protocols for young donor animals in order to enhance the intra-follicular environment and maximize oocyte development inside the follicle. In vitro approaches should focus on amending IVEP procedures to better accommodate the requirements of prepubertal oocytes to maximize meiotic maturation, normal fertilization and embryo development to the blastocyst stage.

9.1. Optimized Gonadotropin Stimulation

Efficient gonadotropin stimulation regimes should increase the size of follicles available for aspiration, as embryo development rates are directly associated with follicular size [35,72,92,96]. As such, gonadotropin stimulation over a longer period of time has been shown to be beneficial in calves. Work in our laboratory compared short (3 FSH injections, 12 h apart, starting 36 h prior to LOPU, total FSH 100 mg) vs. long gonadotropin treatments (6 FSH injections, 12 h apart starting 72 h prior to LOPU, total FSH 96–140 mg) and revealed that not only did the proportion of large follicles aspirated increase (11.2% vs. 34.0%), but cleavage rate (59.0 $\pm$ 23% vs. 72.7 $\pm$ 21%) and blastocyst rate (18.3 $\pm$ 15% vs. 36.7 $\pm$ 26%) were also significantly increased in the longer treatment [53]. Other studies have shown that an even longer stimulation duration of 7 days, compared to 4 days, resulted in a larger proportion (56.4 $\pm$ 8.3% vs. 27.8 $\pm$ 7.5%) and number (13.3 $\pm$ 1.8 vs. 9.0 $\pm$ 1.3) of large follicles ($\geq$9 mm) [142,143]. However, the study focused only on the dynamics of follicular populations by serial ultrasound scanning, and the effects of such a prolonged protocol on oocyte competence and embryo development rates remains to be tested. Similarly, gonadotropin stimulation significantly increased the proportion of medium (4–8 mm) and large follicles ($\geq$9 mm) in buffalo aged between 5 and 9 months [144].

9.2. Oxidative Stress and the Importance of Antioxidants

Oxidative stress caused by reactive oxygen species (ROS) can damage cells by disrupting homeostasis and leading to apoptosis. Glutathione (GSH) is considered the major line of defence against oxidative injury by helping to maintain the redox state within the cell. In addition to its role in preventing oxidative stress, GSH has been shown to play an important role in the transport of amino acids, as well as in DNA and protein synthesis [145]. The tripeptide thiol compound has been shown to be synthesised during oocyte maturation in bovine [146], bubaline [147], caprine [148], and porcine [149] oocytes. GSH is also known to play important roles in the formation of the male pronucleus and early embryonic development [150]. As oxidative stress is known to be pervasive during in vitro manipulation, compared to conditions in vivo, most IVEP protocols use antioxidants aimed at either promoting GSH synthesis (e.g., cysteine), or scavenging ROS (e.g., melatonin) [151]. Since oxidative stress is known to play a significant role in vitro and prepubertal oocytes may be deficient in their ability to combat ROS, it is plausible that they are more susceptible to oxidative stress [151,152]. As such, prepubertal IVEP may require specialized antioxidant treatments tailored to their needs. This may be especially important in buffalo because of the high concentration of lipids within the oocyte and therefore the increased risk of lipid peroxidation.

Although many different antioxidants have been tested and used over the years in adult IVEP schemes, there are fewer studies assessing the efficacy in prepubertal animals, especially in cattle and buffalo. Working with 1–2-month-old goats, Rodriguez-Gonzalez and colleagues found that IVM supplemented with cysteamine increased the GSH concentration, and improved blastocyst yield and total cell number per blastocyst [148]. Similar results were found in adult buffalo by Gasparrini and colleagues [153]. In a subsequent paper by the same group, they showed that supplementation with cysteamine combined

with cystine, was even more advantageous than cysteamine alone, increasing the transferrable embryo rate from 23.8 ± 3.9% to 30.9 ± 5.8% [154]. Whether these findings can be applied to prepubertal animals remains to be determined.

Another antioxidant used in many IVEP schemes across multiple species is melatonin, which has been shown to reduce oxidative damage in the oocyte [155,156]. Melatonin is produced throughout the body, including the ovary, and has been detected in follicular fluid of bovine [157], porcine [158], bubaline [159], and caprine [155] follicles. In prepubertal goats, higher concentrations of melatonin were detected in large follicles (> 5 mm) compared to small follicles (<3 mm) [155]. The same trend was found in adult Murrah buffalo [159]. In prepubertal goats, melatonin supplementation during IVM increased the blastocyst rate [155], decreased intracytoplasmic ROS, improved ATP content, and enhanced mitochondrial activity [156]. Similar results were found in adult Holstein cows [160] and water buffalo [161]. While melatonin supplementation during IVM of COCs from 4–5-week-old lambs was found to have no effect on development rates [162], in 6–10-month-old Holsteins, it was shown to increase blastocyst rates from 11.1 ± 3.5% to 23.1 ± 5.1% [163].

9.3. Endoplasmic Reticulum Stress

Endoplasmic reticulum (ER) stress is a major contributor to embryonic death because physiological and exogenous stressors typically lead to disruptions in protein folding and ROS production in the ER [164]. Induction of ER stress has been shown to impair embryo development rates in multiple species [165,166], while ER stress inhibitors have been shown to improve IVEP development rates [165,167,168]. Tauroursodeoxycholic acid (TUDCA), a bile acid, was shown to inhibit ER stress and improve in vitro embryo development and blastocyst quality in different species [168–172]. TUDCA supplementation was shown to decrease the incidence of DNA double strand breaks in porcine blastocysts [168] and decrease intracellular ROS concentrations in oocytes from adult cattle [173]. In buffalo, treatment with TUDCA decreased cell apoptosis in embryos under ER stress induced by tunicamycin [166]. In prepubertal Holsteins, IVC supplementation with 50 μM TUDCA tended to increase blastocyst rates (30.9 ± 12% vs. 25.7 ± 2%) compared to the control [100]. More studies are needed to better evaluate the impact of TUDCA in prepubertal oocytes, such as testing higher concentrations during IVC. It is also possible that supplementing both IVM and IVC with TUDCA could further impact prepubertal IVEP because of its role in the regulation of calcium metabolism [174,175], which could also favor normal fertilization and embryo cleavage.

9.4. Cytokines and Growth Factors

Cytokines and growth factors are small peptide proteins involved in cellular signalling and communication. Fibroblast growth factor 2 (FGF2), leukaemia inhibitory factor (LIF), and insulin-like growth factor (IGF1) are among the growth factors found in follicular fluid that have regulatory effects on COCs. Working with porcine oocytes, Yuan and colleagues (2017) assessed the impact of adding these growth factors (in a cocktail coined 'FLI') to IVM media and observed a significant increase in oocyte maturation, embryo development and quality, and litter size following embryo transfer [176]. Working with lambs, Tian and colleagues found that combining FLI with insulin-transferrin-selenium (ITS) during IVM increased the blastocyst rate more than two-fold (44.2 ± 5.7% vs. 21.6 ± 4.6%) compared to the control group [162]. How these findings may benefit IVEP systems for prepubertal cattle and buffalo remains unknown.

9.5. Oocyte Pre-Maturation In Vitro

There is evidence that a short 'pre-maturation' period in presence of meiotic inhibitors such as c-type natriuretic peptide (CNP), epidermal growth factor receptor (EGFR) inhibitor, and cAMP prior to IVM may improve oocyte competence. During LOPU, separation of COCs from their follicles causes cAMP concentrations to decrease, resulting in spontaneous resumption of meiosis [177]. During pre-maturation, oocytes are temporarily arrested at

the GV stage, to allow more time for cytoplasmic maturation to occur and promote synchrony among aberrant nuclear and cytoplasmic maturation [177,178]. Several studies have shown pre-maturation protocols able to increase blastocyst rate and quality [179,180]. CNP increases cGMP concentrations in COCs, which inhibits the cAMP hydrolyzing enzyme phosphodiesterase 3A, maintaining meiotic arrest [181]. Pre-maturation of prepubertal goats COCs for 6 h with CNP maintained TZP density, which is essential for cGMP transport into the oocyte and, consequently, meiotic arrest [182]. This treatment significantly increased blastocyst development rates compared to controls (30.2% vs. 17.2%), possibly due to an improved ability of the oocyte to manage oxidative stress, as CNP pre-maturation resulted in increased intra-oocyte glutathione concentrations and decreased ROS [182]. EGFR inhibition can also be used to reversibly arrest bovine COCs at the GV stage [183]. These pre-maturation protocols may represent a new alternative for use in combination with growth factors, antioxidants and inhibitors of ER stress to further improve prepubertal IVEP efficiency. However, these approaches require further validation.

10. Conclusions

Although several obstacles remain to be overcome, the use of prepubertal breeding schemes based on LOPU-IVEP is a powerful method for accelerating genetic gain. In Holsteins, the technology has reached a level of commercial viability, with several large biotechnology companies currently using this technology. Although the potential reward in applying this technology in buffalo is larger due to their prolonged sexual maturity, more work needs to be done for further efficiency optimization. Enhanced stimulation protocols yielding more competent oocytes at collection, coupled with in vitro procedures that will improve cytoplasmic maturation and the oocyte's machinery to fight oxidative and ER stress, are among the improvements that will likely increase the proportion of competent oocytes recovered from prepubertal compared with post-pubertal animals.

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Heat Shock Protein 70 Improves In Vitro Embryo Yield and Quality from Heat Stressed Bovine Oocytes

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Simple Summary: The Holstein cows are among the most thermosensitive farm animals. In this breed, during the heat stress periods, fertility is seriously compromised due to induced alterations of the endocrine status, reduced fertilizing capacity of the oocyte and increased embryo deaths. To combat the deleterious effects of stress, cells synthesize a series of specific molecules that are mainly involved in cellular protection against the heat insult, called heat shock proteins (HSPs). Here, we examined the effects of supplementing HSP70 in in vitro matured bovine oocytes under thermoneutral or heat stress conditions, and we assessed its efficacy on in vitro embryo yield and quality; the latter was determined on the basis of the expression of various genes related to important cellular functions. It was manifested that HSP70 addition into the in vitro maturation medium restores the developmental competence of heat stressed oocytes and improves the quality of the in vitro produced embryos.

Abstract: Heat shock protein 70 (HSP70) is a chaperon that stabilizes unfolded or partially folded proteins, preventing inappropriate inter- and intramolecular interactions. Here, we examined the developmental competence of in vitro matured oocytes exposed to heat stress with or without HSP70. Bovine oocytes were matured for 24 h at 39 °C without (group C39) or with HSP70 (group H39) and at 41 °C for the first 6 h, followed by 16 h at 39 °C with (group H41) or without HSP70 (group C41). After insemination, zygotes were cultured for 9 days at 39 °C. Cleavage and embryo yield were assessed 48 h post insemination and on days 7, 8, 9, respectively. Gene expression was assessed by RT-PCR in oocytes, cumulus cells and blastocysts. In C41, blastocysts formation rate was lower than in C39 and on day 9 it was lower than in H41. In oocytes, HSP70 enhanced the expression of three HSP genes regardless of incubation temperature. HSP70 at 39 °C led to tight coordination of gene expression in oocytes and blastocysts, but not in cumulus cells. Our results imply that HSP70, by preventing apoptosis, supporting signal transduction, and increasing antioxidant protection of the embryo, protects heat stressed maturing bovine oocyte and restores its developmental competence.

Keywords: HSP70; heat stress; in vitro embryos; gene expression; cattle

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1. Introduction

Climate change figures at the top of the challenge list, and could have a potentially devastating impact on the global ecosystem and animal welfare. Due to decreased thermoregulatory capacity, dairy cows are particularly vulnerable to heat stress. During summer heat stress, the dry matter intake is reduced and the general physiology of the cow

is disturbed, leading to a significant decrease in production and fertility; these seriously compromise the sustainability of the dairy industry and the welfare of the animals [1,2]. The maturing oocyte is particularly sensitive to heat stress in a stage-dependent manner. The sensitivity culminates at the second meiotic arrest during metaphase II [3]. Heat stress induces mitochondrial dysfunction, accumulation of reactive oxygen species and increased apoptosis that inhibits the completion of meiosis I and eventually reduces the developmental competence of the oocyte [4–6]. The expression of heat shock proteins (HSPs) is considered the major response mechanism, which the cells operate to maintain their homeostasis against temperature changes [7].

Heat shock protein 70 (HSP70) is a molecular chaperon that protects oocyte against the harmful effects of stress. In mammalian cells, the HSP70 family exists in two isoforms: a constitutively form (HSC70) and a heat-inducible form (HSP70). HSPs were initially associated with the response to heat stress; however, it is now well known that a variety of stressors induce their expression, and therefore they are characterized as "cell stress" proteins [8]. They are normally presented in all cell compartments, such as the cytoplasm, mitochondria, nucleus, endoplasmic reticulum, while during and after heat stress they concentrate mainly in the nucleus [9]. Under normal conditions, HSP70 participates in many important functions such as post translational folding and transportation of cellular proteins through the membranes [10,11]. There is evidence that HSP70 is also involved in fertilization and early embryo development [12]. The addition of antibodies for HSP70 in early embryos cultured in vitro suppresses the blastocyst formation rate both in cattle and in mice [13,14]. Under heat stress, HSP70 plays a vital role by preserving the stability of the cytoskeleton, regulating the cell cycle and the immune response, preventing cell apoptosis and contributing to the thermotolerance of cells [11,15]. Apoptosis is blocked through the interruption of the mechanism of caspase 3 activity [16] as well as by decreasing the phosphorylation of elF-2a, an essential factor for the initiation of protein translation, when reduced protein synthesis during heat stress is needed [15].

Under stress conditions, HSPs are also located in the extracellular space [17], where they regulate functions such as inflammation or acute immune response [18,19]. The secretion of HSPs in the extracellular space can be mediated through a mechanism involving the lysosomes [20] via membrane-bounded particles [21] or by passive leaking from cells undergoing necrotic death, after the disruption of their membranes [17]. Extracellular HSPs are regulators of inflammation [22–24] and their regulatory effect is mediated by Toll-Like Receptors (TLRs), particularly TLR2 and TLR4 [18].

In a recent study [25], we have shown that exposure of in vitro maturing oocytes for only 6 h at 41 °C during the early stages of in vitro maturation disorganizes the expression of many genes in the oocytes, the cumulus cells and the blastocyst; in addition, it impairs embryo yield. Here we sought to examine whether the addition of HSP70 in the in vitro maturation medium would prevent the negative effects that a short-term temperature rise causes to the oocyte and to the embryo production rate and quality and how its effect is mediated by key genes.

2. Materials and Methods

2.1. In Vitro Embryo Production

Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company (Poole, UK). The techniques for in vitro embryo production have been previously described [26,27]. In brief, ovaries from slaughtered mature cows of different breeds (Holstein, Limousine and Holstein crossbreeds) were collected from a local abattoir. The ovaries were transported to the laboratory within 2 h from slaughter, at 37 °C in sterile saline (0.9% NaCl) containing 0.1% Gentamycin. Cumulus oocyte complexes (COCs) were aspirated from 3–8 mm follicles using a syringe with a 18G needle. Only grade 1 and 2 COCs, as morphologically described by de Loos et al. (1989), were used [28]. Selected COCs were washed in phosphate-buffered saline (PBS) and in maturation medium (TCM 199 supplemented with 10% fetal calf serum (FCS) and 10 ng/mL epidermal growth factor (EGF)).

Depending on the experiment, the maturation medium was modified with the addition of 5 ng/mL HSP70. The HSP70 dose was selected on the basis of HSP70 concentration that we measured in the peripheral blood of 164 heat stressed dairy cows [29]. COCs (n = 1933) were randomly allocated to 1 of 4 maturation protocols: 39 °C for 24 h without (group C39, n = 471) or with HSP70 (group H39, n = 353), for 6 h at 41 °C (from the 2nd to 8th hour of IVM) followed by 16 h at 39 °C in the presence (group H41, n = 704) or in the absence of HSP70 (group C41, n = 405). Maturation was carried out in an atmosphere of 5% CO_2, 20% O_2, with maximum humidity.

After 24 h in IVM, matured COCs were inseminated with frozen–thawed, swim-up separated bull sperm at a final concentration of 1×10^6 spermatozoa/mL. Gametes were co-incubated for 24 h in standard IVF medium at 39 °C, under an atmosphere of 5% CO_2, 20% O_2 with maximum humidity. Semen from the same Holstein bull and ejaculation was used for all experiments.

Approximately 20 h post insemination (pi), presumptive zygotes were denuded by gentle vortexing and cultured in groups of 25 in microdroplets (25 μL) under mineral oil. Zygotes were cultured for 9 days in synthetic oviductal fluid (SOF) supplemented with 5% FCS at 39 °C, in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2, in maximum humidity.

Cleavage and blastocyst formation rates were recorded after stereo-microscopic observation at 48 h pi and on days 7, 8 and 9 pi, respectively. The in vitro embryo production experiment was carried out in 10 replicates. From 5 replicates, pools of 12 matured oocytes, with the respective cumulus cells and 12 D7 blastocysts, were snap-frozen in PBS in liquid nitrogen and stored at −80 °C, until gene expression analysis. The oocytes used for gene expression studies were mechanically denuded by sequential passages of the COCs through a fine glass pipette and were washed in PBS.

2.2. RNA Extraction and Reverse Transcription

Total RNA was extracted using PicoPure RNA Isolation Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol and was further treated with DNAfree DNA Removal kit (Thermo Scientific, Waltham, MA, USA) to remove any DNA residuals. RNA's quantity and quality were assessed using a Qubit™ RNA BR Assay Kit. The cDNA synthesis was performed using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA), 15 ng of total RNA and a combination of oligodTs and random primers. cDNA samples were further diluted (1:2 for the oocytes, 1:5 for the cumulus cells and the blastocysts) and were stored at −80 °C.

2.3. Gene Expression Analysis

In oocytes, the gene expression of HSPA1A, HSP90AA1, HSPB11 (heat shock proteins), SOD2, GPX1 (antioxidants), G6PD (metabolism), BCL2 (cell cycle) and TLR2 (inflammation); in cumulus cells the expression of HSPA1A, HSP90AA1 (heat shock proteins), BCL2 (cell cycle), CPT1B, G6PD (metabolism), IGF1 (cell signaling), GSTP1 (antioxidant) and ATP1A1 (osmoregulation); and in blastocysts HSP90AA1, HSPA1A, HSF1 (regulation of HSPs' expression) GPX1, GSTP1, PLAC8A (implantation), TLR2, ATP1A1, BAX, BCL2, DNMT3A (epigenetic regulation), AKR1B1 (metabolism) and IGF1 (signaling), were analyzed. Most of the primer pairs used in this study were from our previous study [25], and the additional pairs that were designed for this analysis are presented in Table 1. For the primer designment, PrimerBLAST and Primer3 were used [30], and their suitability was evaluated using Beacon Designer (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1 (accessed on 5 December 2019)).

Table 1. Primer information: sequence, size of the amplified fragments of transcripts and accession number.

Gene Name	Gene Description	Forward Primer	Reverse Primer	Product Size (bp)
TLR2	Toll like receptor 2	GCTGCCATTCTGATTCTGCT	GCCACTCCAGGTAGGTCTTG	103
BCL2	BCL2 apoptosis regulator	CCCTGTTTGATTTCTCCTGGC	CTGTGGGCTTCACTTATGGC	107
HSF1	Heat shock transcription factor 1	ATGAAGCACGAGAACGAGGC	GCACCAGCGAGATGAGGAACT	112
ATP1A1	ATPase Na+/K+ transporting subunit alpha 1	CGCCAGGGTTTATCCAGTT	AGGGGAAGCCAGTTTTTGTT	80
IGF1	Insulin like growth factor 1	TCACATCCTCCTCGCATCTCTT	AGCATCCACCAACTCAGCC	107
BAX	BCL2 associated X, apoptosis regulator	TTTGCTTCAGGGTTTCATCC	CGCTTCAGACACTCGCTCAG	120

qPCR was performed using KAPA SYBR FAST (Sigma Aldrich, Milwaukee, WI, USA) on an AB Step One Plus Mastercycler (Applied Biosystems, Waltham, MA, USA), in a 20 µL reaction containing 1.5 µL cDNA, gene-specific primers (300 nM final concentration) and 1× KAPA SYBR FAST qPCR Master Mix. The cycling conditions were: 5 min at 95 °C, followed by 40 cycles of 20 s at 95 °C and 20 s at 60 °C for annealing and extension. A melting curve step was performed for each reaction to ensure the specificity of the products. Samples were measured in duplicates and a threshold of ±0.2 in Cq differences between replicates was used to discard samples with discrepancies. Cq values were retrieved for each reaction by setting a constant threshold and the average efficiencies per gene were computed using the LinReg software, as proposed by Ramakers and his colleagues (2003) [31].

The relative gene expression was normalized using the geometric mean of three reference genes: YWHAZ, UBA52 and EEF1A1, which were evaluated using GeNorm, with the respective M value as an indicator of the gene expression stability across samples [32].

2.4. Statistical Analysis

Statistical analyses of in vitro embryo production (IVP) and gene expression studies were performed by R. In IVP, the results are expressed as means ± standard deviations. Data normality was checked using a Shapiro–Wilk test; there was homogeneity of variances, as assessed by Levene's test of homogeneity of variance ($p > 0.05$). Two-way ANOVA was used to show differences between Temperature and HSP70. Moreover, we split the data into four strata to detect the actual relationship between variables. The objective of stratification was to fix the level of the potential effect modifier and produce groups within which the effect modifier did not vary. After that, we conducted an independent Student's *t*-test to assess between-group differences with Temperature and HSP70 treatment in each phase. Significance was determined by a p value of <0.05.

The statistical analysis of differentially expressed genes (DEGs) was performed as follows:

1. A Two-way ANOVA test among all the four groups was used to detect the possible effect of the two factors (Temperature, HSP addition) along with their interaction in the differential gene expression. Pairwise comparisons were conducted between pairs of groups (C39, H39, C41, H41). We focused on the differences between the H and C groups, since our major question is to address the effect of HSP addition to the medium. The significant differences (p-values < 0.05) are presented in Supplementary Tables S2–S4.
2. Correlation coefficients were computed for each pair of genes in two groups (samples supplied with HSP70 and not supplied with HSP70) using the rcorr function, since correlated gene expression may be indicative of a similar regulation mechanism underlying gene expression. Coefficients were plotted using the corrplot function, where positive correlations are displayed in blue and negative correlations in red

color. Color intensity and the size of the circle are proportional to the correlation coefficients.

3. Results

3.1. In Vitro Embryo Production

Cleavage rate in group C39 was significantly higher ($p < 0.01$) compared with groups H41 and C41 and tended to be higher compared with group H39 ($p = 0.068$). No other differences were detected among groups.

On days 7, 8 and 9 blastocyst formation rates in group C41 were significantly lower ($p < 0.03$) than in group C39, and on day 7, it tended ($p = 0.06$) to be lower than in group H39. Similarly, on day 9, the blastocyst formation rate in group C41 was significantly lower ($p = 0.03$) than in group H39. No difference was detected in the embryo yield between groups C39 and H41.

Details on in vitro embryo production are given in Table 2, and in Supplementary Table S1.

Table 2. Cleavage and blastocyst formation rates (mean ± SD) in four groups of COCs matured in vitro at 39 °C without (group C39) or with HSP70 (group H39), at 41 °C for 6 h from the 2nd to 8th hour of IVM without (group C41) or with HSP70 (group H41).

			Blastocysts		
Group	**COCs**	**Cleaved (%)**	**Day 7 (%)**	**Day 8 (%)**	**Day 9 (%)**
C39	519	438 ^{a} (84.4 ± 4.5)	154 ^{a} (29.7 ± 6.6)	172 ^{a} (33.1 ± 6.0)	179 ^{a} (34.5 ± 7.6)
H39	353	286 ^{a,b} (81.0 ± 7.5)	94 ^{ab} (26.6 ± 4.4)	111 ^{ab} (31.4 ± 6.0)	122 ^{ab} (34.5 ± 6.5)
C41	508	401 ^{b,c} (78.8 ± 6.8)	102 ^{b} (20.1 ± 3.7)	123 ^{b} (24.2 ± 7.9)	129 ^{c} (25.5 ± 9.0)
H41	704	551 ^{b,c} (78.2 ± 7.4)	186 ^{ab} (26.4 ± 10.4)	226 ^{ab} (32.1 ± 11.2)	235 ^{abc} (33.4 ± 11.4)

Within columns, values marked with different superscripts (^{a,b,c}) differ significantly ($p < 0.05$).

3.2. Gene Expression

The gene expression analyses were carried out on materials collected from groups C39, H39, C41 and H41.

3.2.1. Oocytes

The Two-Way ANOVA analysis revealed that the expression of four genes (*HSPB11*, *BCL2, GPX1, SOD2)* out of the eight measured were significantly ($p < 0.035$) altered by the presence of HSP70 in the medium and there was a strong tendency towards the differential expression of *G6PD* (p-value = 0.07). The relative expression of genes is presented in Figure 1.

The correlation analysis revealed three genes with a strong positive correlation (*HSPB11, SOD2, GPX1*) in the HSP addition group, and these genes were negatively correlated with two HSP genes (*HSP90AA1, HSPA1A*) in the same group. In the absence of HSP70, these correlations were absent, and *HSP90AA1* was negatively correlated with *HSPB11*, while *BCL2* was positively correlated with *GPX1* (Figure 2).

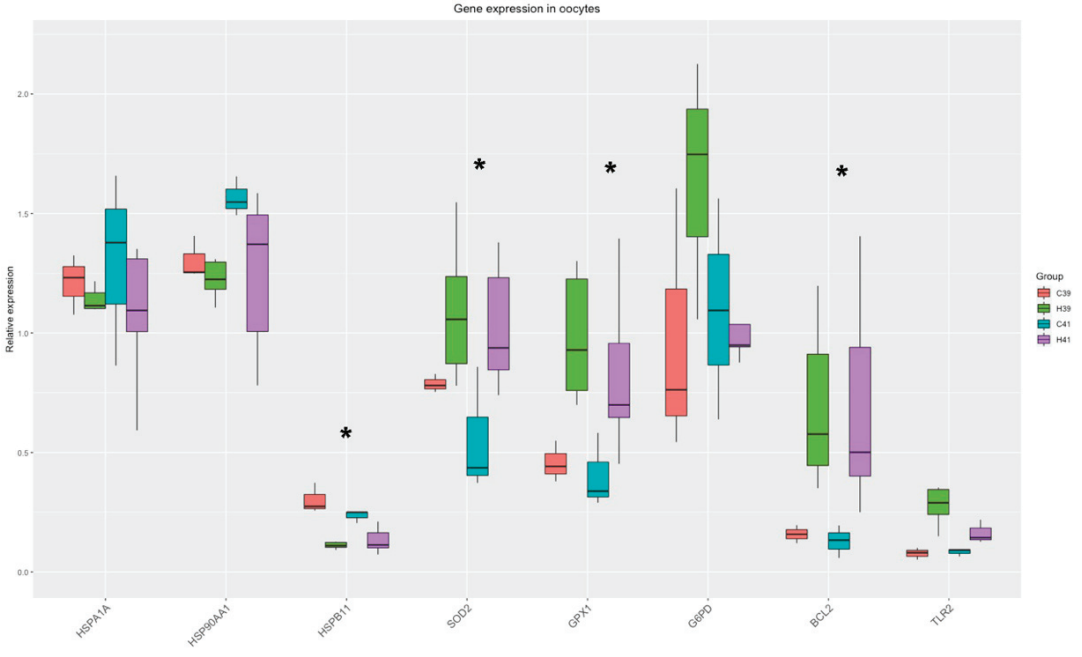

Figure 1. Gene expression in oocytes. Significant changes from the Two-Way ANOVA test are marked with asterisk (*).

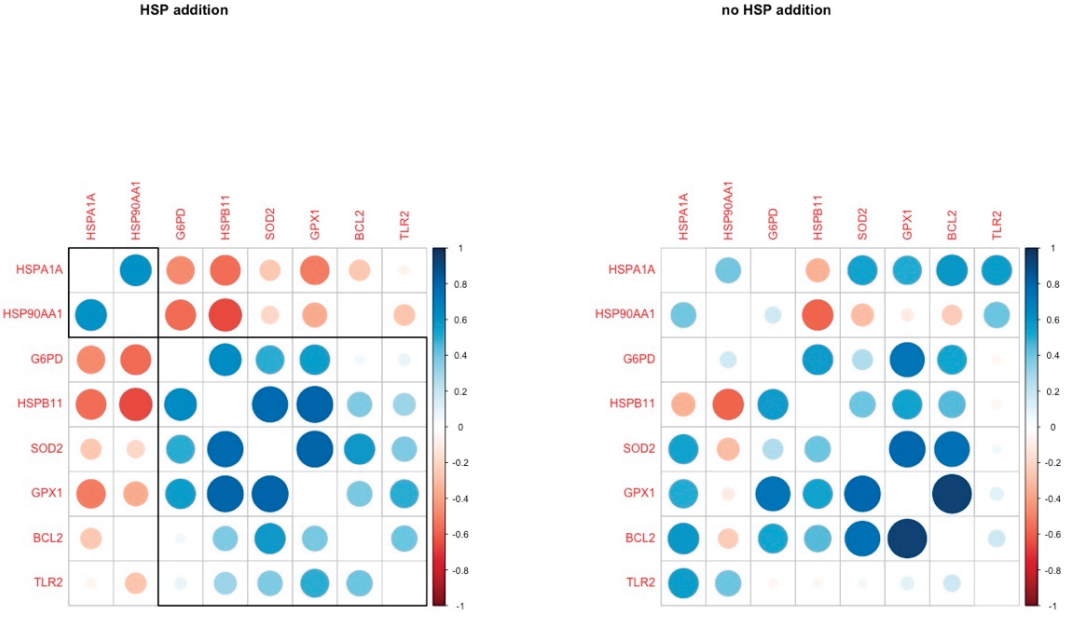

Figure 2. Pairwise correlation coefficients of genes under study in oocytes in H and C groups respectively.

3.2.2. Cumulus Cells

The Two-Way ANOVA analysis revealed that the expression of three genes (*HSPA1A, HSP90AA1, HSF1)* out of the eight measured were significantly altered by the presence of HSP70 in the medium and there was a strong tendency towards differential expression of *GSTP1* (p-value = 0.06). The relative expression of genes is presented in Figure 3. The significant differences between the C and the H groups are presented in Supplementary Table S3.

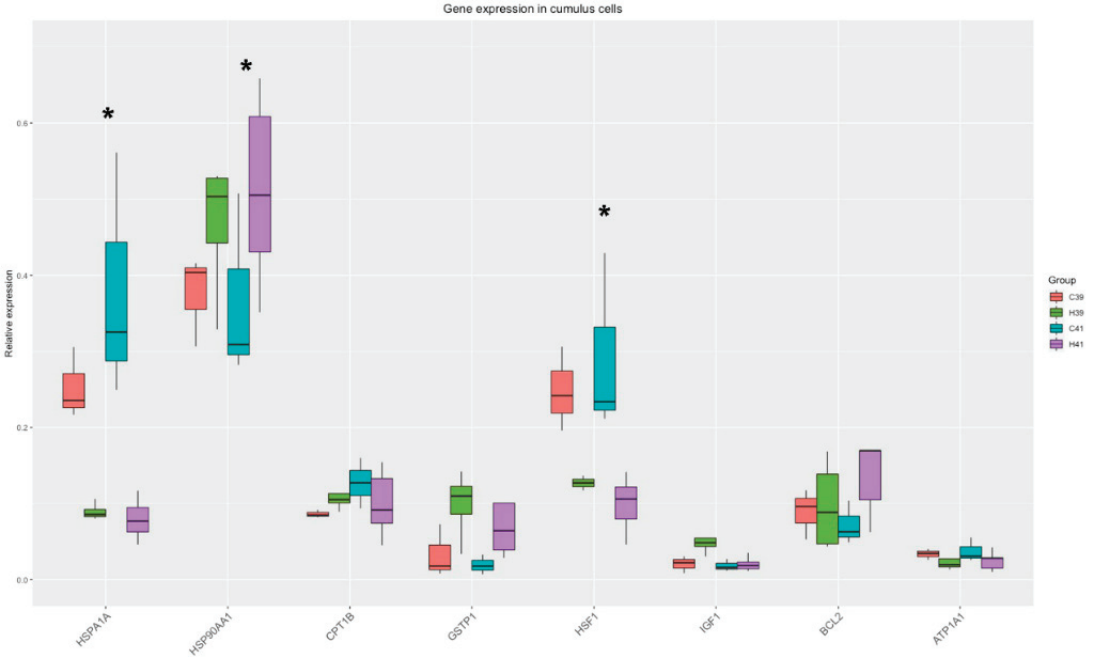

Figure 3. Gene expression in cumulus cells. Significant changes from the Two-Way ANOVA test are marked with *.

The correlation analysis revealed four genes with a strong positive correlation (r > 0.5, *IGF1, GSTP1, HSPA1A, CPT1B*) in the HSP addition group, while HSF1 was negatively correlated with *BCL2*. In the group without HSP addition, *HSP90AA1*, *HFS1*, *ATP1A1* were strongly and positively correlated, and these correlations were absent in the HSP70-treated group (Figure 4).

3.2.3. Blastocysts

HSP70 supplementation in IVM medium induced differential expression of 5 genes (*AKR1B1*, *GPX1*, *HSPA1A*, *IGF1*, *BAX*, *ATP1A1*), and there was a strong tendency towards differential expression in *GSTP1* (Figure 5). The significant differences between the C and the H groups are presented in Supplementary Table S4.

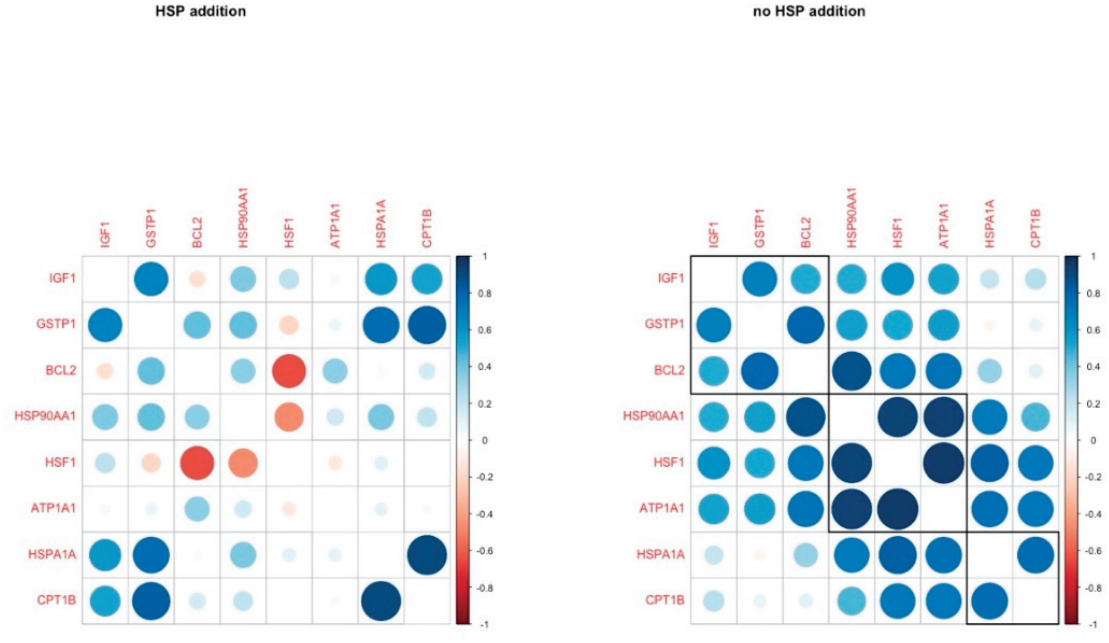

Figure 4. Pairwise correlation coefficients of genes under study in cumulus cells in H and C groups, respectively.

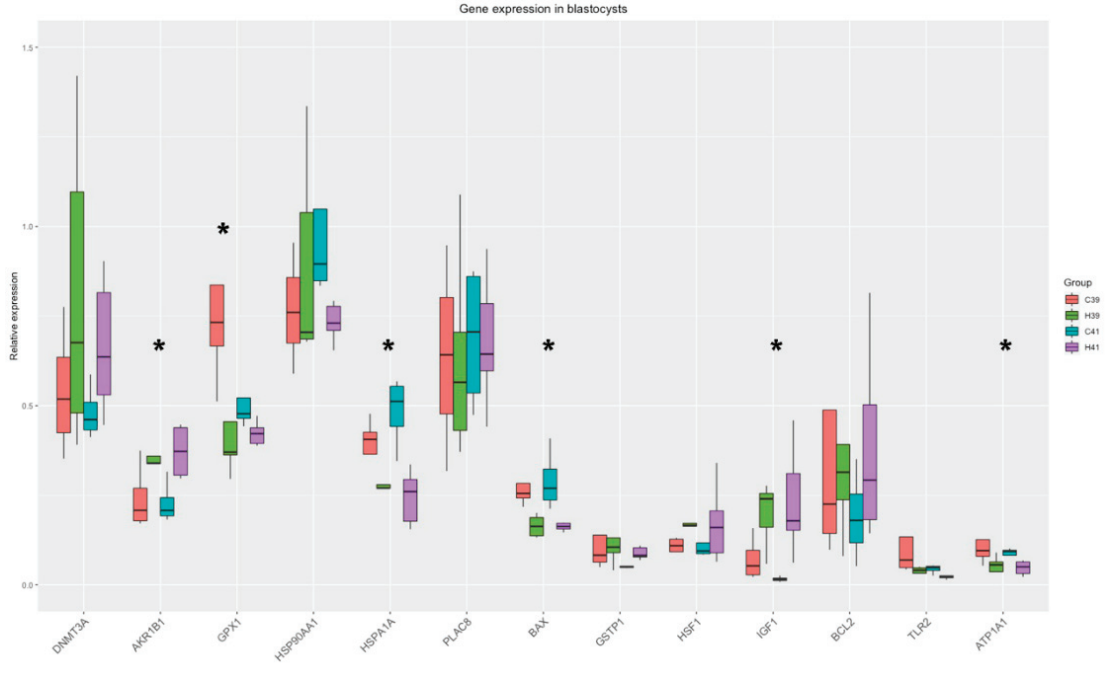

Figure 5. Gene expression in blastocysts. Significant changes from the Two-Way ANOVA test are marked with *.

HSP addition led to a positive correlation in the gene expression of 6 genes (*DNMT3A*, *PLAC8*, *GPX1*, *HSP90AA1*, *GSTP1*, *BCL2)*, while most of these correlations were absent in the other group. However, another group of genes (*HSP90AA1*, *GSTP1*, *BCL2*, *ATP1A1*, *IGF1*, *TLR2)* were tightly correlated in the group without the addition of HSP70. *HSP90AA1* and *GSTP1* were constantly positively correlated in both groups (Figure 6).

Figure 6. Pairwise correlation coefficients of genes under study in blastocysts in H and C groups respectively.

4. Discussion

This study shows for the first time that the presence of exogenous HSP70 in the IVM medium can blunt the deleterious effects of temperature rise on blastocyst yield and preserve oocyte and embryo quality by altering the expression pattern of a number of important genes. In addition, we provide evidence that in comparison to the cumulus cells, the oocyte is more responsive to HSP70 addition, and the compensation effects to the detrimental heat increase remain up to the blastocyst stage.

Here, we confirmed our previous observation [25] that a short lasting exposure of maturing oocytes to heat stress causes a significant reduction in cleavage rate. The addition of HSP70 in the IVM medium could not alleviate the effect of high temperature to cleavage rate. Our hypothesis that an addition of HSP70 in the maturation medium will improve the cleavage rate was not confirmed; on the contrary, HSP70 supplementation without a temperature rise (H39) led to a slightly decreased ($p = 0.068$) cleavage rate in comparison to C39. A good body of evidence suggests that temperature elevation, even for a short period during IVM, brings about an impaired fertilizing capacity and a reduced embryo production rate [33–35]. HSP70 acting on microtubules contributes to the stabilization of meiotic spindle formation in the oocyte. While most HSPs are synthesized under normal conditions, HSP70 is synthesized exclusively under stress [9]. Hence, it could be postulated that under physiological conditions, the presence of HSP70 in the IVM medium was construed as a potential insult stimulus by the oocyte and/or the cumulus cells, triggering defense mechanisms that partly suppressed the cleavage rate.

As was expected, temperature rise during IVM substantially reduced blastocyst formation rate in group C41. This result is consistent with established and published findings [25,36–38] and the underlying mechanisms are extensively discussed in our previous paper [25]. Despite being under heat stress conditions, the blastocyst yield was restored in the presence of HSP70, and it did not differ at any time point from that of group C39. Obviously, this was attributed to the protective role of HSP70. In general, the presence of HSP70 in the maturation medium altered the expression of HSPs (*HSP90AA1*, *HSPB11*) in oocytes, regardless of the temperature. *HSP90AA1* encode for a molecular chaperon that is involved in the proper folding, transport and stability of specific target proteins by use of an ATPase activity, modulated by co-chaperones [39]. *HSP90AA1* is also involved in many other cellular functions, such as cell signaling, transcription, kinase regulation, and DNA replication and repair [40]. HSP72, encoded by *HSPA1A*, also prevents cell death in multiple ways [41]. On the other hand, *HSPB11* that encodes for a small heat shock protein acts as a molecular chaperone that prevents apoptotic cell death [42] via an HSP90-mediated mechanism that stabilizes the mitochondrial membrane [43]. In our previous study [25], the elevation of temperature from 39 to 41 °C for six hours led to an increased expression of *HSPB11* in heat-stressed oocytes, while there was no significant difference in *HSPA1A* and *HSP90AA1*. This finding is in accordance with another study where HSPB11 was the only one among the 16 HSP genes studied that was significantly altered in heat-stressed bovine embryos [44]. Thus, the HSP70 supplementation alone appears to trigger a stronger response to the treated oocytes, which is further supported by the elevated expression of *SOD2* and *GPX1* (antioxidants). *SOD2* is a member of the iron/manganese superoxide dismutase family that catalyzes the conversion of the superoxide radicals of oxidative phosphorylation to hydrogen peroxides and diatomic oxygen [45]. *GPX1* encodes a protein that belongs to the glutathione peroxidase family, members of which transform hydrogen peroxides by glutathione to water [46]. *GPX1* expression increased in both the oocytes and the blastocysts in the presence of HSP70 addition (H39), compared with the oocytes and blastocysts matured at 39 °C (C39), indicating that HSP70 is higher in the hierarchy of the heat response cascade than the protective mechanism against the temperature rise induced oxidative stress. HSP70 supplementation also led to a significant increase in the expression of *BCL2* regardless of the temperature. *BCL2* acts as a protective molecule during heat stress through its anti-apoptotic role, and it has been also been found to be over-expressed in studies of heat-stressed cows [47–50]. *BCL2* encodes an anti-apoptotic protein, which functions as a regulator of cell death by inhibition of the action of pro-apoptotic proteins. Its expression has been associated with high embryo quality [51,52].

Furthermore, the HSP70 supplementation led to a tight coordination of the expression of *G6PD, HSPB11, SOD2* and *GPX1.* It appears that HSP70 is closely linked to the regulation of the oxidative stress mechanism along with HSPB11, and it brings the three antioxidant genes (*G6PD, SOD2, GXP1*) under the same regulation cluster.

In cumulus cells, HSP70 supplementation resulted in modifying the expression of three genes, namely *HSPA1A, HSP90AA1* and *HSF1.* Heat shock factors (HSFs), which are encoded by the *HSF1* gene, are responsible for the induction of HSPs synthesis in stressful conditions [11]. In heat stress conditions, HSFs are activated, enter the nucleus and attach to the heat shock elements (HSEs). This interaction leads to the transcription of HSPs. Exogenous administration of HSP70 possibly leads the cell to a reflective need for modification in the production of HSPs. *HSPA1A* belongs to the HSP70 family of proteins, so we assume that there is a negative feedback in the expression of these proteins due to the external administration of HSP70 in the medium, while HSP90AA1 is a member of the HSP90 family, and it is up regulated to fulfill its cytoprotective roles as mentioned above.

The coordinated patterns of gene expression in cumulus cells are indicative of the processes taking place in every group. Comparing the gene coordination patterns across groups, it was revealed that the expression of IGF1 is tightly coordinated with the expression of *CPT1B* and *GSTP1* regardless of the temperature when HSP70 is supplemented. *CPT1B* encodes for carnitine palmytoltransferase 1, a rate-limiting enzyme of fatty acid β-oxidation,

which is important for the progression of meiosis and the developmental competence of the oocytes [53]. Its close coordination with IGF1 indicates that it might fall within the spectrum of IGF1 regulation of growth mechanisms. It is noteworthy that glutathione S-transferase (GSTP1), key in redox scavenging in the cells, is so tightly linked to energy provision and growth genes. GSTP1 is known to exhibit an anti-apoptotic function through different pathways [54], yet to our knowledge it has not linked before to growth mechanisms. Moreover, *HSF1* and BCL2 are showing a strong negative correlation, since *HSF1* expression is downregulated due to the HSP70 supplementation, while *BCL2* expression is upregulated (though not significantly), especially in the H41 group as reflected in pairwise comparisons (Supplementary Table S3), acting as an anti-apoptotic molecule as mentioned above.

HSP70 supplementation altered the expression of *AKR1B1*, *IGF1*, *GPX1*, *HSPA1A*, *BAX* and *ATP1A1* (Figure 5), while GSTP1 also showed significant differences between the C and the H groups. The expression of *AKR1B1*, which protects against toxic aldehydes derived from lipid peroxidation, is upregulated in HSP70 presence, indicating the protective effects of HSP70. Furthermore, *IGF1* is responsible for the regulation of growth mechanisms and its upregulation can highlight the growth potential of the blastocysts. On the other hand, *BAX* is encoding for an apoptosis activator and is downregulated in the H groups, thus protecting the cells from apoptosis. *HSPA1A* is constantly downregulated in H groups along all cell types (oocytes, cumulus cells, blastocysts) and a possible explanation has already been proposed.

In the HSP70 supplementation group, *BCL2* and *GPX1* cluster with *GSTP1*, *DNMT3A*, *HSP90AA1* and *PLAC8A*. These genes are connected to blastocyst quality and survival (*BCL2* is anti-apoptotic, *GPX1* and *GSTP1* are antioxidants, *PLAC8A* is responsible for normal embryo implantation and *DNMT3A* is responsible for epigenetic reprogramming of the embryo after embryo genome activation (EGA)) and their coordinated expression may orchestrate the blastocysts' survival in the presence of the anti-inflammatory signals (extracellular HSP70). This cluster of correlation did not survive on the C group; *BCL2* and *GSTP1* expression was strongly correlated with *ATP1A1*, *IGF1*, and *TLR2*. These genes are participating in the response to stress (elevated temperature and anti-inflammatory signals), while they are also regulators of apoptosis and growth. As HSP70 contributed to increased embryo yield in H41, it could be hypothesized that it was due to the enhancement of all these protective mechanisms that the fertilization and development of oocytes which lacked some type of inherent thermotolerance to the blastocyst stage was inevitably permitted.

In conclusion, it was demonstrated that the external supplementation of HSP70 can offset the deleterious effects of heat stress on embryo production. In this study, the presence of exogenous HSP70 acted protectively for oocytes and cumulus cells and formed blastocysts, by intercepting apoptosis, promoting signal transduction and increasing the antioxidant protection of the embryo.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11061794/s1, Figure S1: Distribution of blastocysts according to their developmental stage in four groups of COCs matured in vitro at 39 °C without (group C39) or with HSP70 (group H39), at 41 °C for 6 hours from the 2nd to 8th hour of IVM without (group C41) or with HSP70 (group H41), Table S1: Distribution of blastocysts according to their developmental stage in four groups of COCs matured in vitro at 39º C without (group C39) or with HSP70 (group H39), at 41 °C for 6 hours from the 2nd to 8th hour of IVM without (group C41) or with HSP70 (group H41), Table S2: Significant differences in gene expression between C and H groups in oocytes, Table S3: Significant differences in gene expression between C and H groups in cumulus cells, Table S4: Significant differences in gene expression between C and H groups in blastocysts.

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Reproductive Seasonality Affects In Vitro Embryo Production Outcomes in Adult Goats

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Simple Summary: Reproductive seasonality is usually determined by photoperiod and may also be influenced by nutritional sources. Little is known about the effect of season on the efficiency of assisted reproductive technologies, such as in vitro embryo production in seasonal species. This study was conducted to generate an understanding of the seasonality influence on in vitro embryo production outcomes in goats. Overall, the breeding season improved oocyte developmental competence, with higher cleavage and blastocyst yield, while there was no difference in embryo quality throughout the years.

Abstract: Reproductive seasonality may have a considerable influence on the efficiency of assisted reproductive technologies in seasonal species. This study evaluated the effect of season on cleavage, blastocyst rates and quality of in vitro produced (IVP) goat embryos. In total, 2348 cumulus–oocyte complexes (COCs) were recovered from slaughterhouse ovaries and subjected to the same IVP system throughout 1.5 years (49 replicates). The odds ratio (OR) among seasons was calculated from values of cleavage and blastocyst rates in each season. Cleavage rate was lower ($p < 0.05$) in spring (anestrus), in comparison with either autumn (peak of breeding season) or summer, while the winter had intermediate values. Furthermore, lower OR of cleavage was observed in spring. Blastocyst formation rate (from initial number of COCs) was higher ($p < 0.05$) in autumn ($52 \pm 2.5\%$) when compared with the other seasons (combined rates: $40 \pm 1.9\%$). Moreover, its OR was higher ($p < 0.05$) in autumn compared to all other seasons and impaired in the spring compared to winter (OR: 0.54) and summer (OR: 0.48). Embryo hatchability and blastocyst cell number were similar ($p > 0.05$) among seasons. In conclusion, the breeding season leads to improved oocyte developmental competence, resulting in higher cleavage and blastocyst yield, whereas embryo quality remained similar throughout the years.

Keywords: caprine; COC; IVF; IVP; IVEP; oocyte competence; photoperiod; reproductive efficiency; season; seasonal breeder

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1. Introduction

It is well known that although some species have adopted strategies to breed year-round, others exhibit reproductive seasonality, breeding/calving only at a specific period of the year [1]. Reproductive seasonality is generally driven by photoperiod and may also be influenced by nutritional sources availability. In low latitude regions, day length remains steady throughout the year, but reproductive activities variations can be associated

mainly to climatic conditions such as rainfall, impacting feed availability and quality [2]. However, in well-nourished mammals, photoperiod is the strongest reason for reproductive seasonality [3]. This is an adaptation that ensures offspring survival by calving at the period of higher roughage accessibility and nutritional quality [4]. When photoperiod changes from longer to shorter daylight, with more dark hours in a day, the photo receptors of the animal eye send this information to the pineal gland, increasing melatonin secretion, and goats restart their regular estrous cycles [1].

In general, in the northern hemisphere, the transition season in goats occurs in summer (June to September), the peak of breeding season is in the autumn (September to December), in winter (December to March) the occurrence of regular estrus relies more on the breed and animal intrinsic characteristics, while the deepest anestrus is detected in spring (March to June) (Reviewed by Chemineau, et al. [5]). In seasonal breeders, the reproductive seasonality may have a substantial impact on the efficiency of overall assisted reproductive technologies (ARTs, [4]). Considering the strong influence of seasons on small ruminants reproductive function due to hormonal alterations [6], it would be logical to suppose they may also affect the overall success of ARTs, such as in vitro embryo production (IVP).

In bovine, a nonseasonal species, the cleavage and morulae development rates were lower in the autumn compared with all other three seasons, while the blastocyst rate was the least when oocytes were collected during the summer season, probably due to hot weather and lower feed quality in IVP systems [7]. Curiously, the environmental temperature significantly affected women's pregnancy rates after in vitro fertilization (IVF) in a sub-tropical region [8]. In studies assessing the effect of temperature, better results were reported in the cold season in buffalo [9] and in sheep [10], both being seasonal breeders.

In seasonal breeders, most research groups have reported consistent variations in embryo yield throughout the year. For instance, a higher number of oocytes presenting higher quality were recovered from Zandi ewes in the breeding season, allowing them to achieve higher blastocyst development [11]. Overall, higher IVP rates are obtained in the breeding season, compared to anestrus [11–15]. However, in prepubertal goats, after IVF, both cleavage and blastocyst rates were significantly higher in the nonbreeding season, compared to the breeding season (41° N latitude; [16]). It is not clear why these results are different to all others presented before; the reason for such disparity could be species-specific or an effect of the goat category/parity (prepubertal animals).

According to our current knowledge, the seasonal effects on embryo IVP have not been studied in adult goats. Regardless of the season, there are many factors that can directly affect the success of IVP, such as: oocyte origin, either collected from slaughterhouse ovaries or by laparoscopic ovum pick up [17], cumulus–oocyte complexes (COCs) recovery method [11], oocyte grading and selection [18], which is at least in part subjective, and the IVP conditions in the successive steps [17,19,20]. It is reasonable to suggest that all these factors must be controlled in order to specifically assess any season effect throughout the year. Therefore, this study was designed to investigate the effects of reproductive seasonality in adult goats on abattoir-derived oocytes' developmental competence, over a period of 1.5 years.

2. Materials and Methods

All the chemicals were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA) except where otherwise specified.

2.1. Experimental Design

The experiment was performed during the four seasons: spring (nonbreeding season), summer (transition season), autumn (breeding season) and winter (end of breeding season, start of anestrus season) at the Reproductive Physiology and Behaviors Research Unit (PRC) in Nouzilly, France (latitude 47°22′ N, longitude 00°41′ E). Seasons were defined based on periods for equinoxes and solstices in the northern hemisphere. A total of 49 replicates (autumn: 17, spring: 7, summer: 15 and, winter: 10) of goat IVP were performed, using

a total of 2348 COCs (autumn: 811, spring: 404, summer: 639 and, winter: 494). Two straws per replicate of semen from the same ejaculate/buck were used for six months and two straws (same ejaculate) per replicate of semen from another buck were used for 12 months. Data for embryo development were assessed over the period of 1.5 years. Several factors that could certainly affect the IVP outcomes were controlled in this study: only slaughterhouse-derived oocytes were used, the same COC recovery method was applied, the IVP protocol and system conditions were the same and all the steps were conducted by the same technician throughout the entire time.

2.2. Oocyte Recovery

Ovaries from adult dairy goats, regardless the stage of estrous cycle, were collected from a local slaughterhouse and transported to the laboratory in a thermal container with 0.9% NaCl solution at 32 °C within 3–4 h after collection. Ovaries were washed in pre-warmed fresh saline (32 °C), and COCs were aspirated from all follicles between 2 and 6 mm in diameter with an 18-g short bevel needle connected to a conic tube under controlled vacuum (30 mm Hg). The collection tube was previously filled with ∼3 mL of HEPES buffered tissue culture medium 199 (TCM 199, M7528), supplemented with 20 μg/mL heparin (Choay, Glaxo Wellcome Production, Notre Dame de Bondeville, France), 25 μg/mL gentamycin (G1272) and 0.4 mg/mL fraction V bovine serum albumin (BSA; A9647) [21].

2.3. In Vitro Maturation (IVM) of Oocytes

The COCs were recovered under a stereo microscope (Nikon Corporation, Japan) and graded regarding their quality. Only good quality (Grade 1 and 2), i.e., surrounded by at least one complete layer of unexpanded cumulus cells and homogenous cytoplasm, were used for IVM [18]. All COCs were washed four times and transferred into 4-well plates (Nunc, Roskilde, Denmark), containing 40 to 50 oocytes in 500 μL of maturation medium. The maturation medium contained bicarbonate buffered TCM 199 (M4530) supplemented with 10 ng/mL epidermal growth factor (EGF; E4269) and 100 μM cysteamine (M9768). The COCs were incubated for 22 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air [21].

2.4. Sperm Preparation and IVF of Oocytes

Sperm from frozen/thawed semen were centrifuged (15 min at 700 g) on 2 mL of Percoll (Pharmacia, Uppsala, Sweden) discontinuous (45/90%) density gradient. Viable sperm pellet was diluted in the adequate volume of fertilization medium to achieve a final concentration of 2×10^6 sperm/mL, Day 0 being considered as the day of IVF [21].

The matured COCs were transferred into 35 mm Petri dishes and washed in fertilization medium, which consisted of synthetic oviduct fluid (SOF) medium (pH = 7.3, 280 mOsm), supplemented with 10% of heat-inactivated estrus sheep serum, 5 μg/mL heparin (Calbiochem 375 095) and 40 μg/mL gentamycin (G1272). Groups of 40 to 50 oocytes were transferred into 4-well plates containing 450 μL of fertilization medium and, after sperm preparation, 50 μL of sperm suspension were added to each well. Sperm and oocytes were co-incubated for 16–18 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air [21].

2.5. In Vitro Development (IVD) and Embryo Quality

At the end of IVF all presumptive zygotes were placed into 15 mL conic tubes containing 2 mL of TCM 199 medium and 0.4 mg/mL BSA, and vortexed for 2 min (moderate speed) to remove cumulus cells. All presumptive zygotes were recovered in 35 mm plates, washed four times in culture medium (SOF supplemented with 3 mg/mL BSA) to remove spermatozoa and transferred by groups of 20–25 into 4-well plates containing 20–25 μL drops of culture medium covered with 700 μL of mineral oil (M8410). The presumptive zygotes were cultured for eight days at 38.8 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. At Day 2, 10% fetal calf serum (MP Biomedicals, 2916748) was added directly into the culture droplets [21].

Embryos were examined morphologically under a stereomicroscope, and the efficiency of development was assessed as the percentage of cleaved embryos on Day 2, and the percentage of blastocysts on Day 8, expressed either over the initial number of oocytes subjected to IVM or the number of cleaved embryos at Day 2. On Day 8, samples of expanded blastocysts were washed to remove the mineral oil, fixed in ethanol and stained with Hoechst 33258 to count their total number of blastomeres. Cell counting was conducted under an epifluorescence inverted microscope (Diaphot, Nikon, Japan).

2.6. Statistical Analysis

The results of cleavage, blastocyst, and hatching rates were tested for normality by the Shapiro–Wilk test, before being subjected to analysis of variance (ANOVA), followed by Tukey HSD test. Effect of buck semen was evaluated by the comparison of the autumn data in both years using unpaired t test. The odds ratio among seasons was calculated from total values of cleavage and blastocyst rates in each season. Analyses were performed in BioEstat 5.3, with 95% CI. A value of $p < 0.05$ was considered as significant.

3. Results

No differences ($p > 0.05$) were found in the autumn season between the two bucks used in the IVP outcomes (cleavage rate: 76 ± 1.9 vs. 66 ± 3.4%; blastocyst rate/cleaved: 75 ± 2.1 vs. 64 ± 4.4%; blastocyst rate from the initial number of COCs: 58 ± 2.6 vs. 42 ± 2.0%; and hatching rate: 70 ± 4.1 vs. 66 ± 4.1%). Thus, data were pooled regardless of the buck and analyzed together. Results of embryo development for different seasons of the year are presented in Table 1 and Figure 1. The cleavage rate was lower ($p < 0.05$) in spring, in comparison with either autumn or summer, while winter had intermediate values, being similar to all the others. Blastocyst formation rate from the initial number of COCs subjected to IVM was higher ($p < 0.05$) in autumn (52 ± 2.5%) as compared with other seasons (combined rates of the three seasons: 40 ± 1.9%). The hatching rate was similar ($p > 0.05$) among all seasons as well as the average number of cells in expanded blastocysts.

Table 1. Effect of different seasons on developmental competence of adult goat oocytes derived from slaughterhouse ovaries. Percentages of cleavage, blastocysts (Bl), and hatched embryos in relation to the total blastocysts (Hbl/totBl), and blastocyst cell counts (Mean ± S.E.M.).

Season	*n*	Replicates	Cleavage (%)	Bl/COC (%)	Bl/Cleaved (%)	Hbl/totBl (%)	Total Cells
Autumn	811	17	72 ± 2.1 ^{a}	52 ± 2.5 ^{a}	73 ± 2.7 ^{a}	68 ± 2.9	198 ± 4.6
Spring	404	7	51 ± 7.1 ^{b}	28 ± 4.7 ^{b}	55 ± 2.6 ^{b}	65 ± 3.8	187 ± 3.6
Summer	639	15	71 ± 2.0 ^{a}	45 ± 2.3 ^{c}	63 ± 3.3 ^{a,b}	76 ± 5.1	191 ± 3.3
Winter	494	10	66 ± 4.1 ^{a,b}	42 ± 2.1 ^{c}	63 ± 4.1 ^{a,b}	67 ± 4.4	196 ± 4.2
Total	2348	49	67 ± 1.8	44 ± 1.7	65 ± 1.8	66 ± 2.0	193 ± 2.0

n: Number of oocytes submitted to in vitro fertilization and development. a,b,c: Within a column values with different superscripts differ significantly ($p < 0.05$).

Regarding the odds ratio among seasons, the seasonality influence on in vitro embryo production was evident (Figure 2). Cleavage rate was negatively affected ($p < 0.05$) in spring in comparison to all the other seasons, with a greater chance of cleaving in the summer (OR: 2.39) and autumn (OR: 2.43). Similarly, the OR for the blastocyst rate from the initial number of COC was higher ($p < 0.05$) in autumn compared to the rest of seasons, mostly spring (OR: 2.79), and lower in the spring, compared to winter (OR: 0.54) and summer (OR: 0.48). There was a higher possibility to produce blastocysts from cleaved embryos during the autumn compared to the other seasons, mostly when compared to spring (OR: 2.18). The blastocysts had a lower ($p < 0.05$) probability to hatch in the summer.

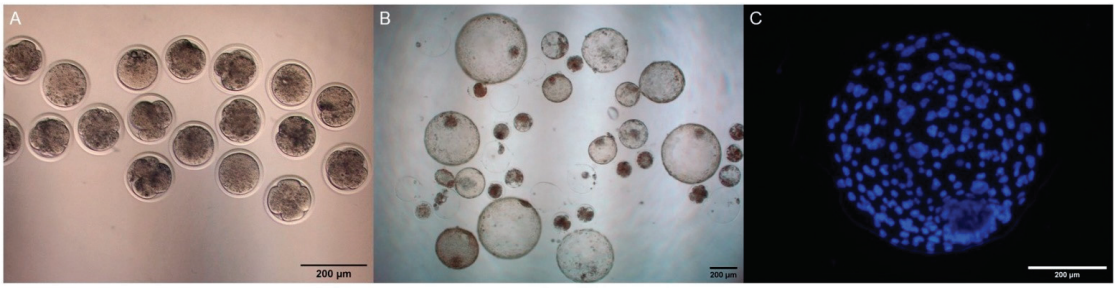

Figure 1. In vitro produced goat embryos. (**A**) Cleavage rate on Day 2 after IVF assessed by stereomicroscope; (**B**) Blastocysts on Day 8 after IVF assessed by stereomicroscope; (**C**) expanded blastocyst stained with Hoechst 33258 assessed by epifluorescence inverted microscope. The scale bar is set at 200 µm.

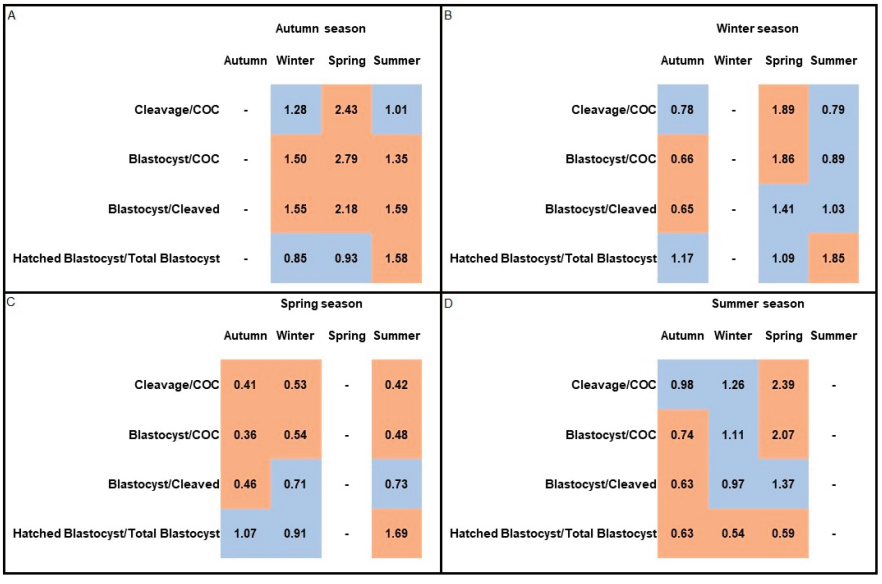

Figure 2. Heat map results of odds ratio (OR) of in vitro embryo production from adult goat oocytes in each season of the year for cleavage and blastocysts rates: (**A**) autumn season compared to all other seasons; (**B**) winter season compared to all other seasons; (**C**) spring season compared to all other seasons; and (**D**) summer season compared to all other seasons. The orange color indicates when the OR value was significant ($p < 0.05$), while the blue color represents OR values that were not significant ($p > 0.05$).

4. Discussion

This study assessed the effects of reproductive seasonality on abattoir-derived oocyte developmental competence and overall IVP efficiency in adult goats over 1.5 years. Considering that caprine is a seasonal breeder species and that there is a great influence of seasons on their hormonal and systemic changes, it would be reasonable to hypothesize that the season could significantly affect the IVP outcomes. Two main conclusions may be drawn from our study. Firstly, a significant effect of the time of year was found, favoring the breeding season. Secondly, the blastocysts produced had a similar quality (cell number) throughout the years. Although it is well known that heat stress can affect the oocyte developmental competence [7], we believe this was not a confounding effect in our study, since winter and summer (most extreme temperatures) had similar and intermediate rates

of embryo development. Of note, the season may reflect different strategies in reducing surplus animals due to farm management and, obviously, the number and status of slaughterhouse animals throughout the year are unknown. Despite this possible bias, the use of slaughterhouse ovaries is practically the only option available when aiming to use a great number of COCs and replicates. In the current study, the differences in the number of replicates across seasons is related to the overall laboratory logistics.

Since we had no difference throughout both years, we pooled data regardless of the years. Our data corroborate a previous study that reported similar rates of cleavage and blastocyst within each season over years in sheep [15]. This was expected since the breeding season is usually stable from year to year, with constant dates of onset and interruption of ovulatory activity (Reviewed by Chemineau, et al. [5]).

In the present study, cleavage rate was significantly lower in the anestrus (spring: 51%), in comparison with the breeding season (autumn: 72%) or transition (summer: 71%) while the winter (66%) had intermediate values. The effect of season on the cleavage rate in photoperiodic species is not unanimous in the literature. Cleavage was higher in the breeding season in the cat [12], plains bison [22], deer [23] and rhesus monkey [24]. Results in sheep varied regarding the effect of season on cleavage rate, since it was either affected [11,13] or not [15]. Overall, these data demonstrate it may be risky to consider cleavage will undoubtedly be affected by season.

In prepubertal goats, an opposite pattern in cleavage was observed, and an increase of 30% was reported in the anestrus, compared to breeding season in IVF-derived embryos (64 vs. 34%). Interestingly, the authors demonstrated that after parthenogenetic activation, cleavage rate was similar throughout the year [16]. The parthenogenetic activation is a great tool to assess the intrinsic competence of oocytes [17], bypassing the male factors. Therefore, it is reasonable to suggest that in such a case anestrus oocytes had a low ability to be fertilized, whereas meiotic maturation and developmental capacities were not affected. It is also important to consider that spermatozoa quality could have affected these results. Altogether, the results of the present study corroborate most literature on seasonal breeders and indicate that the fertilizing competence of the oocytes was probably affected in the anestrus season and/or the IVM-IVF conditions were not adapted to oocytes collected during the anestrus season. In addition, the developmental competence was also decreased in anestrus since both the rates of blastocysts calculated from total or cleaved oocytes were both lower in anestrus than in estrus.

The blastocyst rate from the initial number of COCs subjected to IVM was significantly higher in the breeding (autumn: 52%) as compared with other seasons (other three seasons: ∼40%). This result was corroborated by the OR determined in breeding season, which was significantly higher than the other three seasons. These data are in agreement with a previous study in Sarda sheep, where higher blastocyst rate was achieved during the breeding season [15]. This rate may be considered as an indicator of the success of IVP in terms of embryo yield. The blastocyst rate from the cleaved embryos was higher in the breeding (autumn: 73%) compared to the anestrus season (spring: 55%; OR: 2.18). This parameter is of utmost importance concerning the cytoplasmic competence since it specifically gives information about those embryos that were able to overcome the genome activation stage [25], which occurs in the 8–16 cells stage in goats [26]. Conversely, once cleaved, embryos had similar developmental competence to reach the blastocyst stage in plain bison [22] and in prepubertal goats [16]. Monkey oocytes recovered during the breeding season had similar IVP rates to those recovered during anestrus, even though they had significantly higher developmental competence when the oocytes were subjected to FSH stimulation [24]. It is noteworthy that all seasons produced an adequate and even high rates of cleavage and blastocysts. Perhaps these facts justify the extremely low number of studies proposing alternatives to mitigate the season effect in goat IVP programs throughout the year. For instance, a melatonin implant seemed to improve sheep oocyte developmental competence during anestrus [27]. These data indicate that although oocytes may present lower competence, strategies could be applied in the anestrus season in order

to alleviate the negative effects of season. The identification of molecular approaches for understanding and alleviating seasonal effects may open the way to more adapted methods [4] to maintain the same level of blastocyst production in the anestrus season.

Embryo quality was estimated based on the total number of blastomeres in expanded blastocysts on Day 8, which did not differ significantly among the four seasons. In the present study, the blastocysts had an average of 193 cells, higher than 120 [28], similar to 187 [17] and lower than 243 cells (but counted on Day 9) [29] of all studies in goats. Although the blastocyst morphological appearance is not definitely related to true developmental status in goats [28], all blastocysts allotted to the blastomeres count in the present study had an expected cell number (ranged from 168 to 225) compared to the literature [17,28,29]. Similarly, we found no significant differences between seasons in the number of hatched embryos. However, it was observed that blastocyst hatching was less probable during summer compared to the other seasons. Indeed, it is well known that even though the IVP success in terms of blastocyst production depends on oocyte intrinsic quality and IVM conditions, the quality of the subsequent blastocysts relies on the IVD system [30] and the IVD system remained the same throughout experiments. These data suggest that it is possible to produce goat embryos of similar quality throughout the years, regardless of the season.

5. Conclusions

Results of the present study indicate that the season has a significant impact on the IVP outcomes of adult goats. The breeding (autumn) season leads to improved oocyte developmental competence, resulting in higher cleavage and blastocyst yield, while there is no difference in embryo quality throughout the years. Strategies to mitigate the season effect in the anestrus (spring) season must be proposed to enhance the repeatability of the results throughout the year in goat IVP programs.

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animals

MDPI

Brief Report

Ultrasonic Cutting of Frozen Semen Straws to Optimize the Use of Spermatozoa for In Vitro Fertilization

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Simple Summary: The use of frozen semen is essential for the success of in vitro fertilization in the bovine embryo transfer industry. Therefore, we aimed to maximize the use of a single frozen semen straw by employing a cutting protocol. To the best of our knowledge, the current study is the first to apply ultrasonic cutting to frozen semen. The mechanical damage of spermatozoa was studied in frozen bovine semen by assaying sperm motility, acrosome abnormalities, and developmental competence. These findings may help to optimize the utilization of frozen semen for in vitro fertilization in the bovine industry.

Abstract: The objective of the present study was to establish conditions for using technology that can potentially enhance the efficiency of bovine embryos derived from in vitro fertilization (IVF) with frozen semen. Frozen semen from selected bulls can be stored indefinitely in liquid nitrogen as genetic resources; however, these resources are considered consumable because they cannot be regenerated. Therefore, to optimize the utilization of frozen semen, as many oocytes as possible should be fertilized with one straw. However, a sufficient number of prepared oocytes might not be available for one experiment, which can limit the use of the total spermatozoa population. Thus, an economical method for producing embryos needs to be established by optimizing technology for transplantable embryos. In this study, the utilization of frozen semen was increased by dividing the straw with an ultrasonic cutter. The post-thaw survival rate of uncut straws from Korean Proven Bulls did not differ from that of half cuttings. When ultrasonic cutting was applied to frozen semen, spermatozoa could be prepared for IVF trials at least four times, and blastocysts were produced. Therefore, cutting frozen semen with an ultrasonic cutter represents a potentially useful tool to expand genetic resources from excellent breeding stocks. This approach could also be valuable in the field of IVF of endangered species or rare breeds for their preservation, as well as in ovum pick-up (OPU) techniques.

Keywords: sperm viability; embryo production; fertilization; blastocyst

1. Introduction

In vitro bovine embryo production using in vitro fertilization (IVF) procedures was first introduced for the embryo transfer of livestock [1–3]. Much attention has been drawn to the mass production of transplantable blastocysts to preserve genetic resources [4–6]. For this purpose, spermatozoa

with optimal activity and motility are an essential and indispensable element of IVF using frozen semen [7,8]. However, lab workers and farmers face difficulties in purchasing frozen semen with good genetic backgrounds because of its high demand. Moreover, to prevent the inbreeding of *Hanwoo*, the government of South Koreahas placed limitations on the specific supply of semen from the highly preferred Korean Proven Bull Number (KPN) family.

Since the IVF technique was developed with immature oocytes from slaughterhouse-derived or in vivo ovaries, IVF technicians have become aware of the importance of sperm physiology to produce healthy embryos with suitable culture systems [1,3,9]. Recently, the ovum pick-up (OPU) technique was developed for multiple oocyte recovery from a single cow with 8~15 oocytes per collection, so animal farmers have focused on genetic improvements for subsequent transferable blastocysts [5,10–12]. The estimated number of spermatozoa from a single frozen semen sample needed for IVF or artificial insemination (AI) can differ. Approximately 15×10^6 to 20×10^6 active spermatozoa are required for one bovine AI [13–15]. However, the number of spermatozoa needed for IVF trials has been reported to be approximately 3×10^3 to 5×10^3 active spermatozoa per oocyte [14,15]. These requirements mean that a significant number of spermatozoa are not used in the IVF procedure, and unused sperm is discarded as laboratory waste. Therefore, the ability to leverage multiple uses from one straw of frozen semen could increase the productivity of genetically confirmed spermatozoa, and such a process with high efficiency could lead to a new protocol for IVF researchers.

The ultrasonic cutter, which has a blade that vibrates 20,000–40,000 times per minute, has been developed to gently and accurately cut high-density materials, such as plastic assembly toys [16–18]. In this study, an ultrasonic cutter was tested to obtain multiple uses of frozen semen. It was hypothesized that the characteristics of this instrument would allow a hard frozen straw to be cut quickly and cleanly. In general, materials submerged in liquid nitrogen (LN_2) have increased hardness, show very rigid physical properties, and are not cut easily by scissors [19]. When straw-cutting scissors are used to cut semen straws while they are in the frozen state, cracks are formed on the straw's plastic surface, which leads to semen loss during thawing. Therefore, the present study was designed to offer a new method to produce cuttings with an ultrasonic cutter without thawing; in this approach, the frozen semen straws can be maintained in preservation conditions below the surface of LN_2. To the best of our knowledge, the ultrasonic cutting technique has not been used for cutting frozen semen, and data on the sperm properties from divided frozen semen could not be found. Thus, the present study was conducted to present accurate data for this field and demonstrate the method's feasibility. In this study, the level of damage to spermatozoa from cuttings was investigated by monitoring their motility and activity. Moreover, the production of IVF embryos using the spermatozoa was investigated to confirm the performance of fertilizing activity from frozen semen cuttings.

2. Materials and Methods

2.1. Animals and Ethics Statement

Ovaries from the Korean Hanwoo cow were obtained from a local slaughterhouse (Iksan-si, Jeollabuk-do province). All procedures described were reviewed and approved by the Institutional Animal Care and Use Committee at the National Institute of Animal Science (Approval No. NIAS 2018–406).

2.2. Frozen Bovine Semen and Semen Motility Assay

Commercially available frozen semen from KPN was chosen because of the certified grade of sperm quality. The frozen semen was thawed at 37.5 °C for 45 s, and the sperm was transferred into a 1.5 mL tube on a digital warming block (EppendorfThermoMixer^{TM}, Hamburg, Germany) without shaking. After placing 10 μL of thawed spermatozoa samples on the Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel), a comparative analysis of the motility and activity was

performed using a microscope with a computer-assisted sperm analyzer (CASA) from the Proiser R+D corporation in Spain.

2.3. Bovine Semen Collection, Dilution, and Freezing

Ultrasonic cutting experiments were performed using frozen semen from three Jersey breeding bulls. The semen samples were collected using an electro stimulator (Pulsator IV, Lane Manufacturing Inc., Denver, CO, USA) and cryopreserved. The collected semen samples were transported to the laboratory within 10 min and diluted (1:1) by the dropwise method using Triladyl^{TM}-egg yolk diluent. The diluted semen samples were left at room temperature for 10 min and then diluted again to adjust the final spermatozoa concentration to 80×10^6/mL. A double cooling system was prepared by adding room-temperature water to a ten-fold larger volume of semen in a plastic beaker. The semen-containing vessel was cooled for 2–3 h using slurry ice. After cooling it to 5 °C, the semen was packed into 0.5 mL straws by an automated filling and sealing machine (MPP Uno, Minitube, Tiefenbach, Germany) in a semen processing cabinet (FHK, Tokyo, Japan). The packaged semen samples were frozen by exposure to LN_2 by first placing them 5 cm above the tank for 10 min and then submerging them.

2.4. Ultrasonic Cutting and Thawing of the Frozen Semen Straw

The frozen semen straw fragments were obtained by using an ultrasonic cutter (Honda Electronics, ZO-80, Toyohashi, Japan). First, the intact straw was placed into a tilted Styrofoam box filled with LN_2 that covered half of the tilted bottom. As shown in Figures 1 and 2, the straws were cut smoothly with the ultrasonic cutter for 2–3 s at the surface boundary of LN_2, in which the straws were partially submerged. The 1/2 or 1/3 cuttings of the whole straw remained in LN_2 and were returned to the straw goblet for preservation. The cuttings of frozen semen with two openings were placed into a thin and small plastic bag and submerged in 37.5 °C water for 45 s. The openings of the cutting were blocked and kept horizontal by hand to prevent the spillage of semen. The 1/4 cutting of frozen semen was placed directly into a 15 mL conical tube with 13 mL of S-BO medium, which was warmed at 38.5 °C in the incubator. The tube was inverted 6–8 times to increase the thawing speed of semen samples. The remaining empty straw was removed with sterilized forceps before centrifugation. To fertilize the control oocytes with sperm from uncut frozen straws, the semen was mixed with calcium-/magnesium-free phosphate-buffered saline (CMF-PBS) in a ratio of 1:9 by volume and centrifuged for 10 min at 300 g to remove the diluent component.

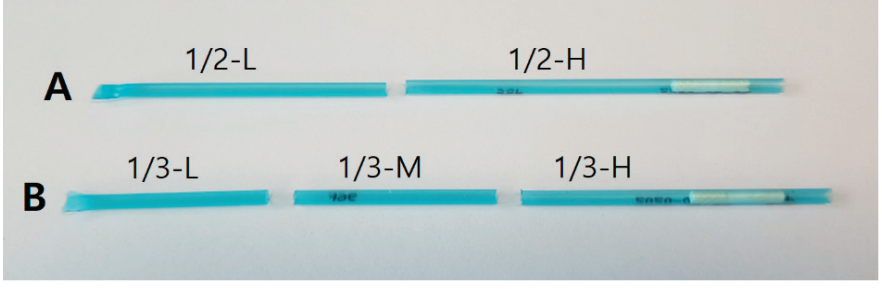

Figure 1. Images of the samples after applying the cutting treatment to hollow 0.5 mL straws. The hollow straw (**A**) is divided into two cuttings, each with one opening;1/2-H is a cutting from the top portion with a cotton seal, and 1/2-L is a cutting from the lower portion with a sealed end. The hollow straw (**B**) is cut twice, producingthree cuttings; 1/3-M is the middle portion of the straw with two openings.

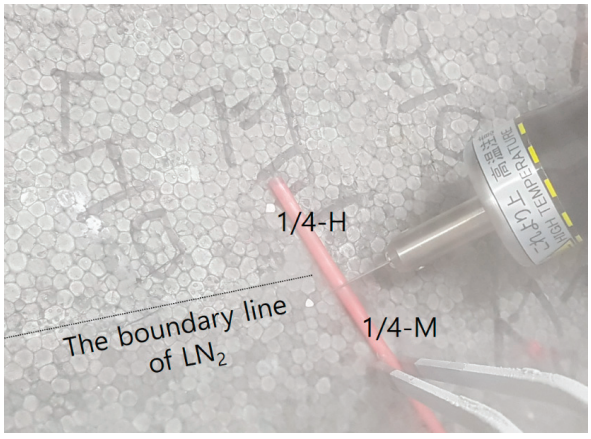

Figure 2. An image of the ultrasonic cutter on the frozen semen straw. The bottom of the tilted Styrofoam box was used to expose the cutting area of frozen semen to the surface of LN2.

2.5. Acrosome Analysis by CBB Staining

The sperm pellets were suspended by gentle tapping. A 5 μL drop of spermatozoa was placed on a glass slide and smeared, dried, and fixed by submersion in PBS containing 3.7% paraformaldehyde (PF-PBS) for 2 min. Coomassie brilliant blue (CBB) stains were used to analyze the acrosome status. The CBB stock was prepared by dissolving 0.3 g of CBB staining reagent in 45 mL of methanol, and a diluent for CBB stock was prepared by mixing 10 mL of acetic acid with 45 mL of double-distilled water. The staining solution was prepared by adding the diluent to CBB stocks, and the final concentration of CBB reagents was 1% CBB R-250 and 1% CBB G-250. A fixed sperm smear was submerged in the staining solution for 2 min and transferred into distilled water for 3–5 s. After drying the stained smears on a slide warmer at 37 °C for 2–3 min, the slide was observed with a microscope (Olympus IX-71, Tokyo, Japan). As shown in Figure 3, the acrosome reaction status was analyzed by counting the number of spermatozoa with intact acrosomes, reacting acrosomes, and reacted acrosomes. The total number of counted sperm was over 200 per examination in each experiment.

2.6. In Vitro Fertilization of Bovine Oocytes with Sperm from the Ultrasonic Cuttings of Frozen Semen

Cumulus–oocyte complexes (COCs) were collected by aspiration from 3–6 mm follicles of slaughterhouse-derived ovaries using a 10 mL disposable syringe with an 18-gauge needle. The pooled COCs were washed three times using tissue culture medium(TCM) 199 culture medium containing 5% FBS to remove the follicular fluid and cells that had detached from COCs. The maturation medium was prepared with TCM 199 culture medium containing 10% FBS, FSH (20 μg/mL), LH (10 μg/mL), and estradiol (1 μg/mL) and incubated overnight in the previous day's oocyte culture. The COCs were cultured for 20–22 h and subsequently fertilized by spermatozoa from the frozen semen straw. As a fertilization medium, Bracket and Oliphant(BO) culture medium was prepared for sperm treatment and fertilization drops for IVF. The fertilization BO drops (F-BO) were prepared by adding 6 mg/mL fatty acid-free bovine serum albumin (FAF-BSA). Drops of culture (25 μL) covered with mineral oil were incubated at 39.5 °C overnight. The semen was transferred to 15 mL centrifuge tubes with S-BO and centrifuged for 5 min at 250 g to remove the diluent of egg yolk and glycerol. To enhance sperm activity and induce capacitation, sperm dilution BO (S-BO) was prepared by adding 0.45 mg/mL theophylline. Approximately 20 COCs were transferred to F-BO, and 25 μL of S-BO containing spermatozoa at a concentration of 4×10^6 to 7×10^6/mL was co-cultured for IVF for 8–16 h.

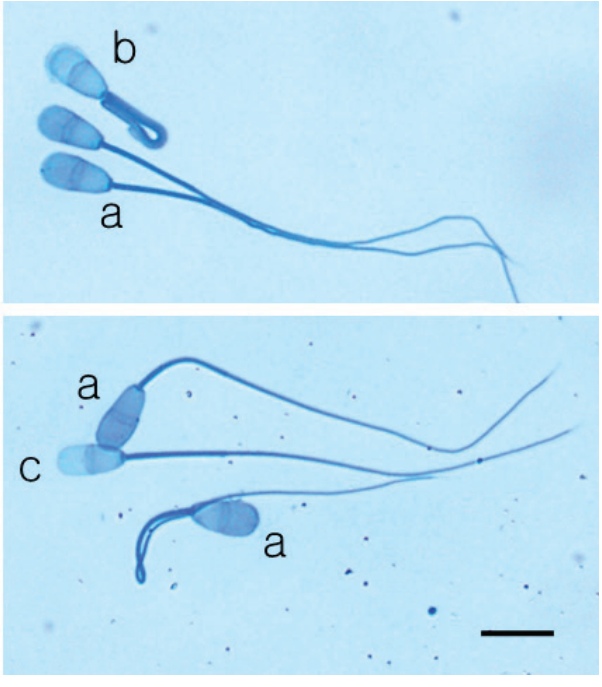

Figure 3. A bovine spermatozoa image stained with a mixed solution of 0.1% coomassie brilliant blue (CBB) R-250 and 0.1% CBB G-250. Spermatozoa with intact acrosomes (**a**) have a bright blue staining pattern over the entire acrosome, whereas damaged spermatozoa have swollen outer membranes (**b**) or no staining pattern over the acrosome (**c**). The black bar is 10 μm.

2.7. Statistical Analysis

The rates of motility and damage to sperm cells were analyzed using a one-way analysis of variance (ANOVA) test, and the significance between the means of analyzed data was evaluated using Duncan's multiple range test.

3. Results

3.1. Effects of Ultrasonic Cutting on Spermatozoa Motility

The motilities of KPN spermatozoa obtained from frozen cuttings were compared with those of the control semen, which was obtained from uncut frozen straws. As shown in Figure 2, the hollow straw samples were cut into 1/2 and 1/3 pieces; 1/2-L indicates the lower portion of the straw, and 1/2-H is the top portion, which resides in a goblet clipped to a metal cane in the preservation chamber of the LN_2 tank. As shown in Table 1, the percentage of motile spermatozoa from 1/2-Ldid not differ from that of uncut frozen spermatozoa (control); however, the motility differed between 1/2-H and the control. The motility of spermatozoa from the lower portion of the frozen semen sample cut by 1/3 was 88.4% and had no significant differences ($p > 0.05$) from the control. However, the motility of spermatozoa from 1/3-H and 1/3-M significantly differed ($p < 0.05$) from that of the control. The percentage of hyperactive spermatozoa differed between 1/2-L, 1/2-H, and the control semen ($p < 0.05$). When the straw was cut by 1/3, the motilities of spermatozoa differed between 1/3-M, 1/3-H, and 1/3-L. There were also differences in hyperactive sperm rates between 1/3-M, 1/3-H, and 1/3-L. The same tendency of hyperactive sperm rates was also observed in the 1/2 cuttings, but those from the lower cutting differed

as compared with the control. The slow motility was not significantly different between the 1/2 cuttings and the control. However, the slow motility of 1/3-M and 1/3-L differed from that of the control. Interestingly, spermatozoa concentrations and motility followed the same trend. The 1/2-H and 1/3-H cuttings contained fewer spermatozoa than 1/2-L, 1/3-L, and the control.

Table 1. The effects of ultrasonic cutting on the sperm motility and activity of Korean Proven Bull Number (KPN) spermatozoa.

Cutting Treatment	Concentration ($\times 10^6$/mL)	% of Spermatozoa		
		Motile	Hyperactive	Slow
Control	115.1 ± 2.0	84.9 ± 1.6	68.5 ± 3.1	16.5 ± 1.9
1/2-H	48.7 ± 7.9 *	77.8 ± 0.6 *	61.1 ± 3.6 *	16.8 ± 4.2
1/2-L	119.0 ± 24.5	88.2 ± 1.5	77.4 ± 4.2 **	10.8 ± 5.1
1/3-H	94.5 ± 4.6 *	76.8 ± 2.5 *	64.7 ± 5.9 *	12.1 ± 4.7
1/3-M	86.3 ± 22.9 *	72.7 ± 1.9 *	61.7 ± 0.7 *	11.0 ± 1.6 *
1/3-L	143.9 ± 25.0	88.4 ± 1.3	78.4 ± 1.4 **	10.0 ± 2.6 *

H: cuttings from the top portion with a cotton sealing component; L: cuttings from the lower portion without a cotton sealing component; M: cuttings from the middle portion with two openings. Mean values that are statistically significant different between treatments are indicated by different superscripts * and ** ($p < 0.05$). Data are expressed as the mean ± SD from three independent experiments.

3.2. Effects of Ultrasonic Cutting on Acrosome Integrity

The acrosome reactions of spermatozoa in the samples were analyzed by CBB differential staining techniques. The acrosome reactions of spermatozoa from KPN frozen semen straws that were ultrasonically cut into 1/2 and 1/3 were compared with those of the control. As shown in Table 2, there were no significant differences in the rates of spermatozoa with intact or reacting acrosomes between the control, 1/2-L, and 1/2-H. However, the rates of reacted acrosomes in the sperm of 1/3-H and 1/3-M were significantly different from those of the control and 1/3-L ($p < 0.05$).

Table 2. The effects of ultrasonic cuttings on the acrosome integrity of KPN spermatozoa.

Cutting Treatment	% of Spermatozoa		
	Intact	Reacting	Reacted
Control	75.4 ± 3.0	20.9 ± 2.5	3.8 ± 0.6
1/2-H	70.6 ± 13.8	19.6 ± 8.6	9.8 ± 5.3
1/2-L	77.6 ± 4.4	17.1 ± 2.8	5.3 ± 2.0
1/3-H	67.5 ± 7.6	21.8 ± 7.4	10.7 ± 0.4 *
1/3-M	71.6 ± 2.4	14.5 ± 4.5	13.9 ± 2.2 *
1/3-L	78.2 ± 4.5	17.8 ± 2.8	4.0 ± 1.8

H: cuttings from the top portion with a cotton sealing component; L: cuttings from the lower portion without a cotton sealing component; M: cuttings from the middle portion with two openings. Mean values that have statistically significant differences between treatments are indicated by different superscripts * ($p < 0.05$). Data are expressed as the mean ± SD from three independent experiments.

3.3. IVF Using the Spermatozoa from 1/4 Cuttings of Frozen Semen

To test the developmental competence of spermatozoa from frozen semen cuttings, IVF was conducted using in vitro matured oocytes, which were collected from slaughterhouse-derived ovaries. As shown in Table 3, the developmental competence of the spermatozoa from the 1/4-H and 1/4-L cuttings of Jersey frozen semen was compared with that of the uncut control semen. The rate of blastocyst production onthe seventh day of culture did not differ between the control and 1/4 cuttings. One hatched and two hatching blastocysts from the sperm of 1/4-L cuttings are shown in Figure 4.

Table 3. The effects of ultrasonic cutting treatment on the developmental competence of spermatozoa.

Cutting Treatment	n	% of Spermatozoa		
		Cleaved	Morula	Blastocyst
Control	66	54.5 ± 11.2	30.2 ± 6.3	22.6 ± 3.5
1/4-H	71	60.1 ± 13.1	28.2 ± 15.6	20.0 ± 6.3
1/4-L	69	53.3 ± 20.7	36.8 ± 10.0	26.2 ± 2.3

H: cuttings from the top portion with a cotton sealing component; L: cuttings from the lower portion without a cotton sealing component. Data are expressed as the mean ± SD from three independent experiments.

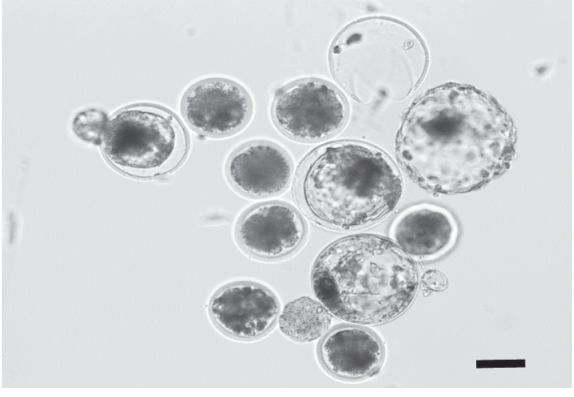

Figure 4. An image of in vitro fertilization (IVF)-derived blastocysts using spermatozoa from the 1/4-L cutting region of the semen straw. The black bar is 100 μm.

4. Discussion

Polyvinyl plastics, which are used as the soft material of semen straws, are characterized by their increased rigidity and strength at ultra-low temperatures (for example, the temperature of LN_2), which are also needed for the cryopreservation of frozen semen. The blade of an ultrasonic cutter vibrates 20,000~40,000 times per second (20~40 kHz), and the friction force from the blade quickly raises the temperature between the blade surface and the object being cut [18]. These properties have been used to cut hard objects such as plastic models and are employed in devices such as butter cutters; as a result of these effects, the cutting force applied to the materials is reduced [16,17]. However, to the best of our knowledge, the application of an ultrasonic cutter has not been studied in cryobiology to cut frozen semen straws.

The present study was based on the potential use of an ultrasonic cutter to cut frozen semen while minimizing damage and preventing cracks that cause spillage during the thawing process. When the cutter is applied to frozen semen, the effects on the spermatozoa in the straws can include lowered motility and acrosome damage. The fertility of damaged spermatozoa in IVF trials may also be compromised. In the present study, the results obtained using semen cuttings from nationally managed KPN Hanwoo and Jersey breeds demonstrate the capability of ultrasonic cutting. Moreover, the fertilizing ability of frozen–thawed spermatozoa derived from the cuttings was evaluated by assessing the developmental competence of blastocysts resulting from IVF procedures. As shown in Figure 2, the frozen semen straw to be cut was only exposed at the surface boundary of LN_2 to reduce the thawing effect from the heat that was generated by friction between the blade and straw. A Styrofoam box with a tilted bottom was used to keep straws submerged in LN_2, which was expected to ensure effective preservation. This assumption can be validated by measuring the motility of spermatozoa from cuttings.

Interestingly, the motility of frozen–thawed semen from the lower positions of straws cut by 1/2 and 1/3 was higher than that from other regions. These results suggest that the motility of sperm from the top region of the goblet may be decreased by temperature fluctuations, which may be caused by LN_2 evaporation. Therefore, the sperm in the lower region of the goblet may have comparatively high motility. Some spermatozoa from the middle portion of the cut straw appeared to be damaged, although the difference was not significant. During the thawing process, the two openings of the middle cuttings can result in the spillage of semen. However, these damages and losses did not affect the motility of the sperm population for the IVF procedure. It was predicted that only a small portion of sperm in the cut area would be lost during the thawing process and that cutting would not affect the characteristics of the overall sperm population. Moreover, the lower region of the goblet was the most protected area by LN_2 because it remained submerged during the transportation of frozen semen. The sperm from the lower region in the goblet had the best motility. The investigation of acrosome damage also verified that sperm from the top portion of the frozen semen straw generally had a greater percentage of damage, as evidenced by acrosome reactions that were in progress or completed. Therefore, the ultrasonic vibration force does slightly damage the spermatozoa from the cutting area, but it does not appear to have a significant effect on the total population of spermatozoa or their availability. As described earlier, the bovine IVF protocol only uses a portion of thawed semen, so only selected spermatozoa are used in fertilization. The results of this study suggest that the proposed method may also be applicable to the bovine OPU technique. Using frozen semen in high demand from bulls with good genetic backgrounds, an elite breeding stock can be produced with the repeated use of a single frozen semen straw. Since this method allows IVF researchers to use one frozen semen sample in four trials or more, the semen cost can be lowered.

5. Conclusions

Recently, in Korea, an increasing number of farms have attempted to transfer OPU-derived blastocysts for breeding as a method for improving the genetic ability of their elite females. However, it is not easy to secure or purchase frozen semen from desirable males for various reasons. By applying the ultrasonic cutting technique proposed in the present study, such problems can be easily resolved with the use of high-quality frozen semen. Therefore, it is concluded that the ultrasonic cutting technique can be applied to frozen semen for IVF and may be used as an efficient method of semen treatment to build an elite breeding stock.

Author Contributions: S.W.K. and B.K. designed the experiments and conceptualized the study; C.-L.K. and I.-S.H. analyzed the data; Y.-G.K. and S.-S.L. performed data curation and methodology; J.-Y.L. performed the investigation; S.W.K. wrote the manuscript; S.W.K. and B.K. supervised and approved the final draft of the document. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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