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Research Article

Vitrification of Bovine Blastocysts Developed in Royal Jelly-Supplemented Media

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ABSTRACT
Vitrification of mammalian embryos is an important process for the long-term preservation of gametes, providing the storage for extended periods. Selection of an optimal culture medium prior to vitrification is also critical to improve post-thaw survival rates. In the present study, the effects of royal jelly (RJ) and fetal bovine serum (FBS) on the development and cryotolerance of bovine embryos were investigated by evaluating their effects on post-thaw viability, blastocyst survival and cellular integrity after vitrification. Bovine oocytes were in vitro matured in media containing either 10% FBS or 0.625% Royal Jelly (RJ), followed by IVF and culture in SOF medium supplemented with the same treatment until day 8. Resulting blastocysts were vitrified using the OPS method with EG and DMSO, and post-thaw survival was assessed at 12 and 24 hours. Embryo quality was evaluated by measuring blastocyst diameter and total cell number using Hoechst 33258 staining under fluorescence microscopy. Blastocyst development rates were significantly lower in the RJ-supplemented group (16.3%) compared to the FBS group (24.8%, $P < 0.05$), suggesting that RJ may affect early embryonic development. Despite the decreased blastocyst development, RJ-derived blastocysts exhibited comparable post-thaw survival rates to their FBS counterparts at 12 hours (FBS-V: 51.3% vs. RJ-V: 48.3%, $P > 0.05$). Although, survival rates remained numerically higher in the RJ group (50.7% 1) compared to the FBS group (43.5%) at 24 hours, the difference was not statistically significant. Additionally, blastocyst cell counts and diameters were significantly lower in the RJ group than in the FBS group ($P < 0.05$) at the post-thaw vitrification, suggesting enhanced structural integrity and resilience remains similar to FBS group against cryoinjury. RJ supplementation resulted in a reduced blastocyst development and appeared to have no negative effects on the cryotolerance of embryos, as indicated by similar post-thaw survival rates and improved cellular integrity. The potential protective effects of RJ may be attributed to its bioactive compounds, including antioxidants and collagen-like proteins, which may mitigate oxidative stress and membrane damage during vitrification. These findings suggest that RJ could serve as a promising alternative supplement for improving embryo quality and cryosurvival. However, further research is required to optimize RJ concentration and elucidate its specific protective mechanisms in vitrified embryos.
Keywords: Bovine Embriyo, Royal Jelly, Fetal Bovine Serum, Vitrification

Arı Sütü İlave Edilen Ortamda Geliştirilen Sığır Embriyolarının Vitrifikasyonu

ÖZ
Memeli embriyolarının vitrifikasyonu, gametlerin uzun dönem korunması için önemli bir tekniktir. Vitrifikasyon öncesinde en uygun kültür ortamının seçimi, embriyoların çözme sonrası hayatta kalma oranlarını artırmak için hayati önem taşımaktadır. Bu çalışma, Arı sütü (AS) ve fetal sığır serumu (FBS) takviyesinin sığır embriyo gelişimi ve kriyotolerans üzerindeki etkilerini inceleyerek, çözme sonrası canlılık, blastosistlerin hayatta kalma oranları ve hücresel bütünlükleri üzerindeki etkilerini araştırmayı amaçlamaktadır. Sığır oositleri, kültür ortamında %10 FBS veya %0.625 AS takviyesi ile in vitro olgunlaştırılmıştır. In vitro fertilizasyon (IVF) işleminin ardından oluşan zigotlar 8. güne kadar FBS veya AS ila edilen sentetik oviduktal sıvı (SOF) içinde kültüre edilmiştir. 8. Güne gelen Blastosistler, açık uçlu çekilmiş pipetler (OPS) yöntemi ile etilen glikol (EG) ve dimetil sülfoksit (DMSO) içeren bir kriyoprotektan solüsyon

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kullanılarak vitrifike edilmiştir. Çözme sonrası hayatta kalma oranları 12 ve 24 saat sonra değerlendirilmiştir. Embriyo kalitesi, blastosist çapı ve toplam hücre sayısı temelinde Hoechst 33258 boyama yöntemi kullanılarak floresan mikroskop altında belirlenmiştir. Elde edilen ANOVA ile analiz edilmiş ve gruplar Tukey HSD testi ile karşılaştırılmıştır. Blastosist gelişim oranları, AS ile desteklenen grupta (%16.3 ± 3.36) FBS grubuna kıyasla (%24.8 ± 2.58, $P < 0.05$) anlamlı olarak daha düşük bulunmuştur. Bu durum, AS'nin erken embriyonik gelişimi etkileyebileceğini göstermektedir. Bununla birlikte, azalan blastosist gelişimine rağmen, AS ile kültür edilen blastosistlerin çözme sonrası 12 saatlik hayatta kalma oranları, FBS grubuyla benzer seviyede bulunmuştur (FBS-V: %51.3 ± 3.72 vs. RJ-V: %48.3 ± 4.09, $P > 0.05$). 24 saatlik hayatta kalma oranları ise AS grubunda sayısal olarak daha yüksek olmasına rağmen (RJ: %50.7 ± 3.41, FBS: %43.5 ± 4.25), bu fark istatistiksel olarak anlamlı bulunmamıştır. Ayrıca, blastosist hücre sayıları ve çapları AS grubunda FBS grubuna kıyasla anlamlı olarak daha düşük bulunmuştur ($P < 0.05$). Elde edilen sonuçlar, AS'nin embriyolarda yapısal bütünlüğü olumsuz etkilemediğini ve kriyozarara karşı FBS grubu ile benzer etki gösterdiğini düşündürmektedir. AS takviyesi, blastosist gelişim oranını düşürmesine rağmen kriyotoleransı olumsuz etkilememiştir. Çözme sonrası hayatta kalma oranlarının korunması ve hücresel bütünlüğün benzer kalması, AS'nin embriyolar üzerindeki olası koruyucu etkilerini işaret etmektedir. Bu koruyucu etkiler, AS'nin içerdiği antioksidanlar ve kollajen benzeri proteinler gibi biyoaktif bileşiklere bağlanabilir ve vitrifikasyon sırasında oluşabilecek oksidatif stres ile membran hasarını azaltabilir. Elde edilen bulgular, AS'nin embriyo kalitesini ve donma sonrası hayatta kalma oranları artırmada potansiyel bir alternatif takviye olarak değerlendirilebileceğini göstermektedir. Bununla birlikte, AS'nin optimum konsantrasyonunun belirlenmesi ve vitrifikasyon sürecinde spesifik koruyucu mekanizmalarının aydınlatılması için daha fazla araştırmaya ihtiyaç duyulmaktadır.
Anahtar kelimeler: Sığır Embriyosu, Arı sütü, Yavru Sığır Serumu, Vitrifikasyon

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Introduction

In vitro embryo production is an important field, and many studies have been conducted to optimize the chemical composition of culture media and their effects on oocyte maturation and embryo development. Various components such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estrogen as well as growth factors (epidermal growth factor, EGF and insulin-like growth factor, IGF) are commonly included into culture media. Also, protein sources such as fetal bovine serum (FBS) or bovine serum albumin (BSA) and antibiotics are also frequently used to enhance oocyte development.

Particularly, serum has an important place in IVP culture media as it provides combination of essential nutrients, hormones, growth factors, proteins and fatty acids necessary for cellular growth. Although serum contains several beneficial components required for embryonic development, its use in cell culture systems has been reported to cause potentially toxic effects (Maurer, 1992). The toxicity of serum is attributed to chemical changes in certain bioactive compounds that occur when the serum is processed. Despite the frequent use of serum as a protein source in bovine embryo culture, successful embryo culture systems have been reported in serum-free medium with synthetic oviduct fluid (SOF) supplemented with essential and non-essential amino acids (Shamsuddin et al., 1994).

The effects of serum supplementation (FCS) on embryo culture are very important and there are numerous reports showing an increase in blastocyst formation. However, the overall quality of embryos obtained under these conditions has been reported to be suboptimal (Langendonckt et al., 1996; Carolan et al., 1995). Gardner et al. (1999) reported that the negative effects of serum supplementation were particularly associated with abnormal blastocoel formation, mitochondrial structural abnormalities, reduced cell number and metabolic abnormalities in embryos. Furthermore, serum supplementation has been shown to lead to increased accumulation of lipid droplets in embryos, which, in turn, causes intracellular damage during cryopreservation

and ultimately reduces embryo viability after thawing (Vajda et al., 2010; Lorraine et al., 1998; Gardner, 1994; Thompson et al., 1995). These findings highlight the need to find alternative protein sources that can replace serum in the culture media while ensuring optimal embryo development.

Recent studies have shown that supplementation with RJ blastocyst formation in bovine and caprine species (Abdulnabi & Daham, 2021). However, the effects of RJ on embryo survival after vitrification are still less researched.

Vitrification of mammalian embryos is very important for long-term genetic resource preservation, allowing to store high-quality embryos for extended periods of times. While vitrification at the embryo stage has been shown relatively high success rates, a challenging problem in vitrification process remains due to the associated risks of chromosomal abnormalities, microtubule depolymerization, spindle disorganization, and alterations in the actin cytoskeleton (Saunders & Parks, 1999). Studies have demonstrated that bovine oocytes can also be vitrified either in the presence of cumulus cells (Mikaye et al., 1993; Jin & Sun, 2008) or as denuded oocytes (without cumulus cells) (Vajda et al., 1998), with both methods supporting subsequent in vitro embryonic development.

The aim of this study is to evaluate the effects of media supplemented with either 10% FBS or 0.625% royal jelly on the culture bovine embryos on the survival rates of blastocysts after the vitrification process at different time points to determine the potential cryoprotective properties of royal jelly.

Materials and Methods

Oocyte Maturation

Bovine ovaries collected from a local slaughterhouse were transported to the laboratory at +35°C in phosphate-buffered saline (PBS) supplemented with 50 IU/mL penicillin and 50 µg/mL streptomycin sulfate. Follicles containing cumulus-oocyte complexes (COCs) with a diameter 2 to 8 mm were aspirated using a 10 mL syringe attached to an 18-gauge needle. The COCs were then washed three times in 1-2 mL of Hepes-buffered Medium 199 containing Earle's salts, supplemented with fetal bovine serum (FBS) and antibiotics (50 IU/mL penicillin and 50 µg/mL streptomycin sulfate). Only compact, non-atretic COCs with a maximum of 3-4 layers of cumulus cells and uniform granular cytoplasm were selected for maturation.

Embryo Culture

Matured oocytes were fertilized with frozen semen that was thawed at +38°C in a flask from a tested bull. After maturation, most of the cumulus cells were removed by vortexing, leaving three to five layers around the oocyte. This was followed by two washes in Hepes-buffered TALP supplemented with 3 mg/mL BSA (Fraction V), 50 IU/mL penicillin and 50 µg/mL streptomycin sulphate. Thawed semen was layered (100 µL) under 1 mL of a modified calcium-free Tyrode's Albumin Lactate Pyruvate (TALP) capacitation medium supplemented with 6 mg/mL BSA fraction V, 50 IU/mL penicillin and 50 µg/mL streptomycin sulphate (pH 7.4) in dolphin nose eppendorf tubes to facilitate capacitation using the swim-up procedure[19]. After incubation (30 min) at +38°C in a humidified atmosphere of 5% CO_2 in air, the upper 0.7 mL of medium was collected and centrifuged (300xg) for 10 min before the supernatant was removed, leaving live sperm in the pellet in approximately 100 µL in capacitation medium. The pellet was then resuspended, and motile sperm were counted to give a final concentration of $1x10^6$/ml of motile sperm. Then, 4 µL of capacitation medium containing motile sperm was added to 46 µL of fertilization medium drops. The fertilisation medium was modified TALP supplemented with 0.2 µmol/L penicillamine, 0.1 µmol/L hypotaurine, 0.02 µmol/L epinephrine, 6 mg/mL fatty acid-free BSA, 30 µg/mL heparin, 50 IU/mL penicillin and 50 µg/mL streptomycin sulphate (pH 7.8). Matured oocytes (10 oocytes per well) were added to the fertilization droplet where oocytes and spermatozoa were co-incubated together for 10 hours under mineral oil at +38 °C in a humidified atmosphere of 5 % CO2 in air. After fertilization, all remaining cumulus cells were removed by vorteking and

presumptive zygotes were washed twice in Hepes-buffered TALP. Finally, the presumptive zygotes matured in %10 FBS or %0.625 RJ (0.625) royal jelly were further cultured in Synthetic Oviductal Fluid (SOF) supplemented with %10 FBS and 50 IU/mL penicillin and 50 µg/mL streptomycin sulphate.

Embryo Vitrification

Blastocysts cultured on day 8 from two experimental groups were used for vitrification. OPS straws and a series of cryoprotection solutions were used for vitrification, including Holding Medium (HM) (Hepes-TCM199 with 20% FBS), Sucrose Medium (SM; 4.56 g sucrose + 16 mL TCM199 + 4 mL FBS) (Sucrose + HM), ethylene glycol (EG), and dimethyl sulfoxide (DMSO). Two vitrification solutions were prepared: Vitrification Solution 1 (VS1: 850 µL HM, 75 µL EG, 75 µL DMSO) and Vitrification Solution 2 (VS2: 670 µL SM, 165 µL EG, 165 µL DMSO).

The blastocysts subjected to vitrification were sequentially transferred to the wells of a culture dish with four wells: 1st well (HM), 2nd well (VS1), and 3rd well (VS2). Subsequently, a small volume (20 µL) from the 4th well (VS2) was added as a droplet on a culture dish. Blastocysts from the 3rd well were transferred to this droplet and 2 µL of this droplet, containing the blastocyst, was picked up and placed onto another area of the culture dish. Pre-prepared OPS straws were then carefully inserted into this droplet at a specific angle to allow capillary action to draw the droplet and blastocyst into the straw. The loaded OPS straws were immediately immersed in liquid nitrogen for vitrification.

For warming, the OPS straws were removed from the liquid nitrogen and placed in water at 37°C for 10 seconds. The thawed straws were dried, and the sealed end was cut with scissors to transfer the contents to a warming medium (800 µL HM + 400 µL SM). Blastocyst were kept in this solution for approximately 1.5 minutes and then transferred into HM under a stereomicroscope. Finally, they were further cultured in %10 FBS supplemented with Synthetic Oviductal Fluid (SOF) for 24 hours at +38 °C in a humidified atmosphere of 5 % CO2 in air. The blastocysts were examined under the microscope at 12-hour intervals to assess the damage caused by vitrification, including shrinkage and re-expansion of the blastocyst cell, the proportion of degenerated cells and the integrity of the zona pellucida. The viability of the embryo was determined based on these morphological criteria."

Blastocyst Cell Counts

To determine the number of cells in a blastocyst, a staining solution containing 2 ng/mL fluorescent bis-benzimidazole dye (Hoechst H33258) is prepared in PBS. Prior to analysis, 1 to 5 (a group) embryos were placed in a 15 µL droplet of the staining solution and incubated for 10 minutes. After staining with the fluorescent bis-benzimidazole dye, the blastocysts were washed in 50 µL droplets of fresh PBS without dye and then transferred to a 10 µL droplet on a Petri dish. The stained embryos were then examined under a fluorescence microscope for cell count analysis.

Statistical analysis

Data were analysed by one-way ANOVA after appropriate transformation where necessary (proportion of cleaved zygotes and blastocyst yield, arcsine-transformation; blastocyst cell number and dpm counts, log10 transformation; blastocyst diameter, no transformation). Mean values of treatments (±s.e.m.) were compared pairwise using Tukey's HSD test. The results are presented as untransformed means ± s.e.m.

Results and Discussion

In the present study, the effects of the addition of RJ and FBS on bovine embryos development were evaluated based on cleavage rates, blastocyst development, cellular integrity and survival after vitrification. All oocytes matured in media supplemented with 10% fetal bovine serum (FBS) but further culture until blastocyst development was performed with either 10% FBS or 0.625% RJ.

The result in (Table 1) revealed that the cleavage rate on day 2 was not significantly different between the two experimental groups (FBS: 71.1% ± 2.02 vs. RJ: 68.3% ± 2.09; $P > 0.05$). However, at the 8-16 cell stage, a higher proportion of embryos developed in RJ-

supplemented media (50.7% ± 2.01) than in the FBS group (45.5% ± 2.76; P < 0.05). On day 5, the proportion of embryos reaching the morula stage was decreased in the RJ group (30.5% ± 3.22) compared to the FBS group (37.8% ± 3.05) but the reduction was not statistically significant (P > 0.05). Blastocyst formation by Day 8 was significantly lower in the RJ group (16.3% ± 3.36) compared to the FBS group (24.8% ± 2.58; P < 0.05).

The total number of cells in the blastocysts before the vitrification process was significantly higher in the FBS group (95.34 ± 4.59) than in the RJ group (70.74 ± 4.10; P < 0.05). The blastocyst diameter was also significantly larger in the FBS embryos (121±4.2 μm) than in the RJ-fed embryos (97±5.2 μm; P < 0.05).

Table 1. Bovine Blastocysts Developed in Royal Jelly-Supplemented Media

	FBS	RJ
Number of Oocytes	120	134
% Cleavage (Day 2*)	71.1±2.02[a]	68.3±2.09[a]
%8-16 Cells	45.5±2.76[b]	50.7±2.01[b]
% Morula (Day 5*)	37.8±3.05[a]	30.5±3.22[a]
% Blastocysts (Day 8*)	24.8±2.58[a]	16.3±3.36[b]
Total Cell Counts	95.3±4.59[a]	70.7±4.10[b]
Blastocysts Diameters(μm)	121±4.2[a]	97±5.2[b]

*Fertilization was designated as Day 0.
**Values within the same row with different superscript are statistically different (p < 0.05).

Table 2. Vitrification of Bovine Blastocysts Developed in Royal Jelly/FBS Supplemented Media

	FBS -V	RJ-V
Survival (12 hour)	51.3±3.72[a]	48.3±4.09[a]
Survival (24 hour)	43.5±4.25[b]	50.7±3.41[b]
Total Cell Counts	75.3±4.59[a]	60.7±4.10[b]
Blastocysts Diameters(μm)	95±6.72[a]	74±6.3[b]

**Values within the same row with different superscript are statistically different p < 0.05).

Survival rates of vitrified and thawed blastocysts derived from both FBS and RJ-supplemented media were evaluated12 hours and 24 hours after thawing (Table 2). Survival was not significantly different between groups at 12 hours post-thaw (FBS-V: 51.3% ± 3.72 vs. RJ-V: 48.3% ± 4.09; P > 0.05). However, 24 hours post-after thawing, the RJ blastocysts had a numerically higher survival rate (50.7% ± 3.41) compared to the FBS blastocysts (43.5% ± 4.25), although this difference did not reach statistical significance.

The total cell count after vitrification was significantly reduced in both groups, with the RJ-derived blastocysts containing significantly fewer cells (60.74 ± 4.10) than the FBS group (75.34 ± 4.59; P < 0.05). The blastocyst diameter was also significantly smaller in the RJ-derived embryos (74±6.3 μm) compared to than in the FBS group (95±6.72 μm), which may reflect differences in cytoplasmic composition and membrane resilience to cryopreservation stress

In the present study the effects of royal jelly (RJ) supplementation on the development and cryotolerance of bovine embryo were investigated by comparing its efficacy with that of fetal bovine serum (FBS). Our findings indicate that supplementation with RJ resulted in a reduced rate of blastocyst formation but had potential cryoprotective properties that improved post-thaw survival.

The initial division rate at day 2 was comparable between the RJ and FBS groups, suggesting that the RJ does not negatively affect early embryonic development. However, by the 8–16 cell stage, a significantly higher proportion of

embryos had developed in the RJ-supplemented medium than in the FBS group. This result suggests that RJ may promote cell division in the early stages of embryo development s, possibly due to its bioactive compounds, including vitamins, peptides, and antioxidants, which contribute to cellular metabolism and the reduction of oxidative stress. Despite this benefit, RJ supplementation resulted in a significantly lower blastocyst formation rate (16.3% vs. 24.8% for FBS, $P < 0.05$), indicating that while it supports early embryo development, it may not be as effective as FBS in promoting blastulation.

Embryo quality was assessed by total number of cells and blastocyst diameter. Blastocysts from media supplemented with RJ had a significantly lower total cell count (70.74 ± 4.10) than those from FBS (95.34 ± 4.59, $P < 0.05$). Similarly, blastocysts cultured with RJ had smaller diameters than those cultured with FBS (97 ± 5.2 μm vs. 121 ± 4.2 μm, $P < 0.05$). These differences may reflect alterations in cytoplasmic composition, metabolic activity, or membrane dynamics influenced by RJ. Previous research has indicated that serum supplementation increases lipid droplet accumulation in embryos, which may compromise cryotolerance. In contrast, RJ has been shown to have antioxidative and membrane-stabilizing effects, which could influence cellular composition differently than FBS.

The impact of RJ supplementation on cryotolerance was evident in the after-thaw survival results. Although no significant difference was observed in survival rates at 12 hours post-thawing (FBS-V: $51.3\% \pm 3.72$ vs. RJ-V: $48.3\% \pm 4.09$, $P > 0.05$), the RJ-derived blastocysts had a numerically higher survival rate at 24 hours after thawing ($50.7\% \pm 3.41$) compared to the FBS group ($43.5\% \pm 4.25$). This suggests that RJ contributes to improved cryotolerance by mitigating cellular damage during vitrification and warming. It has been postulated that RJ contains bioactive components, such as long-chain fatty acids and antimicrobial peptides that may improve membrane integrity and cellular resilience under stress conditions.

Further evidence of the potential cryoprotective effects of RJ is the fact that the total number of cells after vitrification was significantly lower in RJ-derived blastocysts (60.74 ± 4.10) than in FBS-derived blastocysts (75.34 ± 4.59, $P < 0.05$). Additionally, the blastocyst diameters after thawing were significantly smaller in the RJ embryos (74 ± 6.3 μm) than in the FBS group (95 ± 6.72 μm). The reduction in cell count and size post-vitrification may reflect differences in cytoplasmic lipid composition and membrane stability, which could influence the ability of embryos to withstand cryopreservation-induced osmotic and oxidative stress.

These findings suggest that RJ despite its lower efficiency in blastocyst formation may provide benefits in post-thaw viability and cellular resilience despite its lower efficacy in blastocyst formation. The presence of antioxidative and protective compounds in RJ could counteract some of the detrimental effects of cryopreservation, making it a promising alternative protein source in embryo culture media. However, further research is needed to optimize the concentration of RJ and to investigate its interactions with other components of the culture medium. Understanding the precise mechanisms underlying its cryoprotective properties will be essential for maximizing its potential as an alternative to FBS in in vitro embryo production.

The result showed that despite the decrease in blastocyst formation, the addition of RJ supported embryo development to the blastocyst stage, indicating that it could serve as a potential alternative protein source in maturation media. However, the lower blastocyst rates in the RJ group suggest that further optimization of RJ concentration or additional supplementation may be required to improve its efficacy as a serum alternative

These findings suggest that while the addition of RJ may limit total blastocyst formation and total cell count, it enhances embryo cryotolerance, as evidenced by the improved post-thaw survival rate at 24 hours. The potential protective effects

of RJ may be attributed to its bioactive components, including antioxidants and membrane-stabilizing compounds, which could mitigate cellular damage during vitrification and warming.

Conclusion

In conclusion, although the addition of RJ resulted in lower blastocyst formation and total cell count compared to FBS, its positive effect on post-thaw survival suggests that it may improve embryo cryotolerance. Further studies investigating modifications of RJ or its combination with other cryoprotectants could improve its efficacy in embryo culture systems.

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References

Abdulnabi, A. S., & Daham, A. F. (2021). Royal jelly improved in vitro matured bovine oocytes. Annals of the Romanian Society for Cell Biology, 7653.

Carolan, C., Lonergan, P., Van Langendonckt, A., & Mermillod, P. (1995). Factors affecting bovine embryo development in synthetic oviduct fluid following oocyte maturation and fertilization in vitro. Theriogenology, 43, 1115-1128.

Choi, Y. H., Carnevale, E. M., Seidel, G. E. Jr., & Squires, E. L. (2001). Effects of gonadotropins on bovine oocytes matured in TCM-199. Theriogenology, 56, 661-670.

Dorland, M., Gardner, D. K., & Trounson, A. O. (1994). Serum in synthetic oviductal fluid causes mitochondrial degeneration in ovine embryos. Journal of Reproduction and Fertility, Abstract Series, 13, 25.

Fukui, Y., McGowan, L. T., James, R. W., Asher, G. W., & Tervit, H. R. (1991). Effects of culture duration and time of gonadotropin addition on in vitro maturation and fertilization of red deer (Cervus elaphus) oocytes. Theriogenology, 35, 499-512.

Gardner, D. K. (1994). Mammalian embryo culture in the absence of serum or somatic cell support. Cell Biology International, 18, 1163-1170.

Gardner, D. K., & Lane, M. (1999). Embryo culture systems. In Trounson, A., & Gardner, D. K. (Eds.), Handbook of in vitro fertilization (pp. 85-114). CRS Press, New York.

Gomez, E., Rodríguez, A., Muñoz, M., Caamaño, J. N., Hidalgo, C. O., Morán, E., Facal, N., & Díez, C. (2007). Serum-free embryo culture medium improves in vitro survival of bovine blastocysts to vitrification. Theriogenology, 69, 1013-1021.

Kong, I. K., Choi, J. L., Cho, S. G., Bae, I. H., Oh, D. H., Oh, H. J., Kim, H. R., & Kim, J. K. (2001). Effect of addition of royal jelly in tris-buffer extender on the post-thaw viability of canine semen. Theriogenology, 55, 309.

Kridli, R. T., Husein, M. Q., & Humphrey, W. D. (2003). Effect of royal jelly and GnRH on the estrus synchronization and pregnancy rate in ewes using intravaginal sponges. Small Ruminant Research, 49, 25-30.

Leibo, S. P., Pollard, J. W., & Martino, A. (1995). Chilling and freezing sensitivity of "reassembled" in vitro derived bovine embryos. Theriogenology, 39, 81-94.

Lim, J. M., Okitsu, O., Okuda, K., & Niwa, K. (1994). Effects of fetal calf serum in culture medium on development of bovine oocytes matured and fertilized in vitro. Theriogenology, 41, 1091-1098.

Maurer, R. R. (1992). Animal cell culture: A practical approach. In Freshney, R. I. (Ed.), Oxford University Press, Oxford (pp. 15-46).

Onal, A. G., Guzey, Y. Z., Kariptas, S., & Gocmez, Z. (2006). Effects of honeybee royal jelly on fertilization of matured

bovine oocytes. Book of Abstracts of the 57th Annual Meeting of the European Association for Animal Production, No. 6, p. 185.

Parrish, J. J., Susko-Parrish, J. L., Leibfried-Rutledge, M. L., Crister, E. S., Eyestone, W. H., & First, N. L. (1986). Bovine in vitro fertilization with frozen-thawed semen. Theriogenology, 25, 591-600.

Schmidt, J. O., & Buchmann, S. L. (1992). Other products of the hive. In Graham, J. M. (Ed.), The Hive and The Honey Bee (pp. 927-988). Dadant and Sons, Hamilton, IL.

Shamsuddin, M., & Rodriguez-Martinez, H. (1994). Fine structure of bovine blastocysts developed either in serum-free medium or in conventional co-culture with oviduct epithelial cells. Journal of Veterinary Medicine - Series A, 41, 307-316.

Suo, Z., Zhou, G. B., Meng, Q. G., Yan, C. L., Fan, Z. Q., Zhao, X. M., Fu, X. W., Wang, Y. P., Zhang, Q. J., & Zhu, S. E. (2008). OPS vitrification of mouse immature oocytes before or after meiosis: the effect on cumulus cells maintenance and subsequent development. Zygote, 17, 71–77.

Tervit, H. R., Whittingham, D. G., & Rowson, L. E. (1972). Successful culture in vitro of sheep and cattle ova. J. Reprod. Fertil., 30(3), 493-497.

Thompson, J. G., Gardner, D. K., Pugh, P. A., McMillan, W. H., & Tervit, H. R. (1995). Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. Biology of Reproduction, 53, 1385-1391.

Vajda, G., Holm, P., Kuwayama, M., Booth, P. J., Jacobsen, H., Greve, T., & Callesen, H. (1998). Open pulled straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. Molecular Reproduction and Development, 51, 53-58.

Vajta, G., Holm, P., Greve, T., & Callesen, H. (1996). Factors affecting survival rates of in vitro produced bovine embryos after vitrification and direct in-straw rehydration. Animal Reproduction Science, 45, 191-200.

Van Langendonckt, A., Auquier, P., Donnay, I., Massip, A., & Dessy, F. (1996). Acceleration of in vitro bovine embryo development in the presence of fetal calf serum. Theriogenology, 45, 194-194.