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The effectiveness of human SAGE medium compared to other conventional culture media on the ovine early embryonic development, *in vitro*

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Abstract

Developing an optimal *in vitro* embryo culture medium with high blastocyst rates and good-quality embryos remains challenging and controversial, as most culture media directly impact mammalian embryonic development. This study aims to identify the most suitable culture medium for ovine embryo development based on its impact on embryo quality and developmental rates. Ewes' ovaries (n=250) were collected from a local abattoir and transported at 4°C to the laboratory. Oocytes from follicles (3-5 mm) were aspirated. *In vitro* maturation was performed in a 500 µl TCM-199 medium and then incubated for 24 h. The sperm cells were collected from fresh testes-epididymides, then the oocytes were fertilized with sperm concentrations of $7x10^6$/ml. Five embryo culture media were tested to compare embryo quality and development rates. Zygotes were cultured in a synthetic oviductal fluid medium as a control group, Medium 199, Bracket and Oliphant's medium, the bee honey culture medium, or SAGE 1-Step™ medium for seven days. The SAGE medium dramatically raised the embryos' quality compared to their counterparts cultured in synthetic oviductal fluid, medium 199, Bracket and Oliphant, or bee honey medium. In addition, the SAGE medium considerably outperformed the other media regarding embryo compaction rate on day 3. In summary, the rates and speed of embryonic development in ovine varied depending on the culture media used. The availability of glucose and growth factors provided by the culture media influenced the quality and rates of ovine blastocysts.

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Introduction

In vivo, preimplantation embryo development requires a balanced environment enriched with the embryo metabolic needs within the oviduct and uterine fluid, which seems to be changed dynamically as the embryo advances towards the uterus (1). However, *in vitro* embryo development (IVD), the culture medium, is essential for embryonic development. The zygotes undergo a sequence of transformations that allow them to implant in the endometrium; cleavage, compaction, and embryo cavitation are morphological modifications, whereas the biochemical transformations are related to amino acid absorption, energy consumption, and variations in protein synthesis (2,3). Understanding the physiological systems (oviduct and uterus) and the metabolic needs of the embryo allowed the culture media to be modified and support embryo development *in vitro* (4). However, matching the exact conditions between *in vitro* culture media and *in vivo* environments needs further studies (5). Furthermore, culture media can affect the embryonic development rate, the potential of the embryo's post-implantation development, and the fetus's overall health (6,7). There are many *in vitro* ovine culture protocols commercially available; the synthetic oviductal fluid (SOF) medium supplemented with amino acids embryos, CR1 medium, Hams-F-10 medium (8), and M-199 (9). The health and viability of embryos are primarily determined by their metabolic condition (10). Producing adenosine triphosphate

(ATP) from nutrients available in the medium involves a balancing act between metabolic pathways; failure to regulate the equilibrium between these pathways might result in the embryo depleting substrate resources, limiting the capacity for synthesizing ATP (11). In addition, each embryo development stage has different amino acid requirements to develop and function properly (12). Amino acid transporters (AATs) can be found in the oocytes and embryos. Essential amino acids are vital for producing proteins and nucleotides; they are antioxidants, chelators, pH regulators, and osmolytes. Additionally, amino acid consumption has been associated with the embryo's ability to implant in the uterine endometrium (13). The components of the embryo culture media vary in their energy substrate compositions and levels, growth factors, and chemical compounds. For instance, Pyruvic acid, an essential molecule in small ruminants' IVC media, is involved in the cleavage stage, which exhibits a higher concentration than blastocyst stage media (14). Pyruvate metabolic activity remains significantly higher in embryos in the two-cell stage, resulting in higher blastocyst development rates (15). Excess lactate in the embryo culture media can reduce pyruvate oxidation rates. Thus, the medium's pyruvate ratio to lactate can affect metabolic control and developmental competence (16). Glucose is one of the most basic energy needs for embryonic development, mostly at later stages (17), which is necessary for ATP biosynthesis. The Embden-Meyerhof pathway converts glucose into pyruvate in the cytosol through a series of reactions (18). The mouse zygote is unable to utilize glucose as a source of energy. However, at the early blastocyst stage, a mouse embryo development thrives on glucose alone (19). One study found that glucose is essential to bovine embryo viability; glucose absorption begins slowly and rapidly rises after embryo genomic activation. Viable blastocysts consume more glucose than blastocysts that cannot implant (poor-quality embryos) (20).

Much controversy has been in the literature about the most suitable embryo culture medium to optimize high-quality blastocysts *in vitro*. Subsequently, scrutiny of the embryo culture media for *in vitro* ovine embryo development is justified. Our study aimed to compare different optimized culture media in terms of their energy substrate compositions, levels of growth factors, and chemical compounds resembling those found in synthetic oviductal fluid (SOF). Specifically, we examined culture media BO and M-199, readily accessible in the assisted reproductive technology (ART) program. We also included honey, extensively discussed in numerous studies and a commercial SAGE medium.

Materials and methods

Ethical approve

Scientific Ethical Committee on Animal Experimentation at universities and research institutions in Turkey should supervise scientific studies on live vertebrate animals. Since our study was performed using only slaughtered animal material collected from the slaughterhouse, there was no need to apply it to the Scientific Ethical Committee on Animal Experimentation.

In vitro, embryo production

In vitro, embryo production (IVEP) was performed at the Animal Science Laboratories, Ankara University, Turkey. The chemicals for the embryo culture media were requested from Sigma-Aldrich Chemical Company, Frankfurt, Germany. Additional substances, given in the relevant section, were obtained from other companies.

Oocytes collection and maturation

Ewes' ovaries (n=250) were collected from a local abattoir, placed in a cooling box containing 0.9% saline solution, and transported at 4°C to the laboratory within 1-2 h. Oocytes from antral and Graafian follicles (3-5 mm) were aspirated with a 10 ml syringe with 4.0-5.0 ml of aspiration medium and an 18 G needle. Oocytes were assessed under an inverted microscope (LEICA DM IL LED; Wetzlar, Germany), and only those with more than two complete cumulus cell layers were selected (Figure 1) (21). Following the selection of the cumulus-oocyte complexes (COCs) via visualizing morphology, *In vitro* maturation (IVM) was performed in a 500 µl of IVM medium (M199- A supplemented with 10% FBS (16A), 1 IU/ml FSH (Life Technologies), 1 IU/ml LH (Bio98), 750 µM glutamax (35050-06), 0.36 mM sodium pyruvate (P4562), and 5 µl/ml gentamycin (GEN-10B), into a four-well Multi Dish (Nunc) (approximately 30 COCs/ well), and then incubated for 24 h (38.5˚C, 5% CO_2, and 90% humidity) (22).

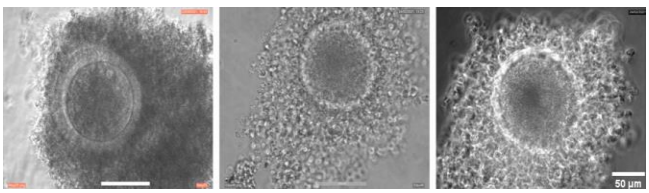

Figure 1: Cumulus-oocyte complexes before *in vitro* maturation.

Semen/ sperm manipulation and fertilization

Post-IVM, expanded COCs (eCOCs) and the oocytes that had the first polar body (Figure 2) were immediately washed two times by the BO culture medium and placed in 500 µl (20-30) (23) of BO medium supplemented with 5 µl/ml gentamycin, 1.25 mM sodium pyruvate, 5 mg/ml BSA (A3311), 1 µl/ml heparin (P4562), 40 µl/ml PHE, 2 mM caffeine and 10% FBS. The sperm cells were collected from fresh testes-epididymides (24); for sperm capacitation, cauda epididymides contents were collected and disseminated in the HEPES-TL medium. Ram sperm were cultured for

roughly 4 h at 37°C in a humidified atmosphere with 5% CO_2. Then examined for sperm quality and assessed according to the individual/collective movement of sperm cells (25). Then the eCOCs were fertilized with sperm concentrations of $7x10^6$/ml. The oocytes and sperm were co-incubated under 38.5°C, 5% CO_2 in high humidity (26).

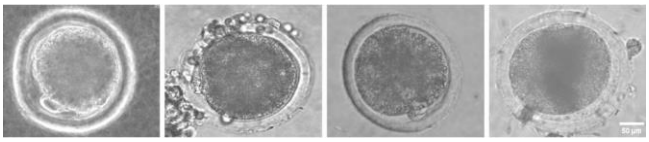

Figure 2: Mature oocyte with (1st PB).

Experimental groups (Embryo culture)

Six hours after fertilization, the oocytes were evaluated as fertilized oocytes with the second polar body or oocytes with sperm heads in the cytoplasm (Figure 3) (27). Cumulus cells were partially removed using 1 µl hyaluronidase (H3884), followed mechanically by pipetting. According to the experimental design, the zygotes were cultured in five different IVD media, as below. The first group was SOF medium supplemented with 0.5 µl/ml Na pyruvate, 4 mg/ml BSA (A3311), 10 µl/ml glutamax, 10 µl/ml sodium citrate, 10 µl/ml Non-Essential AA (M7145), 20 µl/ml Essential AA (B6766), 10 µl/ml myoinositol (P4562), and 5 µl/ml gentamycin (GEN - 10B). In the second group, the M- 199 medium (M199-A) was supplemented with 10% FBS (16A) and 5 µl/ml gentamycin (GEN - 10B). In the third group, BO medium supplemented with 2% Essential AA, 1% Non-Essential AA, 3 mg/ml BSA, 0.6 mM sodium pyruvate, 5 µl/ml gentamycin, and 5 µl/ml gentamycin. In the fourth group, the bee honey culture medium (BH) was prepared by mixing 1 ml of honey bees with 5 ml of D.D. water and used as an IVD medium. Finally, the fifth group used SAGE 1-Step™ (6701) as an IVD medium (28-31).

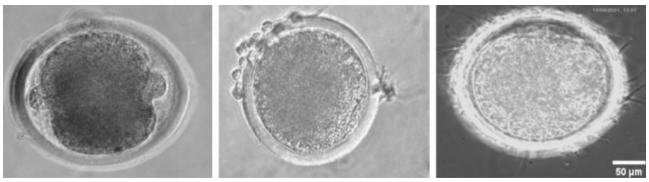

Figure 3: Zygote with the second polar body.

Embryo qualification

Zygotes and embryos were observed under an inverted microscope (LEICA DM IL LED; Wetzlar, Germany) after staining with 2.5 mg/ml Hoechst 3342 for 40 minutes using UV light. The total cell number (TCN), inner cell mass (ICM), and trophectoderm (TE) cells were calculated by staining the blastocyst with 10 mg/ml Propidium iodide (PI) and 25 µg/ml Hoechst (32). Embryos were washed with phosphate buffer solution (PBS). Slides were mounted with 3:1 (vol/vol) glycerol/PBS and coverslipped, sealed with nail polish, and stored at 4°C in the dark (33). On Days 2, 3, and 4, the number and quality of the cells and blastomeres were used to evaluate the embryo's morphology. Image analysis was performed using Image J/Fiji version 1.53t (34).

Statistical analysis

Results are presented as the mean (± SEM) after a one-way ANOVA data analysis using SPSS version 23.0 statistical software. The difference between treatments was compared using Duncan's Multiple Range Test, and a p-value (< 0.05) was considered significant.

Results

More than 80% and 75% of fertilized oocytes reached the cleavage stage in both SAGE and SOF media, respectively; this indicates that the SAGE and SOF media are significantly superior (P<0.05) compared to the other media (Figure 4). The morula rate was significantly higher (P<0.05) in the SAGE and SOF media (71.25% and 63.75%, respectively), followed by the M-199 medium with a morula rate of 41.25%. On the other hand, the overall morula rates were 18.75% and 7.25% (P<0.05) in BO and BH media, respectively (Figure 4). Only the two-culture media of the SAGE and SOF were significantly higher (P<0.05) 62.5 and 45%, respectively compared to those in M-199, BO, and BH culture media 13.75, 3.75, and 2.5%, respectively. The SAGE culture medium showed higher 149±2.4 TCN values than the SOF culture medium 97±1.3. However, there were no differences in ICM cells and TE parameters between the different groups (P>0.05) (Figures 5-7).

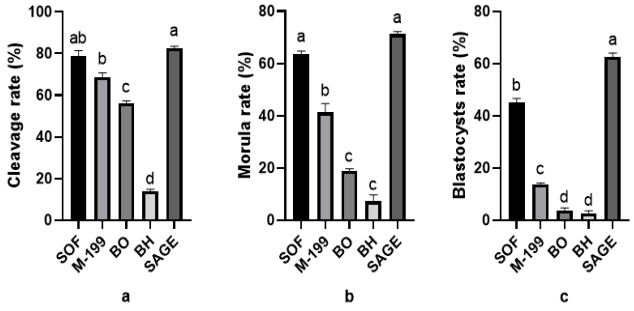

Figure 4: Effect of culture media (SOF, M-199, BO, BH, and SAGE) on cleavage rate (panel A), morula rate (panel B), and blastocysts rate (panel C). Data were presented as the mean (±SEM). The total number of fertilized oocytes was 400 (n=80 for each group). 80 fertilized oocytes were divided into each group. Mean values in the same column with different superscripts are different at P<0.05.

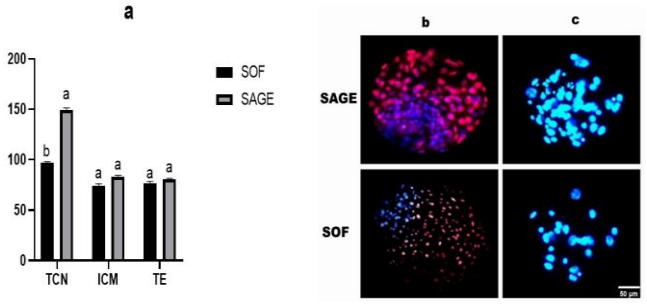

Figure 5: Developmental characteristics analysis of blastocyst development in SAGE medium and SOF culture medium. Panel A represents the total cell number (TCN), inner cell mass (ICM), and trophectoderm (TE) of blastocysts (n=30). Blastocyst stained with Hoechst 33342 and Propidium iodide at 330-385 nm and 400 nm for fluorescence microscopy image (panel B). The blue represents the ICM cells, whereas the red represents TE cells (panel C).

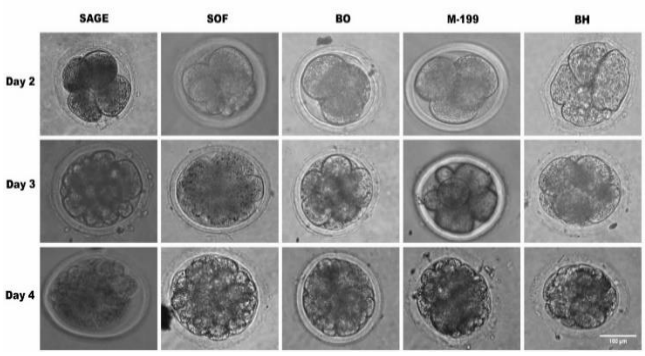

Figure 6: Shows ovine embryonic development in different culture media. The type of culture medium used for embryos affects the compaction of the embryo and the zygote cleavage. On day 2, the zygote cleavages into four cells in the SAGE medium. In addition, the embryo compaction and quality on days 3 and 4 were higher in the SAGE group compared with the other media.

Discussion

In vitro embryo culture composition media have been developed intensively to optimize embryo development to suit the embryo's energy needs. Embryos derived from embryo culture media were shown to have lower quality when compared to *in vivo*-derived embryos (35). Another research study suggests that the current culture media used for *in vitro* development of embryos could not accurately replicate the *in vivo* conditions in terms of mRNA expression (36), and similar concerns apply to the protocols for *in vitro* culture of ovine (37). These findings at the molecular level potentially resulted in disrupted epigenetic control and high DNA methylation levels (38,39).

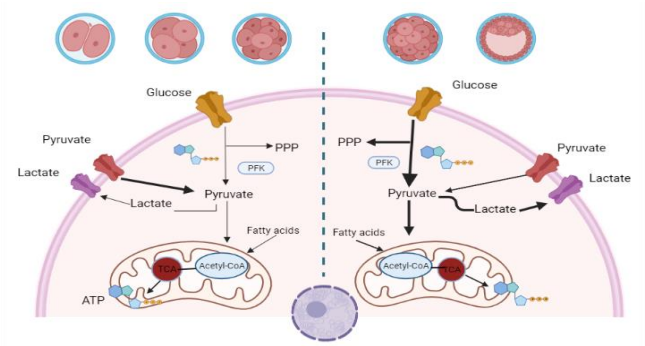

Figure 7: Dynamic changes of ovine embryos on days 2, 3, and 4 of embryonic development for each culture media. The number of cells in high-quality embryos was determined to be 4 on day 2 and 8-12 on day 3. High-quality embryos were selected based on the initiation of early compaction. On day 4, the morphologies of the embryos were assessed descriptively to determine the observed range of morphologies.

Studies on culture media composition indicated that blastocyst development depended on culture media (28). However, such observations have yet to be addressed clearly in every study; for instance, the optimum embryo energy needs, the alternative IVC media, and the actual compositions of the embryo culture media. We hypothesized that specific and alternative IVC culture media, such as SAGE (human embryo culture medium) vs. SOF (the most common ovine culture media), can be used as an alternative for ovine embryo culture media. The SAGE productively promoted embryonic development and blastocyst quality. In contrast, other commercial embryo culture media like M-199 may be less significant in developing *in vitro* embryos (37). Subsequently, embryo development and quality competency may be altered according to the composition of *in vitro* culture media.

Many components like energy sources, amino acids, growth factors, vitamins, minerals, and buffering agents in the culture medium are quintessentially important for embryo development and, to a sufficient extent, affect the development of mammalian embryos (40). In our study, the concentrations of the components within the commercial embryo culture media vary significantly, such as energy sources that play a vital role in regulating metabolism and viability.

SOF medium has been developed to reflect the changing carbohydrate and protein levels of the oviductal fluid within the reproductive tract, which enhances embryonic development, which is not supported by M-199, BO, or BH media. Our study is the first finding of using culture media of SAGE for culturing ovine-derived embryos.

Our findings suggest that the type of culture medium used for embryos affects both the quality of the embryos and the cleavage rate. Moreover, on day 3, the embryo

compaction rate in the SAGE group was considerably higher than that in the other media, where it is considered that early embryo cleavage is a robust biological indicator of *in vitro* ovine embryo development. Importantly, the *in vitro* culture media require nutrients that suit the totipotency of ovine embryonic development, and the variation in the level of energy in the culture media could be accountable for this result in our study; our findings align with previous research on ovine IVF (37,41,42).

In vivo, studies have shown that oviductal and uterine fluids have much higher amino acid levels than those found in other body parts (43). During early embryonic development in the oviduct, circulating concentrations of pyruvate and lactate substrates were high. Low glucose surrounded the embryo during the pre-blastocyst phases (44). Because the oviduct naturally contains glucose, and thus there are certain media in which glucose does not inhibit early preimplantation development, the exclusion of glucose from some culture media appears contradictory (45). However, in the present study, the SAGE group showed a high embryo quality during the subsequent development from the morula stage to blastocyst embryos. This might be due to the optimal concentration of glucose and other medium compositions. Our results match the study conducted by Xie *et al.* (16); the study demonstrated that the energy source of pyruvate is essential for early mouse oocyte maturation and zygote development. As the embryo undergoes cleavages by the 8-cell stage, glucose substrate remains the main energy source for embryo development.

Furthermore, the lactate: pyruvate (L:P) ratio presented in our study embryo culture media was widely disparate. Interestingly, less L:P ratio could lead to pyruvate substrate processing, resulting in a cytosolic oxidant and oxidative effect (46). In addition, a high L:P ratio is also concerning; it may include various factors, such as inefficient utilization of pyruvate, impaired mitochondrial function, inadequate nutrient availability, or suboptimal culture conditions. This metabolic imbalance can have detrimental effects on embryo development and quality. As a result, we suggest that the lactate/pyruvate ratio, which differs significantly between media, could affect the cleavage rate. Our findings align with previous research on mouse IVF (38).

For embryos that develop more quickly, the SAGE medium optimizes a continuous supply of nutrients and growth factors throughout the entirety of the *in vitro* embryonic stages; as a result, compaction can occur at an earlier stage. The discrepancies in embryonic development in culture media could be due to nutrition supply.

Active metabolic pathways during early embryonic development. The embryo development media are supplemented with energy sources consumed by anaerobic glycolysis. The pyruvate supports the zygote to morula transition. Then, the preimplantation embryo at the morula stage switches its metabolism to glucose utilization via glycolysis and pentose phosphate pathways (PPP). Finally, energy sources are irreversibly converted to acetyl-CoA, which enters the tricarboxylic acid cycle to provide electrons for oxidative phosphorylation and produce adenosine triphosphate.

Other studies show that adding growth factors like insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) accelerates embryonic development (47). The variations in embryo development and quality in the SAGE culture medium may be caused by the culture media in terms of components, such as growth factors, energy substrates, and vitamins (especially those belonging to the vitamin B), according to Morbeck *et al.* (48) analysis of the components of this culture medium. In this study, SAGE is the only medium containing growth factors unavailable in other media.

Another notable difference for the SAGE medium was an overall increase in amino acid concentration; this elevation in amino acid levels suggests a deliberate modification in the composition of the SAGE medium to provide enhanced nutritional support for embryonic development (48). One study on human embryos revealed that adding amino acids to the culture medium increases embryo growth *in vitro* (49). These results are consistent with a study in which essential amino acids enhanced H19 expression, which may be involved in controlling the balance between proliferation and differentiation of embryonic cells in porcine embryos, while essential amino acids promoted PEG1 expression, which is involved in controlling embryonic development (50).

The observed data highlights an intriguing aspect regarding the influence of certain culture media components on later embryonic development. Notably, the utilization of M-199 or Bo culture media can subsequently impact late embryonic development, despite these particular media demonstrating satisfactory rates of early embryonic development *in vitro*. Conversely, regarding ovine embryos, the data suggests that SAGE and SOF culture media can achieve sustainable and normal in vitro embryonic development. These findings underscore the significance of considering the initial stages of embryonic development and the long-term consequences of utilizing specific culture media.

The embryo quality can be accomplished by studying the morphology of blastocysts-embryo viability and assessing the total cell number (51), which is positively correlated (52). On the contrary, lower ovine embryonic development rates and a reduced number of cells in the SOF medium were observed. One possible explanation for the observed differences is that the SOF medium used in the study may have been deficient in essential nutrients and growth factors necessary for proper embryonic development, particularly beyond the morula stage. As a result, the quality of the embryos has decreased by reducing the total number of cells.

In our experiment, simple media like BO, M-199, or BH significantly reduces the number of blastomeres at the

morula stage, primarily due to programmed cell death, metabolism, and protein degradation (53,54). The delay of a specific type of energy source for an extended period in the development medium has been observed to impact embryonic development. Subsequent blastocyst development has shown consistent evidence supporting this observation. Moreover, it has been found that certain culture media formulations can disrupt blastocyst development, leading to a reduction in the number of the inner cell mass. Regarding blastocyst development rates, embryos that developed in the SAGE medium significantly showed greater rates than their counterparts cultured in SOF, M-199, BO, and BH media.

Conclusions

This study provided evidence that the media used for *in vitro* embryo culture can profoundly affect sheep's blastocyst and embryonic development. An alternative to the commercial ovine IVC culture media, the human culture medium of SAGE has integral components that prompt the *in vitro* ovine embryonic development. The findings will improve assisted reproduction techniques in ovine species and potentially enhance reproductive outcomes in livestock breeding programs or research applications.

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Conflict of interest

The authors declare that they do not have any known competing financial interests or personal relationships that could appear to have affected the work presented in this paper.

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تقييم فاعلية وسط SAGE البشري ومقارنته بالأوساط الزراعية التقليدية على التطور المبكر لأجنة الأغنام في المختبر

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الخلاصة

صناعة وسط زرعي لتطور الأجنة في المختبر يضمن الحصول على معدلات عالية من الكيسات الأريمية وأجنة ذات نوعية جيدة يمثل تحديًا كبيراً، حيث أن معظم الأوساط الزرعية تؤثر بشكل مباشر على التطور الجنيني للثدييات. تهدف هذه الدراسة إلى تحديد أنسب وسط زرعي لتطور أجنة الأغنام بناءً على تأثيرها على جودة الجنين ومعدلات التطور الجنيني. تم جمع ٢٥٠ مبيض من النعاج المذبوحة في مسلخ محلي ونقلها عند ٤ درجات مئوية إلى المختبر. تم الحصول على البويضات من الحويصلات (٣-٥ مم). تم إجراء عملية إنضاج البويضات في المختبر باستخدام ٥٠٠ مايكروليتر من وسط ١٩٩ ثم حضنت لمدة ٢٤ ساعة. تم جمع الحيوانات المنوية من الخصيتين - البربخ الطازج ثم خصبت البويضات بتركيز ٧*١٠[٦] /مل من الحيوانات المنوية. تم اختبار خمس وسائط زرعيه لمقارنة جودة الأجنة ومعدلات التطور الجنيني. تمت احتضان الأجنة بعد الإخصاب في وسط SOF كوسط تحكم، وسط ١٩٩، وسط BO، وسط عسل النحل، أو وسط SAGE لمدة سبعة أيام. أدى استزرع الأجنة في الوسط الزرعي SAGE إلى رفع جودة الأجنة بشكل كبير مقارنة بالأجنة المستزرعة في الوسط SOF، وسط ١٩٩، BO، أو وسط عسل النحل. بالإضافة إلى ذلك، تفوق وسط SAGE بشكل كبير على الأوساط الزرعية الأخرى فيما يتعلق بمعدل انضغاط الجنين في اليوم الثالث. نستنتج من الدراسة الحالية، تفاوتت معدلات وسرعة التطور الجنيني في الأغنام اعتمادًا على الأوساط الزرعية المستخدمة، بالإضافة الى ذلك، أثر توافر الجلوكوز وعوامل النمو التي توفرها الأوساط الزرعية على جودة ومعدلات الكيسات الأريمية في الأغنام.