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Review article

Practical Considerations for Starting *In Vitro* Embryo Production Programs in Goats: a Systematic Review

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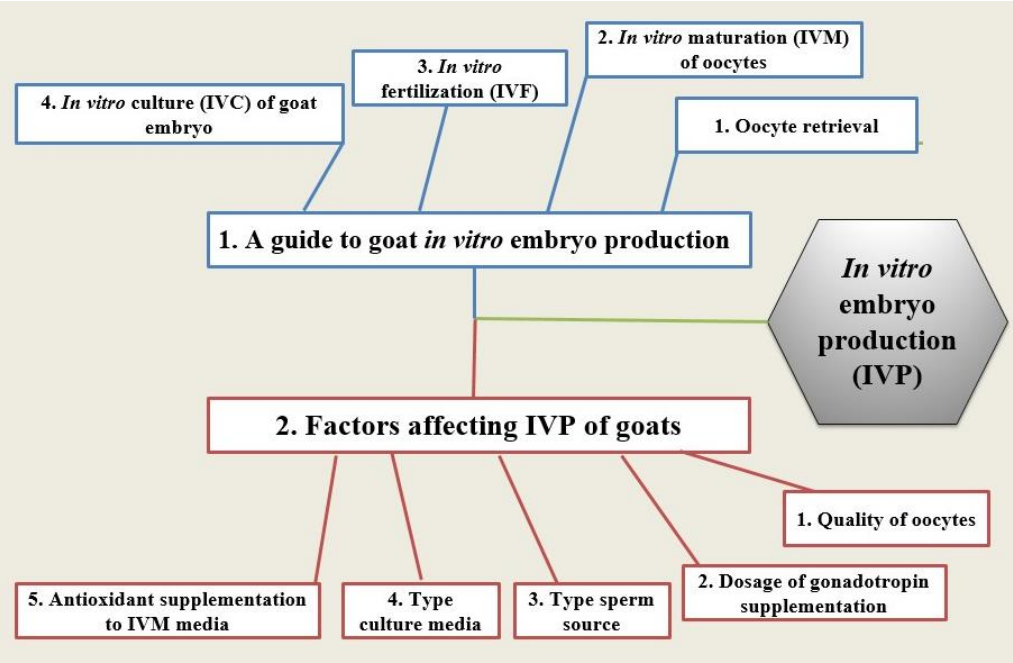
ABSTRACT

The goat is a vital small ruminant livestock species with extensive distribution in several nations. Goats are noted for their unique browsing habits and superior milk, meat, and hides production. Consequently, a substantial rise in demand is expected in the forthcoming years. The efficacy of small ruminant production methods must be deemed sufficient to satisfy global requirements. The *in vitro* production (IVP) of embryos, a facet of reproductive technologies, has enhanced goat production and addressed the limits of multiple ovulation and embryo transfer (MOET). In Egypt, *in vitro* techniques for goats are still a developing concept. Before this review, no research in Egypt focused on the IVP of goats in any laboratory, including oocyte maturation. This review seeks to present an overview of the current status of IVP in goats, with a focus on (i) A statement of the primary methodologies employed for *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) of embryos, and (ii) a particular focus on the key factors influencing the outcomes. The conclusion in this review has shown that supplement variables, the kind of culture media used, the cumulus-oocyte-complexes (COCs) quality, and the sperm source significantly impacted the embryo's growth and maturation.

Graphical abstract



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Contents

1. Introduction

Goats have thrived in impoverished areas and areas of mountains, which has led to the differential distribution worldwide. Although goats have existed in the area known as the Mediterranean basin for thousands of years, the market for meat and milk produced by small ruminants increased during the latter half of the 19th century [1, 2]. Goat meat is widely consumed worldwide and is known for its excellent protein content, low fat and cholesterol levels, and high vitamin and mineral content, including omega-3 fatty acids. Goat milk is also consumed globally and is considered easier to digest than cow's milk. The goat cheese business has grown to be a substantial niche sector in both Europe and the US because of its rich flavor and nutritional value [3]. Prohibiting pork in Muslim countries and the limited consumption of beef in India due to religious beliefs make goats acceptable among diverse populations [3, 4]. Despite the importance of goats, the high demand for goat meat, and their comparative advantages over cattle, goat farming faces challenges. In Egypt, the goat population decreased by about 75% from 3.5 million in 2018 to 1 million in 2021 [1, 5]. Changing farmers' perspectives to see goats as a "productive asset" as opposed to a "saving asset" is still very difficult.

2. A guide to goat in vitro embryo production

This method has dramatically improved goat genetics and output [6]. The IVP has also helped create high-competence embryos for agricultural, medicinal, and animal biotechnology research. The overall process of IVP involves evaluating and grading ovaries and collecting cumulus-oocyte complexes (COCs) from ovaries obtained from slaughterhouses. Also, classification of oocytes, IVM of oocytes, sperm capacitation, *in vitro* fertilization (IVF), and *in vitro* culture (IVC) of presumed zygotes to the morula or blastocyst stage [6, 7, 8]. In Egypt, *in vitro t*echniques for goats (*Hircus* (Baladi)) are still a developing concept due to difficulties accessing ovaries from slaughterhouses. Recent research has focused on identifying the necessary conditions for IVM, IVF, and IVP development processes, as well as beneficial supplementation and positive factors that enhance embryo production [9]. These efforts aim to improve the technique and promote its timely adoption in Egypt.

2. 1. Oocyte retrieval

A pair of goat ovaries comprises thousands of oocytes. Nevertheless, even a minuscule percentage (0.01%) may be employed throughout the female goat's reproductive lifespan, as most follicles experience atresia during their growth and maturity [10]. Oocytes for *in vitro* production can be obtained from living or slain animals [10]. The optimal non-invasive procedure for oocyte collection from live goats is laparoscopic ovum pick-up (LOPU). This treatment is costly, and the quantity of oocytes obtained per ovary is minimal. Conversely, the ovaries of slain animals are the most economical and plentiful supply of primary oocytes for extensive embryo development via IVM and IVF [7, 10].

2.1.1. Methods of oocyte retrieval

Various strategies have been established to retrieve oocytes derived from ovaries collected at a slaughterhouse in domestic animals. The three main methods are follicular aspiration, slicing the ovaries, and puncturing visible surface follicles. Regardless of the oocyte recovery technique, the effectiveness of oocyte recovery is influenced by the type of goat used for oocyte collection [11]. Due to the smaller size of goat ovaries than bovine ovaries, oocytes are closely associated with small to medium-sized follicles before cumulus expansion, complicating the aspiration process. Puncture uses a hypodermic needle to puncture the entire ovarian surface, while slicing uses a scalpel blade to collect follicles of all sizes [12]. Studies on goats have shown that more COCs were recovered from pubertal goats (88%) than from prepubertal goats (77%) [13, 14]. Puncture and slicing techniques produced notably greater total COCs per ovary, with values of 4.22 and 4.14 COCs, respectively, in contrast to the aspiration method, which yielded 3.28 COCs [13, 14]. However, Tsiartas et al. [15] reported that for ewe lambs, there was no significant difference in the number of oocytes per ovary between slicing (4.0 COCs) and aspiration (3.7 COCs).

2.1.2. Classification and evaluation of collected oocytes

Upon retrieval and assembly of oocytes for *in vitro* maturation (IVM), the direct link between each oocyte and its specific follicular environment and physiological context is severed despite the undeniable importance of the originating follicle for subsequent oocyte development. This link to the initiating follicle is essential for future oocyte development [16, 17]. Consequently, evaluating the capacity of oocytes to progress to the *in vitro* blastocyst stage is critical in all IVF programs. Examining oocyte morphological characteristics is now the sole non-invasive criterion for quality evaluation. Before IVM, the shape of the cumulus investment was extensively utilized as a selection criterion due to its substantial influence on oocyte

maturity. More compact layers of cumulus cells (CCs) are considered advantageous. CCs are crucial for oocyte maturation, supplying energy substrates and nutrients while functioning as messenger molecules [18,19,20]. In the majority of IVM studies, oocytes were categorized into four grades according to CCs and nucleus, as briefly outlined: Grade A: oocytes encircled by CCs; Grade B: oocytes partially encircled by CCs; Grade C: oocytes devoid of CCs; Grade D: degeneration noted in both oocytes and CCs. Grades A and B were classified as accepted grads, while C and D were deemed abnormal COCs [21,22]. Other studies established the following criteria for the evaluation and classification of oocytes from I to IV. Oocyte classes I, II, and III were deemed suitable for IVM, while class IV, characterized by poor cytoplasmic oocyte quality (COCs), was excluded from the study under a stereomicroscope (G.X. microscope, UK, Range: 8x to 50x) [10, 23]. Debnath et al. [7] reported that goat oocytes surrounded by more than five cumulus oophorus exhibited higher maturation percentages than those with fewer than five COC layers and denuded oocytes. Mardenli et al. [24] and Souza-Fabjan et al. [25] demonstrated that oocytes with high-quality COCs exhibited improved success rates and IVP following fertilization. The size of an oocyte is significant for achieving maturation. AbdElkhalek et al. [26] determined that the mean quantity of retrieved oocytes per ovary (goats with an average age of 2.5 years) was 6.31. The average number of high-quality oocytes obtained from each ovary was 4.95 oocytes. The percentage of high-quality oocytes was 78.48%, whereas the proportion of low-quality COCs was 21.52%.

2.2. *In vitro* maturation (IVM) of oocytes

The *in vitro* maturation (IVM) process is the initial and crucial step in IVP, enabling oocytes to acquire the capacity for further embryonic development. Successful IVM relies heavily on the follicle size and oocyte quality. The maturation of mammalian oocytes encompasses events from the germinal vesicle (GV) stage leading up to the second meiotic division's completion, which produces the first polar body [27]. Therefore, it is essential to identify an appropriate IVM environment to ensure an effective IVP procedure. The IVM of oocytes can be classified into nuclear and cytoplasmic processes. Nuclear maturation entails the restart of meiosis and advancement to the metaphase II (MII) phase. Cytoplasmic maturation encompasses a range of cellular processes essential for oocyte fertilization and subsequent development into viable embryos, with COCs serving a crucial function. [28]. Enhancing the oocyte IVM mechanism is essential for creating *in vitro* circumstances that mimic the natural environment [29]. Various IVM media have been made for goat oocytes across multiple laboratories. Buffered tissue culture medium-199 (TCM199) often functions as the foundational medium for goat oocytes' IVM. The COCs of adequate quality are typically rinsed 2-3 times in PBS, after that, placed in TCM-199 droplets, and incubated in a CO_2 incubator at 38.5 °C with 5% carbon dioxide in humidified air for 22-27 hours [30]. Oocyte maturation was previously performed in a culture medium of medium-199, supplemented with follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol-17β (E_2), and goat serum. The E_2 may facilitate ooplasmic maturation by boosting DNA polymerase activity and improving the production of male pronucleus growth factors. The presence of E_2 significantly increased blastocyst production in matured oocytes [29]. Typically, the ultimate FSH, LH, and E_2 concentrations differed among researchers. For instance, the final concentration of FSH varied from 0.1 ng/mL to 10 ng/mL. The LH final concentration ranged from 3 ng/ mL to 10 ng mL, and the E_2 final concentration from none to 1 ng/mL [31]. Following cultivation in the maturation medium, the degree of nuclear maturation is assessed by staining oocytes and examining them for germinal vesicle breakdown (GVBD), metaphase I (MI), and metaphase II (MII) stages. Another morphological criterion used to determine oocyte maturation is the expansion of CCs. In IVF, oocytes with moderately to extensively expanded CCs are fully matured [8, 14].

2.3. *In vitro* fertilization (IVF)

In vitro fertilization (IVF) is a complicated process that requires the proper development of oocytes, selection of sperm, capacitation of sperm, and the use of a suitable IVF medium [31]. The initial successful pregnancy in goats through IVF and subsequent ET was reported in 1991, followed by the production of the inaugural goat from IVM, and the fertilization of goat oocytes occurred in 1993 [31]. Under natural conditions, fertile spermatozoa are actively isolated from immotile Spermatozoa fragments and seminal fluid within the female reproductive tract via migration through the mucus of the cervical cavity. Therefore, spermatozoa must be selected and prepared for oocyte insemination before IVF. The ejaculate contains a combination of seminal fluid, mature and immature spermatozoa, non-reproductive cells, and residue [32]. Methods such as swim-up, discontinuous density gradient centrifugation, and Sephadex filtration are commonly employed to separate motile and immotile fractions of spermatozoa from fresh ejaculate or frozen-thawed sperm in bucks. The swim-up is often preferred as it yields higher numbers of highly motile spermatozoa than centrifugation using a density gradient. Nevertheless, no significant variations have been noted regarding oocyte entry and cleavage rates following IVF with fresh goat semen [33]. Typically, chosen spermatozoa are incubated in Tyrode's Albumin Lactate Pyruvate (TALP) medium enriched with heparin for 45 minutes, yielding superior fertilization rates. In several trials, COCs are co-incubated with sperm (either fresh or frozen) in a fertilization medium (e.g., TALP) within a CO_2 incubator at 38.5 °C with 5% carbon dioxide in humidified air [34]. After selecting the most viable and motile spermatozoa, sperm capacitation is performed *in vitro* using specific conditions; this procedure is essential for

mammalian sperm to attain fertilizing capability. The acrosome response produces proteolytic enzymes that facilitate sperm entry into the oocytes. A 90 µl drop of IVF medium containing *in vitro* matured COCs (n=10-20) was mixed with 10 µl of pre-incubated semen. The final concentration of spermatozoa in the fertilization droplets was 1×10^6. After 16 to 18 h, fertilization can be directly checked for fertilization rates [35, 36].

2.4. *In vitro* culture (IVC) of the goat embryo

The last step in IVP involves the cultivation of presumed zygotes in culture media, during which they undergo many divisions until attaining the blastocyst stage, often 6-7 days post-IVF in ruminant species. This post-fertilization culture period significantly impacts blastocyst quality [37]. Significant developmental milestones transpire during this phase, encompassing the primary cleavage division, activation of the embryonic genome, compaction of the morula, and formation of the blastocyst. The blastocyst stage involves the formation of two cell types: the inner cell mass (ICM), which differentiates into the fetus, and the trophectoderm (TE), which facilitates placenta development [38]. *For pre-implantation embryo development, simple, balanced salt solutions with carbohydrates or complicated media like TCM-199 with serum, somatic cells, or a feeder layer of somatic cells can be utilized. Commonly performed, and the most widely used synthetic oviduct fluid (SOF) medium* [39]. Specific laboratories consistently augment SOF medium with serum. Research on bovine indicates that serum exerts a biphasic effect; it inhibits early cleavage divisions while speeding up later developmental stages, leading to the quicker emergence of blastocysts in culture. Consequently, several researchers enhance the IVC medium with 5-10% fetal calf serum (FCS) 2-3 days post-insemination to increase survivability following the transfer of these IVP embryos. Other studies incorporate bovine serum albumin (BSA) into SOF medium [40]. Despite considerable progress in assisted reproduction, *in vitro* embryo generation's efficiency remains inferior to *in vivo* methods. Gametes and embryos undergo spatial and temporal abnormalities during ART, the effects of which remain unclear. In contrast to *in vivo* development, where the cow ovulates one or two oocytes, *in vitro* techniques allow the maturity of almost all oocytes without the dominant follicle's inhibition. However, only 30–40% of such oocytes become blastocysts. It may seem inefficient, but IVM oocytes are derived from small follicles measuring 2–8 mm, which are unlikely to ovulate *in vivo* and are subject to atresia. Furthermore, IVP blastocyst transfer in heifers yields a 40–50% pregnancy rate [41, 42].

3. Factors affecting the IVP of goats

The technique of IVP is widely used to address infertility issues in various mammalian species, and it is a crucial method for generating many progenies with enhanced genetic characteristics. The development of embryos is influenced by events that occur during oocyte maturation. Several environmental factors affect the IVP technique for mammalian oocytes [43, 44]. Thus, mammalian cells can only survive outside their natural *in vivo* environment if the *in vitro* conditions mimic those of a living body. Therefore, culture media must contain the necessary chemical and physiological elements for cell survival [45]**.** Consequently, numerous studies have been conducted to determine the optimal conditions during IVM, fertilization, and cultivation to optimize embryo production. Multiple factors, including follicle size, hormones, serum, and various growth factors in the IVM medium and culture conditions, influence and contribute to the IVM of goat oocytes [46, 47].

3.1 Effect of oocytes' quality

Oocyte maturation is crucial in IVPs' success; its developmental competence is generally lower than that of oocytes grown *in vivo*. Numerous studies indicate that the intrinsic quality of oocytes determines embryo development rates, whereas *in vitro* culture conditions predominantly affect embryo quality [48, 49]. For that reason, multiple research investigations have revealed that oocyte intrinsic quality determines embryo development rates, whereas the IVC environment primarily affects the quality of embryos. *In vitro* cultivation of goat oocytes frequently results in diminished efficiency, developmental arrest, and viability losses relative to previous research, possibly attributable to the initial quality of the oocytes during IVM [50]. Advanced research in goat *in vitro* production has attained increased maturation rates with pre-selected oocytes under particular circumstances [51]. During IVP, embryos are subjected to oxidative stress (OS), which negatively affects embryo development. The poor quality of oocytes may be due to reactive oxygen species (ROS), which are significant factors in diminishing oocyte maturation and embryonic development rates. Standard oxidative biochemical processes continuously produce ROS that impair the fertilization process and growth of embryos [52]. Generally, ROS is naturally produced in all cells but rendered inactive within the cells when they are in a typical physiological condition. The enzymatic antioxidant systems in mammalian cells function as ROS scavengers, safeguarding cells from harm. This enzymatic antioxidant mechanism has been documented in COCs [53, 54].

3.2. Effects of human chorionic gonadotropin (hCG) dosage on IVM outcome

Gonadotropins are the principal regulators of *in vitro* nuclear maturation in mammalian oocytes. The advantageous impact of gonadotropins in the IVM medium is particularly significant for oocytes derived from juvenile or prepubertal females. Luteinizing hormone (LH) and human chorionic gonadotropin (hCG) have traditionally been regarded as similar because they have a common receptor, primarily expressed in the gonads, which activates the classical protein kinase A steroidogenic pathway [55]. Incorporating gonadotropins in the IVM medium has been documented to improve oocyte quality and developmental potential by possibly modifying metabolic pathways. Enhanced maturation rates have been attained using hCG in conjunction with FSH and E_2 from both normal and dysmorphic goat oocytes. Moreover, the incorporation of hormone combina-

tions (PMSG + hCG + E_2) with fetal bovine serum (FBS) into the oocyte culture medium enhanced the IVM [56, 57]. AbdElkhalek et al. [26] concluded that the maturation of goat oocytes in a medium enriched with 20 IU/mL hCG enhanced maturation (26.49%) and fertilization rates by influencing the cytoplasmic distribution and facilitating the usage of oocyte mitochondria.

3.3. Effects of sperm sources on IVF results

The IVF enables the production of offspring from both living and deceased animals. Testicles and ovaries sourced from deceased or slaughtered animals can be utilized for the production of *in vitro* embryos at a cost-effective rate. Caudal epididymal spermatozoa obtained from deceased or significantly compromised animals are crucial for reproducing and preserving animal specimens exhibiting superior genetic traits. Additionally, epididymal sperm collected within 24 to 48 hours post-mortem can be utilized for successful IVF. Epididymal sperm have been used in multiple species, including goats, demonstrating successful fertilization and embryo development rates [60].

3.4. Use of different culture media in embryo development

The culture system used during IVC can significantly impact the development of pre-implantation stage embryos. The viability of embryos during IVC is crucial for successful IVP. Important developmental events occur from fertilization to the blastocyst stage, and the choice of culture media supports these processes [65, 66]. Various culture systems have been examined to facilitate embryo development to the blastocyst stage pre-implantation. Amino acids, found in female reproductive tract secretions, can serve as energy sources for embryos and improve their development by reducing OS and cell fragmentation during IVC. Incorporating amino acids in culture media enhances embryo development, likely due to their antioxidant properties and ability to mitigate stress and cell fragmentation associated with in vitro culture [67, 68]. On the other hand, serum is a common component in undefined culture systems. It provides the embryos with beneficial factors such as amino acids and vitamins. However, serum can also introduce embryotoxic factors into the culture media [69]. Previous studies have primarily focused on single-step culture plus cell culture media, including Ham's F10 and potassium simplex optimized medium with amino acid supplement (KSOMaa) and Bracket-Oliphant (BO), in the context of infertility treatment through IVF. However, limited research explicitly addresses the type of cultural media used. Modifying the culture environment can significantly influence embryo quality [70]. The selection of culture media and supplements at various stages can substantially impact blastocyst production efficiency in vitro. Although modified simple and complex embryo culture media have been used recently, there is no available report on the efficacy of these media during embryonic development from the zygote to blastocyst stages [71]. Recent animal and human research confirms the advantage of including growth factors (GF) in the culture medium. Nonetheless, uncertainty persists over the precise function of GF in embryonic development, the ideal dosage of GF to include in the culture medium, and the synergistic effects of GF on embryonic development. [72]. Several commercial culture media are available on the market for human embryonic culture. Although some of these media have been evaluated using mouse models, limited studies compare these commercially available options [73, 74].

3.5. Antioxidant supplementation for *in vitro* maturation media

The mammalian reproductive system comprises various naturally occurring antioxidants, contrasting with the culture media employed in *in vitro* oocyte and embryo culture. The OS negatively impacts reproductive activities by inhibiting ovulation-sperm fusion, damaging DNA, ribonucleic acid (RNA), and proteins, and inducing blastomere apoptosis, especially in the blastocyst stage [75]. Adding antioxidants to mammalian IVM media has dramatically improved the growth of blastocysts, the proportion of cleaved embryos, and the mean quantity of blastocyst cells. During assisted reproductive procedures, oocytes and embryos undergo initial development stages, which can affect their antioxidant defense systems and make them more susceptible to OS [Figure 1].

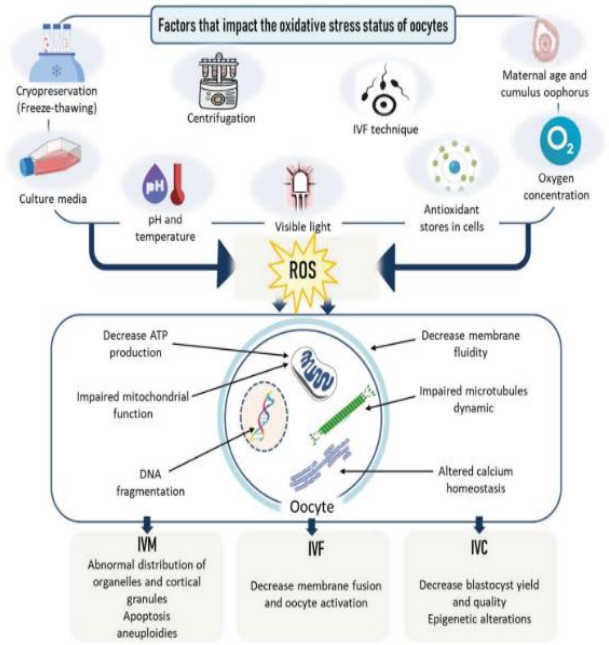

Figure 1. Elements influencing the oxidative stress (OS) state of oocytes/embryos before and during *in vitro* culture (IVC) and the impact of antioxidants. H_2O_2: hydrogen peroxide; O_2: oxygen; OH: hydroxyl group; ROS: reactive oxygen species [82].

Antioxidants are classified as free radical scavengers because they may directly neutralize reactive oxygen species (ROS) and regulate the equilibrium between ROS generation and intracellular antioxidant enzymes [76]. Gap junctional communication (GJC) links the CCs with the oocyte via the COCs. The GJC enables the passage of nutrients and metabolites from CCs to the oocyte, which is essential

for optimal oocyte development, transcription regulation, and meiosis. Insufficient antioxidants exacerbate oxidative stress in the cardiac cells and hinder the opening of cell-to-cell gap junctions. Recent research indicates that the incorporation of antioxidants into the IVM medium enhances the growth of CCs, prolongs GJC, and increases the formation of transzonal projections (TZPs), which are essential for oocyte development and the attainment of meiotic competence [77, 78]. Hardy et al. [52] and Tripathi et al. [54] indicate that successful IVF methods, including antioxidants, are crucial for glutathione synthesis. Consequently, augmenting the culture media with antioxidants may beneficially influence oocyte maturation and embryonic development by improving mitochondrial activity and modulating ROS levels. Researchers have conducted *in vitro* tests on numerous antioxidants to reduce ROS generation and enhance embryonic development; nevertheless, it remains uncertain which antioxidant is the most advantageous for developmental improvement [79]. Researchers hypothesize that OS can hinder oocytes' developmental potential, but specific antioxidant supplementation in culture media can mitigate its adverse effects [80, 81].

4. Conclusion

Reproductive biotechnologies (*in-vitro* embryo production) are essential to increase productivity. The total number and the quality of oocytes recovered from the ovaries of slaughtered goats are critical factors influencing *in vitro* embryo formation. Noteworthy, the culture medium used during the preimplantation of goat embryos requires the inclusion of growth stimulants, antioxidants, and nutrients that support development and enhance embryo quality.

Authors' contributions

Dr. Amira Salem AbdElkhalek fulfilled all assignments of the manuscript. Dr. Maha Ghazi Soliman, Dr. Nehal Ali Abu Elnaga, Dr. Khalid Ahmed El Bahrawy, and Dr. Nasser Ghanem devised the work plan, aided in developing the project schedule, and provided support in the areas of work settings.

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