**In vitro** growth of the ovarian follicle: taking stock of advances in research

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**ABSTRACT**
Several factors are necessary for the growth and survival of healthy follicles in the folliculogenesis process, including endocrine and paracrine glands, and a regulated ratio of granulosa cells to oocytes. One of the most powerful methods for studying folliculogenesis is the culture of ovarian follicles and oogenesis within a completely controlled environment. Follicle culture systems are highly developed and are rapidly evolving. However, the methods for separating the follicles, the cultivation techniques, the culture medium, and the dietary and hormonal supplements vary depending on the species studied. This study made a literature review of follicular culture techniques, and we investigated the heterogeneity among these key variables in follicular culture.

**Keywords:** follicle culture, follicle, folliculogenesis

**INTRODUCTION**
The ovaries produce steroid hormones as well as fertilized eggs. The ovarian function unit is the follicle. Each follicle contains one egg surrounded by granulosa and Theca cells (Edson et al., 2009). Folliculogenesis starts with the transformation of primordial follicles into primary follicles and the transformation of granulosa cells into cube cells. Granulosa cells proliferate, the oocyte grows, and a secondary follicle takes shape. Theca cells produce androgens. They differentiate outside the basal membrane, and the follicles are dependent on gonadotropins. When a cavity filled with follicular fluid forms, this is called the antral follicle. Depending on the species, folliculogenesis completes one or more follicles and ovulation occurs, but the remaining follicles are involved in the growth process, and suffer from atresia (Mesbah et al., 2018; Green & Shikanov, 2016; West et al., 2007; Bahmanpour et al., 2020). Folliculogenesis and oogenesis are controlled by complicated paracrine, autocrine and juxtacrine genetic factors, and are vital to sustainable fertility (Dehghani et al., 2018; Matzuk & Burns, 2012; Richards & Ascoli, 2018) (Figure 1).

A number of in vitro follicular culture systems have been developed to preserve the reproductive ability of threatened species or iatrogenic infertility in women (Marin et al., 2018). In addition, it is used as a method to identify the toxicity of medications and undesirable fertility chemicals in vitro (Xu et al., 2015a). There is now a broad spectrum of culture techniques. Here we investigated the follicular culture variables in detail. Including species differences, age, isolation techniques, two-dimensional (2D) vs. three-dimensional (3D) systems, cultivation medium and hormonal supplementation.

**Follicle culture systems in different species**
Follicle cultures occur in a variety of species. Oocyte growth rate and follicle size (Griffin et al., 2006) vary between species (Pepling et al., 2010). Follicles are usually classified according to diameter. The term “preantral follicles” is used to describe their different phases (Mehrabianfar et al., 2020). Follicles produced in vitro are small compared to follicles produced in vivo (Xiao et al., 2015; Rodrigues et al., 2015). Rodents and mammals are the most prevalent models, approximately one-fifth of the studies use human follicles (Xiao et al., 2015; Telfer et al., 2008), and other mammalian follicles, like the Rhesus monkey (Rodrigues et al., 2015; Xu et al., 2009a; Peluffo et al., 2010; Xu et al., 2011a; Hornick et al., 2012; Xu et al., 2013; Xu et al., 2015b; Xu et al., 2018; Baba et al., 2017); baboon (Xu et al., 2011b); bovine (Yamamoto et al., 2018; Silve et al., 2015; Magalhães et al., 2011); swine (Arunakumari et al., 1999; Rossetto et al., 2013a;b; Araújo et al., 2015); ovine (Arunakumari et al., 2010; Muruvi et al., 2005); caprine (Rossetto et al., 2013a; Ferreira et al., 2018; Silva et al., 2015; Magalhães et al., 2011); swine (Hiraò et al., 1994; Wu et al., 2001); cats (Songssen et al., 2017; Thongkittidlok et al., 2018); dogs (Songssen et al., 2011); horses (Haag et al., 2013); wildcats (Wiedemann et al., 2013).

The main reason for the differences between species is the difference in follicular culture outcomes. For instance, the diameter of the follicles in large mammal species in the preantral stage is much larger than in rodents. In the
in vitro oocyte 2011), and Rhesus monkeys (Peluffo et al., 2007). The enzyme digestion method, the follicles are more likely in house mammals (Araújo et al., 2010), goats (Ferreira et al., 2018; Magalhães et al., 2011), and cats (et al., 2007). Prepubertal follicles have been used in studies of mammalian follicles such as sheep (Thomas et al., 2003; Arunakumari et al., 2010), goats (Ferreira et al., 2018; Magalhães et al., 2011), and cattle (Gutierrez et al., 2000; Itoh et al., 2002; Araújo et al., 2014a,b; 2015). Prepubertal follicles and smaller follicles have been used to evaluate the use of FSH supplementation in cattle and sheep (Wandji et al., 1996; Cecconi et al., 1999; Muruvi et al., 2005). Prepubertal follicles were used in comparison to the follicles of young and adult goats in 2D or 3D culture system (Leal et al., 2018). Prepubertal follicles were used to assess whether smaller preantral follicles could develop into antral follicles in vitro (Wu et al., 2001). In dogs, different stages of the estrus (Songsasen et al., 2011), and in marsupials (Nation & Selwood, 2009) were used in follicular cultures. In the rhesus monkey, the follicles used were primarily of young animals of reproductive age (Rodrigues et al., 2015; Baba et al., 2017; Xu et al., 2018). Small adult follicles were cultivated in adult baboons and were capable of producing live embryos (Xu et al., 2011b). Follicular culture studies have been conducted on different species at different ages and cycle stages; and demonstrate that these factors are chosen based on study objectives and ease of access to ovarian tissue.

Procedures for isolating the follicle

The separation of the follicle from the ovary tissue is the first step in follicle cultivation. Isolated follicles should have a similar morphology (Demeestere et al., 2002). Generally, the techniques of separating the follicles from the ovarian tissue include enzymatic, mechanical, or both. In the enzymatic separation of the follicles, proteolytic extracellular matrix (ECM) digestion such as collagenase, deoxyribonuclease, or liberase is utilized. The number of follicles obtained is typically higher in the enzyme digestion method and in compared to mechanical separation methods, they require less time, particularly for fibrous tissues in house mammals (Araújo et al., 2014a,b). However, in the enzyme digestion method, the follicles are more likely to be damaged. In mice, for example, collagenase leads to the production of preantral granulosa cell-oocyte complexes (PGOCs) and cell-oocyte complexes (COCs) from ovarian tissues, rather than whole follicles. In the mechanical separation method, special needles are used to separate the follicles of the ovarian stroma or tissue grinders, homogenizers, and cell strainers (Songsasen et al., 2017; Mahalingam et al., 2016a;b; Craig et al., 2010). The mechanical separation method results in less damage to the follicle than the enzyme method, and provides improved protection to the theca layer and follicular morphology (Araújo et al., 2014a;b), but the worst problem is that this method takes a lot of time (Demeestere et al., 2002). Usually, the selection of the isolating method depends on the follicular stage and the species used in the study. Generally, a short enzymatic digestion step and mechanical separation are used to maintain the structure of the follicle and obtain the maximum number of follicles (Table 1).

Culture systems

Follicular culture systems are known as two-dimensional (2D) or three-dimensional (3D) (Figure 2). In 2D cultures, the follicles are static, but in 3D cultures, the follicles float in biomaterial matter (West et al., 2007). 2D-systems include the droplet method, substrate method (ECM coating), and membrane insert systems. In general, the 2D-method is used for small culturing follicles, hormonal studies, and gene expression studies. It is difficult to evaluate folliculogenesis and oocyte maturation in the 2D-method, because during oocyte proliferation, granulosa cells migrate to the surface of the culture medium (Kreeger et al., 2006). Logout of the communication between follicular cells stops follicular growth, inhibits ovulation, and meiosis in the egg (Green & Shikano, 2016; West et al., 2007). In general, the follicles may be maintained for a short period of time in the 2D-culture.

A - 2D-culture systems

1. Droplet culture

Within the droplet system, each follicle is implanted into a drop of culture medium, and each drop is covered with oil. There are drop methods for different stages and different species including mice (Adam et al., 2004; Wycherley et al., 2004; Adriáns et al., 2004), Rhesus monkey (Peluffo et al., 2012), sheep (Arunakumari et al., 2010), marsupial (Nation & Selwood, 2009), goat (Rossetto et al., 2013a; Ferreira et al., 2018) and cows (Araújo et al., 2014a,b) have been used. It typically takes about 6-18 days for the droplet method (Nation & Selwood, 2009; Arunakumari et al., 2010), A18 (Ferreira et al., 2018; Magalhães et al., 2011) and 32 (Araújo et al., 2014a;b) (Figure 2-A).

2. 2D-culture

In the two-dimensional method, the follicles are grown directly on a surface covered by ECM compounds, such as collagen, laminin, or Matrigel. ECM plays an important role in folliculogenesis and affects cellular behavior, differentiation, and secretory activity (Desai et al., 2010). Collagen compounds have elasticity properties and contribute to intercellular communication, while Matrigel promotes cell proliferation and differentiation (Belli et al., 2012). Larger follicles such as preantral and antral follicles, PGOCs, and COCs have been used more in systems with 2D plastic substrates (Zhou & Flaws, 2017; Xu et al., 2018; Araújo et al., 2015; Patel et al., 2016; Mahalingam et al., 2016a;b; Peluffo et al., 2010). In large mammals, the follicles are larger and require more time in the culture environment to grow. Thus, the growing time of larger follicles may be reduced (Araújo et al., 2014a;b), and the duration of the culture varies in hours and days. For example, some studies have used the method to grow mammalian follicles such as those of Rhesus and cattle (Xu et al., 2018; Gutierrez et al., 2000). A fibronectin-coated plate was also used to culture the primordial and primary follicles of sheep. The growth of follicles was not much different from...
### Table 1. Summary of follicular isolation methods in follicular culture studies.

| Isolation | Species | Follicle - Stage | References |
|-----------|---------|------------------|------------|
| **Enzymatic** | **Bovine** | Preantral (60–179µm) | Wandji et al., 1996 |
| | | Preantral (90–240µm) | | |
| | | Immature and secondary (176.46±7.20µm) | | |
| | | Class I and II (90µm and <90µm) | | |
| | | Primordial/primary follicle (≥60µm) | | |
| | | Primary/early secondary follicle (>60–120µm) | | |
| | | Small preantral follicles (42.98±9.06µm) | Amorim et al., 2009 |
| | **Canine** | Preantral (100–500µm) | Songsasen et al., 2011 |
| | | Immature and secondary (100–500µm) | | |
| | | Primordial (100–130µm) | | |
| | | Small follicles | | |
| | **Human** | Preantral (60–179µm) | | |
| | | Immature and secondary (176.46±7.20µm) | Laronda et al., 2014 |
| | | Class I and II (90µm and <90µm) | Roy & Treacy, 1993 |
| | | Primordial/primary follicle (≥60µm) | | |
| | | Primary/early secondary follicle (>60–120µm) | | |
| | | Small preantral follicles (42.98±9.06µm) | Amorim et al., 2009 |
| | **Mechanical** | **Bovine** | | |
| | | Preantral (≥190µm) | Araújo et al., 2015 |
| | | Preantral (166±2.15µm) | Gutierrez et al., 2000 |
| | | Preantral (190.0±6.6µm) | Araújo et al., 2014 |
| | | Preantral (268.6±4.5µm) | Antonino et al., 2019 |
| | | Preantral (145–170µm) | Itoh et al., 2002 |
| | | Preantral (≥150µm) | Rossetto et al., 2013a |
| | **Caprine** | Preantral (>200µm) | Magalhães et al., 2011 |
| | | Preantral and early antral (~250μm, ~350µm) | Ferreira et al., 2016 |
| | | Preantral (150–250µm) | Silva et al., 2015 |
| | **Human** | Secondary (≥100µm) | McLaughlin et al., 2014 |
| | | Secondary (100–150µm) | McLaughlin et al., 2018 |
| | | Preantral (66–132µμm) | Telfer et al., 2008 |
| | | Preantral (>120µm) | Abir et al., 1997 |
| | **Murine** | Secondary (≥150µm) | Rossetto et al., 2013a |
| | | COC | | |
| | **Marsupial** | Primordial (63.6–215.5µm) | Nation & Selwood, 2009 |
| | | Secondary (111–137µm) | Jin et al., 2010 |
| | | Preantral (85–115µm) | Hornick et al., 2013 |
| | | Two-layered: (100–130µm); multi-layered: (150–180µm) | Kreeger et al., 2005; 2006 |
| | | Two-layered secondary (100–130µm) | Shikanov et al., 2009 |
| | | Primary (60–80mm); two-layered (90–100µm) | Tagler et al., 2014 |
| | | Secondary (~90, 100–105, or 120µm) | Tingen et al., 2011 |
| | | Secondary (180–210µm) | Skory et al., 2015 |
| | | COC | Buccone et al., 1990 |
| | | Antral (360.94±16.1µm) | Craig et al., 2010 |
| | | Antral (200–350µm) | Craig et al., 2013 |
| | | Antral (250–400µm) | Hannon et al., 2015 |
| | | Antral (250–400µm) | Hannon et al., 2015 |
| | | Antral (250–400µm) | Peretz & Flaws, 2013 |
| | | Antral (250–400µm) | Zhou & Flaws, 2017 |
| | | Antral (250–400µm) | Patet et al., 2016 |
| | | Antral (250–400µm) | Peretz et al., 2013 |
Continued Table 1.

| Species   | Stage Description                        | Reference(s)       |
|-----------|------------------------------------------|--------------------|
| Ovine     | Preantral small (130±10µm)               | Cecconi et al., 1999 |
|           | Preantral medium (185±14µm)              |                    |
|           | Preantral large (250±10µm)               |                    |
|           | Preantral (161±2µm)                      | Thomas et al., 2003 |
|           | Preantral (250–400µm)                    | Arunakumari et al., 2010 |
| Porcine   | Preantral (296±9µm)                      | Wu et al., 2001    |
| Rhesus    | Secondary (100–300µm)                    | Xu et al., 2009a   |
|           | COC                                       | Peluffo et al., 2012 |
|           | Small antral (≥0.5mm)                    | Peluffo et al., 2013 |
|           | Secondary (140–225µm)                    | Xu et al., 2018    |
|           | Secondary (125–250µm)                    | Baba et al., 2017  |
|           | Secondary (125–225µm)                    | Rodrigues et al., 2015 |
|           | Secondary (125–250µm)                    | Ting et al., 2015  |
|           | Primary (80–120µm)                       | Xu et al., 2013    |
|           | secondary (125–225µm)                    |                    |
|           | Secondary (130–220µm)                    | Xu et al., 2015b   |
| Feline    | Secondary (100–200µm)                    | Songtsasen et al., 2017 |
|           | Secondary (208±7.9µm diameter)           |                    |
|           | Early antral (329.8±5.4µm)               | Thongkittidilok et al., 2018 |
| Baboon    | Preantral (270–300µm)                    | Xu et al., 2011b   |
| Human     | Secondary (74–260µm)                     | Skory et al., 2015  |
|           | Primary (47.0±8.2µm)                     | Abir et al., 1999  |
|           | Preantral (190±30µm)                     | Aziz et al., 2017  |
|           | Secondary (~170µm)                       | Xu et al., 2009b   |
| Murine    | Preantral (~60–69µm)                     | Oktay & Oktay, 2007 |
|           | Preantral follicles and COC              | Vanderhyden et al., 1992 |
|           | Immature secondary (140–150µm)           | Shikanov et al., 2011 |
|           | Multi-layered secondary (150–180µm)      | Xu et al., 2006a   |
| Porcine   | Preantral (200–300µm)                    | Hirao et al., 1994 |
| Rhesus    | COC                                       | Peluffo et al., 2010 |
|           | Secondary (125–225µm)                    | Xu et al., 2011a   |
|           |                                           | Xu et al., 2010    |

the follicles cultured in fibronectin-free plates (Muruvi et al., 2005) (Figure 2B).

3 - Membrane insert culture

Membrane insertion systems function in the same way as 2D-systems, and may contain ECM protein coatings, but in this method, the follicles are in an insert within a well of a culture plate and immersed in the environment. The mice follicles were cultured using a membrane inserting system, which improved the growth and ovulation of the follicles (Adam et al., 2004). For the first time, human follicles were cultured with a membrane insert system for 4 weeks. COC culture studies using membranes coated with ECM proteins (Sugiura et al., 2010) were also reported. Other 2D methods of follicle culture, include the use of glass coverslips coated with various ECM components. Although the earliest methods for cultivating ovarian follicles are 2D-systems, the 2D-methods damages the structure of the follicles, so that it is better suited for short-term cultures and small follicles (Figure 2C).
3D-culture systems

3D-culture acts as *in vivo* and is adapted to long-term follicle culture. A major disadvantage of two-dimensional systems is that it damages the structure of the follicle surrounded by granulosa cells. This system is problematic for the culture of large mammalian follicles, which require culture and long-term communication among cells. In a 3D culture system, the structure of the intact follicles retains, in which the follicles are surrounded by biomaterials or have little access to a substrate. There are different types of 3D-systems, some using different scaffolding and encapsulation follicles, others using floating culture, or using in situ culture. To encapsulate the follicles, several matrices are used, which, in vivo, creates a very restricted environment, similar to that of the ovary and maintains the follicular structure and intercellular communication (Belli *et al.*, 2012). Matrix compounds include natural substances such as collagen, alginate, or matrigel, or synthetic compounds such as polyethylene glycol (PEG) hydrogels that bind to protein-sensitive peptides (Figure 2).

1. **Suspension culture**

In this 3D-system, there is no scaffold and the structure of the follicles is protected by a system of rolls, inversion, or magnetic grains (Nation & Selwood, 2009; Wycherley *et al.*, 2004). In marsupials, using inverted droplets, mature oocytes were obtained, which, in vivo, were more effective than follicles cultivated in different systems such as vertical droplets and roller systems. In tubes containing polypropylene, rat follicles produced eggs capable of performing meiosis, and were fertilized with intra cytoplasmic sperm injection (ICSI). Using a 3D magnetic system, cattle follicles produced live eggs that resumed meiosis after *in vitro* maturation (IVM) (Antonino *et al.*, 2019) and follicle survival was higher than in the 2D-system (Figure 2D).

2. **Encapsulated culture**

In these culture systems, a biocompatible substance such as agar and collagen surround the follicle and protects its 3D structure. These materials are placed in layers on culture sheets to insert the follicles between these layers. In the first report of using the collagen gel matrix in the three-dimensional method, due to the stiffness of the matrix, no antrum was formed. Other studies have used collagen and agar matrices to grow follicles in mice (Vanderhyden *et al.*, 1992) and pigs (Hirao *et al.*, 1994), which, in comparison to 2D-systems, has maintained follicle structure and extended culture. In human studies, the use of collagen and agar in the 3D system made it possible to maintain the structure of the follicle and the egg for only 24 to 120 hours.

Brown algae are capable of producing a hydrogel called alginate that is biocompatible and can be used as a matrix in follicle culture (Belli *et al.*, 2012). Alginate was first used in the culture of mice COCs. The results showed that alginate maintains intercellular communication, the proliferation of granulosa cells, and increases egg volume. Usually, ovarian cortex follicles move from the hard medulla to softer layers as they develop. Results of studies have demonstrated that concentrated alginate contributes to
the growth of mice primary follicles, but it is not suitable for the development of larger follicles and the formation of antrum (Xu et al., 2006a;b; Skory et al., 2015). Also, studies of follicular culture in a 3D-system containing alginate have shown that low levels of alginate contribute to follicular growth, but, concentrated alginate is appropriate for hormone production (Songsasen et al., 2011). Alginate encapsulation was used in other mammals, such as the Rhesus monkey, which could produce embryos by the cleavage stage (Xu et al., 2011a). By culturing the follicles in the combination system, the first mature human metaphase II (MII) oocytes were produced. First, the preantral follicles were cultured in 0.5% alginate for 10-15 days, and then the antral follicles were placed in low attachment plates for up to 40 days (Xiao et al., 2015). Supplements can impact 2D and 3D-culture systems. For example, one study found that vascular endothelium growth factor (VEGF) contributes to the growth of bovine secondary follicles in the 2D-system, and the growth hormone (GH) induces estradiol production in the 3D-alginic system (Araújo et al., 2014a;b). In a study using the caprine model, the encapsulation of 3D alginate was compared to the 2D substrate system that increased follicular survival and increased the number of eggs appropriate for IVM and IVF in the 3D-system. But in the 2D-culture, the follicles produced higher levels of progesterone.

Using the combination of alginate and fibrin, a dynamic permeable fibrin-alginate (IFN) network was developed (Shikanov et al., 2009). Within this matrix, follicular proteases degrade fibrin, reducing alginate concentration and matrix rigidity. This matrix mimics the internal environment of the ovary, as in ovarian tissue, follicles smaller than the hard cortex move into the soft marrow (Shikanov et al., 2011). With IFN in rodents, high meiotic follicles were developed (Jin et al., 2010) but in monkeys, it did not increase secondary follicle production. Embryonic stromal cells and fibroblasts (MEF) in mice were also grown with alginate-encapsulated follicles (Tagler et al., 2014). Ovarian stromal cells are involved in the growth, survival, and production of androgens in primary and secondary mouse follicles. Culture of MEF cells with primary follicles containing alginate enhanced growth but decreased cell survival. Matrigel matrix is also used in three-dimensional culture, which in addition to maintaining the structure of the follicle, creates a protein-rich environment for folliculogenesis. In matrigel, with fibrin and alginate, baboon follicles were enclosed, grew, and were able to produce mature eggs. The hyaluronan matrix was also used to grow follicles (Belli et al., 2012). The hyaluronan-ECM (no alginate) matrix on rat follicles increased follicular survival and increased the steroid hormone (Desai et al., 2012). The synthetic matrix of polyethylene glycol (PEG) acts like fibrin and is degraded by follicular proteases. Using the PEG matrix increased follicle growth in mouse models by 17 times (Shikanov et al., 2011) (Figure 2E).

3. Multi-step culture

Multi-step methods have been developed for follicle growth and the creation of a more similar physiological environment that primordial, primary, and early-secondary stage follicles can be cultured. First, the small follicles are grown in situ in the ovarian natural environment, and then the cultured follicles are separated from this tissue (McLaughlin et al., 2014; Jin et al., 2010; McLaughlin et al., 2018; Telfer et al., 2008). This method helps grow human follicles until they become mature gametes. For example, in one study using human ovarian tissue, secondary follicles were isolated and encapsulated in alginate. As the follicles grew and the antrum formed, they were released from the alginate matrix and transferred to low attachment plates for 40 days. Which turned human follicles into mature eggs (Xiao et al., 2015).

In the next study, an alternative multistage method was used. In the first stage, cortical strips were cultured for 8 days. Secondly, the follicles were cultured for 8 days, and the COC cells were cultured on the membranes for 4 more days (Step 3). In the fourth stage, eggs larger than 100 μm were selected for IVM (McLaughlin et al., 2018). Also, a multi-step method was used for follicle growth in rodents. Generally, these systems have shown very useful for long-term cultures of large mammalian follicles. Therefore, the introduction of microfluidic systems or other natural scaffolding can be very useful in healthy in vitro follicles (Gargus et al., 2020) (Figure 2F).

Media composition and supplements

To grow the follicles, it is necessary to enrich the growing medium with nutrients, growth supplements, and hormone compounds. The selected culture medium should protect the growth of follicles and the maturation of eggs. As a result, the main media used in follicular culture typically include minimal essential medium (MEM), Dulbecco’s modified eagle medium (DMEM), Waymouth’s medium, McCoy’s 5a medium. Supplemental salt solutions (EBSS), or mixed media (DMEM + F12, a-MEM + Glutamax).

Also, supplements are added to the follicular culture medium. For example, glucose as a source of carbon energy (Nation & Selwood, 2009), L-glutamine or fetuin as a source of amino acids (Asadi et al., 2017); ascorbic acid for reducing apoptosis and maintaining follicular structure, penicillin, streptomycin, and kanamycin as antibiotics (De-mestre et al., 2005) is used. Additionally, for the growth of follicles in vitro from the combination of ITS (insulin, transferrin, selenium) to increase the absorption of amino acids (Abedelahi et al., 2008). Protein supplements such as fetal calf serum (FCS), fetal bovine serum (FBS), and bovine serum albumin (BSA) are used in culture medium. Results from a mice model study showed that over a 10-day period, a-MEM, DMEM, and DMEM + F12 media had a better effect on antrum formation, follicle growth than Waymouth, M199, IMDM, and RPM1640. Also resulted in an increase in the number of MII oocytes (Simon et al., 2020). For culturing the human ovarian cortical tissue over a 10-day period, the MEM medium enriched with 10% human serum and 300 mlU/mL FSH may have a greater effect on follicular growth than the Waymouth and EBSS media (Wright et al., 1999). In another study, TCM-199 enriched with 10 ng/ml EGF was used over a 7-day period and had a better effect on the growth of goat and sheep follicles than a-MEM with the EMF (Andrade et al., 2014).

The TCM199 medium also increased the rate of antrum formation from bovine preantral follicles, relative to a-MEM or McCoy 5a medium (Rossetto et al., 2013a;b). Another factor affecting folliculogenesis is oxygen stress. Oxygen 5% is near the physiological oxygen levels. High oxygen stresses may produce reactive oxygen radicals (ROS) with cytotoxic effects (Rajabi et al., 2018). In one study, oxygen stress was induced in the follicle culture environment in rats. Which resulted in the production of mature eggs with higher performance in terms of static control. The rate of antrum formation in culture with 5% oxygen from caprine, ovine, and bovine (Gigli et al., 2006) follicles had more than 20% oxygen. Also, the culture of dog COCs in 5% oxygen decreased cell apoptosis compared to that in 20% oxygen (Silva et al., 2009). Low-oxygen stress along with high FSH and high fetuin in rhesus monkey, increased follicle growth, and antrum formation (Xu et al., 2011a). In general, these studies show that the selection of a suitable culture medium for follicle growth depends on the species. Furthermore, the protective effect of oxygen is much more important at the physiological level (Table 2).
| Culture Medium          | Species | Follicle Stage                  | References                          |
|-------------------------|---------|---------------------------------|-------------------------------------|
| Whitten's medium        | Murine  | PGOC                            | Eppig, 1980                         |
| Bicarbonate buffered M199 | Murine  | Small follicles                 | Torrance et al., 1989               |
|                         | Murine  | PGOC                            | Eppig, 1991                         |
| Waymouth’s medium       | Human   | Immature                        | Laronda et al., 2003                |
|                         | Ovine   | Primordial and primary (40–60μm) | Muruvi et al., 2005                 |
|                         | Porcine | Preantral (200–300μm)           | Hirao et al., 1994                  |
| Way/IBMX/ITS/BSA medium | Bovine  | Preantral (60 to 179μm)         | Vandjij et al., 1996                |
| DMEM                    | Murine  | Preantral and COC               | Vanderhyden et al., 1992            |
|                         | Human   | Class 1 (90μm)                  | Roy & Treacy, 1993                  |
|                         |         | Class 2 (<90μm)                 |                                     |
|                         |         | Preantral (90–240μm)            | Yuan & Guidice, 1999                |
|                         | Marsupial | Primordial and primary (63.6–215.5μm) | Nation & Selwood, 2009 |
|                         | Baboon  | Preantral (270–300μm)           | Xu et al., 2011b                    |
|                         | Bovine  | Preantral (190.0±6.6μm)         | Araújo et al., 2014                 |
|                         | Caprine | Secondary (≥150μm)              | Rossetto et al., 2013a              |
|                         | Caprine | Secondary (≥150μm)              | Rossetto et al., 2013a              |
|                         | Caprine | Preantral (≥200μm)              | Magalhães et al., 2011              |
|                         | Caprine | Preantral (150–250μm)           | Silva et al., 2015                  |
|                         | Caprine | Preantral (~250μm)              | Ferreira et al., 2018               |
|                         | Canine  | Pre- and early antral (100–500μm) | Songsasen et al., 2011              |
|                         | Feline  | Secondary (208±7.9μm)           | Songsasen et al., 2017              |
|                         |         | Early antral (329.8±5.4μm)      |                                     |
|                         | Human   | Secondary (100–200μm)           | Thongkittidilok et al., 2018        |
|                         | Human   | Pre- and early antral (≥120μm)  | Abir et al., 1997                   |
|                         | Human   | Secondary (170–178μm)           | Xu et al., 2009b                    |
|                         |         | Secondary (176.46±7.20μm)       | Laronda et al., 2014                |
|                         | COC     | Pangas et al., 2003             |                                     |
|                         | Preantral (150–200μm)           | Adam et al., 2004                  |
|                         | Preantral (180–240μm)           | Wycherley et al., 2004             |
|                         | Antral (≥200 μm)                | Miller et al., 2005                |
|                         | Two-layered (100–130μm)         | Kreeger et al., 2005; 2006         |
|                         | Multi-layered (150–180μm)       |                                     |
|                         | Murine  | Two-layered (100–130μm)         | Xu et al., 2006b                    |
|                         |         | Multi-layered secondary (150–180μm) | Shikanov et al., 2009               |
|                         |         | Preantral (~60–69μm)            | Oktem & Oktay, 2007                 |
|                         |         | Secondary (111–137μm)           | Jin et al., 2010                    |
|                         |         | Antral (360.94±16.1μm)          | Craig et al., 2010                  |
|                         |         | Immature secondary (140–150μm)  | Shikanov et al., 2011               |
|                         |         | Secondary (~90, 100–105, or 120μm) | Tingen et al., 2011               |
| Species | Stage Description | Culture Media | References |
|---------|-------------------|---------------|------------|
| Ovine   | Preantral Medium  | αMEM + TCM199B | Arunakumari et al., 2010 |
| Rhesus  | Preantral (100–130µm) | αMEM + Glutamax | Xu et al., 2011a |
|         | Secondary (100–200µm) | αMEM + Glutamax | Xu et al., 2010 |
| Human   | Preantral (160±30µm) | αMEM + Glutamax | Aiz et al., 2017 |
| Murine  | Preantral (166±2.15µm) | McCoy’s 5a | Gutierrez et al., 2000 |
| Bovine  | Preantral (145–170µm) | McCoy’s 5a | Telfer et al., 2008 |
|         | Preantral (40–70µm) | McCoy’s 5a | Schotanus et al., 1997 |
|         | Secondary (268.6±4.5µm) | McCoy’s 5a | Antonino et al., 2019 |
|          | Preantral (161±2µm) | McCoy’s 5a | Thomas et al., 2003 |
| Porcine | Preantral (190±30µm) | αMEM + F12 | Xiao et al., 2015 |
| Ovine   | Preantral (150–160µm) | αMEM + F12 | Xu et al., 2009a |
|         | Preantral (140–170µm) | αMEM + F12 | Xu et al., 2009 |
|         | Early secondary (100–130µm) | αMEM + F12 | Mainigi et al., 2011 |
| Human   | Preantral (190±30µm) | αMEM + F12 | Aiz et al., 2017 |
|         | Preantral (100–200µm) | αMEM + F12 | Xu et al., 2010 |
|         | Primary (60–80 µm) | αMEM + F12 | Peretz et al., 2013 |
| Murine  | Preantral (165±14µm) | αMEM + F12 | Peretz et al., 2013 |
| Bovine  | Preantral (145–170µm) | McCoy’s 5a | Gutierrez et al., 2000 |
|         | Preantral (66 to 132µm) | McCoy’s 5a | Telfer et al., 2008 |
|         | Primordial/primary follicle (≤60µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Primary/early secondary follicle (>60–120µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Secondary (>120–250µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Secondary (100–150µm) | McCoy’s 5a | McLaughlin et al., 2014 |
| Porcine | Preantral (190±30µm) | αMEM + F12 | Aiz et al., 2017 |
| Rhesus  | Preantral (150–160µm) | αMEM + F12 | Xu et al., 2009 |
|         | Preantral (140–170µm) | αMEM + F12 | Xu et al., 2009 |
|         | Early secondary (100–130µm) | αMEM + F12 | Mainigi et al., 2011 |
| Human   | Preantral (190±30µm) | αMEM + F12 | Aiz et al., 2017 |
| Murine  | Preantral (165±14µm) | αMEM + F12 | Peretz et al., 2013 |
| Bovine  | Preantral (145–170µm) | McCoy’s 5a | Gutierrez et al., 2000 |
|         | Preantral (66 to 132µm) | McCoy’s 5a | Telfer et al., 2008 |
|         | Primordial/primary follicle (≤60µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Primary/early secondary follicle (>60–120µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Secondary (>120–250µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Secondary (100–150µm) | McCoy’s 5a | McLaughlin et al., 2014 |
| Porcine | Preantral (190±30µm) | αMEM + F12 | Aiz et al., 2017 |
| Rhesus  | Preantral (150–160µm) | αMEM + F12 | Xu et al., 2009 |
|         | Preantral (140–170µm) | αMEM + F12 | Xu et al., 2009 |
|         | Early secondary (100–130µm) | αMEM + F12 | Mainigi et al., 2011 |
| Human   | Preantral (190±30µm) | αMEM + F12 | Aiz et al., 2017 |
| Murine  | Preantral (165±14µm) | αMEM + F12 | Peretz et al., 2013 |
| Bovine  | Preantral (145–170µm) | McCoy’s 5a | Gutierrez et al., 2000 |
|         | Preantral (66 to 132µm) | McCoy’s 5a | Telfer et al., 2008 |
|         | Primordial/primary follicle (≤60µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Primary/early secondary follicle (>60–120µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Secondary (>120–250µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Secondary (100–150µm) | McCoy’s 5a | McLaughlin et al., 2014 |
| Porcine | Preantral (190±30µm) | αMEM + F12 | Aiz et al., 2017 |
| Rhesus  | Preantral (150–160µm) | αMEM + F12 | Xu et al., 2009 |
|         | Preantral (140–170µm) | αMEM + F12 | Xu et al., 2009 |
|         | Early secondary (100–130µm) | αMEM + F12 | Mainigi et al., 2011 |
| Human   | Preantral (190±30µm) | αMEM + F12 | Aiz et al., 2017 |
| Murine  | Preantral (165±14µm) | αMEM + F12 | Peretz et al., 2013 |
| Bovine  | Preantral (145–170µm) | McCoy’s 5a | Gutierrez et al., 2000 |
|         | Preantral (66 to 132µm) | McCoy’s 5a | Telfer et al., 2008 |
|         | Primordial/primary follicle (≤60µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Primary/early secondary follicle (>60–120µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Secondary (>120–250µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Secondary (100–150µm) | McCoy’s 5a | McLaughlin et al., 2014 |
| Porcine | Preantral (190±30µm) | αMEM + F12 | Aiz et al., 2017 |
| Rhesus  | Preantral (150–160µm) | αMEM + F12 | Xu et al., 2009 |
|         | Preantral (140–170µm) | αMEM + F12 | Xu et al., 2009 |
|         | Early secondary (100–130µm) | αMEM + F12 | Mainigi et al., 2011 |
| Human   | Preantral (190±30µm) | αMEM + F12 | Aiz et al., 2017 |
| Murine  | Preantral (165±14µm) | αMEM + F12 | Peretz et al., 2013 |
| Bovine  | Preantral (145–170µm) | McCoy’s 5a | Gutierrez et al., 2000 |
|         | Preantral (66 to 132µm) | McCoy’s 5a | Telfer et al., 2008 |
|         | Primordial/primary follicle (≤60µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Primary/early secondary follicle (>60–120µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Secondary (>120–250µm) | McCoy’s 5a | McLaughlin et al., 2014 |
|         | Secondary (100–150µm) | McCoy’s 5a | McLaughlin et al., 2014 |
| Porcine | Preantral (190±30µm) | αMEM + F12 | Aiz et al., 2017 |
| Rhesus  | Preantral (150–160µm) | αMEM + F12 | Xu et al., 2009 |
|         | Preantral (140–170µm) | αMEM + F12 | Xu et al., 2009 |
|         | Early secondary (100–130µm) | αMEM + F12 | Mainigi et al., 2011 |
CONCLUSIONS
The general process of follicular culture has changed a lot from the past until now, and the main purpose of these changes has been to imitate the natural ovarian environment. By identifying the structure of the ovarian scaffold, information about 3D printing of the ovary was obtained. Ovarian function was thoroughly investigated by making 3D-printed scaffolds (Laronda et al., 2017). In addition, depending on physiological needs of the cell, other technologies such as microfluidics can be used to grow follicles. In static models, the use of a microfluidic system can be very effective. Because in addition to oxygenation, nutrient exchange and cellular communication, it provides a three-dimensional environment for the follicles (Desai et al., 2010). In order to reconstruct the human ovarian environment in vitro, factoring plays a major role in the menstrual cycle. Therefore, in the context of a microfluidic chip (Scaramuzzi et al., 2011), alginate encapsulation (Gomes et al., 2015) was used to mimic the hormonal changes of the menstrual cycle in follicle culture. Microfluidics have made possible the successfully recombine the 28-day human menstrual cycle by fusion of tissues, such as mice ovaries and human fallopian tubes, ectopic uterus, and liver (Xiao et al., 2017). Microfluidic operating systems should be readily available and promote follicular culture among different species. Follicle culture methods vary depending on the species, the age of the animals, and the stage of the follicle.

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CONFLICT OF INTEREST
The authors have no conflict of interest to declare.

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