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GIULIANA DE AVILA FERRONATO

Produção *in vitro* de embriões bovinos em diferentes sistemas de cultivo 3D: efeitos na morfologia nuclear, padrão de metilação, transcrição gênica e vesículas extracelulares

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Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Produção in vitro de embriões bovinos em diferentes sistemas de cultivo 3D: efeitos na morfologia nuclear, epigenética, vias de mecanotransdução e vesículas extracelulares", protocolada sob o CEUA nº 5343150721 (ID 001759), sob a responsabilidade de **Juliano Coelho da Silveira** *e equipe; Giuliana de Avila Ferronato* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais da Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo - FZEA/USP (CEUA/FZEA) na reunião de 09/10/2021.

We certify that the proposal "In vitro production of bovine embryos in different 3D culture systems: effects on nuclear morphology, epigenetics, mechanotransduction pathways and extracellular vesicles", utilizing 60 Bovines (60 females), protocol number CEUA 5343150721 (ID 001759), under the responsibility of **Juliano Coelho da Silveira** *and team; Giuliana de Avila Ferronato* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **APPROVED** by the Ethic Committee on Animal Use of the School of Animal Science and Food Engineering - (São Paulo University) (CEUA/FZEA) in the meeting of 10/09/2021.

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Origem:	Faculdade de Zootecnia e Engenharia de Alimentos (FZEA/USP)						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	3 a 15 anos	N:	30
Linhagem:	Nelore			Peso:	360 a 500 kg		
Origem:	Faculdade de Zootecnia e Engenharia de Alimentos (FZEA/USP)						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	3 a 15 anos	N:	30
Linhagem:	Nelore			Peso:	360 a 500 kg		

Local do experimento: Nos experimentos 1, 2, 3 e 4 amostras serão coletadas de abatedouro local e transportadas ao laboratório. No experimento 4 também será realizado um grupo in vivo, o qual será realizado no FZEA/USP Curral da Teriogenologia (Prof. Luciano Andrade Silva). As vacas serão mantidas à pasto de capim Braquiária (Brachiaria brizantha), com acesso em livre demanda de água e sal proteinado.

Pirassununga, 02 de novembro de 2022

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Folha de aprovação

GIULIANA DE AVILA FERRONATO

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RESUMO

FERRONATO, G. A. **Produção *in vitro* de embriões bovinos em diferentes sistemas de cultivo 3D: efeitos na morfologia nuclear, padrão de metilação, transcrição gênica e vesículas extracelulares.** 2022. 97 f. Dissertação (Mestrado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2022.

A produção *in vitro* de embriões (PIV) é uma técnica amplamente utilizada para melhorar os parâmetros reprodutivos em bovinos e humanos. No entanto, a PIV ainda apresenta algumas limitações, como menores taxas de blastocisto e prenhez em comparação com embriões produzidos *in vivo*. Além disso, os embriões obtidos através da PIV possuem alterações epigenéticas que podem afetar o estabelecimento da prenhez e da saúde pós-natal. *In vitro,* os embriões são cultivados em placas, que podem ser seis vezes mais rígidas do que o ambiente *in vivo*, o que causa diferentes estímulos externos que geram diferentes respostas biológicas intracelulares nesses embriões. Uma alternativa bem estabelecida por ser mais fisiológica são os cultivos tridimensionais (3D), que consistem na criação de um microambiente onde as células interagem melhor entre si e com o meio de cultivo. Esses cultivos podem ser produzidos de diferentes formas e materiais. As *liquid marbles* (LM), são um tipo de cultivo 3D que consiste na criação de um ambiente através de uma pequena gota de líquido encapsulada por partículas de uma sustância hidrofóbica. Outro material constantemente utilizado é o alginato, que possui a capacidade de gerar um hidrogel estável a partir da adição de um íon bivalente. Baseado nisso, a nossa hipótese é que os sistemas 3D por LM ou hidrogéis de alginato possam contribuir positivamente durante as etapas de maturação e cultivo dos embriões produzidos *in vitro.* Para isso, no experimento 1 (capítulo 2), a maturação *in vitro* (MIV) foi realizada no sistema de LM e quando avaliada a taxa de maturação nuclear observamos que foi semelhante à do cultivo convencional. Contudo, a expansão das células do cumulus e a expressão de genes importantes para o processo de maturação dos oócitos nas células do cumulus foram diminuídos no grupo LM. O cultivo de prováveis zigotos também foi realizado no sistema LM. No dia 7, foi observada uma menor taxa de blastocisto, diminuição na expressão do bta-miR-615 e aumento na metilação e hidroximetilação global de DNA nos blastocistos do grupo LM. No experimento 2 (capítulo 3), o sistema de cultivo 3D por hidrogéis de alginato foi utilizado no cultivo de prováveis zigotos. Neste experimento, o cultivo convencional foi denominado 2D; uma superfície de hidrogel de alginato foi o grupo 2.5D; e o grupo 3D foi aquele em que os embriões foram encapsulados nos hidrogéis de alginato. No dia 7 do cultivo, foi observado uma diminuição na taxa de blastocisto em ambos os grupos 2.5D e 3D em comparação ao controle. Também

houve uma diminuição da expressão dos miRNAs miR-1245 e miR-1260b no grupo 2.5D em relação aos demais. Além disso, o miR-541 foi menos expresso nos blastocistos do grupo 3D em relação ao 2D e 2.5D. Os padrões de metilação e hidroximetilação foram aumentados apenas nos embriões do grupo 2.5D. Além disso, também foi observado uma mudança no perfil transcricional global dos blastocistos cultivados em 2.5D e 3D quando comparados com o grupo 2D, em que genes importantes para o desenvolvimento embrionário inicial foram diferentemente expressos entre os embriões dos diferentes grupos. Juntos, estes resultados sugerem que os cultivos 3D utilizados nos nossos experimentos tiveram um impacto negativo na taxa de produção de embriões bovinos. Entretanto, mais estudos são necessários, visto que, para obtenção dos resultados de embriões produzidos *in vitro* que temos atualmente foram necessários mais de 40 anos de estudo e essa técnica continua sendo constantemente aprimorada. Portanto, em pesquisas futuras precisamos considerar a hipótese de que com a utilização do cultivo 3D será possível obter blastocistos de PIV semelhantes aos produzidos *in vivo*.

Palavras-chave: liquid marbles, hidrogel de alginato, produção *in vitro* de embriões, bovino.

ABSTRACT

FERRONATO, G. A. ***In vitro* production of bovine embryos in different 3D culture systems: effects on nuclear morphology, methylation pattern, gene transcription and extracellular vesicles.** 2022. 97 f. Dissertação (Mestrado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2022.

The *in vitro* embryo production (IVP) is a technique widely used to improve the reproductive parameters in bovines and humans. However, the IVP still have some limitations, such as lower blastocysts and pregnancies rates in comparison with the *in vivo* produced embryos. Moreover, the embryos produced by IVP harbor epigenetic alterations which can affect the conception rates and the health of the offspring. IVP embryos are cultured in petri dishes, that are ~6 x more rigid than the *in vivo* environment, causing different external stimuli and generating different intracellular responses in the embryos. An alternative to circumvent this and create a more physiological culture environment is to use the 3D cultures, which consists in the creation of a microenvironment where the cells can interact themselves and with the culture medium. These culture systems can be produced using several methodologies and materials. The Liquid Marbles (LM) are a type of 3D culture that consists in the creation of an environment trapping a small droplet of culture medium using a hydrophobic substance. An alternative material used is the alginate, that possess the capacity to generate a stable hydrogel after the addition of a bivalent ion. On this basis, our hypothesis was that the 3D systems produced using LM or alginate hydrogels can benefit the *in vitro* maturation and *in vitro* culture of bovine oocytes and embryos, respectively. To this end, in the experiment 1 (chapter 2), the *in vitro* maturation was carried out in LM. We observed that the nuclear maturation rate was similar between the LM and control group. However, the cumulus cells expansion and the expression of genes involved in the maturation processes were decreased in the LM group. The culture of presumptive zygotes was also carried out in LM. As a result, on the day 7 was observed lower blastocysts rate, decrease in the expression of the miRNA bta-miR-615 and increased in the global levels of DNA methylation and hydroxymethylation in the LM blastocysts compared with the controls. In the experiment 2 (chapter 3), the 3D system using alginate hydrogels were used to culture the embryos. In this experiment, the conventional culture was termed 2D; the embryo culture on top of an overlay of alginate termed 2.5D; and the culture with the embryos encapsulated in an alginate hydrogel termed 3D. After the culture for 7 days in each system, the blastocysts rates were examined. We observed a decrease in the blastocysts rates in the embryos cultured in the 2.5 and 3 D systems compared to the control. Regarding the miRNas expression, the miRNAs miR-1245 and miR-1260b

were decreased in the 2.5D group compared with the other groups. Moreover, the miR-541 presented lower expression in the 3D group when compared with the other groups. The global levels of DNA methylation and hydroxymethylation increased only in the embryos from the 2.5D group. In addition, the transcriptome from embryos from the 2.5D and 3D differed from the control group, and are more similar between them. Also, several genes critical for embryo development, pluripotency, placentation were decreased in the embryos cultured in the alginate hydrogel. Altogether, these results suggest that the 3D culture systems used in our experiments impaired the embryo development. However, more studies are necessary to fully understand the effects caused by these molecules on embryos. The IVP technique has constantly evolved in the past 40 years, and always a new approach was attempted a lot of drawbacks had to be surpassed. In the future, we envision that the embryos will be cultured in more physiological systems and they will be more similar to the *in vivo* ones.

Keywords: liquid marbles; alginate hydrogel*; in vitro* embryos production; bovine.

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Introdução

A produção *in vitro* de embriões (PIV) é uma alternativa que surge frente a problemas reprodutivos ou ensejos de aumentar a eficiência reprodutiva, por isso possui extrema relevância e é amplamente utilizada em inúmeras espécies, como bovinos e humanos. No Brasil, a PIV é uma biotécnica muito utilizada para melhorar os índices reprodutivos em rebanhos bovinos de leite e corte. Mais de 90% dos embriões bovinos são produzidos por meio dessa biotécnica, sendo o Brasil um dos maiores produtores de embriões PIV do mundo (Viana et al., 2018). Apesar da dimensão na utilização dessa biotécnica, a PIV ainda possui algumas limitações que precisam ser contornadas.

Em comparação com os embriões produzidos *in vivo*, os embriões bovinos PIV apresentam menores taxas de blastocisto (Sartori et al., 2016), menores taxas de prenhez e são menos resistentes à criopreservação (Mello et al., 2016). Além disso, os embriões PIV possuem alterações epigenéticas (Canovas et al., 2017), sendo essas reguladas pela metilação do DNA, modificações pós-traducionais de histonas e por microRNAs (miRNA), podendo influenciar no desenvolvimento dos embriões e até mesmo na sua saúde após o nascimento (Bouillon et al., 2016). Essas alterações podem ocorrer porque durante o desenvolvimento embrionário inicial uma intensa reprogramação epigenética ocorre, coincidindo com a etapa do cultivo *in vitro*.

In vivo, a reprogramação epigenética ocorre principalmente no oviduto, que é um ducto sinuoso responsável por fornecer os nutrientes necessários para a mantença e desenvolvimento inicial do embrião (Killian, 2004). Por outro lado, o cultivo *in vitro* é realizado em placas, com os embriões livres no meio de cultivo e sem comunicação com o ambiente materno. Além disso, *in vivo* existe também a estrutura física do tecido, que exerce funções mecânicas, como a movimentação do embrião pelo oviduto até chegar ao útero (Li & Winuthayanon, 2017). Já *in vitro,* as placas de cultivo apresentam uma rigidez seis vezes maior do que a encontrada no ambiente materno (Kolahi et al., 2012).

Frente a esses fatores, surge a necessidade de buscar novas metodologias para a realização do cultivo de embriões PIV. Uma alternativa interessante é o cultivo *in vitro* em sistemas 3D, que é uma técnica baseada na criação de um microambiente celular adequado e mais parecido com o encontrado *in vivo*, permitindo que a célula interaja com seu entorno nas suas três dimensões (Langhans, 2018). O sistema 3D também é capaz de simular a força mecânica exercida pelos tecidos *in vivo,* pois a rigidez é mais semelhante (Kolahi et al., 2012).

Diferentes tipos de materiais podem ser utilizados na produção dos sistemas de cultivo 3D, como substâncias hidrofóbicas e polímeros solúveis em água. As *liquid marbles* (LM) são uma

forma de cultivo 3D, que se baseiam na criação de esferas através da utilização de uma substância altamente hidrofóbica (Aussillous & Quéré, 2001). O sistema das LM apresenta uma série de vantagens (Vadivelu et al., 2017), como a utilização de um baixo volume de meio de cultivo, com variação na quantidade de meio utilizada, permitindo cultivos individuais ou em grupos. Também apresenta uma boa permeabilidade gasosa e um baixo custo de produção (Avramescu et al., 2018; Oliveira et al., 2017). Esse cultivo vem sendo utilizado para uma série de aplicações, principalmente na formação de esferoides (Lin et al., 2019). Já na reprodução, o sistema das LM foi utilizado durante a etapa de maturação *in vitro* em ovinos, apresentando resultados bem sucedidos (Bebbere et al., 2021; Ledda et al., 2016).

Outra forma de cultivo 3D é por meio da utilização dos hidrogéis, que são estruturas formadas por polímeros solúveis em água, que formam redes tridimensionais reticuladas (Determan et al., 2007). Existe um grande número de polímeros que formam os hidrogéis, sendo o alginato o mais utilizado em cultivos 3D (Jones & Shikanov, 2019). Esse polímero é capaz de gerar um hidrogel estável a partir da adição de um íon bivalente, como o cálcio (Lee & Mooney, 2012). Em células-tronco espermatogoniais foi demonstrado que o alginato não altera a morfologia das células, não é citotóxico e ainda possui características antioxidantes que aumentam a viabilidade das células (Jalayeri et al., 2017). Em reprodução, o hidrogel de alginato é muito utilizado para cultivo de folículos ovarianos (Vanacker & Amorim, 2017) e também já foi bem sucedido quando utilizado para elongação de embriões bovinos (Zhao et al., 2015).

Portanto, tendo em vista a importância da PIV, das suas limitações e frente as vantagens apresentadas pelos cultivos 3D, nosso principal objetivo foi avaliar a aplicação do sistema de *LM* na maturação de oócitos bovinos *in vitro* e a utilização do sistema de LM ou de hidrogéis de alginato durante o desenvolvimento embrionário inicial de prováveis zigotos cultivados *in vitro*. Assim, esta dissertação está organizada em 3 capítulos: o capítulo I, que consiste em uma revisão de literatura abordando diferentes sistemas de cultivo 3D e os principais achados acerca da utilização destes na reprodução; o capítulo II, no qual é apresentado um artigo original, onde investigamos o sistema de LM como uma nova abordagem para maturação (MIV) e cultivo *in vitro* de embriões (CIV) em bovinos e; o capítulo III, em que foi utilizado 3 diferentes dimensões de cultivo (2D, 2.5D e 3D), produzidas a partir de hidrogéis de alginato, durante o processo de desenvolvimento embrionário inicial no cultivo *in vitro* de embriões bovinos.

Capítulo 1: 3D culture applied to reproduction in females: possibilities and perspectives

Artigo de revisão de literatura preparado para submissão na revista *Animal Reproduction* (Impact Factor: 1.807)

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Abstract

In reproduction, gametes and embryos can be cultivated, as well as other reproductive tissues, such as ovary and endometrium. These cultures are important for a wide purpose, such as embryology studies, understanding signaling pathways, developing drugs for reproductive diseases and *in vitro* embryo production. Although many cultures are successful, they still have some limitations that need to be overcome. As a more physiological alternative we have the three-dimensional cultures systems, which allow a greater interaction between cells and cell with environment, maintenance of the cells natural morphology, expression of genes and proteins similar to *in vivo*, stimulation of the extracellular matrix and thus the mechanical force produced by it. Different types of matrices can be used to build 3D systems, such as hydrogels, magnetic nanoparticles, liquid marbles, and also techniques that use microfluids, 3D printing, hanging drop, among others. These systems demonstrate satisfactory results in follicle cultivation, allowing the culture from the pre-antral to antral phase, maintaining the follicular morphology as well as increasing development rates of embryos. Here we review some of the different types of 3D systems and their applications in the culture of follicles and embryos, bringing new possibilities to the future of assisted reproduction.

Keywords

In vitro embryo; follicles; mechanotransduction; 3D systems matrix; tissue tension.

1. Introduction

Cell culture is a routinely used procedure performed in laboratories which aims to keep living cells in controlled conditions (1). This procedure has numerous applications, including in what is associated with assisted reproduction techniques, whether in basic embryology studies to understand pathways or mechanisms, or in commercial *in vitro* embryo production (IVP). Although the monolayer (2 dimensional; 2D) system is the most used in cell culture, it has some limitations, in the 2D culture, cells lose their original shape, interact with the culture medium with only one side, as the other side is adhered to the plate dish, and this can influence cell proliferation and differentiation. Based on that, three-dimensional culture systems have emerged as an alternative to improve the *in vitro* culture techniques (2).

The first description of 3D culture was in 1912, using a surface made of silk threads for cardiomyocytes culture (3). This culture system allowed greater interaction between cells and the surrounding environment. During the early 40s, Holtfreter (4) performed different studies in developmental morphology describing a method to generate spherical cell aggregates applying agar to the surface of petri dishes, thus preventing cells from adhering to the bottom of the dish. In 1951, Joseph Leighton realized that 3D culture allowed cells to acquire their natural shape *in vitro*, thus presenting an increase in cell surface distinct from the monolayer cultures (5). Due to Leighton's contributions he can be called the father of 3D cell and tissue culture (6).

A 3D culture system is based on creating a cell microenvironment suitable and similar to that found *in vivo*, which allows cells to explore its three dimensions, generating an increase in their interaction with the environment. Therefore, the 3D culture technique presents advantages over 2D culture, as it allows the natural cell shape (7), cell-to-cell communication and levels of protein expression (8), gene expression (9) as well as cells mechanical stimuli, since 3D culture stimulates the extracellular matrix and inducing cells to respond biologically to these physical signals (1).

The advantages of 3D culture systems attracted laboratories involved in the most varied areas, such as, in new drugs development, since it can reduce animal experimentation (7), as well as in stem cells research, since it allows greater aeration and nutrients (10). Besides that, there are studies using 3D culture in experiments associated with reproduction, most of them related to early folliculogenesis (11-13), and a few others related to embryonic development and embryo culture (14, 15).

The 3D culture has come as an alternative to conventional cell cultivation and it has been widely used in the most diverse areas for its practicality, versatility and favorable results. In this review we will make an overview about 3D culture systems applied to animal reproduction, mainly in follicle, oocyte, and embryo culture, as well as the mechanical stimulus, main types of culture matrix, possibilities, and the perspectives.

2. Mechanical stimuli and reproductive tissue stiffness

Mechanotransduction is the ability of cells to respond to mechanic stimuli, allowing cells to transform external physical stimuli into biological responses inside the cytoplasm, inducing pathways activation and modulating gene transcription (16). Thus, depending on the type of tissue, the extracellular environment can be soft or stiff, thus influencing cells' response (17).

The reproductive tract presents tissues with soft tensions as the oviduct and the uterine epithelium which demonstrated stiffness between 100–1000 Pa (18). These mechanical stimuli are important for gametes and embryos, as these tensions form physical forces according to the needs of these cells. In the ovary, tissue stiffness plays a role in follicle development impacting cell proliferation, differentiation and growth (19). The oviduct is important for oocyte fertilization, early embryo development and embryo transport to the uterus. In mouse embryos, for example, a higher blastocyst rate and also a greater number of cells were obtained when *in vitro* produced embryos were induced by a continuous and uniform mechanical stimuli; mimicking what occurs naturally within the oviduct (20). Additionally, uterine tissue stiffness is involved in the establishment of pregnancy by embryo adhesion as well as in the adaptation of the organ as the fetus enlarges (19).

Conventional IVP or gametes culture, occurs on petri dishes, which have stiffness of 1 GPa, six times greater than that found in the uterine epithelium and oviduct (18). Some 3D culture systems, on the other hand, try to overcome the high stiffness through the use of different type of matrix, as is the case of several materials based on hydrogels, which have a natural elasticity similar to the reproductive tract (around 1 kPa) (18).

3. Types of 3D cultures used in reproduction

3.1. Hydrogels matrix

Hydrogels are structures formed by water-soluble polymers, which form reticulated three-dimensional networks and have the ability to respond to external stimuli inducing the substance to become gel or liquid state, such as temperatures, pH, osmotic pressure, among

other physical characteristics (21). There are several sources of hydrogels matrices, which are polymers or even components already present in the living cells extracellular matrix.

The most commonly used matrix found in 3D studies is alginate (22), which is a natural polymer obtained from different species of brown algae. Alginate hydrogel is formed by the addition of a divalent ion, such as calcium, with properties to form an stable gel similar to the extracellular matrix (23). This polymer is well known for its non-cytotoxic proprieties, cell morphology maintenance and antioxidant characteristics increasing cell viability (24). Some components can be added to alginate matrix improving the 3D environment, such as fibrin, which will allow cellular proteases to degrade the matrix and create a dynamic mechanical environment throughout the culture, since alginate it is not susceptible to this degradation and the forces remain constant in the culture (22).

Other types of elements are also used to form the hydrogel in studies applied to reproduction, such as collagen, which is naturally present in the uterine extracellular matrix, and has an elasticity that mimics the uterine environment allowing the construction soft gels, similar to the uterine tissue (25). Based on these reasons hydrogels are the subject of several studies in follicle and embryo culture in different species of domestic animals (14, 18, 26, 27).

3.2. Levitation

Levitation culture systems are based on magnetic nanoparticles with a size less than 1 μm composed by magnetic elements such as iron, nickel, among others. These magnetic elements are placed in the culture media containing cells to be cultivated, after a while these nanoparticles adhere to the cell membrane and from there cells can be placed in culture (28). Subsequently, cell and magnetic beads mixture is subjected to a magnetic field to induce cells to aggregate by levitation, forming three-dimensional spheroids or ring-shaped structures. This culture system allows a practical and efficient 3D culture that has already demonstrated good results in ovarian follicles (29).

3.3. 3D Microfluidic system

Microfluidics can be characterized as the study of certain fluid behavior in contact with tissues or cells in laboratory and based in very small volumes (30). The microfluidics technique has been associated with 3D cultivation systems to mimic cellular environment (31). The microfluidic technique has been used with hydrogels to mimic a cellular microenvironment during angiogenesis, with the ability to monitor in real time the response of cell dynamics to changes within this environment (30). The 3D printing has also been crucial

in the use of the microfluidic technique, as it allows the setting up of an environment with greater precision and reproducibility (32).

3D printing was associated with hydrogel and microfluidic for human differentiation Neuronal Stem Cells (33). However, what has attracted the most attention are the organ-on-a-chip models, which are three-dimensional structures usually composed of optically clear plastic, glass or flexible polymers, such as polydimethylsiloxane (PDMS), where cells are cultivate in a microfluidic system (34). This type of system allows physiological responses to be more realistic, which is why these systems have been used in several areas of biological studies, as well as in reproduction, with the construction of an oviduct-on-a-chip system for fertilization and early embryonic development in cattle (15, 35). It can be used for endocrinology studies and to improve *in vitro* research mimicking what happens on natural and complex tissues, using less experimental animals, for example.

3.4. Liquid marble

Liquid marble (LM) is the result of an interaction between solid particles (external phase) and a particular liquid (internal phase), LM is also known as "dry water" (36). The solid part is formed by extremely hydrophobic micro or nanoparticles, which allows molecules to adhere to a drop of liquid forming a 3D system; importantly, these solid particles are not adhesive allowing cells to be free for interaction with other cells and with medium (37). This technique has several advantages, such as, easy handling for changing or collecting culture medium, droplet size can be variable allowing multiple or individual cultures depending on the needs of each study, allows the addition of drugs to be tested and the use of low volumes since the system is small, reducing experimental costs (38). LM has multiple applications and has already been used in embryonic stem cell cultures (39) and also in sheep oocytes during *in vitro* maturation, using a total of 10 cumulus-oocyte complexes (COCs) per drop (38). Thus, LM system can improve our knowledge regarding COC maturation in small media droplets.

3.5. Hanging drop

The hanging drop (HD) method was initially developed for microbiology studies (40). However, HD reveal good results when it was adapted for animal embryology studies, and has been used extensively in embryoid bodies (41-43). This technique is quite simple; it consists in use gravity to form spheroids from the aggregation of cells. The cells are suspended together with the culture medium in an inverted plate and are incubated, thus forming the spheroid (44).

The HD can also be associated with other materials to improve the technique, such as methocel and matrigel (also called "reconstituted basement membrane") because some cell lines need these conditions to form spheroids (45). HD has already been used in granulosa cell cultures in buffaloes to mimic the intra-follicular environment (46) and porcine oocyte to investigate antioxidant factors for maturation (47). Therefore, it can be a simple and economical method to obtain 3D structure in laboratories for cell study.

3.6. 3D printing

3D prints are capable of making scaffolds of high precision and accuracy, they can form complex structures that promote cell attachment, proliferation, distribution and differentiation (48). These scaffolds can be formed from different materials such as metals, ceramics, polymers and composite materials, since they are biocompatible, biodegradable, present adequate pore size and mechanical properties (49). The 3D printing technique is usually associated with that of other matrices, precisely because scaffolds can be used as molds only. It has already been associated with hydrogels for the construction of ovarian implants in mice (50) and also with microfluidics for mimicking a bovine oviduct *in vitro* (35). Thus, the use of 3D printing allows to create experimental settings mimicking stiffness as well as morphology of natural tissues.

4. 3D ovarian follicle culture

Ovarian follicles culture is an important technique for a number of purposes, such as understanding folliculogenesis, cryopreservation of follicles to preserve fertility, toxicology tests for fertility related drugs, among others (51-53). Normally in 2D culture, follicles lose their original architecture due to adhesion of granulosa cells to the bottom of the plate, inducing changes in the cell-to-cell interaction and also between somatic cells and the oocyte (54, 55). Thus, several researchers use 3D systems formed from different types of matrices (table 1) to mimic the physiological ovarian environment to improve follicle culture (22, 26, 29, 56).

Alginate has been the most used biomaterial tested for ovarian follicles 3D culture systems in domestic animals (26). This is due to alginate satisfactory results combined with easy manipulation, *in vivo* biocompatibility and *in vitro* non-cytotoxicity (11, 13, 23, 24, 57). In non-human primates, it was demonstrated the ability of secondary follicles to grown in alginate to reach oocyte maturation until metaphase II (MII) (58). In the same study, using the right concentrations of fetuin, FSH and 5% of O_2 in the culture medium, they also obtained a

greater survival of follicles and greater production of AMH after the antrum formation. In goats, the use of alginate allowed follicle activation to continue growth of primordial follicles (59). In cattle, secondary follicles were able to reach the antral phase in alginate culture for 32 days (60); additionally, this same study observed that the addition of growth hormone (GH) was able to increase the estradiol production. Besides that, Rossetto, Saraiva (52) also observed that bovine pre-antral follicles cultured in medium without insulin remained in the primordial stage after 10 days, suggesting an important role for this hormone in the activation follicles. In addition to that, bovine pre-antral follicles presented higher survival rates, growth and production of estradiol after treatment with a combination of 10 ng/mL insulin and 100 ng/mL FSH, corroborating the results also observed in goats with similar concentrations of insulin and FSH, showing higher oocyte maturation rates (61).

In a different approach, a study demonstrate a microfluidic system to culture secondary mouse follicles, using both alginate and collagen due to their differences in rigidity, to mimic the cortical part of the ovary with alginate (harder) and medullary part with collagen (softer), as a result they were able to observe ovulation of oocytes (62). In contrast, in conventional 2D culture, the authors did not obtain any secondary follicles development to antral follicle, precisely because of the natural architecture loss of the follicles (62). Although individual culture reveal good results, when multiple primary follicles were cultured in the same alginate matrix, it was possible to observe a higher survival and growth rate in the largest number of follicles group, probably due to the increase in soluble factors, secreted within the medium from surrounding follicles, which are beneficial to the folliculogenesis process (63). It is well known that alginate has been a great matrix for growing follicles in 3D systems, but despite that, some studies achieve great results using other materials. Kim, Yang (27) compared extracellular matrix-derived soft hydrogel (ES-hydrogel) to alginate and observed greater antrum formation, higher maturation rate, normal spindle morphology in oocytes as well as normal E2 production in ES-hydrogel. In order to obtain these results authors compared lower rigidity of ES-hydrogel to alginate, which in addition to maintain the structure of the follicle also allows a better exchange of nutrients and hormones with the medium.

To cultivate secondary bovine follicles, Antonino, Soares (29) used levitation system with magnetic nanoparticles. This approach was able to obtain greater follicular growth, antrum formation, better morphology, oocyte viability and higher oocytes resumption rate after *in vitro* maturation. In a new attempt to develop a new method Shen, Xu (56) cultured buffalo oocytes in 3D utilizing a glass scaffold system. Based on this method, they obtained

higher oocyte maturation, cleavage and blastocyst rates as well as greater blastocysts cell numbers. They also found higher levels of proteins related to oocytes maturation (COL1A1, COL2A1, COL3A1, and FN) in cumulus cells as well as cell connection-related proteins such as N-cadherin, E-cadherin and GJA1, indicating that 3D culture might promote oocyte maturation due to improvement in cell-to-cell connection.

A crucial point around ovarian follicles is the preservation of fertility in females, which seeks to maintain the oocyte viability for future use. Hydrogels are considered good encapsulation material to protect cells during cryopreservation, as they create barriers preventing formation of harmful ice crystals (64). Some studies illustrate success upon vitrification of follicles with alginate capsules (51, 65), although others studies still demonstrating low viability of these follicles compared with fresh follicles (66-68), demonstrating the need to improve follicle vitrification protocols. Based on this information, 3D culture systems might be good tools to preserve ovarian follicles; however, there still room to improve these techniques using both, different concentrations of hormones and different types of materials, to achieve better results.

5. 3D embryo culture

In vivo embryos in the early stage of development are allocated in the oviduct, which is a critical organ for the embryo development (69), demonstrated by the comparison between *in vitro* and *in vivo* produced embryos (70, 71). During the *in vitro* embryo culture, the idea is to try to replicate an environment with physiological functions similar to the oviduct allowing embryo development as efficient as possible (35). In laboratory, embryos are normally grown in 2D culture systems, using only culture dishes and media different from the *in vivo*. Although the *in vitro* production techniques of embryos in 2D are well-established, the obtained results suggest the need for improvements, demonstrated by the low rates of blastocysts to live births (72) and decreased resistance to cryopreservation (73). In addition, embryos produced *in vitro* have epigenetic changes when compared to embryos produced *in vivo* (74), which are regulated by DNA methylation, post-translational modifications of histones and by microRNAs (miRNA), which can influence embryo development and even individual health after birth (75). There are several efforts to improve the IVP technique to obtain better rates and embryos similar to the *in vivo* ones. The 3D culture systems have been used for embryogenesis studies (table 1), since they are ethically and economically viable as well as the ability to allow embryo hatch.

Mouse embryos cultivated in a 3D system made of type I collagen, presented higher cleavage, blastocyst and hatching rates as well as an increased number of trophoblast cells compared to conventional *in vitro* embryo culture (18). This can be explained by the mechanical properties of the environment, since collagen is already part of the extracellular matrix of the uterus, thus its use can provide a natural elasticity to the *in vitro* culture, similar to the uterine environment (1 kPa) (17, 76). Interestingly, embryos produced in 3D and conventional culture systems generate the same number of fetuses after transfer to recipient cows; however, the weight of the placenta was greater in the 3D group than in the conventional culture group. This result demonstrates that in addition to affecting the initial embryonic development by the difference in the number of trophoblast cells, the environment in which the embryo is inserted can also affect embryos after implantation, due to the difference in placental weights (18).

The beneficial effects of 3D systems are also associated with the mechanical pressure naturally exerted by the pellucid zone. In mice, a study removed the zone pellucid (ZP) of embryos prior to culture in an alginate matrix combined with calcium and was able to obtain better blastocyst rates in comparison to ZP-free embryos cultured without matrix (57). In bovine, Zhao, Liu (14) used alginate as a method to maintain embryos architecture after hatching to build a system for embryo elongation and implantation studies, since it is challenging to maintain embryos normal morphology *in vitro*, due to cell adhesion to the culture plate and interruption of cell-to-cell interactions. In this study, embryos were cultured for 18 days and those that were encapsulated with alginate had a higher survival rate, and were able to present expansion and elongation. Once embryos were placed back in conventional culture, they still showed growth, until the 26th and 32nd day of culture as well as demonstrated the presence of binuclear cells and expression of genes associated with placental tissue differentiation.

Moreover, in cattle, using a 3D culture system based on microfluid, called “oviduct-on-a-chip”, authors where able to mimic the functionality of an oviduct in terms of its hormonal levels and nutrients circulation (15). This same manuscript demonstrated that the global DNA methylation of zygotes cultured in this system was similar to embryos produced *in vivo* and differed from embryos produced *in vitro*, similar to changes observed at the transcription level of genes linked to DNA (de)methylation. These results are promising for the study of *in vitro* fertilization and embryonic development which can help us to understand what happens physiologically, thus improving existing IVP techniques as well as development rates of *in vitro* produced embryos.

Table 1. 3D culture systems and its main results in livestock reproduction studies.

Type of Matrix	Material	Structure	Results	References
Hydrogel	Alginate	Follicle	Greater viability and differentiation of pre antral into antral follicles in countless animal species	(**26**)
		Embryo	Viable for longer after hatching and embryo elongation	(**14**)
	Extracellular matrix-derived	Follicle	Greater antrum formation, maturation rate and normal spindle	(**27**)
	Collagen + Alginate	Follicle	Oocytes ovulation	(**62**)
	Collagen	Embryo	Higher cleavage, blastocyst and hatching rates. Greater number of trophoblast cells	(**18**)
Levitation	Magnetic nanoparticles	Follicle	High follicular growth, oocyte viability, morphology and maturation rate.	(29)
Microfluidic system	Oviduct-on-a-chip	Embryo	Global DNA methylation similar with *in vivo* embryo	(**15**)
Hanging drop	-	Granulosa cell	CYP19 gene longer expression, simulating preovulatory follicle stage	(**46**)
3D printing	Combination with hydrogel matrix	Ovary	Ovarian bioprosthetic able to return fertility in sterile mice	(**50**)

6. Additional 3D applications in female reproduction

In addition to ovarian follicles and embryos, 3D culture systems are also present in other studies related to reproduction, whether for the understanding of biological pathways and studying cell biology or even for transplants and fertility recovery. In that sense domestic animals as well as mice are good models for this type of studies. MacKintosh, Serino (77) used a 3D model of the endometrium to investigate pathophysiological diseases and to develop new therapies. Primary, bovine endometrium epithelial and stromal cells were cultivated in an electrospun polyglycolide (PGA) scaffold, resembling *in vivo* cell organization (77). In mice, a study was able to perform a bioprosthetic ovary using 3D printing together with hydrogel matrices and was even able to achieve implantation in sterile mice (50). Follicles in the ovary managed to develop normally, were able to ovulate, oocytes

were fertilized, and puppies were born healthy and fertile. These studies demonstrate the versatility of 3D culture systems, suggesting their use in several different approaches to contribute to a basic and applied science.

7. Future perspectives

The 3D culture of cells is a century old technique; however, new studies still bringing new technology to these systems to improve its application to different cell types and tissues. A number of different matrices can be used to generate the 3D system, each with its advantages and limitations, which must be considered according to the culture necessity. 3D systems are used for numerous applications, including cultures associated with reproduction, where it has already been successfully applied to culture secondary follicles until the development of the antral follicles (58), in the extension of embryo viability (14), mimicking the endometrium (77) and synthetic ovaries for transplantation (50). The 3D culture systems have a great number of benefits, in addition to increasing the cell-to-cell interaction as well as between cells and the environment, many of the matrices used are already known to mimic the tension present in the natural extracellular matrix (18), making the environment similar to that found in the tissue, inducing cells to respond similarly to these mechanical stimuli.

The 3D culture systems have demonstrated relevant promising results, and there may be a transition from 2D to 3D culture in the near future, especially with regard to co-cultures or for new drugs development (2). In embryology, 2D culture is still routinely used, due to the lack of 3D systems validations, especially in early embryo development, where studies are quite scarce and need additional tests with different types of matrices as well as in different species. Thus, is important to understand the mechanisms by which 3D culture can benefit cumulus-oocyte complexes and embryos.

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Capítulo 2: Bovine *in vitro* oocyte maturation and embryo culture in liquid marbles 3D culture system

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Abstract

Despite the advances in *in vitro* embryo production (IVP) over the years, the technique still has limitations that need to be overcome. In cell cultures, it is already well established that three-dimensional culture techniques are more physiological and similar to the *in vivo* development. Liquid marble (LM) is a three-dimensional system based on the use of a hydrophobic substance to create *in vitro* microbioreactors. Thus, we hypothesized that the LM system improves bovine *in vitro* oocyte maturation and embryo culture. In experiment I, bovine cumulus-oocyte complexes (COCs) were placed for *in vitro* maturation for 22h in two different groups: control (conventional 2D culture) and LM (three-dimensional culture). We found that oocyte nuclear maturation was not altered by the LM system, however it was observed a decrease in expression of genes important in the oocyte maturation process in cumulus cells of LM group (*BCL2, EIF4E,* and *GAPDH*). In experiment II, the COCs were conventionally matured and fertilized, and at the time of cultivation, they were divided into LM or control groups. There was a decrease in blastocyst rate and cell counting, a down-regulation of miR-615 expression, and an increase in the DNA global methylation and

hydroxymethylation in embryos of LM group. Therefore, for the bovine *in vitro* embryo production, this specific three-dimensional system did not present the advantages that we expected, but demonstrated that the embryos changed their development and epigenetics according to the culture system.

Keywords: three-dimensional culture; microbioreactors; cumulus cell expansion; DNA global methylation and hydroxymethylation.

1. Introduction

In vitro embryo production (IVP) is a worldwide used technique as an alternative to numerous reproductive issues. In livestock, the IVP is responsible for a large part of bovine embryos produced by assisted reproductive techniques (Viana et al., 2018). In humans, it is estimated that more than 8 million babies are born in the world through this tecnique (European Society of Human Reproduction and Embryology, 2018). Therefore, the IVP has a substancial importance for domestic species and human reproduction.

Despite its relevance, the IVP has some limitations that need to be overcome. The rate of oocytes *in vitro* matured that develop to the blastocyst stage is in average 30% (Sartori et al., 2016), and these *in vitro* produced blastocysts have lower pregnancy rates as well as low resistance to cryopreservation (Mello et al., 2016). *In vitro* produced embryos also show epigenetic differences when compared to *in vivo* produced embryos (Canovas et al., 2017), which can be regulated by DNA methylation, post-translational modifications of histones and/or microRNAs (miRNAs), influencing during embryonic development and after birth (Bouillon et al., 2016).

Faced with the IVP limiting factors, the need arises to seek new perspectives that contribute positively to the *in vitro* culture of oocytes and embryos. The three-dimensional (3D) culture systems, are based on a cellular microenvironment similar to that found *in vivo*, which allows cells to interact with its surroundings on its three dimensions keeping the cells original structure during the *in vitro* culture (Langhans, 2018). Liquid Marbles (LM) is one type of 3D culture system, which is produced from highly hydrophobic particles that adhere to a drop of liquid, making a sphere that remain stable (Aussillous & Quéré, 2001). The droplet size can be variable, allowing for group or individual cultures, depending on the needs of each study. Also, a very low amount of culture medium volume can be used in addition to the ability that the drops allow good gas permeability at a low cost (Avramescu et al., 2018; Oliveira et al., 2017).

The LM system has been widely used in cell cultures for different applications, such as to formation of organoids and spheroids (Rychecký et al., 2016; Vadivelu et al., 2017; Vadivelu et al., 2015); to embryonic body formation, where it demonstrated excellent cell viability in 7-day cultures by live/dead assay and also in 48-hour cultures by MTT (Lin et al., 2019). In studies focused on reproduction, it has already been used in sheep, where the nuclear maturation rates, cleavage, and blastocyst was similar between conventional maturation or using LM (Ledda et al., 2016). Moreover, in prepubertal ovine females where the maturation

rates were similar, but there was an increase in the blastocysts rate from the oocytes matured in LM group (Bebbere et al., 2021). Together these studies suggest that the LM system has no toxic effect on the cells and appears to improve *in vitro* embryo production.

Considering the importance of oocyte maturation for the IVP technique success and the importance of viable blastocysts production, we hypothesized that the LM system improve *in vitro* oocyte maturation and embryo culture in bovine. For that, the study was divided in two sets of experiments, which evaluated in an independent way the impacts of the LM culture on oocyte maturation and embryo developmental rates. Our findings demonstrate that the LM system does not alter the oocytes nuclear maturation rate, but may have a negative effect on cumulus cells from these oocytes and can also impair early embryonic development in bovine, contrary to our hypothesis.

2. Results

2.1. Experiment 1: *In vitro* maturation of cumulus-oocyte-complexes

2.1.1. The effects of liquid marble culture applied to cumulus-oocyte complexes during in vitro maturation

To observe the effects of the LM system during IVM (figure 1), COCs were submitted to IVM for 22 hours using the LM system and evaluated in comparison to a control group cultured in the conventional system. After IVM, the oocytes from both groups were denuded and the nuclear maturation rate was evaluated by the first polar body extrusion. The maturation rate did not differ (P=0.2192) between the control (61.19%; n=123/201) and LM systems (68.14%; n=77/113) groups (figure 2). Although, the cumulus cells showed a lower expansion in the LM group after maturation (P=0.0453; figure 3). Thus, these results demonstrate that the culture system by LM may affect the oocyte maturation supported by a lower cumulus cells expansion.

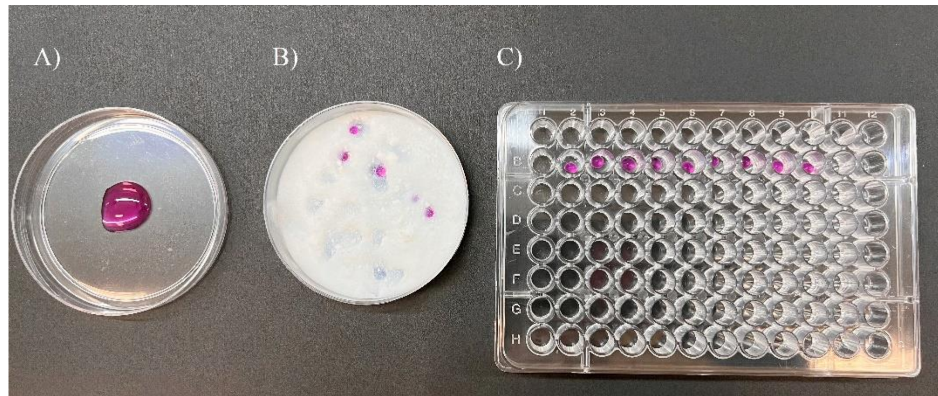

Figure 1. Liquid marble (LM) culture system. For both experiments (IVM or IVC), the LM system was performed in the same way, just changing the number of structures inside the microbioreactor. **A)** The structures to be cultured (COCs or presumptive zygotes) were all placed inside the same drop of culture medium in a petri dish. **B)** A total if 6 COCs (experiment I - IVM) or 3 presumptive zygotes (experiment II - IVC) were picked up in a 30 µl of culture medium and placed in contact with the CAB-O-SIL®TS-530 fumed silica powder to coat the drops. **C)** The drops were transferred using a 1000 µl pipette to a 96-well plate to be incubated at 38,5ºC in 5%CO_2.

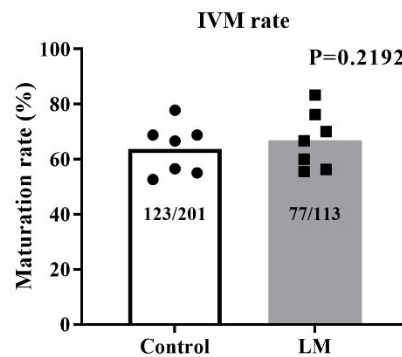

Figure 2. ***In vitro*** **maturation rates.** Nuclear maturation rate of oocytes cultured in conventional (control; 61.19%, 7 replicates) or in LM (68.14%, 7 replicates) systems. Within the bars there are mature/total oocytes numbers. Analysis were performed by Chi-square test with a statistical, no statistical difference was found between the groups (P>0.05).

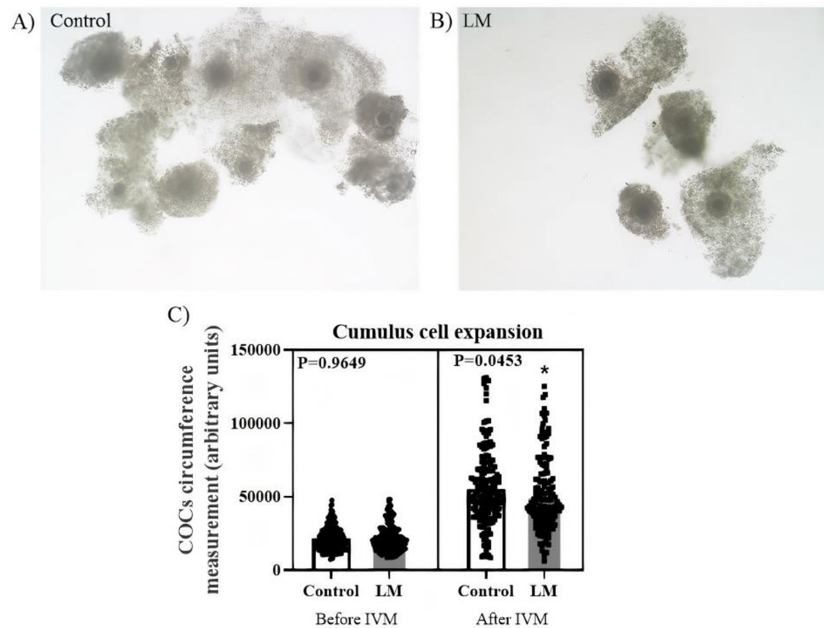

Figure 3. Cumulus cells expansion analysis. A) Cumulus-oocyte-complexes after IMV in control and **B)** LM systems. **C)** Cumulus cells expansion before IVM in control (n=180) or LM system (n=180), and after IVM in control (n=172) or LM (n=154) systems. Circumference of COCs were measured using ImageJ software. Analysis were performed using Wilcoxon test and asterisk represent statistical difference, considered when P<0.05.

2.1.2. Target transcripts in cumulus cells and oocytes matured in different systems

To evaluate the effects of the LM microbioreactors during IVM, we investigated the expression of target genes in cumulus cells and denuded oocytes isolated from the COCs maturated in control or LM systems. In cumulus cells (figure 4 A-H), we observed decreased levels of three target genes (P<0.05), which had their expression reduced in LM when compared to the control group: *EIF4E*, *BCL2* and *GAPDH* (Figures 4 B, D, and G). Interrestingly, in denuded oocytes (figure 5 A-H), we did not observed significant difference in the levels of the evaluated transcripts (P>0.05). In summary, the LM system may negatively modulate target transcripts in cumulus cells.

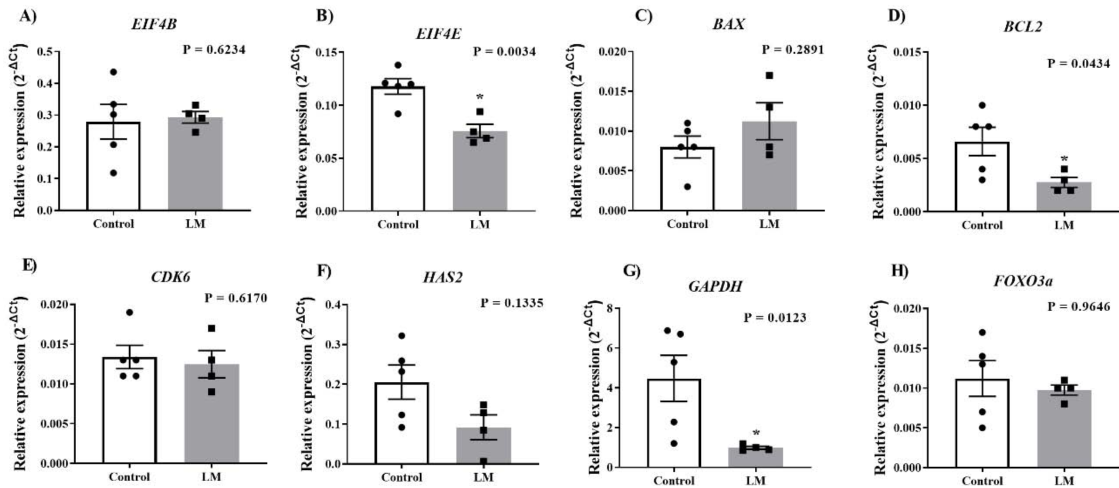

Figure 4. Target transcripts analysis in cumullus cells. Relative expression of **A)** *EIF4B;* **B)** *EIF4E*; **C)** *BAX;* **D)** *BCL2;* **E)** *CDK6;* **F)** *HAS2;* **G)** *GAPDH* and; **H)** *FOXO3a* genes in cumulus cells from COCs matured for 22h in conventional or LM systems (5 biological replicate). Transcripts levels analysis were compared using Student's t test and asterisk means statistical difference, considered when P<0.05. Standard bars represents the SEM.

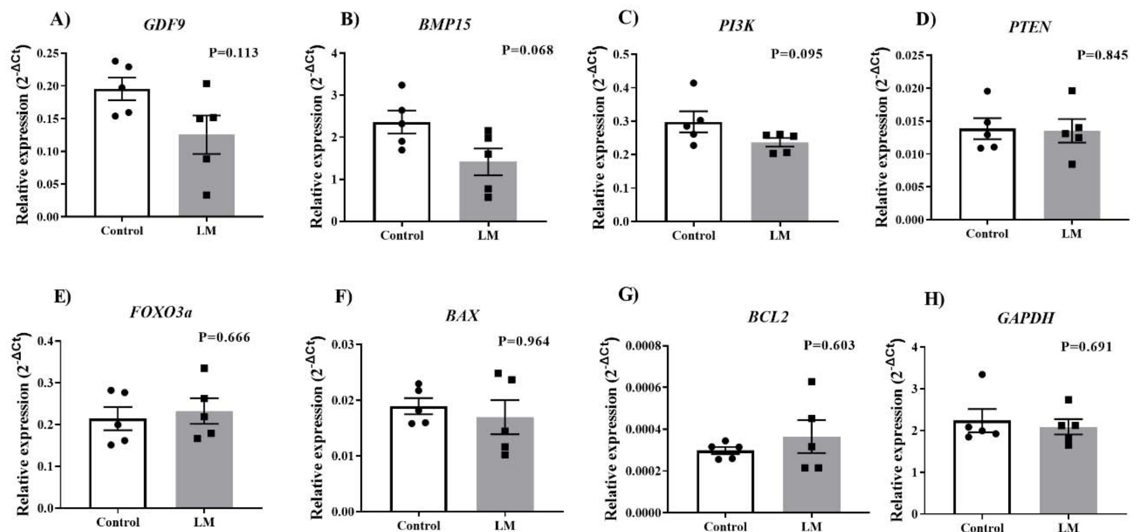

Figure 5. Target transcript analysis in oocytes. Relative expression of **A)** *GDF9;* **B)** *BMP15*; **C)** *PI3K;* **D)** *PTEN;* **E)** *FOXO3a;* **F)** *BAX;* **G)** *BCL2* and; **H)** *GAPDH* genes in oocytes matured for 22h conventional or LM systems (n=10 oocytes/replicate, 5 biological replicates). Statistics was performed using Student's t test, no statistical difference was found between the groups (P>0.05). Standard bars represents the SEM.

2.2. Experiment 2: *In vitro* culture of prezumptive zygotes

2.2.1. Blastocysts cultured inside LM microbioreactors had their development negatively affected

In view of the results obtained in the IVM, we wanted investigate how this LM system would work affect early embryo development. For that, we carried out an experiment to evaluate the use of LM only in the *in vitro* embryo culture (IVC). Thus, the COCs were matured and fertilized conventionally and the prezumptive zygotes were cultured in LM or control systems until day 7 (D7). First, we aimed to evaluate the development of these blastocysts. We observed a lower blastocyst rate (P=0.0030; Figure 6A) in the LM (18.09%; n=34/188) compared to the control group (29.28%; n=178/608). Although the overall blastocyst rate was different, the morphological classification of these blastocysts (early blastocyst, blastocyst, expanded blastocyst, and hatched blastocyst) appears to be homogeneous between the groups (figure 6B). Additionally, the blastocysts did not present different sizes between groups (P=0.1294; figure 7A). However, the total number of cells of the blastocysts were significantly decreased in the LM group compared to the control (P=0.004; figure 7B). Therefore, these results suggest that the LM system negatively impacts blastocyst development.

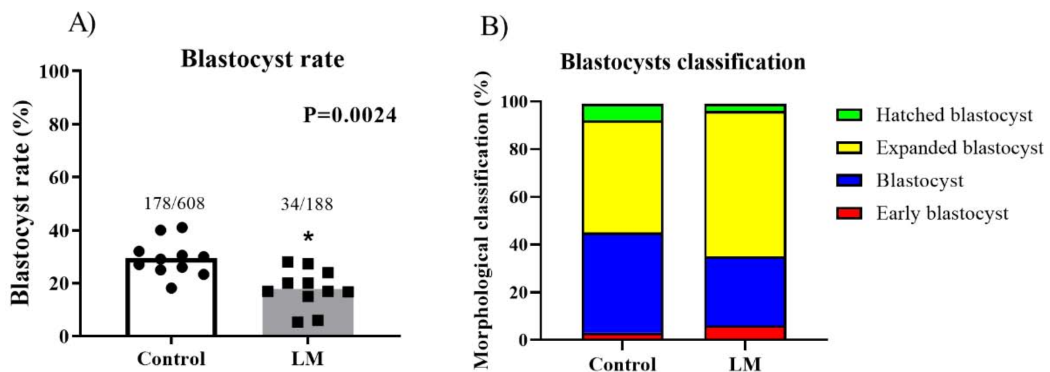

Figure 6. *In vitro* culture of prezumptive zygotes. A) Total blastocysts rate obtained from the presumptive zygotes cultured for 7 days in conventional (control; 29.28%, 11 biological replicates) or in LM system (18.09%, 11 biological replicates). Statistics performed by chi square test, asterisk means statistical difference (P<0.05). **B)** Blastocysts morphological classification Bó and Mapletoft (2013), from the 11 different routines. Standard bars represents the SEM.

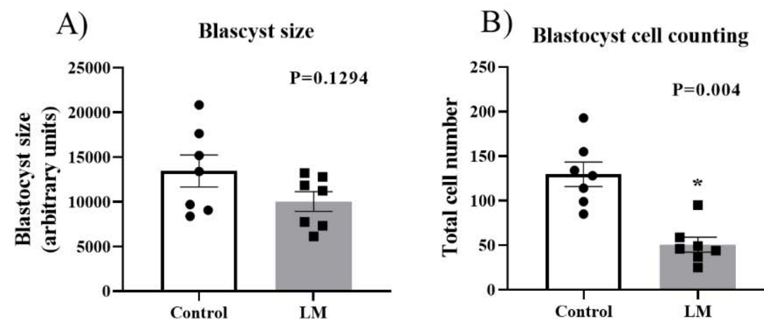

Figure 7. Blastocysts analysis. A) Blastocysts size (n=7); and **B)** Total cell counting of day 7 blastocysts (n=7) from control or LM group. Statistics performed by Student's t test, asterisk means statistical difference (P<0.05). Standard bars represents the SEM.

2.2.2. Bta-miR-615 was down-regulated in blastocysts cultured in liquid marbles system

In order to evaluate the molecular effects of these two different culture systems at the level of post-trancriptional regulators, we investigated bta-miR-615 relative expression as a biomarker for embryo quality and development (Quah & Holland, 2015). Bta-miR-615 was differently expressed (P=0.0449), its expression was decreased in the LM group in comparison to the control group (figure 8). Thus, indicating that LM affects embryo quality at the levels of post-trascriptional regulator as miRNAs.

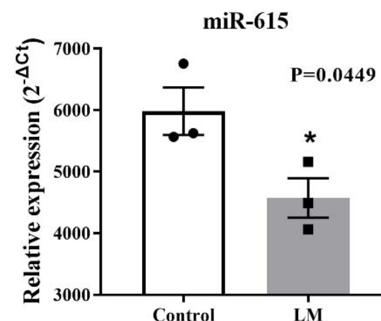

Figure 8. Bta-miR-615 analysis in blastocysts. Bta-miR-615 relative expression miRNA in blastocysts cultured conventionally (control) or in LM system. Statistics was performed using Student's t test. Asterisk means statistical difference (P<0.05). Standard bars represents the SEM.

2.2.3. Liquid marbles culture system increased blastocyst global DNA methylation and hydroxymethylation

To investigate the relation between culture environment (Control or LM) and epigenetic status we evaluated the levels of global DNA methylation in blastocysts using an antibody against 5-methylcytosine (5mC) and global DNA hydroxymethylation using an antibody against 5-hydroxymethylcytosine (5-hmC) in bovine blastocysts (Figure 9A). The results demonstrated that embryos cultured in the LM system had a higher levels of both DNA methylation (P<0.0001; Figure 9B) and hydroxymethylation (P<0.0001; Figure 9C) than those cultured in the conventional system. Thus, these results indicate that a series of genes may be differently regulated in the experimental groups, supported by the increase of both methylation and hydroxymethylation in the LM system.

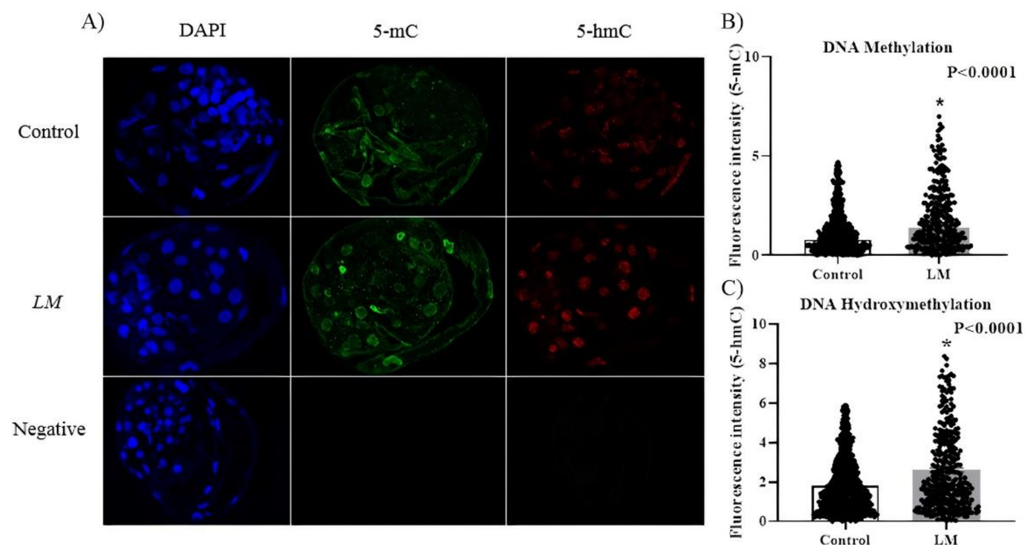

Figure 9. Global levels of DNA methylation and hydroxymethylation in blastocysts cultured in different culture systems. A) Confocal images of blastocysts submitted to different culture systems, conventional (control) or Liquid marble (LM), also a negative control was added to the experiment, without primary antibody labeling (40x objective). **B)** Global DNA methylation levels in nuclei (n=909) of D7 blastocysts (n=7) cultured in conventional system and in nuclei (n=354) of D7 blastocysts (n=7) cultured in LM systems. **C)** Global DNA hydroxymethylation levels in D7 blastocysts. Data are presented as mean ± SEM. Analysis were performed using Wilcoxon test. Asterisk represents statistical difference between groups (P<0.05).

3. Discussion

The oocyte quality directly influences the embryo development. Among the different steps involved in oocyte quality, the maturation is the most important process, allowing this cell

acquire the necessary competence to be fertilized and develop into a blastocyst. Although *in vitro* nuclear maturation rates are high in the literature (Izadyar et al., 1996; Luo et al., 2002)**,** most of these oocytes do not develop into a blastocyst *in vitro*. Thus, the *in vitro* culture plays a pivotal role determining the oocyte and embryo quality. In this sense. Based on that, in the present study we evaluated a different type of *in vitro* culture system, based on liquid marbles (LM). In order to investigate the effects of LM system we performed experiments evaluating the IVM and IVC independently. For our knowledge, this is the first manuscript to evaluate different molecular responses (mRNA, miRNAs, global DNA methylation and hydroxymethylation, respectively) of bovine oocytes and embryos submitted to the LM culture system.

Initially we evaluated the impacts of the LM system during the IVM period. The IVM rate did not differ between groups, corroborating the finding by Ledda et al. (2016) in a study in sheep, where IVM rate was also similar between control and LM group. However, we observed a lower cumulus cells expansion in the LM group compared to the control, and this expansion is highly related to the oocyte's ability to mature and fertilized (Eppig, 2001; Salustri et al., 1992). Next, we investigated transcript levels in cumulus cells collected from these oocytes, and observed a downregulation in three genes expression in the LM group compared to the control group: *EIF4E, BCL2* and *GAPDH*. *EIF4E* is associated with cell differentiation and proliferation, due its function to mediate protein translation (Mamane et al., 2004)**,** thus the observed decrease in transcripts could be associated with a low response to IVM. *GAPDH* is a gene involved in cell metabolism, impacting cell energy production (Seidler, 2013). Recent studies demonstrated that addition of iodoacetate, a GAPDH inhibitor, during IVM could influence in embryo development, through a lower rate of cleavage and blastocyst, demonstrating the importance of the glucose metabolism mediated by *GAPDH* (Lipinska et al., 2021). For this, the observed results in cumulus cells are possibly associated to a low cell metabolism during IVM. *BCL2* is a gene involved in blocking apoptosis in different cells type (Chiou et al., 1994). In women, *BCL2* mRNA expression was significantly higher in cumulus cells associated with mature oocytes than those associated with immature oocytes (Filali et al., 2009), suggesting that the reduced expression identified in our study could indicate that COCs did not have a proper *in vitro* maturation. Additionally, the investigated transcripts in the oocytes did not demonstrate to be affects by the LM culture system. Therefore, these gene expression results suggest that the IVM was negatively affected by the LM system, mainly due to decreased expression of important transcripts in cumulus cells.

Next, we investigated the effects of LM system applied during IVC. For that, presumptive zygotes were placed in the LM and conventional culture until day 7. The blastocyst rate was considerably reduced in the LM group. Another difficulty was to maintain the droplets intact until the end of the culture period. Due to that, the number of structures in LM system was smaller than in the conventional system. The same also occured with the drops during the IVM, however, as it was only 1 day in culture, we had a smaller loss of these microbioreactors, but during the 7 days culture this fragility in the system was more noticeable. The blastocyst rate obtained in control group corroborates with other studies (Bridi et al., 2021; Sartori et al., 2016), demonstrating that the performance of the technique was efficient. In addition to the lower blastocyst rate, the blastocysts subjected to the LM system presented a lower number of cells, another indication of the effect of this system on early embryo development (Rodríguez-Alvarez et al., 2013). This was the first study to cultivate embryos inside microbioreactors produced by LM and we observed that the technique is feasible, since we obtained developed blastocysts, however it can limit embryonic development by reducing blastocyst rate.

In order to further understand the effects of the LM system during IVC, we investigated the bta-miR-615 and the global DNA methylation and hydroxymethylation. Our results demonstrated a decrease in the levels of miR-615 in blastocysts cultured in the LM system compared to the convetional system. The miR-615 has its transcript located in an intron region of the *Hoxc5* gene, this in turn is one of the best characterized genes belonging to a gene family called homeobox. In other words, genes that code for transcription factors (Quah & Holland, 2015). These transcription factors regulate several other genes, especially at the embryogenesis phase, having a direct role in embryo development (Holland, 2013).

Interestingly, the global DNA methylation and hydroxymethylation was increased in embryos cultured in the LM system compared to the conventional system. The global DNA methylation and hydroxymethylation profile is influenced by the culture environment, as was demonstrated by Li et al. (2016) and Bomfim et al. (2017), where in both studies, global DNA methylation was increased in bovine embryos produced at high oxygen tension compared to low oxygen tension, demonstrating that culture environment can affect these epigenetics marks. Additionally, embryos developed *in vitro* show hypermethylation at several genomic loci compared to *in vivo* (Salilew-Wondim et al., 2015), which may partially corroborate with the differences we observed in the *in vitro* blastocyst development between our LM system. These results may indicate the modulation of embryo epigenetic marks according to the

culture system, which can lead to an abnormal expression of certain genes, either repression or overexpression.

In summary, the LM system presents great challenges to be applied in the IVM and IVC. The IVM rate did not appear to be affected by the LM system; however, we observed changes in transcripts related to COCs function and lower expansion of the cumulus cells. Similarly, we were able to produce blastocysts using the LM system even with the seven days culture challenge. However, the LM system decrease the number of blastocyst cells and miR-615 expression, while inducing an increase in global DNA methylation and hydroxymethylation. Thus, the LM system needs to be improved in order to be used in commercial laboratories of IVP.

4. Materials and methods

This work had two completely independent studies: experiment I: COCs *in vitro* maturation (IVM) and experiment II: *in vitro* embryo culture (IVC). Unspecified chemical brand is from Sigma-Aldrich/Merck Chemical Company.

4.1. Experimental design I - IVM

For this experiment, 120 COCs were selected according to their morphology (Grade I and II) and divided into two different groups for *in vitro* maturation. The experimental groups were: Control or LM. For the control group, 60 COCs were maturated in groups of 20 in a 100 µl drop of maturation medium (TCM 199 – GIBCO, buffered with 25 mM sodium bicarbonate, supplemented with 10% FBS, 0.2 mM sodium pyruvate, 50 µg/mL gentamicin sulfate, 0.5 µg/mL FSH - Folltropin and 5 U/mL hCG), submerged in 4 mL of mineral oil. In the LM group, 60 COCs were maturated drops of 30 µl of maturation medium (figure 1), containing 6 COCs, coated using CAB-O-SIL®TS-530 fumed silica (chemical name: Silanamine, 1,1,1-trimethyl- N-(trimethylsilyl)-silica hydrolysis products) and placed for cultivation in 96-well plates containing one drop in each well. Seven biological replicates were performed for IVM rates. From these, 5 biological replicates were used for gene expression of oocytes and cumulus cells. The oocytes were denuded together and snap frozen in pools containing 10 oocytes. The cumulus cells from these oocytes were briefly centrifuged and also snap frozen. Then the samples were stored at -80ºC for further RNA isolation.

4.2. Experimental design II – IVC

IVM and IVF were performed conventionally for both groups as describe below. After 18 hours of the IVF, the presumptive zygotes were denuded through multiple pipetting, washed and divided into groups: control group and LM Group. The control group (n=60/replicate) was conducted conventionally, on 4-well plates, with 500 µl SOFaaci culture medium (containing 2.5% FBS, 5 mg/ml BSA, 22 µg/ml sodium pyruvate and 50 µg/ml gentamicin) and 150 µl mineral oil per well, containing 30 presumptive zygotes per well. The LM group (n=60/replicate) was conducted in microbioreactors (figure 1), created with drops of 30 µl of SOFaaci culture medium, containing 3 presumptive zygotes per drop, coated with CAB-O-SIL®TS-530 fumed silic and placed for cultivation in 96-well plates containing one drop in each well. They were cultivated for 7 days at 38.5°C, 5% CO_2, 5% O_2 and 90% N_2. The blastocysts were individually frozen by snap frozen and stored at -80 ºC for miR-615 expression or were fixed in 4% PFA and stored at 4ºC for immunofluorescence analysis. Seven replicates were performed for miRNA expression and 5 replicates were performed for immunofluorescence (global DNA methylation and hydroxymethylation, blastocyst size, and cell counting).

4.3. *In vitro* maturation

Cumulus-oocyte complexes (COCs) were aspirated from antral follicles (3-6 mm in diameter) from local slaughterhouse ovaries. The COCs were selected in TCM 199 medium modified with Hepes (supplemented with 0.001 g/mL of BSA, 0.2 mM of pyruvate and 50 µg/mL of gentamicin sulfate). For each repetition, 120 COCs were selected according to their morphology (Grade I and II) and maturated for 22h, at 38.5°C, 5% CO_2 in controlled humidity. For the control group, COCs were maturated in groups of 20 in a 100 µl drop of maturation medium (described above), submerged in 4 mL of mineral oil. For the LM system 6 COCs were placed in a 30 µl of media and used to creat the microbioreactor droplets. For experiment I, the oocytes were denuded to evaluate the maturation rate, then the oocytes and cumulus cells were collected, snap frozen in liquid nitrogen, and stored at -80ºC for further analysis. For experiment II, after 22h of IVM, the COCs proceeded to the *in vitro* fertilization (IVF) stage.

4.4. Maturation rate

After 22h maturation, the oocytes from 7 replicates of experiment I (IVM) were denuded by multiple pipetting using TrypLE™ Express Enzyme (ThermoFisher). The completely

denuded oocytes were washed in PBS 1% PVP and the first polar body extrusion was observed. The samples were stored in pools of 10 oocytes and the cumulus cells were centrifuged twice at 300 xg for 5 minutes and the supernatant was removed. Samples were first frozen by snap freezing in liquid nitrogen followed by storage at -80ºC until RNA isolation.

4.5. Cumulus cells expansion

This evaluation was performed in three replicates (n=60 COCs/group/replicate) from experiment I. Images of the morphology of cumulus expansion were assessed using an inverted microscope (Nikon Eclipse TI) under 4× magnification of all the oocytes of each group before placing for maturation and after maturation. The cumulus cells expansion was measured using the Image J software circling the entire COC circumference.

4.6. *In vitro* fertilization

For the experiment 2, after the IVM, the oocytes were washed and transferred to 100 µl drops of IVF-TALP (Tyrode-lactate stock) medium, supplemented with 6 mg/ml BSA, 5.5 IU/ml heparin, 40 µL/ml PHE (2 mM D-penicillamine , 1 mM hypotaurine, and 245 µM epinephrine), 22 µg/ml pyruvate and 50 µg/ml gentamicin, submerged in mineral oil. The semen straw (from the same bull and same batch) was previously processed in a Percoll gradient (45% and 90% concentration) to obtain a approximately final concentration of $1x10^6$ viable sperm/mL to be added to the drops where the oocytes were located and incubated at 38.5°C, 5% of CO_2 in a controlled humidity.

4.7. *In vitro* culture

The presumptive zygotes were denuded all together by multiple pipetting, into the same IVF drops they were placed before, and washed using the TCM 199 medium modified with Hepes described above. For each replicate, 60 presumptive zygotes were divided into two groups, previously described. In control group, they were divided into 2 wells on nunc plates, containing 30 presumptive zygotes per well. Each well contained 500 µl SOFaaci culture medium (containing 2.5% FBS, 5 mg/ml BSA, 22 µg/ml sodium pyruvate and 50 µg/ml gentamicin) and 150 µl mineral oil. The LM group was performed, using 3 presumptive zygotes per microbioreactor droplet using 30 µl of the SOFaaci medium. After that, they were incubated for 7 days at 38.5°C, 6% CO_2, 5% O_2 (regulated by 90% N_2) and controlled humidity. On day 7 of the culture (D7) the blastocysts were classified morphologically,

according with Bó and Mapletoft (2013) in early blastocyst, blastocyst, expanded blastocyst, and hatched blastocyst. We performed 11 replicates in total, and the IVC rate was evaluated in all of them. Seven replicates were used for gene expression analysis; thus, the blastocysts were individually frozen by snap frozen and stored at -80 ºC until RNA isolation. Moreover, the others 5 replicates were performed to obtain blastocysts for immunofluorescence analysis, thus the blastocysts were fixed in 4% PFA and stored at 4ºC.

4.8. RNA isolation

Total RNA was isolated from 5 pools of oocytes/group (n=10 oocytes/pool). From these same oocytes pools, the cumulus cells samples were recovered (5 samples/group) and total RNA was isolated. For blastocysts, the RNA was isolated from 3 pools containing 5 blastocysts in each pool (2 blastocysts and 3 expanded blastocyst), from 7 biological replicate. Total RNA from all samples was isolated with QIAzol Lysis Reagent (Qiagen), following the manufacturer's protocol, in combination with 1.33 µl GlycoBlue co-precipitant (Thermo Fisher Scientific). The quality and concentration of the RNA was evaluated in NanoDrop One (Thermo Fisher Scientific) and the total RNA was treated with DNAse I (Invitrogen; Carlsbad, CA) to avoid DNA contamination. One pool of RNA isolated from cumulus cells of the LM group was excluded from the analysis because of the result obtained in the nanodrop.

4.9. cDNA synthesis and RT-qPCR for gene expression analysis

The cDNA was synthesized using the commercial iScript Synthesis kit (BIORAD®, Hercules, USA) following the manufacturer's instruction. Real-time PCR was conducted with 7.5 ng per well analysed using the GoTaq qPCR Master Mix (Promega®, USA). In the experiment I (IVM), we evaluated the following genes in cumulus cells: *EIF4B*; *EIF4E*; *BAX*; *BCL2*; *CDK6*; *HAS2*; *GAPDH* and; *FOXO3a.* In oocytes: *GDF9*; *BMP15*; *PI3K*; *PTEN*; *FOXO3a*; *BAX* and; *BCL2*. The *ACTB* and *PPIA* genes were used as endogenous controls. Primers sequences are listed on table 1. Statistics was performed with the normalized data and the transformed data ($2^{-\Delta Ct}$) were used in the graphs.

Table 1. Bovine primer sequences used in the RT-qPCR amplification.

Gene symbol	Primer sequences (5'-3')	Accession number	Reference
ACTB	**F:** CAGCAGATGTGGATCAGCAAGC **R:** AACGCAGCTAACAGTCCGCC	NM_173979.3	(Sangalli et al., 2014)
BAX	**F:** CCCGAGTTGATCAGGACCAT **R:** CACTCCAGCCACAAAGATGG	NM_173894.1	Andrade et al. (2017)
BCL2	**F:** CTTTGTGGAGCTGTATGGC **R:** CCAGATAGGCACCCAGGG	NM_001166486.1	Andrade et al. (2017)
BMP15	**F:** GCCTCGGATCTTAGGGCATC **R:** TATGTGCCAGGAGCCTCTGA	NM_001031752.1	Designed by authors
CDK6	**F:**CTCCGAGGCCTGGACTTTCT **R:** TAGATGCGAGCAAGGCCGAA	NM_001192301.1	Andrade et al. (2017)
EIF4B	**F:** ACGACTCCAGATCTGCACCTG **R:** TCTTCACCGTCAATGGCGAGA	NM_001035028.2	Andrade et al. (2017)
EIF4E	**F:** TTAATGCCTGGCTGTGACTAC **R:** ACGATCGAGGTCACTTCGTCT	NM_174310.3	Andrade et al. (2017)
FOXO3	**F:** GCAGGGAGCGCGATATTG **R:** CGGGCACCATGAATCTGAA	NM_001206083.1	Andrade et al. (2017)
GAPDH	**F:** GCCATCAATGACCCCTTCAT **R:** TGCCGTGGGTGGAATCA	NM_001034034.2	Designed by authors
GDF9	**F:** CAGCCAGATGACAGAGCTTTGAG **R:** CACTGATGGAAGGGTTCCTGCT	NM_174681.2	Designed by authors
HAS2	**F:** CCTAAACATTTGAGACTCCCCC **R:** CACAATGCATCTTGTTCAGCTC	NM_174079.3	Designed by authors
PI3K	**F:** TCAAACGTGAAGGCAACGAG **R:** CGCCTGCTTCTTCAAGTCCT	NM_174575.1	Designed by authors
PPIA	**F:**GGTCCTGGCATCTTGTCCAT **R:** TGCCATCCAACCACTCAGTCT	NM-001077866.1	(Santos et al., 2018)
PTEN	**F:** GCCACAAAGTGCCTCGTTTACC **R:** AGAAGGCAACTCTGCCAAAC	XM_613125.6	(Santos et al., 2018)
Bta-miR-541	**F:** TGGTGGGCACAGAATCCGGCCT	NR_031188.1	(Bridi et al., 2021)

4.10. cDNA synthesis and RT-qPCR for miRNA expression

The cDNA was synthesized from 100 ng of total RNA with the miScript II RT kit (Qiagen), following the manufacturer's instructions, using the miScript HiSpec Buffer for selection of mature miRNAs. For the bta-miR-615 expression, the RT-qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems), miScript Universal Primer (Qiagen), 0,05 pg cDNA per miRNA evaluated, and specific forward primer (10µM; the sequence is listed on table 1). The device used was the QuantStudio 6 Flex (Applied Biosystems), under the following conditions: 95°C for 15min, followed by 45 cycles of 15 s at 94°C, 30 s at 55°C, and 30 s at 70°C and finally the melting curve. Ct values were normalized from bta-miR-99b to calculate the relative expression. The data were transformed through $2^{-\Delta Ct}$ to obtain the graphs.

4.11. Global DNA methylation and hydroxymethylation

On day 7, the blastocysts were collected, fixed in 4% PFA and stored at 4ºC for further analysis. Changes in global DNA methylation and hydroxymethylation were evaluated by immunofluorescence to detect 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC). The embryos were incubated with PBS containing 1% Triton X-100 for 30 min, incubated in 4N HCl for 10 min, neutralized in 100 mM Tris-HCl (pH 8.5) for 20 min. Next, the embryos were placed in PBS with 3% BSA and 0.3 M Glycine for 1 hour. Finally, the embryos were incubated with mouse-specific anti-5-mC (Abcam; ab10805) and rabbit-anti-5-hmC (Abcam; ab214728, 1:500) antibodies, diluted at a concentration of 1:1000 in PBS overnight at 4°C. After 6 washes, the embryos were incubated with goat/anti-Mouse IgG-AlexaFluor 488 (Life Tech, cat. #: A-11029), and goat/anti-rabbit IgG-AlexaFluor 594 (Life Tech, cat. #: A-11012) secondary antibody for 1 h. In total, 7 blastocysts per group were analyzed by Leica SP5 confocal microscopy. All images were captured under the same parameters, performing sequential acquisitions. For visualization of methylation excitation and emission, it was set to 488 nm and 516 nm, respectively. For hydroxymethylation excitation and emission, it was set at 543 nm and 574 nm, respectively. Confocal images of the blastocysts were captured under a 40x objective, with 3 slices at different points for each blastocyst. The analyzes were performed using ImageJ software, measuring the fluorescence of all blastomeres present in each image, discounting the background fluorescence.

4.12. Statistical analysis

Data are displayed in text and graphs as mean ± standard error of the mean. Statistical analyzes were performed using the GraphPad Prism 7 software. For all analysis outliers were identified and excluded, and the normality test was performed in the clean data by Shapiro-Wilk test. For normal data Student's t test was used: miR and mRNA expression, blastocyst size and cell counting. . For non-normal data Wilcoxon test was used: cumulus cells expansion, global DNA methylation, and hydroxymethylation. For frequency analysis (maturation and blastocyst rate) chi-square was used. A statistical difference was considered when $P<0.05$.

5. Conclusion

This was the first study in cattle that performed the IVM and IVC steps using the LM microbioreactors. Despite the difficulty we had in producing the LM droplets due to their fragility, which resulted in the loss of oocytes and embryos, we observed that the IVM rate was not altered. However, the expression of transcripts important for COCs quality and development were altered in cumulus cells. In embryo culture experiment, we had lower rate of blastocyst and lower number of blastocyst cells. This system was also able to decrease the expression of miR-615 , requiring further studies to understand the role of this modulation in the development of blastocysts. Additionally, we had an increase in the patterns of global DNA methylation and hydroxymethylation in the LM group, which can modify the accesssability of genes to be transcribed. These results are promising, considering that, even less than in the control, the blastocysts were able to develop when placed in the LM system. However, the system production technique itself needs adjustments to make its use more viable.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Capítulo 3: Bovine embryos development in *in vitro* 2.5D and 3D culture systems using alginate hydrogels

Artigo original preparado para submissão na revista *Molecular Reproduction and Development* (Impact Factor: 2.609)

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Abstract

Cells sense external signals and have mechanisms to respond them. *In vitro* cultured cells are exposed to different stimulus when compared with the tissues. Blastocysts produced *in vitro* are cultured in a sturdy petri-dish, while *in vivo* embryos develop in a soft and dynamic structure, the oviduct. Three-dimensional (3D) culture systems arise as an alternative to propitiate a more physiological environment. Thus, here we hypothesized that embryo cultures with different dimensions produced using alginate hydrogels, could improve bovine blastocysts development *in vitro*. We did conventional *in vitro* maturation followed by in vitro fertilization, obtained the prezumptive zygotes and divided them into three experimental groups: 1) Control (termed 2D) – a conventional *in vitro* culture; 2) 2.5D – embryos cultivated on top of an alginate hydrogel overlay; 3) 3D – embryos encapsulated inside of the alginate hydrogel sphere. As a result, we observed a decrease in blastocyst rate in 2.5D and 3D compared with the control. Profiling 383 bovine miRNAS, we found 3 miRNAs (miR-1246; miR-1260b, and miR-541) involved in cell proliferation being differently modulated by the 2.5D and 3D cultures. Analyzing global levels of DNA methylation and

hydroxymethylation, we observed increased levels in the 2.5D group when compared with the 2D and 3D systems. RNA-seq analysis carried out in the 3 groups of blastocysts showed alteration in several developmentally important genes altered among the groups. Altogether, these results showed that while alginate hydrogels are suitable for embryo culture, they affected the global levels of epigenetic marks, and caused alterations in the transcriptome in the resulting blastocysts. Several of the affected genes are critical for embryo development and pregnancy establishment, suggesting caution in the use of these culture system before we fully understand their effects on the health of the embryos.

Keywords: RNAseq; blastocyst; DNA methylation; DNA hydroxymethylation.

1. Introduction

In vitro embryo production (IVP) systems generate blastocysts that differ morphologically and molecularly from blastocysts produced *in vivo* (Holm & Callesen, 1998). Blastocysts produced *in vitro* have lower pregnancy rates and resistance to cryopreservation when compared with *in vivo* (Mello et al., 2016). In addition, they also have a different pattern of epigenetic marks (DNA methylation, post-translational modifications of histones) and microRNAs (miRNAs) signature caused by the culture systems (Canovas et al., 2017). These alterations can affect the embryo and even their health after birth (Bouillon et al., 2016).

The early embryonic development is marked by intense chromatin remodeling and epigenetic reprogramming, making this period particularly sensitive to any type of stressors (e.g., culture media, oxygen tension, temperature, type of culture plate). While *in vivo* embryos develop in a complex 3D environment inside the oviduct, *in vitro* usually they are cultured in petri dish plates. Plates are generally composed of polystyrene, with the embryos free in the culture medium, without physical and endocrine stimuli that *in vivo* are caused by contact with the maternal tissues. It has been demonstrated that embryos and somatic cells are capable of responding to mechanical stimuli and transforming them into biological signals within the cytoplasm, inducing activation of mechanosensitive pathways that can cause changes in gene transcription within nucleus (Martino et al., 2018).

These external physical stimuli are important, because they affect cell proliferation, differentiation and growth (Jorge et al., 2014). Petri or culture dishes are six times more rigidity than the uterine epithelium and oviduct (Kolahi et al., 2012). Thus, is important to understand the role of external stimuli during *in vitro* embryo culture. An alternative may be the three-dimensional (3D) systems, which can generate cellular microenvironments similar to that found *in vivo*, allowing the cells to interact with their surroundings in three dimensions (Woodruff & Shea, 2007).

The 3D culture systems can be produced using different types of materials, such as the hydrogels, which are structures generated from water-soluble polymers, capable of forming three-dimensional networks in response to a given stimulus (Determan et al., 2007). There are a large number of polymers that form hydrogels, and the most used in 3D cultures is the alginate (Jones & Shikanov, 2019). Alginate is a natural polymer obtained from different species of brown algae, which by the addition of a divalent ion, such as calcium, can form a stable hydrogel similar to the extracellular matrix present *in vivo* (Lee & Mooney, 2012). Moreover, alginate hydrogels have a rigidity similar to that found in reproductive tissues

(Kolahi et al., 2012). In spermatogonial stem cells, it has been shown that alginate does not change cells morphology, does not present cytotoxic and it has antioxidant characteristics, increasing cell viability (Jalayeri et al., 2017). For these reasons, this polymer has been the subject of many studies in cell and tissue engineering (Lee & Mooney, 2012). The use of alginate hydrogels to culture oocytes from goats increased the levels of progesterone and estradiol in the media, considered a good sign of oocyte competence (Brito et al., 2014). In bovine, the alginate hydrogels propitiated a platform to study the process of embryo elongation in bovine, which is normally not achieved *in vitro* (Zhao et al., 2015).

Due to the large use of *in vitro* embryo production, we aimed to investigate a possible more physiological system to culture mammalian embryos. To do that we used alginate polymer as a hydrogel, and the objectives of this study were: 1- to evaluate the development of *in vitro* produced blastocysts in two different culture systems, on top of an alginate surface (2.5D) or inside an alginate sphere (3D); 2- To investigate whether the alginate systems affect epigenetic marks in the resulting embryos; 3- To analyze the expression of candidate miRNAs in the blastocysts; 4- To sequence the mRNAs to monitor all the differences in gene expression caused by the different culture environments.

2. Results

2.1. Bovine embryo culture in an alginate decreased blastocyst rates

To observe the bovine blastocyst development in different culture systems using alginate, on day 1 after *in vitro* fertilization (IVF), we divided the presumptive zygotes in three groups to be cultivated per 7 days. Embryo culture was performed in a conventional culture system, called herein 2D group, an alginate overlay (2.5D group) and an alginate sphere to encapsulate the zygotes (3D group). We carried out 12 biological replicates and the blastocyst rate (figure 2A) was lower ($P<0.05$) in the 2.5D and 3D groups (8.8% and; 14%, respectively) compared with the 2D group (29.71%). Furthermore, we classified the morphology of these blastocysts on day 7, as described by Bó and Mapletoft (2013), and observed that the 2D and 3D groups had a similar proportion of embryos in each stage, while the blastocysts from the 2.5D group had a slightly delayed development compared with 2D and 3D groups (figure 2B).

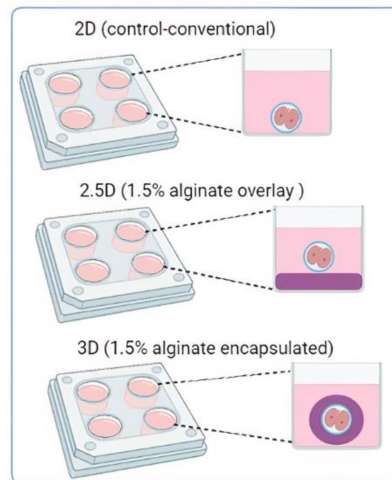

Figure 1. Experimental design demonstrating the three study groups: 2D (Control-conventional); 2.5D (1.5% alginate overlay); and 3D (1.5% alginate encapsulated). Ovaries from local slaughterhouse were aspirated for COCs *in vitro* maturation (IVM), after 22h, these COCs were *in vitro* fertilized for 18h. Then, the presumable zygotes were divided in the three experimental groups. They were cultivated until day 7 (D7) and finally collected for molecular or immunofluorescence analysis.

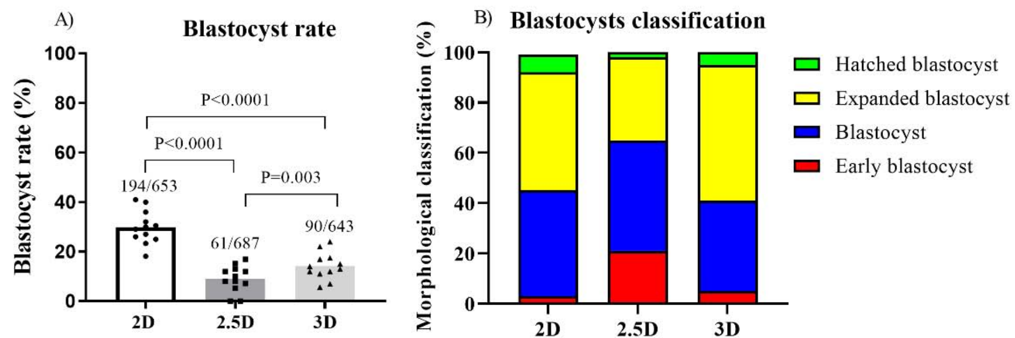

Figure 2. A) Blastocysts rates in percentage after culture in 2D, 2.5D, and 3D culture systems. P value calculated using chi square test between control vs each group. B) Proportions of blastocysts in each stage.

2.2. Alginate culture systems alter the expression of miRNAS Bta-miR-1246, miR-1260b, and miR-541 in blastocysts

To evaluate the effects of 2D, 2.5D, and 3D systems in post-trancriptional regulators in the D7 blastocysts, we performed a custom miRNAs profiler plate and observed 28 miRNAs present in all groups (figure 3A). The miRNAs miR-1246 and miR-1260b were downregulated in the 2.5D compared to the 2D group (Figure 3B-C; P=0.0269 and; P=0.0490, respectively), but in the 3D group they were similar to the control. On the other hand, miR-

541 had its expression downregulated in the 3D compared to the 2D group (Figure 3D; P=0.0331), and did not differ in relation to the 2.5D group.

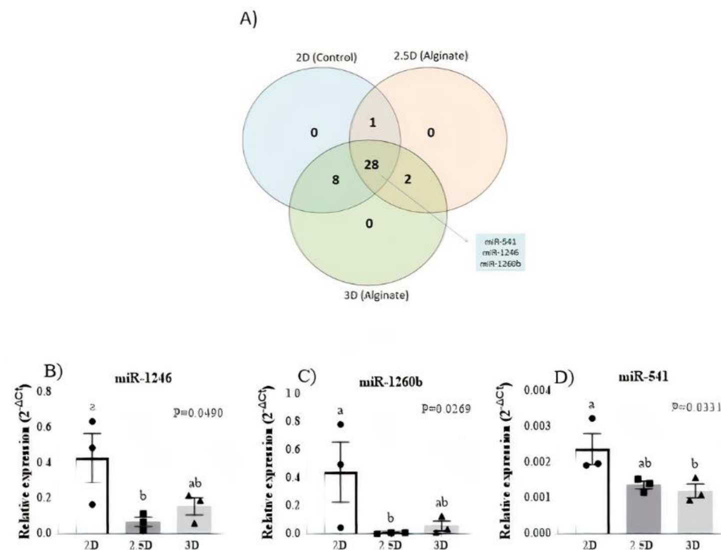

Figure 3. A) Venn diagram demonstrating the distribution of miRNAs found in blastocysts (D7) in 2D, 2.5D and 3D groups. B) miR-1246; C) miR-1260b; and D) miR-541 relative miRNAs expression in blastocysts (D7) from each experimental groups (2D, 2.5D, and 3D. P value calculated using one-way ANOVA followed by Tukey. Different letters mean statistical difference (P<0.05).

2.3. Global DNA methylation and hydroxymethylation were increased in embryos culture in 2.5D system

To determine whether the culture environment had an effect in the embryos' epigenetic marks, we investigated the global levels of DNA methylation (5-methylcytosine -5mC) and global DNA hydroxymethylation (5-hydroxymethylcytosine - 5-hmC). We observed that both global DNA methylation and hydroxymethylation (Figure 4A-C) were increased in 2.5 group compared with 2D and 3D group (P<0.0001). The 2D and 3D groups did not differ between them (P>0.05).

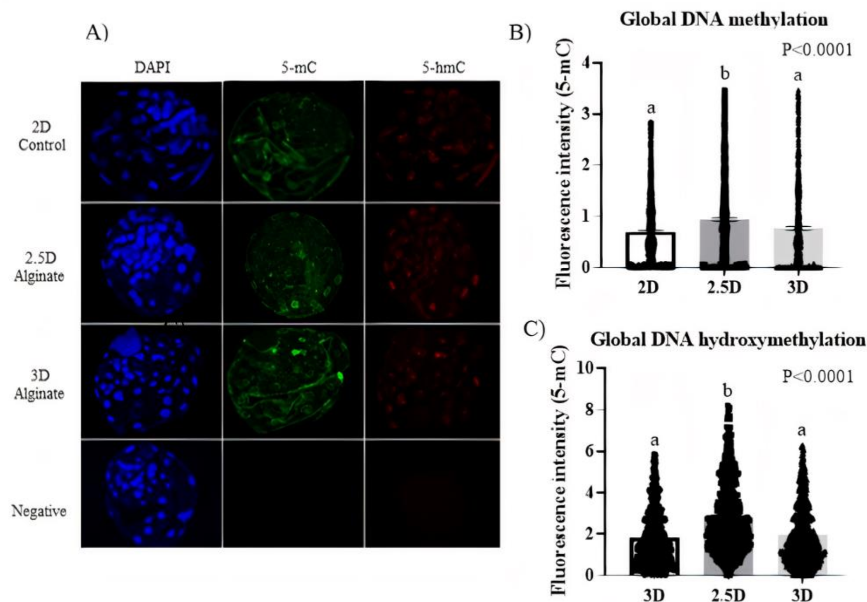

Figure 4. Global levels of DNA methylation and hydroxymethylation in blastocysts cultured in different culture systems. A) Confocal images of blastocysts from different culture systems, 2D – Control, 2.5D – Alginate and 3D – Alginate, and also a negative control was added to the experiment, without primary antibody labeling (40x objective). B) Global DNA methylation in D7 blastocysts. C) Global DNA hydroxymethylation in D7 blastocysts. Different letters represent statistical difference between groups (P<0.05), tested by one-way ANOVA.

2.4.Blastocysts transcriptomes were differently modulated depending on the culture system type (2D, 2.5D, and 3D)

To investigate the transcriptional differences in blastocyst after their development in each culture system (2D, 2.5, and 3D), we carried out the RNA-seq analysis. Firstly, we carried out Principal component analysis (PCA), and observed that the samples are distinct (figure 5A). After counting the transcripts, we had at least 22,0000 genes were commonly identified between groups. Next, we analyzed the differently expressed genes (DEGs), and as a result we observed that the gene expression profiles were divergent in blastocysts produced in different culture systems, as shown by the volcanos plot (Figure 5B-D).

Initially, blastocyst cultivated in 2D versus 2.5D, demonstrated 129 DEGs, where 67 were upregulated in the 2D group, and the most differently expressed genes were the *BOLA DMB* and *PDZD4*. On the other hand, 62 were upregulated in the 2.5D, being *EFCAB9* and *LOC510351* the most differently expressed genes. Comparing the transcriptome of 2D with the 3D group, we had 103 DEGs. In the control embryos 17 genes were upregulated in relation to 3D, being the *ENSBTAG00000001442* and *SLC14A2* the genes with the highest

difference. The 3D group had 86 upregulated genes, with the *GGT7* and *ENSBTAG00000023666* the top two most differently expressed genes. Comparing the two groups cultured in alginate hydrogels (2.5D versus 3D), we found 28 DEGs, where 9 were upregulated in the 2.5D, with the *ZBTB37* and *LOC786372* genes having the most distinct expression, and 19 genes upregulated in the 3D group, with *ENSBTAG00000003047* and *LOC510351* genes the most highly expressed between the groups. All DEGs can be found in the supplementary tables (S1-6). All together, these results showed us that the type of culture system can impact the D7 blastocysts RNA profile (figure 5E).

To gain insights about the pathways altered by the culture of embryos in alginate hydrogels, we submitted the list of DEGs to the platform DAVID Bioinformatics Resource 2021 version. As a result, we obtained 1 KEGG pathway and 6 biological process Gene Ontology (GO) for the genes up regulated in 2.5D compared with 2D system. In the genes downregulated in 2.5D compared with 2D system we found 5 KEGG pathways and 14 biological processes being regulated. In the upregulated genes in blastocysts cultured in 3D compared with 2D system, we observed 7 KEGG pathways and 4 biological processes. In the other contrasts (3D downregulated genes compared with 2D, and DEGs in 2.5D versus 3D), as we had only few DEGs, it was not identified KEGG pathways or biological processes. All KEGG pathways, biological processes, and its respective genes can be found in the supplementary tables (S7-12).

We also did a gene-by-gene search in the literature with all the DEGs, seeking for critical genes involved in pluripotency, embryo development, pregnancy establishment and cell allocation. Analyzing the 2.5D vs 2D contrasts, we found genes as *POU5F1, STC1, TXNIP*, and *MEG3* upregulated in 2.5D, while NANOG was downregulated, showing opposite effects on crucial genes involved in pluripotency. The *ISG20* gene was upregulated in 3D group compared with both 2D or 2.5D systems. Additionally, we also found the transcripts: *IFI44, RSAD2, and PARM1*, upregulated in 3D compared with 2D, while: *IL10, PTGS1* and *ELF5* were downregulated. The smallest number of DEGs (28 in total) were found when we compared 2.5D versus 3D systems. The *ISG20* is only upregulated in blastocysts from 3D system. Overall, the RNA-seq results are in accordance with the other data, showing that the 2.5D and 3D are more similar between them compared to embryos cultured in the regular 2D systems.

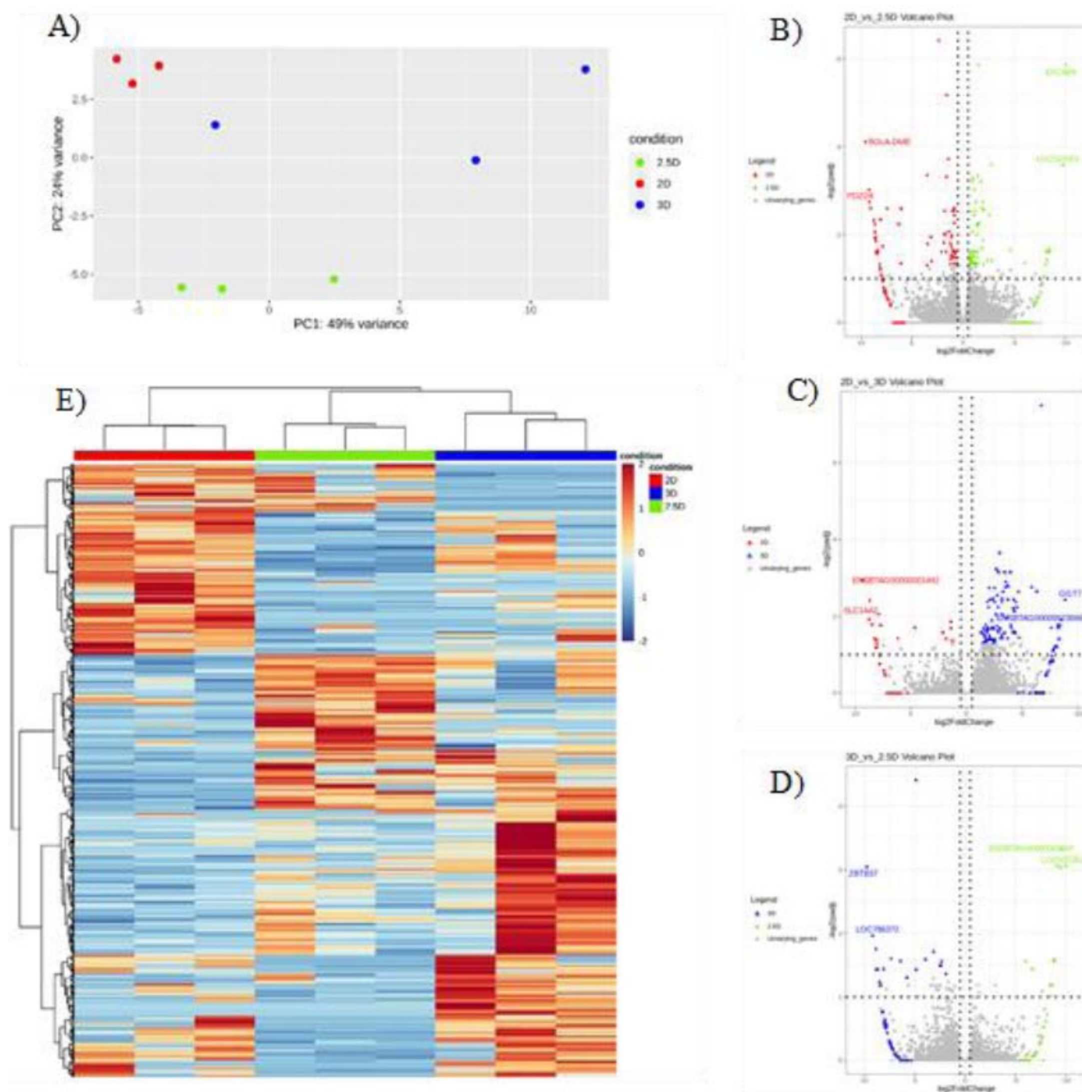

Figure 5. A) Principal component analysis (PCAs) among the groups. **B)** Volcano plot between the groups 2D versus 2.5D. **C)** Volcano plot between the groups 2D versus 3D. **D)** Volcano plot between the groups 2.5D versus 3D. **E)** Heatmap showing all DEGs among the groups.

3. Discussion

Cell's ability to interact with its surroundings can generate physical signals as a biological response through a cascade of signals, this phenomenon is known as mechanotransduction (Martino et al., 2018; Riquelme et al., 2021). Conventional *in vitro* culture (2D) is usually carried out in plastic dishes that have a greater rigidity than that found in *in vivo* tissues, such as in the reproductive tract (Kolahi et al., 2012). Therefore, we hypothesized that using a softer environment during the embryo culture could affect the blastocysts development due to the physical similarity to the reproductive tract. To test this hypothesis, we cultured the

embryos in 3 different systems: 1- conventional embryo culture (2D) in petri dishes, 2- embryo culture on the top of an alginate layer (2.5D), and 3- embryo encapsulation in alginate (3D) spheres. Through these cultures, we evaluated the developmental rates, expression of candidate miRNAs, DNA methylation and hydroxymethylation patterns, and transcriptome from the blastocysts produced by these three different systems. We observed that the blastocyst production rate dropped in cultures with alginate, being the lowest in the alginate overlayer system. In addition, alginate overlay (2.5D) increased the levels of DNA global methylation and hydroxymethylation. Also, 2.5D and 3D led to altered levels of few miRNAs and several mRNAs' transcripts.

Alginate hydrogels was used in bovine blastocysts culture to study the elongation process and did not show signals of toxicity (Zhao et al., 2015). Also, alginate is largely used in several types of cell culture (Brito et al., 2014; Jalayeri et al., 2017; Lee & Mooney, 2012). In our experiments, the blastocysts rate obtained were considerably reduced in the 2.5D and 3D groups compared to the 2D (control) group, suggesting a suboptimal condition. This evidence suggests the necessity to investigate if the hydrogels are trapping nutrients, reducing its availability for the embryos at the initial stages of development. Another possible mechanism possibly affecting embryo production can be alteration in gas diffusion caused by the alginate sphere, leading to alterations in the oxygen availability and in the pH inside the sphere, compromising embryo development. Thus, additional experiments will be necessary to answer all these questions.

To investigate the epigenetic and molecular alterations caused by the alginate hydrogels, we carried out a series of experiments to investigate the possible mechanisms. First, we carried out a miRNA profile in D7 blastocysts cultivated in different systems. We found 28 miRNAs commonly expressed in the 3 groups. Among those, the miRNAs bta-miR-1246 and bta-miR-1260b had their expression reduced in blastocysts cultured in the 2.5D system compared to the 2D and 3D systems. Regarding the miR-1246 function, it was found upregulated in heat-stressed sperm (Alves et al., 2021) and it was down-regulated in extracellular vesicles secreted by blastocysts produced *in vivo* on day 9 compared to *in vitro* (Bridi et al., 2021), suggesting that the low levels identified in our experiments could indicate a positive effect of the culture system. Furthermore, overexpression of miR-1246 in LPS-treated cells decreased cell viability, and its inhibition alleviates LPS-induced inflammatory damage (Wu et al., 2017). All these observations suggest that miR-1246 is involved in stress-responses, what can explain its upregulation in the embryos cultures under the 2.5D systems, that showed reduced embryo development. Regarding the miR-1260b, it was found

downregulated under hypoxia and its overexpression promoted cell proliferation (Seong & Kang, 2020). Since we observed it downregulated in 2.5D, supports our findings of impaired development, that can be caused by reduced capacity of the embryonic cell to proliferate. In relation to the miR-541, it is associated with suppression of cell proliferation and invasion in cancer cells (Xu et al., 2018). Herein, there was also a reduction in miR-541 in blastocysts in the 3D alginate group compared to the other systems. The proportions of expanded and hatched embryos in the 3D were smaller than the control, suggesting that the miRNAs can be modulating genes involved in cell proliferation. All these altered miRNAs are predicted to regulate important pathways that are crucial for embryo development, demonstrating that alginate hydrogels can modulate the expression of these molecules. Finally, these data suggests that embryo are able to respond differently to the 2.5D and 3D environment; although our data do not demonstrate if the decrease in blastocyst rates is negative or positive for embryo quality, since the miRNAs indicate control of cell proliferation and similarity to the *in vivo* situation.

As for the epigenetic marks, both DNA methylation and hydroxymethylation global levels were increased only in the 2.5D system. In the literature, hypermethylation is always associated with poorer developmental potential, since blastocysts produced at high oxygen tension have higher global DNA methylation than blastocysts produced in low tension (Bomfim et al., 2017; Li et al., 2016), and *in vivo*-produced blastocysts have lower DNA global methylation than *in vitro*-produced blastocysts (Salilew-Wondim et al., 2015). These results suggest that the gene expression profile among the systems can be different.

Next, we performed the transcriptome analysis using RNAseq to determine the effects of the culture systems in gene expression. The analysis showed distinct RNA profile for blastocysts produced in the three culture systems. The DEGs analysis demonstrated that a significant number of upregulated genes in 2D compared 2.5D while a larger number of DEGs upregulated in 3D group in comparison with 2D, meaning that alginate layers or capsule somehow affect the embryos in different manner.

We searched in the literature, gene-by-gene looking for genes that had already been associated with important functions in D7 blastocyst or genes well-known to be regulated in reproductive tract. As a result, we found 62 upregulated DEGs in 2.5D compared with 2D. Among them, we observed that the *POU5F1* gene (also known as *OCT4*). *POU5F1* is strongly associated with early embryo development, being essential for blastocyst formation, pluripotency, and cell differentiation in bovine (Daigneault et al., 2018; Simmet et al., 2022). In this same contrast, we also found the *STC1* gene upregulated, which has already been

related as a predictor gene for blastocysts rate and blastocysts with better kinetics and morphology in granulosa cells (Mazzoni et al., 2017), Furthermore, the *SCT1* gene was more affected in day 8 bovine *in vitro*-produced blastocysts that were transferred into utero from day 6 to day 8 than *in-vitro*-produced ones. Since its function is to regulate Ca^{2+} in tissues and cells (Muñoz et al., 2017), its downregulation can affect embryo development. *TXNIP* has an important role in apoptosis and oxidative stress (Chen et al., 2008; Schulze et al., 2004). Because it was upregulated in 2.5D system compared with 2D, this gene can be trying to protect the embryos by the stress caused by the hydrogels. Supporting this, in mice it was associated as a preimplantation stress marker, and its dysregulation is maintained even in in adipose and muscle adult tissues (Feuer et al., 2014). Another developmentally gene altered was the *MEG3*. *MEG3* is highly associated with trophoblast migration, invasion, and its inhibition may be the cause of unexplained abortions (Tao et al., 2019; Wang et al., 2021; Zhang et al., 2021). Since it was upregulated, we can suggest that culture in hydrogels can induce changes within the trophoblast layer possibly placentation and developmental competence.

Additionally, we found 67 genes being downregulated in blastocysts from 2.5D system in contrast with 2D. The most interesting gene was the *NANOG* because of its well-known essential role in forming the epiblast, that will later on to originate the embryo (Hatano et al., 2005; Miyanari & Torres-Padilla, 2012; Silva et al., 2009), meaning that the 2.5D system can affect the embryo growth beyond the blastocysts stage.

Interestingly, the *ISG20* showed higher expression in the 3D group compared to the 2D and 2.5D groups. *ISG20* is a gene regulated by IFNT (Forde et al., 2011; West et al., 2019). In mice, the expression of this gene in uterine epithelium can be restricted to the time of implantation (Pan et al., 2006), and its expression in endometrium might early indicate conceptus presence in cows (Forde et al., 2011). The fact it was increased in alginate encapsulated embryos, suggests these three-dimensional systems can stimulate genes that are essential to receptivity in cows.

In relation to the 86 genes which were upregulated in blastocysts from 3D system compared with blastocysts from 2D we found some developmentally important genes. Among them, we found the genes: 1-*IFI44,* that is involved in the blastocyst's implantation process (Manjari et al., 2018); 2- *RSAD2* that had its expression increased in D8 *in vitro* blastocysts exposed to endometrial explants, demonstrating that it probably also have a role in embryo-maternal interaction (Passaro et al., 2018); 3- *PARM1* gene, that was strongly associated with blastocyst development, number of trophectoderm and inner cell mass cells in bovine (Zolini

et al., 2019). Regarding the downregulated ones, we found 17 genes decreased in blastocysts from 3D system compared with 2D system. One of them was the *IL10*, that may also be associated with or serve as a marker of implantation process, since its increase has already been observed in the blood of women 6 days after embryo transfer (Zhao et al., 2021). We also found a downregulation in *PTGS1*. Its expression is induced by estrogen and P4, and it may have a role in implantation in mice (Wang et al., 2004). Finally, the *ELF5* was also decreased, and it is an important gene in trophectodermal lineage specification in mice, being essential for implantation and survival of the embryos (Donnison et al., 2005; Zhou et al., 2005). Thus, these results together demonstrate that the 3D system can also induce responses in the bovine blastocyst, which can be associated with an embryo quality.

Altogether, these results showed the while alginate hydrogels are suitable for embryo culture, they affected the global levels of epigenetic marks, and caused alterations in the transcriptome in the resulting blastocysts. Although several of the affected genes are critical for embryo development and pregnancy establishment, our data interpretation suggests caution in the use of these molecules before we fully understand their effects on the health of the embryos.

4. Materials and Methods

4.1. Oocyte collection and *in vitro* maturation

Cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (3-6 mm) of ovaries from a local slaughterhouse. The COCs were selected in TCM 199 with Hepes (supplemented with 0.001 g/mL of BSA, 0.2 mM of pyruvate, and 50 µg/mL of gentamicin sulfate). For each repetition, 180 COCs were selected according to their morphology (Grade I and II) and incubated for 22 hours at 38.5°C, 5% CO_2, and controlled humidity, in 100 µl drops of IVM medium (TCM 199 - GIBCO, buffered with 25 mM sodium bicarbonate, supplemented with 10% FBS, 0.2 mM sodium pyruvate, 50 µg/mL gentamicin sulfate, 0.5 µg/mL FSH – Folltropin, and 5 U/mL hCG), containing 20 COCs per drop, submerged in 4 mL of mineral oil.

4.2.*In vitro* fertilization

After 22h of IVM, the oocytes were washed with TCM 199 with Hepes and transferred to 100 µl drops of IVF-drops medium, supplemented with 6 mg/ml BSA, 5.5 IU/ml heparin, 40 µL/ml PHE (2 mM D-penicillamine, 1 mM hypotaurine, and 245 µM epinephrine), 22 µg/ml pyruvate and 50 µg/ml gentamicin, submerged in 3 mL of mineral oil. A semen straw was

previously processed in a Percoll gradient (45% and 90% concentration) to obtain a final concentration of $1x10^6$ viable sperm/mL, to be added to each drop, where the oocytes were located, and incubated at 38.5°C, 5% of CO_2 in controlled humidity for 18h.

4.3. *In vitro* culture and experimental groups

The presumptive zygotes were stripped all together into the IVF drops by multiple pipetting and washed using TCM 199 with Hepes and placed in the experimental groups. The three experimental groups were: 2D; 2.5D; and 3D. For each replicate, 60 presumptive zygotes were divided into three groups (figure 1), and each group was divided into 2 wells on 4-well plates, containing 30 presumptive zygotes per well. Each well contained 500 µl SOFaa culture medium (containing 2.5% FBS, 8 mg/ml BSA, 22 µg/ml sodium pyruvate and 50 µg/ml gentamicin) and 150 µl mineral oil. After that, they were incubated for 7 days at 38.5°C, 5% CO_2, 5% O_2 (regulated by 90% N_2), and controlled humidity. On day 7 (D7), the blastocysts were classified morphologically according with Bó and Mapletoft (2013), snap frozen in liquid nitrogen, and stored at -80ºC for further analysis. The 2D group was performed conventionally, as described above. The 2.5D group was performed using a thin layer of 1.5% alginate on the surface of the 4-well plate, this layer was adhered to the bottom and the embryos were cultured conventional media on top. In the 3D group, the embryos were encapsulated inside the hydrogel formed by the 1.5% alginate and cultured in 4-well plates covered with conventional media.

4.4. Preparation of alginate hydrogel and crosslinking solution

The alginate solution was prepared the day before the IVC. The Sodium Alginate from Sigma Aldrich-Merck (W201502) was weighed and eluted in the SOFaaci culture medium, in order to obtain a 1.5% solution. This solution was stabilized in the culture incubator. The crosslinking solution was prepared using Mili-Q water with 50 mM $CaCl_2$ and 140 mM NaCl and preheated in the incubator before cultivation. For the 2.5D group, 150 µL of 1.5% alginate solution was added to the bottom of the 4-well plate and 125 µL of the crosslinking solution was slowly added to the top, after it was placed in the incubator for 6 minutes. Then, the 125 µL of the crosslinking solution was removed with the pipette, since a firm layer of hydrogel had already formed at the bottom of the well, and it was washed twice with the SOFaaci medium. Then 500 µL of culture medium and 150 µL of mineral oil were added and the plate was incubated to stabilize. For the 3D group, first a 4-well plate were already stabilized with the SOFaaci medium. Then, the presumptive zygotes were placed in a drop of

1.5% alginate and with a volume of 7 μL, 5 denuded presumptive zygotes within the 1.5% alginate media were transferred to the crosslinking solution. In a few minutes, spheres of alginate containing embryos were solid enough for them not to fall apart. These spheres were washed again in SOFaaci and placed for culture in the previously stabilized 4-well plate, with 150 μL of mineral oil on top. On D7 the spheres were broken by gently pipetting for blastocyst collections.

4.5. RNA isolation

From the 8 experimental routines performed, 3 pools of 10 blastocysts were obtained. Total RNA from all samples was extracted with QIAzol Lysis Reagent (Qiagen), following the manufacturer's protocol, in combination with 1.33 µl GlycoBlue co-precipitant (Thermo Fisher Scientific). The purity and concentration of the RNA was evaluated in NanoDrop One (Thermo Fisher Scientific) and the total RNA was treated with DNAseI (Invitrogen).

4.6. miRNA expression

In 3 pools of blastocysts per group, a profile of 382 miRNAs was analyzed. For this, cDNA was synthesized from 100 ng of total RNA with the miScript II RT kit (Qiagen), following the manufacturer's instructions, using the miScript HiSpec Buffer, selecting just the mature miRNAs. For RT-qPCR, Power SYBR Green PCR Master Mix (Applied Biosystems), miScript Universal Primer (Qiagen), 0.05 pg cDNA per miRNA evaluated, and specific forward primers for each miRNA were used (10 µl; supplementary table). The analysis was conducted in the QuantStudio 6 Flex (Applied Biosystems), under the following conditions: 95°C for 15min, followed by 45 cycles of 15 s at 94°C, 30 s at 55°C, and 30 s at 70°C, and after that it was performed the melting curve. Ct values greater than 37 or that had more than one peak in the melting curve were excluded. Ct values were normalized with the bta-miR-99b expression to calculate the relative expression. To obtain the graphs, data were transformed using $2^{-\Delta Ct}$.

4.7. Global DNA methylation and hydroxymethylation

The blastocysts were first fixed in 4% PFA and stored at 4ºC for further immunofluorescence analysis. Changes in global DNA methylation and hydroxymethylation were evaluated by the detection of 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC). The embryos were incubated with PBS containing 1% Triton X-100 for 30 min, incubated in 4N HCl for 10 min, neutralized in 100 mM Tris-HCl (pH 8.5) for 20 min. Next, the embryos were placed in PBS with 3% BSA and 0.3 M Glycine for 1 hour. Finally, the

embryos were incubated with mouse-specific anti-5-mC and rabbit-anti-5-hmC antibodies, diluted at a concentration of 1:1000 in PBS overnight at 4°C. After 6 washes, the embryos were incubated with goat/anti-Mouse IgG-AlexaFluor 488, and goat/anti-rabbit IgG-AlexaFluor 594 secondary antibody for 1 h. In total, 7 blastocysts per group were analyzed by Leica SP5 confocal microscopy. All images were captured under the same parameters, performing sequential acquisitions. For visualization of methylation excitation and emission, it was set to 488 nm and 516 nm, respectively. For hydroxymethylation excitation and emission, it was set at 543 nm and 574 nm, respectively. Confocal images of the blastocysts were captured under a 40x oil objective, with 3 slices at different points for each blastocyst. The analyzes were performed using ImageJ software, measuring the fluorescence of all blastomeres present in each image, discounting the background fluorescence.

4.8. RNA library preparation and sequencing

Three blastocyst pools per group (each pool containing ten blastocysts) were sequenced. RNA integrity and quantity were assessed using the RNA 6000 Pico Kit and the 2100 Bioanalyzer (Agilent Technologies). RNA sequencing (RNAseq) was performed by cDNA synthesis and amplification based on the use of the SMART-Seq HT Kit RNAseq library amplification (Takaha Bio). As per manufacturer's recommendations and depending on input RNA amount, cDNA was subjected to 19 cycles of amplification. Libraries were then prepared using the Nextera XT DNA Library Prep (Illumina) and sequenced on a NextSeq 550 (Illumina) with 75-bp single-end reads. A minimum of 10 million reads was considered per sample.

4.9. Global transcripts quantification and comparison among experimental groups samples.

Data were visualized using R software, in which we primarily observed the classification, intensity, and difference in expression between groups. The quality of the reads was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The 76 bp reads were trimmed with Trimgalore and then mapped using Star (Dobin et al., 2013), and identification and quantification were performed using ARS-UCD1.2 (Ensembl and NCBI) as a reference genome using the featureCounts implemented in Rsuberead package (Liao et al., 2014, 2019) for gene count. Once the genes were identified, differential expression analysis was performed between groups using DESEq2 (Love et al., 2014) considering a padj<0,1 and an absolute log2Folchange >0.5. Additionally, we considered genes as differentially

expressed if they were exclusive, expressed in one group (expressed in all samples from the same group), and not expressed in the other group (zero counts in all samples from the same group) within comparison and using the function filterByExpr from edgeR package (Robinson et al., 2010). We estimate the hub genes using CeTF (Oliveira de Biagi et al., 2021) based on RIF—Regulatory Impact Factor and PCIT—Partial Correlation and Information Theory (Reverter & Chan, 2008; Reverter et al., 2010). Gene ontology analyses were performed using clusterProfiler (Yu et al., 2012) and pathways were explored using Pathview (Luo & Brouwer, 2013).

4.10. Statistical analysis

Data are presented as mean ± standard error of the mean. For statistical analysis of frequency, chi-square was used. To evaluate the expression of miRNAs, ANOVA was performed using the JMP7 software, followed by Tukey's test. For the analysis of global DNA methylation and hydroxymethylation, blastocyst size and cell counting it was used ANOVA followed by Tukey in the GraphPad Prism 7 software. A statistical difference was considered when $P<0.05$.

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Conflicts of interest

The authors have no conflicts of interest.

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Conclusão geral

Esta dissertação teve como objetivo principal avaliar a utilização de dois diferentes sistemas 3D (LM ou hidrogéis de alginato) na produção *in vitro* de embriões bovinos. No sistema de LM, a taxa de maturação nuclear foi semelhante com a do cultivo convencional, contudo tivemos uma diminuição na expansão e na expressão de genes importantes para o processo de maturação oocitária nas células do cumulus. Além disso, utilizamos esse sistema durante a etapa de cultivo *in vitro* e obtivemos uma menor taxa de blastocisto, diminuição na expressão do bta-miR-615 e aumento na metilação e hidroximetilação global de DNA nos embriões. No sistema de cultivo produzido a partir de hidrogéis de alginato, observamos uma diminuição na taxa de blastocisto, alterações no perfil de miRNAs, nos padrões globais de metilação e hidroximetilação, e no transcriptoma dos blastocistos entre os diferentes grupos (2D, 2.5D e 3D). Portanto, estes resultados sugerem que os cultivos 3D utilizados nos nossos experimentos tiveram um impacto negativo na taxa de produção de embriões bovinos.

Entretanto, este foi o primeiro estudo a conduzir a etapa de CIV no sistema de LM e a realizar a MIV de complexos cumulus-oócito bovinos neste sistema também. Além disso, foi o primeiro a realizar o cultivo de embriões bovinos até o dia 7 no sistema de hidrogéis de alginato. Assim, apesar dos cultivos 3D já serem bem estabelecidos em cultivos celulares, no cultivo de embriões ainda é uma alternativa muito nova. Com isso, mais estudos são necessários para estabelecer qual será o melhor tipo de cultivo 3D para ser utilizado durante as diferentes etapas da PIV. Em bovinos, a PIV vem sendo realizada a 40 anos e os resultados de produção de blastocistos que temos hoje é fruto de muitas pesquisas realizadas com o intuito de melhorar o desenvolvimento no sistema bidimensional. Por isso, os resultados obtidos aqui com o sistema 3D são muito precoces, podendo o avanço desta tecnologia ainda tornar o sistema *in vitro* mais fisiológico e melhorar o desenvolvimento e a produção dos blastocistos produzidos *in vitro*.

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Apêndices

Tabelas suplementares do capítulo 3:

Supplementary Table S1. MicroRNAs primers sequences used in quantitative RT-PCR analysis.

miRNA	Sequence	MiRNA	Sequence
bta-let-7a-3p	CTATACAATCTACTGTCTTTC	bta-miR-33b	GTGCATTGCTGTTGCATTGC
bta-miR-103	AGCAGCATTGTACAGGGCTATGA	bta-miR-340	TCCGTCTCAGTTACTTTATAGCC
bta-let-7a-5p	TGAGGTAGTAGGTTGTATAGTT	bta-miR-365-3p	TAATGCCCCTAAAAATCCTTAT
bta-miR-105a	TCAAATGCTCAGACTCCTGTGGT	bta-miR-342	TCTCACACAGAAATCGCACCCATCT
bta-let-7b	TGAGGTAGTAGGTTGTGTGGTT	bta-miR-365-5p	AGGGACTTTTGGGGGCAGATGTG
bta-miR-105b	TCAAATGCTCAGACTCCTTGGT	bta-miR-345-3p	CCTGAACTAGGGGTCTGGAG
bta-let-7c	TGAGGTAGTAGGTTGTATGGTT	bta-miR-367	GAATTGCACTTTAGCAATGGTGA
bta-miR-106a	AAAAGTGCTTACAGTGCAGGTA	bta-miR-345-5p	GCTGACTCCTAGTCCAGTGCT
bta-let-7d	AGAGGTAGTAGGTTGCATAGTT	bta-miR-369-3p	AATAATACATGGTTGATCTTT
bta-miR-106b	TAAAGTGCTGACAGTGCAGAT	bta-miR-346	TGTCTGCCCGCATGCCTGCCTCT
bta-let-7e	TGAGGTAGGAGGTTGTATAGT	bta-miR-369-5p	ATCGACCGTGTTATATTCGC
bta-miR-107	AGCAGCATTGTACAGGGCTATC	bta-miR-34a	TGGCAGTGTCTTAGCTGGTTGT
bta-let-7f	TGAGGTAGTAGATTGTATAGTT	bta-miR-370	GCCTGCTGGGGTGGAACCTGGT
bta-miR-10a	TACCCTGTAGATCCGAATTTGTG	bta-miR-34b	AGGCAGTGTAATTAGCTGATTG
bta-let-7g	TGAGGTAGTAGTTTGTACAGTT	bta-miR-371	AAGTGCCGCCATGTTTTGAGTGT
bta-miR-10b	TACCCTGTAGAACCGAATTTGTG	bta-miR-34c	AGGCAGTGTAGTTAGCTGATTG
bta-let-7i	TGAGGTAGTAGTTTGTGCTGTT	bta-miR-374a	TTATAATACAACCTGATAAGTG
bta-miR-122	TGGAGTGTGACAATGGTGTTTG	bta-miR-361	TTATCAGAATCTCCAGGGGTAC
bta-miR-1	TGGAATGTAAAGAAGTATGTAT	bta-miR-374b	ATATAATACAACCTGCTAAGTG
bta-miR-124a	TAAGGCACGCGGTGAATGCCAAG	bta-miR-362-3p	AACACACCTATTCAAGGATTC
bta-miR-100	AACCCGTAGATCCGAACTTGTG	bta-miR-375	TTTGTTCGTTCGGCTCGCGTGA
bta-miR-124b	TAAGGCACGCGGTGAATGCCAAG	bta-miR-362-5p	AATCCTTGGAACCTAGGTGTGAGT
bta-miR-101	TACAGTACTGTGATAACTGAA	bta-miR-376a	ATCATAGAGGAAAATCCACGT
bta-miR-125a	TCCCTGAGACCCTTTAACCTGTG	bta-miR-363	ATTGCACGGTATCCATCTGCG
bta-miR-125b	TCCCTGAGACCCTAACTTGTGA	bta-miR-376b	ATCATAGAGGAAAATCCATGTT
bta-miR-133b	TTTGGTCCCCTTCAACCAGCTA	bta-miR-376c	GTGGATATTCCTTCTATGTTTA
bta-miR-126-3p	CGTACCGTGAGTAATAATGCG	bta-miR-382	GAAGTTGTTCGTGGTGGATTCG
bta-miR-133c	ATTTGGTTCCATTTTACCAGC	bta-miR-376d	ATCATAGAGGAAAATCCACAT
bta-miR-126-5p	CATTATTACTTTTGGTACGCG	bta-miR-383	AGATCAGAAGGTGATTGTGGCT
bta-miR-134	TGTGACTGGTTGACCAGAGTGG	bta-miR-376e	AACATAGAGGAAAATCCACATT
bta-miR-127	TCGGATCCGTCTGAGCTTGGCT	bta-miR-409a	AGGTTACCCGAGCAACTTTGCAT
bta-miR-135a	TATGGCTTTTTATTCCTATGTGA	bta-miR-377	ATCACACAAAGGCAACTTTTGT
bta-miR-128	TCACAGTGAACCGGTCTCTTT	bta-miR-409b	GGGGTTCACCGAGCAACATTC
bta-miR-135b	TATGGCTTTTCATTCCTATGTGA	bta-miR-378	ACTGGACTTGGAGTCAGAAGGC
bta-miR-129	CTTTTTGCGGTCTGGGCTTGCT	bta-miR-410	AATATAACACAGATGGCCTGT
bta-miR-136	ACTCCATTTGTTTTGATGATGGA	bta-miR-378b	ACTTGACTTGGAGTCAGAAGGC
bta-miR-129-3p	AAGCCCTTACCCCAAAAAGCAT	bta-miR-411a	ATAGTAGACCGTATAGCGTACG
bta-miR-137	TTATTGCTTAAGAATACGCGTAG	bta-miR-378c	ACTGGACTTGGAGTCAGAAGT
bta-miR-129-5p	CTTTTTGCGGTCTGGGCTTGCT	bta-miR-411b	TGGTCGACCATAAAACGTACGT
bta-miR-138	AGCTGGTGTTGTGAATCAGGCCG	bta-miR-378d	CTGGACTTGGAGTCAGAAGACC

bta-miR-130a	CAGTGCAATGTTAAAAGGGCAT	bta-miR-411c-3p	TGTATGTCAACTGATCCACAGT
bta-miR-139	TCTACAGTGCACGTGTCTCCAGT	bta-miR-379	TGGTAGACTATGGAACGTAGG
bta-miR-130b	CAGTGCAATGATGAAAGGGCAT	bta-miR-411c-5p	GGTTGATCAGAGAACATACATT
bta-miR-140	TACCACAGGGTAGAACCACGGA	bta-miR-380-3p	TATGTAATGTGGTCCACGTCT
bta-miR-132	TAACAGTCTACAGCCATGGTCG	bta-miR-412	ACTTCACCTGGTCCACTAGCTGT
bta-miR-141	TAACACTGTCTGGTAAAGATGG	bta-miR-380-5p	TGGTTGACCATAGAACATGCGC
bta-miR-133a	TTTGGTCCCCTTCAACCAGCTG	bta-miR-421	ATCAACAGACATTAATTGGGCGC
bta-miR-142-3p	AGTGTTTCCTACTTTATGGATG	bta-miR-381	TATACAAGGGCAAGCTCTCTGT
bta-miR-142-5p	CATAAAGTAGAAAGCACTAC	bta-miR-423-3p	AAGCTCGGTCTGAGGCCCCTCAGT
bta-miR-151-3p	CTAGACTGAAGCTCCTTGAGG	bta-miR-423-5p	TGAGGGGCAGAGAGCGAGACTTT
bta-miR-143	TGAGATGAAGCACTGTAGCTCG	bta-miR-449c	AGGCAGTGCATCTCTAGCTGG
bta-miR-151-5p	TCGAGGAGCTCACAGTCTAGT	bta-miR-424-3p	CAAAACGTGAGGCGCTGCTAT
bta-miR-144	TACAGTATAGATGATGTACTAG	bta-miR-449d	GAAGGCTGTGTGCTGTGGAG
bta-miR-152	TCAGTGCATGACAGAACTTGGG	bta-miR-424-5p	CAGCAGCAATTCATGTTTTGA
bta-miR-145	GTCCAGTTTTCCCAGGAATCCCT	bta-miR-450a	TTTTGCGATGTGTTCCTAATAT
bta-miR-153	TTGCATAGTCACAAAAGTGATC	bta-miR-425-3p	ATCGGGAATGTCGTGTCCGCCC
bta-miR-146a	TGAGAACTGAATTCCATAGGTTGT	bta-miR-450b	TTTTGCAATATGTTCCTGAATA
bta-miR-154a	TAGGTTATCCGTGTAGCCTTCG	bta-miR-425-5p	ATGACACGATCACTCCCGTTGA
bta-miR-146b	TGAGAACTGAATTCCATAGGCTGT	bta-miR-451	AAACCGTTACCATTACTGAGTTT
bta-miR-154b	AGAGGTCTTCCATGGTGCATTCG	bta-miR-429	TAATACTGTCTGGTAATGCCGT
bta-miR-147	GTGTGCGGAAATGCTTCTGCTA	bta-miR-452	TGTTTGCAGAGGAAACTGAGAC
bta-miR-154c	AGATATTGCACGGTTGATCTCT	bta-miR-431	TGTCTTGCAGGCCGTCATGCAGG
bta-miR-148a	TCAGTGCACTACAGAACTTTGT	bta-miR-4523	GACCGAGAGGGCCTCGGCTGT
bta-miR-155	TTAATGCTAATCGTGATAGGGGT	bta-miR-432	TCTTGGAGTAGGTCATTGGGTGG
bta-miR-148b	TCAGTGCATCACAGAACTTTGT	bta-miR-453	AGGTTGTCCGTGGTGAGTTCGCA
bta-miR-15a	TAGCAGCACATAATGGTTTGT	bta-miR-433	ATCATGATGGGCTCCTCGGTGT
bta-miR-149-3p	GAGGGAGGGACGGGGGCTGTGC	bta-miR-454	TAGTGCAATATTGCTTATAGGGT
bta-miR-15b	TAGCAGCACATCATGGTTTACA	bta-miR-448	TTGCATATGTAGGATGTCCCAT
bta-miR-149-5p	TCTGGCTCCGTGTCTTCACTCCC	bta-miR-455-3p	GCAGTCCATGGGCATATACACT
bta-miR-16a	TAGCAGCACGTAAATATTGGTG	bta-miR-449a	TGGCAGTGTATTGTTAGCTGGT
bta-miR-150	TCTCCCAACCCTTGTACCAGTGT	bta-miR-455-5p	TATGTGCCTTTGGACTACATC
bta-miR-16b	TAGCAGCACGTAAATATTGGC	bta-miR-449b	AGGCAGTGTATTGTTAGCTGGC
bta-miR-17-3p	ACTGCAGTGAAGGCACTTGT	bta-miR-483	TCACTCCTCTCCTCCCGTCTT
bta-miR-188	CATCCCTTGCATGGTGGAGGGT	bta-miR-484	TCAGGCTCAGTCCCCTCCCGAT
bta-miR-17-5p	CAAAGTGCTTACAGTGCAGGTAGT	bta-miR-496	TGAGTATTACATGGCCAATCTC
bta-miR-18a	TAAGGTGCATCTAGTGCAGATA	bta-miR-485	AGAGGCTGGCCGTGATGAATTCG
bta-miR-181a	AACATTCAACGCTGTCGGTGAGTT	bta-miR-497	CAGCAGCACACTGTGGTTTGTA
bta-miR-18b	TAAGGTGCATCTAGTGCAGTTA	bta-miR-486	TCCTGTACTGAGCTGCCCCGAG
bta-miR-181b	AACATTCATTGCTGTCGGTGGGTT	bta-miR-499	TTAAGACTTGCAGTGATGTTT
bta-miR-190a	TGATATGTTTGATATATTAGGT	bta-miR-487a	AATCATACAGGGACATCCAGT
bta-miR-181c	AACATTCAACCTGTCGGTGAGTTT	bta-miR-500	TAATCCTTGCTACCTGGGTGAGA
bta-miR-190b	TGATATGTTTGATATTGGGTT	bta-miR-487b	AATCGTACAGGGTCATCCACTT
bta-miR-181d	AACATTCATTGTTGTCGGTGGGT	bta-miR-502a	AATGCACCTGGGCAAGGATTCA
bta-miR-191	CAACGGAATCCCAAAAGCAGCTG	bta-miR-488	TTGAAAGGCTGTTTCTTGGTC
bta-miR-182	TTTGGCAATGGTAGAACTCACACT	bta-miR-502b	AATCCACCTGGGCAAGGATTC
bta-miR-192	CTGACCTATGAATTGACAGCCAG	bta-miR-489	GTGACATCACATATATGGCGAC

bta-miR-183	TATGGCACTGGTAGAATTCACTG	bta-miR-503-3p	GGAGTATTGTTTCTGCTGCCCGG
bta-miR-193a	GGGACTTTGTAGGCCAGTT	bta-miR-490	CAACCTGGAGGACTCCATGCTG
bta-miR-184	TGGACGGAGAACTGATAAGGGT	bta-miR-503-5p	TAGCAGCGGGAACAGTACTG
bta-miR-193a-3p	AACTGGCCTACAAAGTCCCAGT	bta-miR-491	AGTGGGGAACCCTTCCATGAGG
bta-miR-185	TGGAGAGAAAGGCAGTTCCTGA	bta-miR-504	AGACCCTGGTCTGCACTCTGTC
bta-miR-193a-5p	TGGGTCTTTGCGGGCGAGATGA	bta-miR-493	TGAAGGTCTACTGTGTGCCAGG
bta-miR-186	CAAAGAATTCTCCTTTTGGGCT	bta-miR-505	CGTCAACACTTGCTGGTTTCCT
bta-miR-193b	AACTGGCCCACAAAGTCCCGCTTT	bta-miR-494	TGAAACATACACGGGAAACCTC
bta-miR-187	TCGTGTCTTGTGTTGCAGCCGG	bta-miR-532	CATGCCTTGAGTGTAGGACCGT
bta-miR-194	TGTAACAGCAACTCCATGTGGA	bta-miR-495	AAACAAACATGGTGCACTTCTT
bta-miR-195	TAGCAGCACAGAAATATTGGCA	bta-miR-539	GGAGAAATTATCCTTGGTGTGT
bta-miR-200c	TAATACTGCCGGGTAATGATGGA	bta-miR-541	TGGTGGGCACAGAATCCGGCCT
bta-miR-196a	TAGGTAGTTTCATGTTGTTGGG	bta-miR-582	TTACAGTTGTTCAACCAGTTACT
bta-miR-202	TTCCTATGCATATACTTCTTT	bta-miR-542-5p	TCGGGGATCATCATGTCACGAG
bta-miR-196b	TAGGTAGTTTCCTGTTGTTGGGA	bta-miR-584	TGGTTTGCCTGGGACTGAG
bta-miR-204	TTCCCTTTGTCATCCTATGCCT	bta-miR-543	AAACATTCGCGGTGCACTTCTT
bta-miR-197	TTCACCACCTTCTCCACCCAGC	bta-miR-592	ATTGTGTCAATATGCGATGATGT
bta-miR-205	TCCTTCATTCCACCGGAGTCTG	bta-miR-544a	ATTCTGCATTTTTAGCAAGTTC
bta-miR-199a-3p	ACAGTAGTCTGCACATTGGTTA	bta-miR-599	GTTGTGTCAGTTTATCAAAC
bta-miR-206	TGGAATGTAAGGAAGTGTGTGG	bta-miR-544b	ATTCTGCATTTCTAACAAGTTC
bta-miR-199a-5p	CCCAGTGTTCAGACTACCTGTT	bta-miR-615	GGGGGTCCCCGGTGCTCGGATC
bta-miR-208a	ATAAGACGAGCAAAAAGCTTGT	bta-miR-545-3p	ATCAACAAACATTTATTGTGTG
bta-miR-199b	CCCAGTGTTTAGACTATCTGTTC	bta-miR-628	ATGCTGACATATTTACTAGAGG
bta-miR-208b	ATAAGACGAACAAAAGGTTTGT	bta-miR-545-5p	TCAGTAAATGTTTATTGGATG
bta-miR-199c	TACAGTAGTCTGCACATTGG	bta-miR-631	AGACCTGGCTTAGACCTCAGC
bta-miR-20a	TAAAGTGCTTATAGTGCAGGTAG	bta-miR-551a	GCGACCCAATCTTGGTTTCCA
bta-miR-19a	TGTGCAAATCTATGCAAAACTGA	bta-miR-652	AATGGCGCCACTAGGGTTGTG
bta-miR-20b	CAAAGTGCTCACAGTGCAGGTA	bta-miR-551b	GGCGACCCATACTTGGTTTCAG
bta-miR-19b	TGTGCAAATCCATGCAAAACTGA	bta-miR-653	GTGTTGAAACAATCTCTGTTG
bta-miR-21-3p	AACAGCAGTCGATGGGCTGTCT	bta-miR-562	AAAGCAGCTGTACCATTTAC
bta-miR-200a	TAACACTGTCTGGTAACGATGTT	bta-miR-654	TATGTCTGCTGACCATCACCTT
bta-miR-21-5p	TAGCTTATCAGACTGATGTTGACT	bta-miR-568	ATGTATAAATGTATACACAC
bta-miR-200b	TAATACTGCCTGGTAATGATG	bta-miR-655	ATAATACATGGTTAACCTCTCT
bta-miR-210	ACTGTGCGTGTGACAGCGGCTGA	bta-miR-574	TGAGTGTGTGTGTGTGAGTGTGTG
bta-miR-211	TTCCCTTTGTCATCCTTTGCC	bta-miR-656	AATATTATACAGTCAACCTCT
bta-miR-22-5p	AGTTCTTCAGTGGCAAGCTTTA	bta-miR-658	GGCGGAGGGAAGCGGGTCCGTTGGT
bta-miR-212	ACCTTGGCTCTAGACTGCTTACT	bta-miR-758	TTTGTGACCTGGTCCACTAACC
bta-miR-221	AGCTACATTGTCTGCTGGGTTT	bta-miR-660	TACCCATTGCATATCGGAGCTG
bta-miR-214	ACAGCAGGCACAGACAGGCAGT	bta-miR-759	GCAGACTGCAAACAATTTTGAC
bta-miR-222	AGCTACATCTGGCTACTGGGT	bta-miR-664a	CAGGCTGGGGTGTGTGTGGATG
bta-miR-215	ATGACCTATGAATTGACAGACA	bta-miR-760-3p	CGGCTCTGGGTCTGTGGGGA
bta-miR-223	TGTCAGTTTGTCAAATACCCCA	bta-miR-664b	TATTCATTTATCTCCCAGCCTAC
bta-miR-216a	TAATCTCAGCTGGCAACTGTGA	bta-miR-760-5p	CCCCTCAGTCCACCAGAGCCCG
bta-miR-224	CAAGTCACTAGTGGTTCCGTTTA	bta-miR-665	ACCAGTAGGCCGAGGCCCCT
bta-miR-216b	AAATCTCTGCAGGCAAATGTGA	bta-miR-761	GCAGCAGGGTGAAACTGACACA
bta-miR-23a	ATCACATTGCCAGGGATTTCCA	bta-miR-669	TGTGGGTGTGTGCATGTGCGTG

bta-miR-217	TACTGCATCAGGAACTGATTGGAT	bta-miR-763	CCAGCTGGGAGGAACCAGTGGC
bta-miR-23b-3p	ATCACATTGCCAGGGATTACCAC	bta-miR-670	TCCCTGAGTATATGTGGTGAA
bta-miR-218	TTGTGCTTGATCTAACCATGTG	bta-miR-764	GGTGCTCACTCGTCCTTCT
bta-miR-23b-5p	GGGTTCCTGGCATGCTGATTT	bta-miR-671	AGGAAGCCCTGGAGGGGCTGGAG
bta-miR-219	AGAGTTGAGTCTGGACGTCCCG	bta-miR-767	TGCACCATGGTTGTCTGAGCATG
bta-miR-24	GTGCCTACTGAGCTGATATCAGT	bta-miR-677	CTCACTGATGAGCAGCTTCTGAC
bta-miR-219-3p	AGAATTGTGGCTGGACATCTG	bta-miR-769	TGAGACCTCCGGGTTCTGAGCT
bta-miR-24-3p	TGGCTCAGTTCAGCAGGAACAG	bta-miR-7	TGGAAGACTAGTGATTTTGTTGTT
bta-miR-219-5p	TGATTGTCCAAACGCAATTCTT	bta-miR-873	GCAGGAACTTGTGAGTCTCCT
bta-miR-25	CATTGCACTTGTCTCGGTCTGA	bta-miR-708	AAGGAGCTTACAATCTAGCTGGG
bta-miR-22-3p	AAGCTGCCAGTTGAAGAACTG	bta-miR-874	CTGCCCTGGCCCGAGGGACCGA
bta-miR-26a	TTCAAGTAATCCAGGATAGGCT	bta-miR-744	TGCGGGGCTAGGGCTAACAGCA
bta-miR-26b	TTCAAGTAATTCAGGATAGGTT	bta-miR-875	TATACCTCAGTTTTATCAGGTG
bta-miR-29d-3p	TAGCACCATTTGAAATCGATTA	bta-miR-876	TGGATTTCTTTGTGAATCACCA
bta-miR-26c	AGCCTATCCTGGATTACTTGAA	bta-miR-98	TGAGGTAGTAAGTTGTATTGTT
bta-miR-29d-5p	TGACCGATTTCTCCTGGTGTT	bta-miR-877	GTAGAGGAGATGGCGCAGGG
bta-miR-27a-3p	TTCACAGTGGCTAAGTTCCG	bta-miR-99a-3p	CAAGCTCGCTTCTATGGGT
bta-miR-29e	TAGCATCATTTGAAATCAGTGTTT	bta-miR-885	TCCATTACACTACCCTGCCTCT
bta-miR-27a-5p	AGGGCTTAGCTGCTTGTGAGCA	bta-miR-99a-5p	AACCCGTAGATCCGATCTTGT
bta-miR-301a	CAGTGCAATAGTATTGTCAAAGCAT	bta-miR-9-3p	ATAAAGCTAGATAACCG
bta-miR-27b	TTCACAGTGGCTAAGTTCTGC	bta-miR-99b	CACCCGTAGAACCGACCTTGCG
bta-miR-301b	CAGTGCAATGATATTGTCAAAGCAT	bta-miR-9-5p	TCTTTGGTTATCTAGCTGTATG
bta-miR-28	AAGGAGCTCACAGTCTATTGAG	bta-miR-1179	AAGCATTCTTTCATTGGTTGG
bta-miR-302a	AAGTGCTTCCATGTTTTAGTGA	bta-miR-92a	TATTGCACTTGTCCCGGCCTGT
bta-miR-296-3p	GAGGGTTGGGCGGAGGCTTTCC	bta-miR-1185	AGAGGATACCCTTTGTATGTT
bta-miR-302b	TAAGTGCTTCCATGTTTTAGTAG	bta-miR-92b	TATTGCACTCGTCCCGGCCTCC
bta-miR-296-5p	GAGGGCCCCCCCCAATCCT	bta-miR-1193	TAGGTCACCCGTTTGACTATC
bta-miR-302c	TAAGTGCTTCCATGTTTCAGTGG	bta-miR-93	CAAAGTGCTGTTCGTGCAGGTA
bta-miR-299	TGGTTTACCGTCCCACATACAT	bta-miR-1197	TAGGACACATGGTCTACTTCT
bta-miR-302d	TAAGTGCTTCCATGTTTTAGT	bta-miR-935	CCAGTTACCGCTTCCGCTACCGC
bta-miR-29a	CTAGCACCATCTGAAATCGGTTA	bta-miR-122	TGGAGTGTGACAATGGTGTTTG
bta-miR-3064	TTGCCACACTGCAACACCTTACA	bta-miR-940	AAGGCTGGGCCCCCGCTCCGC
bta-miR-29b	TAGCACCATTTGAAATCAGTGTT	bta-miR-1224	GTGAGGACTCGGGAGGTGGAG
bta-miR-30a-5p	TGTAAACATCCTCGACTGGAAGCT	bta-miR-95	TTCAACGGGTATTTATTGAGCA
bta-miR-29c	TAGCACCATTTGAAATCGGTTA	bta-miR-1225-3p	CCGAGCCCCTGTGCCGCCCCCAG
bta-miR-30b-3p	CTGGGAGGTGGATGTTTACTT	bta-miR-96	TTTGGCACTAGCACATTTTTGCT
bta-miR-30b-5p	TGTAAACATCCTACACTCAGCT	bta-miR-1246	AATGGATTTTTGGAGCAGG
bta-miR-328	CTGGCCCTCTCTGCCCTTCCGT	bta-miR-1247-3p	CGGGAACGTCGGGACTGGAGC
bta-miR-30c	TGTAAACATCCTACACTCTCAGC	bta-miR-1296	TTAGGGCCCTGGCTCCATCTCC
bta-miR-329a	AACACACCTGGTTAACCTTTTT	bta-miR-1247-5p	ACCCGTCCCGTGCGTCCCCGGA
bta-miR-30d	TGTAAACATCCCCGACTGGAAGCT	bta-miR-1298	TTCATTCGGCTGTCCAGATGTA
bta-miR-329b	AGAGGTTTTCTGGGTTTCTGTTT	bta-miR-1248	ACCTTCTTGTATAAGCACTGTGCTAAA
bta-miR-30e-5p	TGTAAACATCCTTGACTGGAAGCT	bta-miR-1301	TTGCAGCTGCCTAGGAGTGATTTC
bta-miR-330	GCAAAGCACACGGCCTGCAGAGA	bta-miR-1249	ACGCCCTTCCCCCCCTTCTTCA
bta-miR-30f	TGTAAACACCCTACACTCTCAGCT	bta-miR-1306	CCACCTCCCCTGCAAACGTCC
bta-miR-331-3p	GCCCCTGGGCCTATCCTAGAA	bta-miR-1260b	ATCCCACCACTGCCACCA

bta-miR-31	AGGCAAGATGCTGGCATAGCT	bta-miR-1307	ACTCGGCGTGGCGTCGGTCGTG
bta-miR-331-5p	TCTAGGTATGGTCCCAGG	bta-miR-1271	CTTGGCACCTAGTAAGTACTCA
bta-miR-32	TATTGCACATGACTAAGTTGCAT	bta-miR-1343-3p	CTCCTGGGGCCCGCACTCTC
bta-miR-335	TCAAGAGCAATAACGAAAAATGT	bta-miR-1277	TACGTAGATATATATGTATTTT
bta-miR-320a	AAAAGCTGGGTTGAGAGGGCGA	bta-miR-1343-5p	TGGGGAGCGGCCCCCGGGCGGG
bta-miR-338	TCCAGCATCAGTGATTTTGTTGA	bta-miR-1281	TCGCCTCCTCCTCTCCC
bta-miR-320b	AGCTGGGTTGAGAGGGTGGT	bta-miR-1388-3p	ATCTCAGGTTTGTCAGCCCGCA
bta-miR-339a	TCCCTGTCCTCCAGGAGCTCAC	bta-miR-1282	TCGTTTGCCTTTTTCTGCTT
bta-miR-323	GCACATTACACGGTCGACCTCT	RNT43 snoRNA	CTTATTGACGGGCGGACAGAAAC
bta-miR-339b	TCCCTGTCCTCCAGGAGCTC	bta-miR-1284	TCTGCACAGACCCTGGCTTTTC
bta-miR-324	CGCATCCCCTAGGGCATTGGTGT	Hm/Ms/Rt T1 snRNA	CGACTGCATAATTTGTGGTAGTGG
bta-miR-33a	GTGCATTGTAGTTGCATTGCA	bta-miR-1287	TGCTGGATCAGTGGTTTGAGTC
bta-miR-326	CCTCTGGGCCCTTCCTCCAG	bta-miR-1291	TGGCCCTGACTGAAGACCTGCAGT

Supplementary table 2. DEGs upregulated in 2.5 group compared with 2D group.

ENTREZ_GENE_ID	Gene Symbol	Log2FoldChange	p-adjusted
280726	ASS1	-1.28182123369624	0.00181334586052221
281056	CD247	-4.64984767773921	0.0223350608695638
281409	PLOD1	-2.07232651603002	0.00287795461712212
282015	PSMC5	-0.583564845835522	0.049868331179072
282214	CYP3A5	-1.76765463351396	0.000618568266244194
282316	POU5F1	-0.649420475703755	0.0314182155829802
282326	HTRA1	-1.55395507726355	0.00537260192171739
282462	MEA1	-0.619496770029273	0.0419439872323889
282856	PCK2	-1.05621399894537	0.0247916557708796
338078	STC1	-1.91616924066044	0.0025267487664919
359715	TRIM21	-1.12639046938994	0.0452497316100024
404187	CTSZ	-1.42737654826301	0.000456978252662449
504800	APOBR	-0.696090859834712	0.0324134768210337
505438	ARFGAP2	-0.741347141191272	0.00300326766889341
505794	KLHL41	-8.24592470530832	0.0379430487631599
506560	MYG1	-0.906872305057483	0.0247916557708796
506759	IFI16	-2.79691857065936	0.0395497420919867
506790	TXNIP	-2.38687844105742	0.0380014151765604
507473	CNPPD1	-2.01354092456356	0.0481300318783325
507839	PRMT2	-1.05232426274035	0.0454192355539663
507858	SQOR	-2.78625663014948	0.000258101397837374
509255	CDK5RAP3	-1.09113095989406	0.003536309770115
513617	ZNF622	-0.701685837588947	0.0442698060999488
513621	VAMP1	-1.43023280592551	0.0247916557708796

514669	WRAP73	-1.10870202708404	0.0308825939194644
515472	RECQL4	-1.10163055622308	0.000949292308008263
516934	RPUSD4	-1.0560788044235	0.0247916557708796
518906	GPR153	-1.36680340202452	0.00642207964083551
522144	C5AR2	-8.21852212727869	0.0247916557708796
525414	DNAH8	-1.41989710151434	0.00581574424319183
527488	SNTA1	-1.8418663908841	0.0204876818383834
533145	PHLDB3	-1.22072139717579	0.0442698060999488
533356	WDR18	-0.731965270459376	0.0379430487631599
535109	PACSIN3	-0.922360317483158	0.00863845848214188
535992	RAB11FIP5	-1.95280950089108	0.0131776178972863
536153	GPC6	-2.56370135899589	0.00362238700101371
537379	RBP1	-1.44297678365868	0.032974345687821
538998	FKBP2	-0.75208732352853	0.0256475601051833
539976	P3H1	-1.38355010684836	0.0100355626037502
540229	HSD3B7	-1.36783463332751	0.0248041186692876
613571	FUOM	-0.664135299290581	0.0283463964916156
614194	CTU2	-0.739240108071606	0.0390733116251348
614673	NUPR1	-1.49330625721758	1.42413816372017e-06
618333	EFCAB9	-10.0734557934217	1.42413816372017e-06
787106	SERPINB12	-8.49019693206925	0.0247916557708796
787811	LGALS7	-0.914158786630521	0.000618568266244194
788323	OR2T29	-8.41268400609522	0.0247916557708796
788925	PRR5	-0.77605363824037	0.0360981407957021
100125591	IRF7	-0.974055101557263	0.000527010519706765
100139548	SKIV2L	-0.942399518869576	0.0325380956070901
100298130	LGALS7B	-0.883290223525817	0.0411005113674903
100335527	MEG3	-0.890450389598758	0.0455514308707844
100337422	SIM2	-1.37748410337243	0.0452497316100024
107132821	LOC107132821	-8.41944370818012	0.0212974363721606
112445956	LOC112445956	-1.13682425795628	0.00287795461712212
C5H12orf10	C5H12orf10	-0.905061743929376	0.0247916557708796
ENSBTAG00000045898	ENSBTAG00000045898	-8.10448088825526	0.0472765497502034
ENSBTAG00000050807	ENSBTAG00000050807	-6.09497804135329	0.0442698060999488
ENSBTAG00000051369	ENSBTAG00000051369	-1.9686994410877	0.00181334586052221
LOC100139548	LOC100139548	-0.972331549137166	0.0242718592012257
LOC510351	LOC510351	-9.79928056120396	0.000258101397837374
LOC788323	LOC788323	-8.41268400609522	0.0247916557708796

Supplementary table 3. DEGs upregulated in 2D group compared with 2.5D group.

ENTREZ_GENE_ID	Gene Symbol	Log2FoldChange	p-adjusted
280832	KIT	0.85441331693115	0.0452497316100024
281170	FRZB	6.30665199518012	0.00583082281672741
281233	IBSP	8.63562132849618	0.0118196040904949
281760	FABP5	1.23687700446521	0.015663990557753
281832	HSP90AA1	0.557087807269018	0.0454192355539663
282023	PTGS2	0.994842136031616	0.0223350608695638
282191	COL4A1	0.81236668547163	0.0339872906832597
282301	PDGFRA	1.61085927900814	6.70305358372421e-06
282491	BOLA-DMB	9.57622907748399	7.77155795679665e-05
286800	CACNG2	3.15739628748593	0.0113142993554135
407767	HMGCS1	0.709940034026529	0.0247916557708796
504220	SLC7A7	0.994285634903316	0.0311951768871797
504440	EXOC1	0.940432626678191	0.0247916557708796
504657	ARHGAP29	3.52109278891545	0.000446699087448344
506945	BHLHE40	1.80839120361504	0.00287795461712212
509805	LOC509805	8.41876269652628	0.0243552457888442
510366	NIN	1.13427042193159	0.0185700424499032
511167	NRM	1.24681703673015	0.0476303154723586
512512	DNASE1L3	8.40602647474521	0.0223350608695638
512700	FLT3	1.44222685313785	0.00018882961883593
513774	AKAP12	0.859224650342642	0.00541595677826451
513856	A2M	1.39782027102093	0.00863845848214188
514255	SLC20A1	0.863345915277968	0.0025267487664919
514261	KIF5B	0.870147485010398	0.00287795461712212
515333	LOC515333	0.970249905637036	0.0247916557708796
526535	SQLE	0.909964727836089	0.0247916557708796
527740	CBFA2T2	0.973429015287242	0.0325380956070901
529416	FLRT3	1.6671595449296	0.0247916557708796
531682	CAT	1.09845098273371	0.0102657871154471
532600	TMEM165	0.916333454769726	0.0247916557708796
532836	AHCYL2	1.04310048995684	0.00287795461712212
533129	ETV4	9.19872897121618	0.00181334586052221
534319	NID1	1.63315606507379	0.000488168580846659
535194	GPHN	0.963705770609429	0.0247916557708796
536753	CPN1	8.55326558111619	0.0137711073706202
538475	PDZD4	9.21847309776031	0.000949292308008263
538951	NANOG	2.09977637297397	0.0247916557708796
539638	FAM124A	1.77297872288196	0.0102163721833037

540187	EML4	1.17920286914634	0.0354534599619758
614007	SH3BGRL	1.11866298611983	0.0293835560448444
614456	KRT6A	3.47480312518008	0.0161988408726133
615304	CPNE3	0.738088970969995	0.0457768637762308
615323	GRP	1.87663725698095	0.0152181006879066
782386	NLRP14	9.09512348719037	0.0025267487664919
785309	LOC785309	6.06300083336434	0.0025267487664919
100297935	TMEM132D	1.82036656314094	0.00942867238600327
101902345	LOC101902345	8.54670923012809	0.0247916557708796
101903068	LOC101903068	8.76315088118228	0.0047567544158746
101906311	LOC101906311	3.51233432332272	0.049868331179072
101906545	LOC101906545	7.44218888036709	0.0025267487664919
104970173	LOC104970173	1.10471130709815	0.0113142993554135
104972133	LOC104972133	8.46519927945911	0.0272056067302826
107131398	LOC107131398	0.836216437736744	0.00362238700101371
107131637	LOC107131637	8.16988899673858	0.0476303154723586
112443766	LOC112443766	8.6391067415767	0.00863845848214188
112443788	LOC112443788	8.09130576449352	0.00455387759179149
112445782	LOC112445782	2.38887221408625	3.86927717694403e-07
112447077	LOC112447077	7.88903892008004	0.0185700424499032
112449040	LOC112449040	3.07439287541075	0.0399143414726624
ENSBTAG00000012748	ENSBTAG00000012748	8.41876269652628	0.0243552457888442
ENSBTAG00000017978	ENSBTAG00000017978	8.68880966629609	0.00583082281672741
ENSBTAG00000040504	ENSBTAG00000040504	6.0495390579515	0.00259471197539851
ENSBTAG00000043567	ENSBTAG00000043567	0.764340902589985	0.0260715716188983
ENSBTAG00000043570	ENSBTAG00000043570	0.71404946830421	0.0326498395466801
ENSBTAG00000050318	ENSBTAG00000050318	6.09135518904841	0.0450549648834077
ENSBTAG00000053113	ENSBTAG00000053113	8.37791306174914	0.0248041186692876
ENSBTAG00000053900	ENSBTAG00000053900	8.56457790657961	0.0113571064503454

Supplementary table 4. DEGs upregulated in 3D group compared with 2D group.

ENTREZ_GENE_ID	Gene Symbol	Log2FoldChange	p-adjusted
280752	CNP	-1.48024534258046	0.0267671453348643
280890	PAM	-2.69223125957605	0.000587104390443897
281153	FATE1	-8.24964164905078	0.0165811067725598
281254	INHA	-4.21617918808899	0.00616519788191649
281511	SYT1	-2.35865416872822	0.0407801696473033
281611	AKAP4	-8.33304545392209	0.0387201867269703
282013	PSMB8	-3.54668874557084	0.00127012596387676

282603	PRSS2	-3.23606491190031	0.0375135933009271
287026	PLCB1	-2.06132644306084	0.0324740625442519
338078	STC1	-1.88550579992378	0.0205263414967662
353510	IFITM1	-3.94569651558334	0.000701313817965669
404163	ST3GAL4	-2.14458415700886	0.0373561637338938
404187	CTSZ	-1.48297480302979	0.0496893841636882
407107	COL4A4	-2.47389408689241	0.0205263414967662
444863	GYPC	-3.74070078243492	0.00369302621387483
504407	SPSB1	-1.53886925962288	0.0205263414967662
505184	SERPINB2	-6.34279449465267	0.00222994781490459
505632	MARCO	-3.19637565770419	0.0317650268914302
506415	RSAD2	-3.4437007312741	0.000701313817965669
506604	ISG20	-2.27139354002232	0.0126509397019394
506759	IFI16	-3.49041356251477	0.00175013053000843
507432	MAP1LC3C	-3.71385617651416	0.0373601369986782
507858	SQOR	-2.6749248080686	0.00889384205669585
508224	SCG5	-4.21457585231567	0.025003228900464
508268	CLDN11	-8.44705159185017	0.0170455340423395
508348	IFI44	-3.1955972881167	0.00347458223568008
509678	IFIT3	-6.73683415358899	3.25834255919767e-08
509823	PALMD	-4.68062300444556	0.00229147455807307
510203	CAVIN3	-3.02324389359259	0.0205263414967662
510359	TSC22D1	-1.67986788070547	0.0440650857935916
510496	PLAC9	-2.8818232595547	0.000701313817965669
510658	SLC2A6	-4.3079439898393	0.00754885918462556
510773	PID1	-2.02066182679198	0.0212590282099714
511575	GPX8	-2.37830656620437	0.00347458223568008
511799	P3H2	-1.46511136355834	0.0496893841636882
512106	MAP7D1	-2.53161031243187	0.00429667176247899
512999	MANSC1	-3.72471209337387	0.00162840085976585
514394	UCHL1	-2.09220176286788	0.00369302621387483
514474	TP53BP2	-1.29199563159002	0.0262034058239338
514889	IFNGR2	-4.02080581780257	0.0202866413822475
515224	HAPLN3	-2.52456179395406	0.0440650857935916
515367	FSTL3	-1.84260251721589	0.0252000783864851
515593	MAP1A	-2.01755025848115	0.00183024867340268
515731	PLXDC2	-1.63805128324987	0.0343953179310027
517545	RGS6	-2.82043633182319	0.0232807434293413
517768	HS6ST2	-3.17751207185448	0.00429667176247899
518368	PARM1	-1.89408585467031	0.00429667176247899

518845	PRSS50	-4.19795791948248	0.0266755501867102
519758	ATP1B1	-1.85804030571383	0.0223070282954232
522049	CDC20B	-1.80347629720769	0.046363513592155
522392	MXRA8	-2.27620288015729	0.0205263414967662
526865	PVR	-2.77621911212913	0.00283462082444857
528099	RAB32	-2.09026429933745	0.0407801696473033
530164	SLC17A5	-1.57141439302074	0.0496893841636882
532442	RTP4	-4.23621253004267	0.00369302621387483
534129	CREB5	-1.98454403695126	0.00582239253574006
534626	GRIA3	-1.81285293190426	0.0399899323158629
535439	SMARCA1	-2.76340326630871	0.0178071277165469
536607	ACTN2	-4.39956032612458	0.0267671453348643
537379	RBP1	-2.52275330227786	0.00183024867340268
538062	EDIL3	-3.29223195580721	0.00226688373582511
538437	GEM	-3.63556438212541	0.010603280269374
538466	TECTB	-8.22874523859037	0.0202866413822475
540318	ZNF664	-1.7423885573535	0.0170455340423395
613787	PDGFC	-2.3540059179062	0.0196774650713736
614348	RGCC	-3.99236869148379	0.0170455340423395
615813	ITPKA	-2.41602267701928	0.0496893841636882
615929	GGT7	-8.88572791268194	0.00369302621387483
768028	BEX2	-1.29446366284484	0.0293655420011335
781681	GLDN	-4.73341194502748	0.0359288262160204
781806	FNDC4	-4.44397350905126	0.00429667176247899
784747	ZFPM2	-2.88988627795985	0.0266755501867102
785475	CDH11	-2.54532949590765	0.0394178685396362
785924	ROR2	-2.98867130813389	0.000221595020301482
100125309	TSC22D3	-3.23505230827477	0.00889384205669585
100125760	DPP7	-2.86066514974358	0.00585042831481341
100140421	GCKR	-2.47254720869902	0.046363513592155
100300400	CLOCK	-1.64952843442841	0.0317650268914302
104968447	HS3ST4	-3.42776775382814	0.0267671453348643
112441657	LOC112441657	-7.64248491284883	0.0202866413822475
112442048	LOC112442048	-8.27875843277875	0.0196774650713736
112448058	LOC112448058	-5.86944403741009	0.00175013053000843
ENSBTAG00000023666	ENSBTAG00000023666	-8.48185183849812	0.0122743818150494
ENSBTAG00000051369	ENSBTAG00000051369	-1.9294103649497	0.0266755501867102
ENSBTAG00000053138	ENSBTAG00000053138	-4.53876229138195	0.00582239253574006
ENSBTAG00000054391	ENSBTAG00000054391	-3.06476597962763	0.0373601369986782

Supplementary table 5. DEGs upregulated in 2D group compared with 3D group.

ENTREZ_GENE_ID	Gene Symbol	Log2FoldChange	p-adjusted
281246	IL10	8.10921947640496	0.0399899323158629
282022	PTGS1	4.64405974793318	0.0196774650713736
505671	RFTN2	1.83186329425103	0.0387201867269703
506404	CENPK	1.25372267241712	0.0442470315738893
528021	ANKRD13C	8.4860558507193	0.0164913687494165
528647	VSX2	8.06325191824682	0.0496893841636882
537150	SLC14A2	8.74134604472184	0.0120885054006015
538731	ALG8	1.38464074908324	0.0201847849441337
539420	ELF5	6.15973746379416	0.0373601369986782
613358	MT1E	2.39003051721811	0.00120049834862865
784785	ZNF473	2.12624056238199	0.0266755501867102
104975030	LOC104975030	8.28692061237668	0.0373601369986782
112448786	LOC112448786	7.89078503023794	0.0086706369404078
ENSBTAG00000001442	ENSBTAG00000001442	9.32711479622556	0.00114707017836248
ENSBTAG00000017978	ENSBTAG00000017978	8.69387861901925	0.00386490838973485
ENSBTAG00000048434	ENSBTAG00000048434	7.70894311236953	0.0170455340423395
ENSBTAG00000053747	ENSBTAG00000053747	1.3920242962898	0.0138858598428916

Supplementary table 6. DEGs upregulated in 2.5D group compared with 3D group.

ENTREZ_GENE_ID	Gene Symbol	Log2FoldChange	p-adjusted
515382	SCRG1	-8.88563194311224	0.0257743173024902
516043	APOBEC1	-9.51826074262124	0.000907580496405638
520023	LOC520023	-9.13187493145086	0.000868991889497393
520133	ADAMTS16	-6.6954494182941	0.0364380155610718
617830	POU3F1	-6.07150572446827	0.0275780255702947
ENSBTAG00000003047	ENSBTAG00000003047	-9.63165099269118	0.000468084854840669
ENSBTAG00000035530	ENSBTAG00000035530	-8.74586494211748	0.0275780255702947
ENSBTAG00000048900	ENSBTAG00000048900	-8.82319847884434	0.0275780255702947
LOC510351	LOC510351	-10.0286209547863	0.000868991889497393

Supplementary table 7. DEGs upregulated in 3D group compared with 2.5D group.

ENTREZ_GENE_ID	Gene Symbol	Log2FoldChange	p-adjusted
353510	IFITM1	4.87127928705006	3.87785299463253e-05
506604	ISG20	2.33124013482553	0.0275780255702947
507432	MAP1LC3C	2.36901220662002	0.0318208285093466
507859	NCF4	8.82434791282612	0.0373133047828006
521580	LOC521580	6.41737729813933	0.0275780255702947
531084	PADI6	8.68464401919553	0.0368859112730354
531659	C1QTNF3	3.1253713779423	0.0193993147175048

535376	TGFBR2	1.90982364205933	0.0433531822649895
538691	HS3ST1	3.9380982608576	0.0257743173024902
615254	PHF24	4.85171209316065	0.0373133047828006
617314	ZBTB37	9.75984922598326	0.000907580496405638
786372	LOC786372	9.18509077518692	0.0109241447935517
100295249	HOXC8	8.68562105184465	0.0364380155610718
101902345	LOC101902345	8.85594387098515	0.0177007373513034
101906545	LOC101906545	7.34710068889098	0.0249637555942031
112443766	LOC112443766	8.69286296635011	0.0364380155610718
112446374	LOC112446374	2.46257869978965	0.0318163699622503
112449112	LOC112449112	8.09945734184364	0.0364380155610718
ENSBTAG00000053070	ENSBTAG00000053070	5.77887961544633	0.0499891835779205

Supplementary table 8. KEGG pathways of genes **upregulated** in blastocysts cultured in **2.5D** compared with 2D system.

KEGG pathway	Genes	P-Value
NOD-like receptor signaling pathway	*IFI16/IRF7/TXNIP*	8,0E-2

Supplementary table 9. KEGG pathways of genes **downregulated** in blastocysts cultured in **2.5D** compared with 2D system.

KEGG pathway	Genes	P-Value
Pathways in cancer	*EML4/KIT/COL4A1/FLT3/HSP90AA1/ PDGFRA/PTGS2/LOC104970173*	6,7E-4
PI3K-Akt signaling pathway	*KIT/COL4A1/FLT3/HSP90AA1/ IBSP/PDGFRA*	4,1E-3
Central carbon metabolism in cancer	*KIT/ FLT3/ PDGFRA*	1,6E-2
Hematopoietic cell lineage	*KIT/ FLT3/ BOLA-DMB*	4,0E-2
MAPK signaling pathway	*KIT/ CACNG2/ FLT3/ PDGFRA*	5,0E-2

Supplementary table 10. KEGG pathways of genes **upregulated** in blastocysts cultured in **3D** compared with 2D system.

KEGG pathway	Genes	P-Value
Thyroid hormone synthesis	*ATP1B1/ CREB5/ GPX8/ PLCB1*	2,9E-3
Dopaminergic synapse	*CREB5/ CLOCK/ GRIA3/ PLCB1*	1,4E-2

Insulin secretion	*ATP1B1/ CREB5/ PLCB1*	4,1E-2
Aldosterone synthesis and secretion	*ATP1B1/ CREB5/ PLCB1*	5,1E-2
Pancreatic secretion	*ATP1B1/ PLCB1/ PRSS2*	5,6E-2
Protein digestion and absorption	*ATP1B1/ COL4A4/ PRSS2*	8,6E-2
Relaxin signaling pathway	*CREB5/ COL4A4/ PLCB1*	8,7E-2

Supplementary table 11. Biological process in GO of genes **upregulated** in blastocysts cultured in **2.5D** compared with 2D system.

Biological process	Genes	P-Value
Negative regulation of calcium ion transport	*PACSIN3/STC1*	1,1E-2
Negative regulation of NF-kappaB transcription factor activity	*CDK5RAP3/PRMT2/TRIM21*	1,4E-2
Positive regulation of proteasomal protein catabolic process	*NUPR1/PSMC5*	3,8E-2
Positive regulation of cell cycle	*NUPR1/TRIM21*	5,1E-2
Regulation of cyclin-dependent protein serine/threonine kinase activity	*CDK5RAP3/CNPPD1*	8,7E-2
Skeletal muscle cell differentiation	*KLHL41/NUPR1*	1,0E-1

Supplementary table 12. Biological process in GO of genes **downregulated** in blastocysts cultured in **2.5D** compared with 2D system.

Biological process	Genes	P-Value
Positive regulation of kinase activity	*KIT/ FLT3/ PDGFRA*	9,3E-3
Postsynaptic neurotransmitter receptor diffusion trapping	*CACNG2/GPHN*	1,8E-2
Transmembrane receptor protein tyrosine kinase signaling pathway	*KIT/ FLT3/ PDGFRA*	2,0E-2
Regulation of cell proliferation	*KIT/ PTGS2/ SQLE*	2,5E-2
Mast cell degranulation	*KIT/ GRP*	2,7E-2
Centrosome localization	*KIF5B/ NIN*	3,8E-2
Extracellular matrix organization	*COL4A1/ IBSP/ PDGFRA*	4,1E-2
Digestive tract development	*KIT/ GRP*	4,5E-2
Positive regulation of cell migration	*KIT/ CPNE3 / PDGFRA*	5,0E-2

Neuromuscular junction development	*CACNG2/ COL4A1*	5,8E-2
Stem cell differentiation	*KIT/NANOG*	6,0E-2
Cellular response to growth factor stimulus	*CPNE3/IBSP*	6,6E-2
Phosphatidylinositol-mediated signaling	*EXOC1/ PDGFRA*	8,1E-2
Cellular response to amino acid stimulus	*COL4A1/ PDGFRA*	1,0E-1

Supplementary table 13. Biological process in GO of genes **upregulated** in blastocysts cultured in **3D** compared with 2D system.

Biological process	Genes	P-Value
Negative regulation of viral genome replication	*IFITM1/ ISG20/ RSAD2*	8,2E-3
Cell adhesion	*ATP1B1/ CLDN11/ COL4A4/ HAPLN3/ MXRA8*	3,0E-2
Defense response to virus	*IFIT3/ IFITM1/ ISG20/ RSAD2*	3,5E-2
Embryonic organ development	*PDGFC/ ZFPM2*	9,6E-2