Open Veterinary Journal, (2023), Vol. 13(2): 143–149
ISSN: 2226-4485 (Print)
ISSN: 2218-6050 (Online)
Review Article
DOI: *10.5455/OVJ.2023.v13.i2.2*

Submitted: 01/11/2022 Accepted: 17/01/2023 Published: 04/02/2023

Analysis of mice (*Mus musculus* L.) and hamster embryo development using culture and vitrification medium: Systematic review

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Abstract

Background: The success of embryo production is largely determined by the accuracy of making medium formulations that are adapted to the age of embryo growth. It is known that the cryopreservation method is widely used for the vitrification of embryos frozen at −196°C.

Aims: This study aimed to analyze the embryonic development of mice (*Mus musculus* L.) and hamsters using culture and vitrification media.

Method: This method uses the preferred guide to reporting items for systematic review and meta-analysis.

Results: Based on the search results, a total of 700 articles were obtained which then entered the elimination stage, resulting in 37 articles on the development of mice embryos (*M. musculus* L.) and hamsters using culture and vitrification media.

Conclusion: Thus, it can be concluded that the identification of the embryonic development of mice (*M. musculus* L.) and hamsters can be used with the use of culture media and the development of vitrification methods.

Keywords: Embryo, *Mus musculus*, Hamster, Culture, Vitrification media.

Introduction

In the practice of embryo production to obtain embryos with superior genetic potential, the aggregation method is often used, because this method is considered the cheapest and easiest method only by utilizing the potential of the embryo. Embryos to be aggregated can come from two sources; first are non-clonal embryos, namely embryos which are growths from zygotes without any treatment, while the second is clonal embryos obtained from splitting results. Several problems are often encountered in vitro embryo production, both related to internal and external factors. One example of a problem that is often encountered is the low level of viability of aggregated embryos. This low viability value is often caused by the absence of a match in the selection of embryonic stages that will be used as a source for the aggregation process with the medium to be used. The biggest obstacle to in vitro embryo production through the aggregation method is the occurrence of the "cell block" phenomenon in embryo growth. The success of embryo production is again largely determined by the accuracy of making a medium formulation that is adapted to the growth age of the embryo to be used. There are several types of medium as a source of nutrients to support the growth and development of embryos, ranging from simple compound media, such as M16 (Verschoor *et al.*, 2003; Kurniawati, 2006) used for the growth of mouse embryos, CR1AA medium (Rosenkrans and First, 1994) for embryonic growth of sheep and goats (Verschoor *et al.*, 2003) to more complex ones such as tissue culture medium 199 which is widely used for embryo growth from laboratory animals and livestock (Boediono *et al.*, 1995; Verschoor *et al.*, 2003). In preparing the compound medium, there are basic things that must be considered, namely the source of water used as a solvent, and physical properties such as osmolarity and pH, and the composition of the media that is adjusted based on the needs for embryo growth and development.

The most important benefit and application of double vitrification are related to the right time of embryonic development to be transferred to each patient. For example, if there is an excess number of zygotes in an IVF program patient, these zygotes can be frozen immediately or cultured in vitro until the blastocyst stage of development, and then frozen again for future transfer. Pregnancy failure due to OHSS can be overcome by repeating the transfer of previously frozen embryos after the patient's uterus is in normal condition. The indicator of the success of vitrification is the viability of the post-vitrified embryo. Embryo viability includes survival (survival rate) and successful development of each stage of the embryo (development

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rate). The research results of Murakami *et al.* (2011) showed that double vitrification in human embryos with cryotop containers still had high viability, which was around 98.1%, and resulted in normal live births. The purpose of this study was to analyze the embryonic development of mice (*Mus musculus* L.) and hamsters using culture and vitrification medium.

Materials and Methods

This review uses a Systematic Review approach that is based on the Preferred Report Items for Systematic Reviews and Meta-analysis. The literature search was carried out in August to September 2022, in several databases, namely PubMed, ScienceDirect, NCBI, and Elsevier, with an article publication time span of 1973–2022. The inclusion criteria consisted of articles reporting "development of mouse embryos, (*Mus musculus* L.), hamsters, culture media, vitrification." Based on the search results, 37 articles were found which were then included in the review results in this article. Then, the writing of this manuscript reviews articles based on abstracts and full texts for further description in order to find similarities and differences in each study and draw conclusions.

Results

Based on the results of a search conducted in PubMed, ScienceDirect, NCBI, and Elsevier, a total of 700 articles were obtained which then entered the elimination stage, resulting in 37 articles. Figure 1 showed the disintegration of the zona pellazida of 8-cell embryos with 0.25% pronase in the drop of KSOMaa medium. Figure 2 showed the preparation of embryo pairs ready for aggregation in KSOMaa culture medium. Figure 3 showed the aggregation of embryos in KSOMaa culture medium. Figure 4 showed the morphology of the blastocyst in the freezing process with double vitrification. On the other side, this result of this research also showed the percentage of embryonic quality of mice (*M. musculus*) before and after vitrification and thawing (Table 1), the percentage of zygotes able to pass cell block on M16 and HTF medium (Table 2), and development of 8-cell stage mouse and hamster embryos after 48 hours of culture (Table 3).

Discussion

Embryo aggregation in 8-cell division stage on KSOMaa culture medium

Embryo aggregation or embryo fusion to produce embryos with high viability values can be carried out at an early stage of embryonic development until before the embryo reaches the compact morula stage (Kelly *et al.*, 1978). In this study, the embryos used in the aggregation process were embryos with an 8-cell division stage and were proven to be capable of producing embryos with high viability values.

The blastomeres of the 8-cell stage embryo usually form a loose configuration with plenty of space between the blastomeres. After the blastomeres undergo the next division, the blastomeres will undergo dramatic physiological changes to grow and develop into the next stage. During the growth and development stage, the agglomerated embryos continuously undergo changes, namely an increase in diameter, transcriptional activity, and cytoplasmic content. During cytoplasmic maturation, molecular and structural changes occur, namely a rapid increase in the number and size of organelles such as ribosomes, fat globules, Golgi apparatus, and mitochondria. Based on the research that has been done, it has been proven that the KSOMaa medium is able to stimulate the growth and development of the embryo at the stage of 8-cell division in the aggregation process so that it can produce aggregated embryos with high viability (Nieminen *et al.*, 2004).

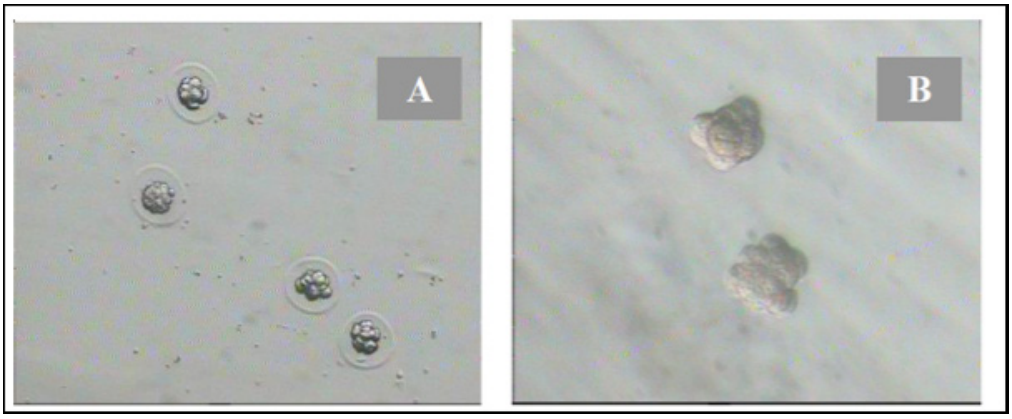

Fig. 1. Disintegration of the zona pellazida of 8-cell embryos with 0.25% pronase in the drop of KSOMaa medium. (A): 8-cell-stage embryos with the decay of the zona pellucida under the influence of pronase. (B): 8-cell stage of embryos without zona pellucida that were ready to be aggregated (Nieminen *et al.*, 2004).

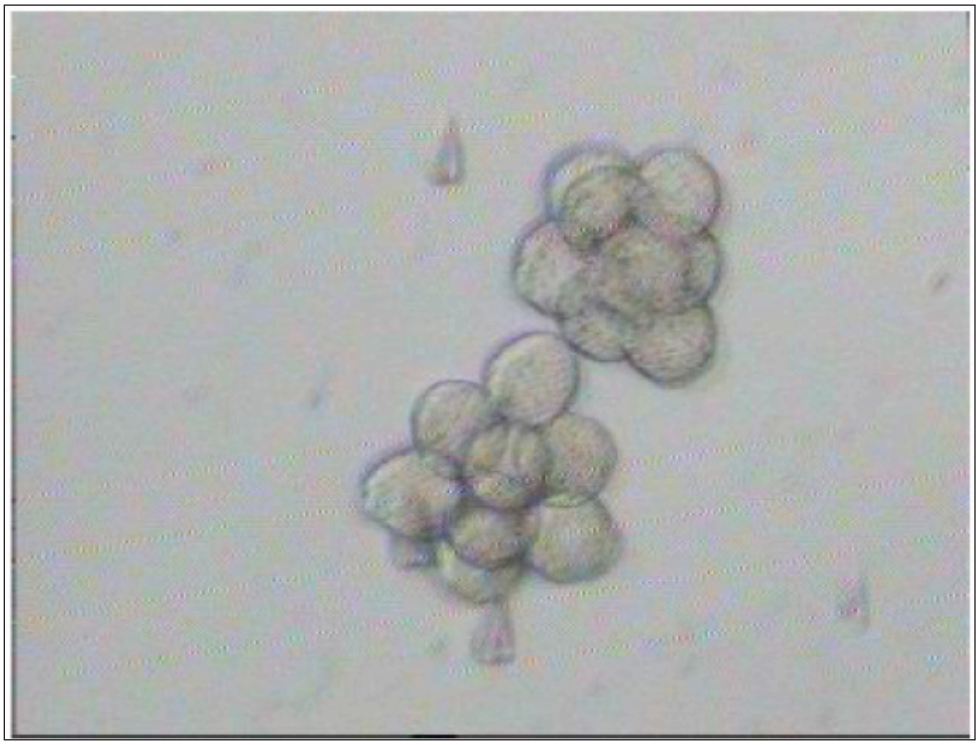

Fig. 2. Preparation of embryo pairs ready for aggregation in KSOMaa culture medium (Nieminen *et al*., 2004).

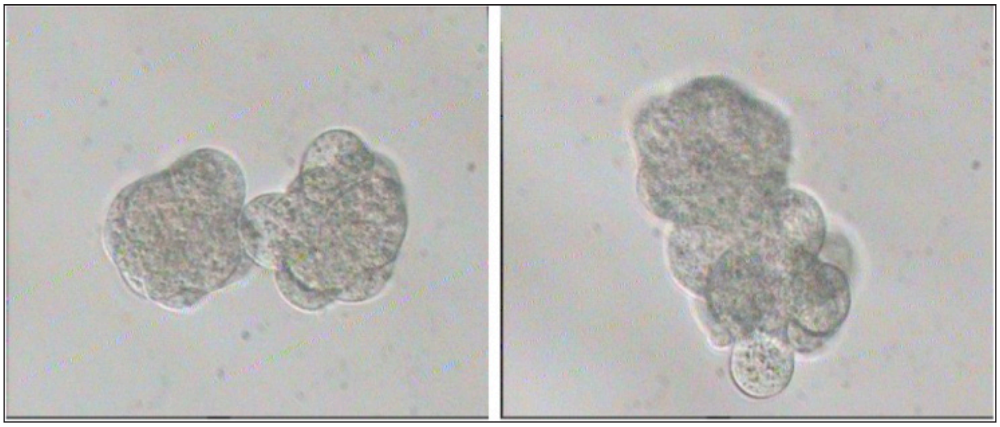

Fig. 3. Aggregation of embryos in KSOMaa culture medium. Left: early aggregation. Right: advanced aggregation (Nieminen *et al*., 2004).

Viability of mice (M. musculus albinus) embryo after cryopreservation with double vitrification

The success of vitrification cannot be separated from the optimization of each vitrification stage, starting from equilibration, vitrification, and heating, to in vitro culture. Equilibration of embryos in cryoprotectants prior to freezing was carried out to withdraw water and replace water with intracellular cryoprotectants. In mouse embryos, water and intracellular cryoprotectants will seep slowly by diffusion through the aquaporin protein on the plasma membrane (Kasai and Edashige, 2007) so that the perivitelline space will appear looser, the period and temperature of equilibration depending on the cryoprotectant used, taking into account the factors to minimize toxicity and osmotic pressure caused by cryoprotectants. The percentage of survival after multiple vitrification obtained in this study was 92.5%. Double vitrification has been proven to be successful because its viability is more than 80% (Saftiany, 2011), although, it is lower than the study of Murakami *et al.* (2011) who performed double vitrification with a cryotop container in embryos at

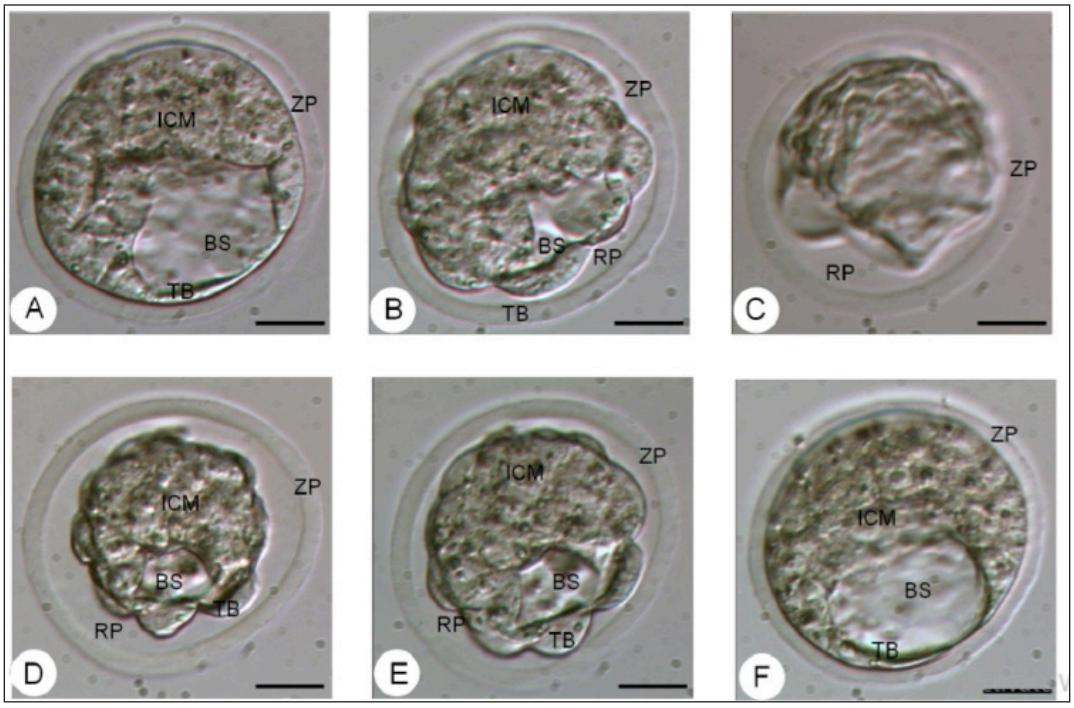

Fig. 4. The morphology of the blastocyst in the freezing process with double vitrification. (A): Blastocyst before vitrification. (B): Blastocyst exposed to equilibration medium, shrinkage occurs which is indicated by the widening of the perivitelline cavity (RP). (C): Blastocyst exposed to vitrified medium, maximal shrinkage occurs. (D–E): Blastocyst exposed to medium warming. (F): Blastocysts after two hours of in vitro culture. RP: Perivitelin Room; ZP: Zona Pellucida; BS: Blastosul; ICM: Inner Cell Mass; TB: Trophoblast (Bar: 20 µm).

Table 1. Percentage of embryonic quality of mice (*M. musculus*) before and after vitrification and thawing (Kusumaningtyas *et al.*, 2005).

Embryo quality	Number of embryos (%)	Number of Morula (%)			Number of Blastocysts (%)			Number of Embryo shrinking)
		Cannot be compressed	incompressible	Total	Beginning	Carry on	Total	
Before vitrification	204 (100)	13 (6.38)a	22 (10.78)a	35 (17.16) a	102 (50)a	67 (32.84)a	169 (82.84)a	0 (0)a
After vitrification	174 (85.3)	10 (5.75)a	11 (6.32)a	21 (12.07) a	58 (33.33) a	37 (21.27)b	95 (54.60)b	58 (33.33) b
Embryo missing	30 (14.7)	-	-	-	-	-	-	-

the pronuclear development stage and continued at the blastocyst stage at 98.1%.

Quality, implantation ability, and in-vivo viability of mice embryo (M. musculus) Swiss Webster strain after freezing by vitrification method

The results of the study (Kusumaningtyas *et al.*, 2005) showed a decrease in embryo quality after vitrification and thawing up to 33.33%. The decline in embryo quality is quite high compared to the research of Lopatářová on bovine embryos (Lopatářová *et al.*, 2002). By using the medium added 16.5% EG + 16.5% DMSO + 0.5 M sucrose, Lopatarova showed a high recovery rate of around 92.9% for the bovine embryos used. In this experiment, Lopatarova used a longer incubation time of 72 hours, compared to the time we used in this study (24 hours) (Lopatářová *et al.*, 2002). When the embryo is exposed to a vitrification medium, water will undergo exosmosis and a certain amount of ethylene glycol (EG) and dimethyl sulfoxide (DMSO) will enter the embryo cells. At the same time, sucrose will help the dehydration of cells, as a result, the cells will shrink (Saha *et al.*, 1994). At the time of thawing the embryos were gradually exposed to some hypertonic medium containing sucrose. This hypertonic medium can prevent a very fast influx of water into the cells. At the same time, EG and DMSO will leave the cell

Table 2. Percentage of zygotes able to pass cell block on M16 and HTF medium (Pusporini and Arifin, 2012).

Medium treatment	Number of zygotes	Develop into 2 cells	Not Developing/Degenerating
M16	148	132 (85.09 ± 15.88)a	16 (14.91 ± 16.69)a
HTF	136	118 (83.36 ± 22.74)a	18 (16.54 ± 11.32)a

M16: medium 16; HTF: human tubal fluid. The same superscript in the same column is not significantly different ($p > 0.05$).

Table 3. Development of 8-cell stage mouse and hamster embryos after 48 hours of culture (Rosadi *et al.*, 2008).

Treatment	Number of embryos	Developmental stage and number of developing embryos (%)				
		MK	BAw	Bl	BLj	H/ed
Mice-KSOaa	140	140 (100)a	140 (100.0a	129 (92.1)a	129 (92)a	32 (22.9)b
Mice-HECM-6	82	82(100.0)a	82(100.0)a	82(100.0)a	48(92.3)b	30(87.7)c
Hamster-KSOaa	60	60(100.0)a	60(100.0)a	81(88.0)a	29(48.3)a	0(0.0)a
Hamster-HECM-6	46	46(100.0)a	46(100.0)a	46(100.0)a	46(100.0)a	34(73.9)a

MK: Morula compact; Bottom: early blastocyst; Bl: blastocyst; BLj: late blastocyst; H/ed: hatching/hatched. Different superscripts in the same column showed significantly different ($p < 0.05$).

thereby decreasing the concentration gradient between intracellular and extracellular. This flow of EG and DMSO can stop the movement of water across the cell membrane, thus preventing cell lysis during the diffusion of EG and DMSO outward (Ozkavukcu and Erdemli, 2002). The embryo expands again because water enters the cell and the cell will return to its normal size after the embryo is cultured for 1–2 hours in an M2 medium. Embryos that remain shriveled after vitrification and thawing are caused by the embryo not being able to return to its original state, due to large changes in osmotic pressure during vitrification and thawing. A large change in osmotic pressure will increase the intracellular ion concentration, cause damage to the plasma membrane and reduce the permeability of embryonic cells resulting in embryonic cells failing to expand (Lopatářová *et al.*, 2002).

Vitrification of morula and blastocyst stage mouse embryos using VABEDS medium (15% EG, 15% DMSO, and 0.5 M sucrose) exposed to room temperature (± 27°C) for ± 1 minute proved to be successful. This technology can be used to support reproductive technology programs with or without embryo engineering. Reproductive technology in the form of embryo vitrification, thawing, and embryo transfer is indispensable in overcoming cases of infertility in humans. In the future, embryo vitrification (freezing) technology, thawing, and embryo transfer can be developed to store gamete cells (sperm and egg cells) as well as embryos of rare mammals such as jungle cats, tigers, Javan rhinoceros, Sumatran rhinos, deer and deer whose population is large, and getting smaller (Kusumaningtyas *et al.*, 2005).

Development of mice embryo cultured in M16 medium and human tubal fluid

The medium used for embryo culture is arranged based on the purpose of using tissue or organ culture medium, while embryo tissue until the blastocyst stage is relatively more homogeneous as long as other basic needs for culture are met (El-Sohaimy and Hafez, 2010). M16 medium and HTF medium used as culture medium can meet the basic needs of gamete cells to develop into embryos so as not to affect their morphology. In general, embryos require the same environment as other cultured mammalian cells. The elements needed for in vitro culture of embryos contained in M16 and HTF medium include a substrate, air, water, and temperature. The substrate in the M16 culture medium and HTF medium consisted of organic and inorganic chemicals. The amount of substrate added to the culture medium is adjusted to the composition of the substances in the Fallopian tube fluid where the embryo develops. The substrate consists of nutrients and other substances in dissolved form and the nutrients provided are a source of energy and protein (Summers *et al.*, 2005; Lloyd *et al.*, 2009).

In M16 medium and HTF medium, the added energy sources are glucose, pyruvate, and lactate. The energy in the substrate is added to support the development of the preimplantation embryo from the single-cell stage to the blastocyst stage. In general, the pH in the cell culture medium was 7.4 beyond that pH the cells did not grow well, although the optimum pH for growth had little variation among different cell types. Ca, Mg, K, and PO4 ions contained in M16 and HTF medium are required for enzyme activity and maintaining the osmolarity of single-cell mouse embryos ranging from 250 to 280 mOsm and for two cells ranging from 272 to 280 mOsm (Biggers *et al.*, 2000).

Carbon dioxide is an important and beneficial element in embryonic development. Carbon dioxide is associated with bicarbonate buffer, which is very important to regulate the pH balance, so to regulate the balance of CO_2 pressure inside and outside the medium can be given 5% CO_2 in the air. The balance between CO_2 carbonate which is related to pH balance is also

related to temperature. Temperature can affect pH by increasing the solubility of CO_2 at low temperatures and possibly through changes in ionization and pH of the buffer. The balance between these three things must be maintained because they have a direct influence on cell growth (Steeves *et al.*, 2001; Feil *et al.*, 2006).

Water in cell culture is used as a solvent for chemicals that make up the medium, besides that water is also a medium for introducing substances needed for embryos to develop. The chemical elements that make up the culture medium if mixed with metals or other elements that can be present in the water will be toxic to the culture. Another element added in M16 medium and HTF medium is BSA . Serum is a biological fluid that is proven to support cell growth outside the body. Serum functions to provide a number of growth factors, contains hormones and provides binding proteins that carry and bind small elements. Serum is also a source of various fats which are generally needed by cells to live and develop (Leese *et al.*, 2001) (Summers *et al.*, 2005).

From the results of the study, in both M16 and HTF medium, the fertilization rate and the ability of the zygote to pass through the cell block were not significantly different, this was because the basic composition of M16 and HTF medium was almost the same. The ability of the zygote to pass through the cell block is seen by the ability of the zygote to divide into two cell stages. In both treatment groups, the number of embryos dividing into two-cell stages was quite high. Therefore, HTF medium can be used for culturing mouse embryos because of the similarity in the basic composition of the medium (Pusporini and Arifin, 2012).

Development of mice and hamster embryos in KSOMaa and HECM-6 medium

Schini and Bavister (1988) found that in hamster embryos the 8-cell stage of phosphate alone did not cause inhibition of embryonic development, but at an earlier stage, low concentrations of up to 800 nM inhibited the development of hamster embryos (Schini and Bavister, 1988; Ludwig *et al.*, 2001). A similar phenomenon occurs in mouse embryos (Lawitts and Biggers, 1991). KSOMaa was quite effective in supporting the growth of 8-cell stage (T1) mouse embryos but not sufficient for hamster (T3) embryos until advanced blastocyst stage. This medium is known to be able to support high blastocyst acquisition from mouse zygotes with strains undergoing 2-cell block. The number of cells obtained was more than using other media, even equivalent to the number of blastocyst cells produced in vivo (Erbach *et al.*, 1994; Summers *et al.*, 1995).

Amino acids added to this medium stimulate embryonic cell proliferation, especially ICM and ICM differentiation (Summers *et al.*, 2005). What is interesting from this study is the ability of HECM-6 to support the development of 8-cell stage mouse embryos. This medium does not contain glucose and sodium lactate is used as an energy source. Embryos that hatched were higher ($p < 0.08$) than those cultured using the KSOMaa medium. The absence of the KH2PO4 component in the medium was thought to reduce the negative effect of phosphate on the organization of mitochondria in the cytoplasm, thereby improving the metabolic process. The high hatching rate indicates that the embryo has sufficient energy to break down and exit the zona pellucida. It is not known whether this is related to the use of lactate as an energy source. Hamster embryos cultured in HECM-6 medium also had a high hatching rate (73.9%). The high hatching success indicates the metabolism is running well, and the composition of the medium can fully support the development of the embryo (Rosadi *et al.*, 2008).

Conclusion

Based on the results of a search conducted in PubMed, ScienceDirect, NCBI, and Elsevier, a total of 700 articles were obtained which then entered the elimination stage, resulting in 37 articles. Further studies are needed, especially the identification of the embryonic development of mice (*M. musculus* L.) and hamsters using various culture mediums and the development of vitrification methods.

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