Challenges to Embryo Selection

Assisted reproductive technologies (ARTs) have made many advancements since the birth of Louise Brown in 1978. Success rates have improved within the past four decades, but currently remain only between 40-60% successful and fertility treatments cost $12,000-$15,000 [1]. To improve pregnancy rates, two to three embryos are often transferred into the uterus of the patient or recipient in hopes of one surviving. This results in high fecundity, which is a current challenge ART is facing [2-4]. The practice of transferring multiple embryos into the uterus of non-litter bearing animals or humans is not the safest technique to the mother or fetus because it can increase likelihood of preterm labor, low birth weights, uterine growth restrictions, preedampsia, gestational diabetes, placental abruption, fetal demise and cesarean delivery [5]. Single embryo transfer can reduce these risks but requires the ability to select the embryo with the highest chance of establishing a pregnancy which survives to term. Choosing which embryo is “best” is of much disagreement amongst embryologist, with one study demonstrating persons evaluating embryos agreed in only 68.5% of cases [6]. This indicates that even the most expert, professional embryologist cannot always determine which embryo has the greatest likelihood of establishing a pregnancy, surviving to term and creating a healthy baby. The objective of the present review is to provide an overview of methods currently employed in ART which will help predict high quality embryos.

Morphological Analysis

The zygote is the first stage of embryonic development after fertilization. Early stage development suggests some predictive value for the embryo's implantation potential based on an evaluation of the embryos morphology and cleavage stage [7]. The zygote scoring system attempts to differentiate normal embryos with more developmental potential from abnormal embryos by evaluating the pronuclei [7]. However, no correlation between zygote morphology and implantation has been identified [7]. Other minimally invasive methods are needed to predict embryo quality.

Past studies have suggested that early stage development has some predictive value for the embryos implantation potential based on an evaluation of the embryos morphology and cleavage stage [7]. These criteria are evaluated based on embryo appearance, which is considered to be the “gold standard” of assessing embryo development. Zygotes ideally have the same number of small nucleolar precursor bodies distributed in the nucleus or large nucleolar precursor bodies with polar distribution between the pronuclei. Zygote abnormalities frequently observed can be a large, perivitelline space, dark zona pellucida, dark incorporations, spots, vacuoles and irregular shape [8,9]. The second criterion observed in the morphological analysis is the occurrence of meiotic division, which can be observed by the presence of two blastomeres [5]. Continued morphological assessment relies on symmetry of blastomeres, cellular fragmentation and developmental rate [8-12]. While a number of studies support use of early embryo scoring systems, others cannot demonstrate a strong relationship between these measures and pregnancy [7].

A more recent means of assessing development has been the addition of cleavage rate. Increased pregnancy rates were observed with early cleaving zygotes in both IVF and ICSI fertilized embryos [13]. This is likely because early cleaving zygotes derive from oocytes with adequately synchronized cytoplasmic and nuclear maturation. Evaluating cleavage is not limited to the first division but should be examined critically up to the blastocyst stage and prioritized as a high selection factor when choosing the “best” embryo to transfer. Studies by Ebner et al. [13] even “confirmed that on day 2 after transfer, 4 cell embryos - even when showing minor fragmentation - should be preferred to good quality 2 cell embryos”. This enunciates the high developmental potential in a rapidly dividing embryo, because heavy fragmentation has been described as an undesirable quality that can indicate chromosomal abnormalities, mosaicism, programmed cell death and potential for apoptosis.

An embryo which has reached the blastocyst stage has proven more developmental competency over those which stall in early development [14]. A blastocyst has proven the ability to develop into a more organized structure than an embryo in the more primitive stages of development. Blastocyst transfer also correlates more timely with the natural physiological timeline of implantation. Gardner and Lane state that “it is an accepted global practice in human IVF to transfer embryos on day 2 (around the 4 cell stage) or on day 3 (around the 8 cell stage) of development [14]. However, in vivo, such cleavage stage embryos reside in the Fallopian tube and not the uterus”. This implies that implantation
rates will be improved if embryo transfer aligns with *in vivo* development, though pregnancies can occur with earlier transfers. It is logical to assume mimicking conditions found in nature will yield more successful outcomes. Research supports this theory, as blastocysts cultured in favorable conditions routinely result in successful implantation and pregnancy rates as high as 60%. It is important to remember all blastocysts are not of equal quality and only the highest quality blastocyst should be selected for transfer. Formation of a distinct blastocoele, trophectoderm and tightly compacted inner cell mass indicate high quality blastocysts with the trophectoderm grade being the strongest morphological predictor [13,15]. These attributes are visually apparent and observed during morphological assessment.

It is understood practical use of ARTs demand the embryo remain viable throughout all embryo quality assessments and selection techniques. While morphological analysis allows embryo survival and does not hinder embryonic development. However, the morphological assessment is “highly subjective and the morphological classification of oocytes and embryos is not always compatible with their ability to grow and develop” [16]. Much can be learned from an embryos morphology in regard to embryo quality, but Cruz et al., warns “embryo selection based on discrete routine assessment of embryology morphology is not always associated with a higher implantation or pregnancy rate” [17]. Therefore, other criteria should be considered in the recognition of embryos with a good projection for implantation or pregnancy” [17].

**Time Lapse Imaging**

Time-lapse imaging is often incorporated into both research and clinical embryology settings. While time-lapse imaging is simply an advanced take on the standard morphological analysis, it provides more frequent observation in a controlled environment. It can improve embryo selection “based on the reasonable assumption that more frequent observation will provide substantially more information to the relationship between development, timing and embryo viability” [18].

The EmbryoScope is a commercially available embryo time-lapse imaging system. The EmbryoScope provides optimal incubation conditions for early developing embryos. This enables the time-lapse imaging system to get detailed and precise information about morphological characteristics and cleavage status of growing embryos while contained in a controlled environment [17]. This is beneficial because developmental events can be linked to specific time points, which can predict developmental capacity with minimal risks to the embryo because incubation and inspection is integrated into one system. In essence, the EmbryoScope can allow a morphological assessment much more advanced than by human eye under a standard microscope, and can prevent dangers involved in removing embryos from culture for examination. These dangers include shifts in media pH, temperature fluctuations and risk during transportation from incubator to microscope platform (e.g. dropping or sloshing culture dish). For these purposes, time-lapse imaging provides a safer and more thorough embryo assessment.

Though time-lapse imaging allows many benefits, it is expensive technology. It also requires light, a known potential stressor, to acquire digital images. However, studies comparing embryos cultured in the EmbryoScope versus standard incubation conditions conclude the EmbryoScope provides an adequate culture environment that does not hinder embryo quality, blastocyst development or viability. However, a series of studies presented at the 2016 Congress of the American Society for Reproductive Medicine were almost equally split as to the efficacy of the technique in embryo selection [19].

**Lipid Composition**

Embryo biochemical information is not indicated in a morphological analysis. Lipid content is a variable component in embryos and important in effecting energy utilization. Lipid is also known to affect cryopreservation outcomes. Cytoplasm of oocytes and embryos differs between species, as well as within the same mother. Typically, mammals such as cattle and pigs have higher embryo lipid content than mice and humans [20]. There is a higher energy demand, and therefore lipid demand, for embryos in species with longer intervals between ovulation and implantation as compared to species with short intervals, such as the mouse. Differences in lipid densities also occur amongst animals of different breeds of the same species. When comparing Jersey and beef embryos, electron microscopy determined that Jersey embryos have approximately 36% lipid as compared to 8% in beef cattle embryos [21]. This data suggests high intracellular lipid content in Jersey embryos contribute to low conception rates of cryopreserved Jersey embryos, because high intracellular lipid is known to cause cryodamage [21]. Therefore, lipid can be used to indicate embryo quality and as means to predict an embryo’s ability to survive cryopreservation. Typically, lipid is commonly evaluated though lethal staining techniques. A morphological assessment cannot provide information about lipid content, but a specific gravity device (SGD) has provided estimation of an embryos’ lipid status based on embryonic density [22]. Different embryo densities, as measured by SGD, can reflect differences in lipid composition. This suggests SGD is a non-invasive method to evaluate lipid content in embryos. Sequential studies with SGD demonstrates that highly buoyant embryos fail to develop at a significantly higher rate as compared with the rest of the cohort, likely due to a large incorporation of lipids in the embryo cytoplasm [2]. Therefore, biochemical properties, such as lipid, can be evaluated by SGD. An inverse association between embryo lipid content and quality has been identified, so lipid evaluation can be used as an excellent predictor of quality [20].

An understanding of normal embryo lipid composition is required to select against embryos with abnormal lipid content. In an individual embryo, total lipid content remains constant up to the morula stage and decreases at the blastocyst stage [22]. There is a low energy demand as the embryo develops from a zygote to morula, but as more cleavage occurs, intracellular lipids are used as an economical energy source. However, while total lipid is constant during zygote to morula stages, changes in specific types of lipids
do occur and can be indicative of quality. Triglycerides are known to be the most constant in early stage embryos and are the major type in the bovine oocyte, with palmitic and oleic acid accounting for 32% and 25%. In a study regarding porcine embryo cytoplasm, diverse classes of unsaturated and saturated fatty acids were found with high levels of triglycerides, free fatty acids and phospholipids. This suggests triglycerides are present in both oocytes and zygotes, and do not alter much post-fertilization. The consistency of triglycerides remains constant through the late blastocyst stage [22]. Therefore, triglyceride content can be an early or late detector of embryo quality, because it is not expected to change during early embryo development. While triglycerides remain apparent in embryos at a steady state, free fatty acids are dynamic. By staining of embryos with OD blue, it was shown that free fatty acids in zygotic droplets were high, but significantly decreased in 2-4 cell embryos and remained unchanged in the morula. In the late blastocyst, droplets with free fatty acids and phospholipids were undetectable. Cleavage of blastomeres increase as an embryo develops and causes a decrease in free fatty acids.

Increased embryonic lipid composition decreases embryo survival of cryopreservation, because increased lipid accumulation aggravates vitrification injury and increases the production of free radicals. This suggests lipid content can be used to predict an embryo’s ability to maintain viability after cryopreservation. Methods to predict embryo survival of cryopreservation would increase pregnancy rates after frozen embryo transfer.

**Genetic Selection**

There is a demand to select for embryos based on genetic criteria. This can include selecting for a particular sex or against genetic disease. The desire to control the sex ratio is not a modern concept as folklore to select gender have been documented for thousands of years. In 456 B.C. it was thought that females came from the left testicle and males from the right; In 50 A.D Pliny thought bulls dismounted to the right after siring a male and to the left after siring a female; the Geoponica stated that sex was determined by wind direction at the time of copulation; and George Tuberville believed that mating after the moon had passed full and precession of the sun indicated offspring sex. These methods were used for bred Jersey heifers due to the continued demand for high protein milk for making cheese. These reasons all support using embryo genetic screening to improve the health, well-being, and control sex of offspring. This enunciates the potential of embryo genetic screening to improve the health, well-being, and gene pool of a species.

When PGT is used to target genetic conditions, it can be used to select against disease. Couples at high risk for genetic disease can be assured their baby will not have a lethal or crippling heritable disease [25]. It is known some diseases are more prevalent in a particular sex. In humans, PGT is currently indicated and considered appropriate in case of: autosomal recessive diseases in which both parents are known genetic carriers (e.g. cystic fibrosis, Tay-Sachs disease and sickle cell disease), autosomal dominant diseases in one or both parents (e.g. Huntington’s disease), genetic mutations causing important consequences (e.g. BRCA gene), X linked diseases (e.g. hemophilia), and certain balanced chromosomal translocations or inversions [24]. In case of X-linked disease, there is a medical advantage of embryo sex selection, rather than simply parent’s preference of gender or family balancing.

PGT is not solely used in humans, but is advancing into animal industries. Sex selection can offer economic advantages to producers but cannot be efficiently manipulated in well managed breeding programs. Seidel states how animal breeders in a traditional breeding program must that the probability is a 51% chance that each bovine conception will result in a bull calf, and that the probability is independent of the sex of other conceptions [26]. This makes it nearly impossible to select for sex in a breeding program. Herrera et al. [27] explains that “in some equine breeds, like Polo Argentino, females are preferred to males for their ease of training and agility, so the availability of PGD for gender determination may allow only transferring embryos from the desired sex”. While embryo sex selection is a benefit of incorporating PGT into a breeding program, PGT for the diagnosis of genetic mutations associated with specific genetic diseases such as hypokalemic periodic paralysis and hereditary equine regional dermal asthenia, polysaccharide storage myopathy, myotome (neuromuscular disorder) and genes for coat color have also been reported. This enunciates the potential of embryo genetic screening to improve the health, well-being, and gene pool of a species.

In the dairy industry, heifer calves are preferred to allow herd expansion and sale to others [28]. Females can lactate, making them an integral gender within an expanding dairy. Seidel explains sale to others creates a demand for heifer calves born because exported heifers have exceeded 100,000/year. In a dairy, lower birth weights of heifer calves reduce dystocia and a strong domestic market exists for bred Jersey heifers due to the continued demand for high protein milk for making cheese. These reasons all support using embryo sex selection to increase economic gain in the dairy industry.
Conversely, bull calves provide more economic advantages in the beef industry. Bull calves have higher weights at weaning and higher feed efficiency during fattening, which is economically advantageous to beef producers. Therefore, embryo sexing technologies are used to select for male offspring in beef operations. PGT promises economic gains to the cattle in industry but added costs, time, decreased pregnancy rate and increased numbers of discarded embryos make producers hesitant to embrace this technology. Noninvasive methods such as enzymatic determination and an immunological approach to sex sorting bovine embryos has been studied, but has not proven effective in embryo sex determination for commercial purposes [29]. Because PGT increases cost of fertility treatments by $4000-$7000, and only approximately 20% of PGT embryos result in pregnancy, humans are often reluctant to use these technologies as well [30]. The invasive, expensive, and time consuming nature of PGT creates a demand for an inexpensive, noninvasive, and quick technique predict genetic properties of both human and animal embryos.

**Selection after Cryopreservation**

Cryopreservation of embryos has allowed for delayed transfer of embryos to preserve genetics, promote single embryo transfer and is necessary for PGT. However, cryopreservation does not guarantee embryo viability and often reduces pregnancy success rates 15-20% [31].

It is well documented cryopreservation decreases embryo viability due to two main causes:

1. Intracellular ice crystal formation causing freeze-fracture and
2. Toxicity and osmotic shock from cryoprotectants necessary to cryopreserve embryos [32-34].

There are no current methods routinely used to determine embryo survival of cryopreservation, but studies by Wessels et al. [32] demonstrate a noninvasive embryo assessment technique detects differences in mouse and ovine embryo buoyancy post-thaw in blastocysts that survived cryopreservation and continued development from those that did not. This suggest new methods to select for embryo survival of cryopreservation which will reduce the transfer of non-viable embryos and increase pregnancy rates of frozen embryo transfer and PGT.

**Microfluidics as a Means of Embryo Culture**

Many improvements have been made in ARTs since the birth of Louise Brown in 1978, but embryos are still stored in culture media inside the controlled environment of an incubator. While this embryo culture method routine establishes pregnancies, the in vitro environment commonly used in IVF contrasts vastly to the in vivo environment of the female reproductive system. The lumen of the oviduct is a complex environment which supports fertilization of oocytes, early embryo development and facilitates embryo transport to the uterus due to the flux generated by cilia and peristaltic movements from the contraction of the smooth muscle layer which generates a turbulent flow toward the uterine horn [35]. However, in vitro culture systems do not emulate the dynamic environment of the female reproductive system [36]. While embryos are commonly cultured under static conditions, innovative, microfluidic culture system are currently being studied to determine if a more optimal culture system exists to improve embryo development and produce healthier offspring. Current methods to generate fluid dynamics in embryo culture systems are currently composed of platform/ dish movement, wave fluid movement and designed fluid dynamics.

Kinetic system can be created with a rotating and tilting platform. Studies show platform movement increased blastomere formation but not blastocyst formation. Other methods to cause wave fluid movement use vibratory movement in which the “mechanical stimulus through vibration seems to increase the proliferation rate and behavior of some types of somatic cells” as well as enhance oocyte maturation in pigs and increase rates of blastocyst formation and pregnancy in human.

Designed fluid dynamics is a broad category which contains many methods to create a kinetic environment from using a gravity gradient to pumping media through a micro channel, all in attempt to create a laminar or peristaltic flow suitable for embryo development. The most current, state-of-the-art microfluidic lab-on-chip device is a “computer controlled, integrated microfluidic control system with up to hundreds of on-chip pumps and valves, powered individually by Braille pins on a portable, refreshable display” [37]. In essence, lab-on-chips are a miniature Jacuzzi hot tubs designed for embryo culture, as they are a temperature controlled environment with jets, pumps and fluid circulation. In theory, this chip allows for new media to circulate around embryos which wash away toxins, provide precise flow around the embryo to appropriately induce mechanical stress, and allow for analysis of the medium to identify secreted factors from the embryo to create successful, individual embryo culture. The ability to identify and track developmental progress and secretions of each individual embryo could allow for better embryo selection and increase pregnancy rates. The potential for these lab-on-chips to automatically sample and monitor embryos and culture medium in an atmospheric controlled environment can reduce the need for human manipulation and create very consistent culture [36].

It is believed mechanics play a role in embryonic development and applied mechanical forces in vitro mimics the oviduct’s physical stimulation as it peristaltically pumps the embryo in to the uterus [38]. Currently, the in vitro produced embryo undergoes these forces through pipetting, tweezers or the atomic forces of microscopy, but culture systems inducing these biomechanical forces are potentially beneficial as well. This has stimulated much research in attempt to develop more ideal culture systems to induce beneficial mechanical stress on embryos.

Though microfluidic embryo culture systems appear to be promising, it is known that autocrine and paracrine factors of embryo co-culture is beneficial for embryo development (embryo development thrives in small groups of 2-3 embryos rather than individual culture). Goovaerts poses the dilemma that while the
microfluidic devices allow a dynamic environment where the fluid flow can wash toxic metabolites away, it also simultaneously dilutes important autocrine/paracrine factors [39]. While this a valid concern, Kim et al. [40] demonstrated significantly higher development of bovine embryos, greater percentage of implantation and increased ongoing pregnancies by using a microfluidic device with channel constructions and direct mechanical stimulation. Alegretti et al. [41] demonstrates human embryos cultured in dynamic system produced significantly higher top quality embryos and good quality embryos with significantly less fragmentation and poor quality embryos over embryos cultured in traditional static conditions. These studies suggest culturing embryos in a dynamic system, which better emulates the female reproductive tract, may enhance embryo development and further experimentation to improve these systems should continue.

**Challenges to in vitro Fertilization and Embryo Transfer**

Morphological analysis, developmental rate, time-lapse imaging and triglyceride analysis are all methods to predict which embryo is of the highest quality, but they are all qualitative, and triglyceride analysis is inhibitory to embryo survival. PGT has allowed major advancements in embryo genetic selection but its high costs and invasive properties are not conducive to its mainstream use in animal industries. New methods which are noninvasive and inexpensive will improve embryo selection techniques to increase pregnancy rates and promote single embryo transfer.

**References**

1. www.IHR.com
2. Prien SD, Wessels CE, and Penrose LL. (2015) Preliminary Trials of a Specific Gravity Technique in the Determination of Early Embryo Growth Potential. Hum Reprod 30(9): 2076-2083.
3. http://www.cdc.gov/art/
4. Gerris J, De Neubourg D, Mangelshotts K, Royen EV, Nan de Meersche M, et al. (1999) Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: a prospective randomized clinical trial. Hum Reprod 14(10): 2581-2587.
5. http://americanpregnancy.org/multiples/complications/
6. Farin PW, Britt JH, Shaw DW, Slenning BD (1995) Agreement among evaluators of bovine embryos produced in vivo or in vitro. Theriogenology 44(3): 339-349.
7. Wittmer CK, Bettahar-Lebugle J, Ohl C, Rongieres I, Nisand, et al. (2000) Zygote Evaluation: An Efficient Tool for Embryo Selection. Human Reproduction 15(12): 2591-2597.
8. Nashiri A and Eftekhari-Yazdi P (2015) An overview of the available methods of morphological scoring of pre-embryo implantation in in vitro fertilization. Cell J 16(4): 392-405.
9. De Sutter P, Dozortsev D, Qian C, Dhont M (1995) Oocyte morphology does not correlate with fertilization rate and embryo quality after intracytoplasmic sperm injection. Hum Reprod 11(3): 595-597.
10. Scott LA, Smith S (1998) The successful use of PN embryo transfers the day following oocyte retrieval. Hum Reprod 13(4): 1003-1013.
11. Kattera S and Chen C (2004) Developmental potential of human pronuclear zygotes in relation to their pronuclear orientation. Hum Reprod 19(2): 294-299.
12. Ebner T, Moser M, Sommergruber M, Gaiswinkler U, Wiesinger R, et al. (2003) Presence, but not type or degree of extension, of a cytoplasmic halo has a significant influence on preimplantation development and implantation behavior. Hum Reprod 18(11): 2406-2412.
13. Ebner T, Sommergruber M, Tews G (2003) Selection Based on Morphological Assessment of Oocytes and Embryos at Different Stages of Preimplantation Development: A Review. Hum Reprod Update 9(3): 251-262.
14. Gardner D and Lane M (1997) Culture and Selection of Viable Blastocysts: A Feasible Proposition for Human IVF? Hum Reprod Update 3(4): 367-382.
15. Hill MJ, Richter KS, Heitmann JR, Graham JR, Tucker MJ, et al. (2013) Trophoectoderm Grade Predicts Outcomes of Single-blastocyst Transfers. Fertility and Sterility 99(5): 1283-1289.
16. Bukowska D, Kempsisty B, Piotrowska H, Walczak R, Sniadek P, et al. (2012) The Invasive and New Non-invasive Methods of Mammalian Oocyte and Embryo Quality Assessment: A Review. Veterinarni Medicina 57(4): 169-1676.
17. Cruz M, Blanca G, Garrido N, Pedersen KS, Martinez M, et al. (2011) Embryo Quality, Blastocyst and Ongoing Pregnancy Rates in Oocyte Donation Patients Whose Embryos Were Monitored by Time-lapse Imaging. J Assist Reprod Genet 28(7): 569-573.
18. Kirkegaard K, Ahlström A, Ingerslev HJ, Hardarson T (2015) Choosing the Best Embryo by Time Lapse versus Standard Morphology. Fertility and Sterility 103(2): 323-332.
19. (2016) ASRM Scientific Congress and Expo: Scaling New Heights in Reproductive Medicine 106(3): e1-e104.
20. Sutton-McDowall ML, Feil D, Roblier RL, Thompson JG, Dunning KR (2012) Utilization of Endogenous Fatty Acid Stores for Energy Production in Bovine Preimplantation Embryos. Theriogenology 77(8): 1632-1641.
21. Weiders JD, Prien SD (2014) Estimation of Weight and Lipid Composition in Preimplantation Embryos from Jersey and Beef Breeds of Cattle. Open Journal of Veterinary Medicine 4(11): 261-266.
22. Romek M, Gajda B, Krystofowicz E, Smorag Z (2010) Changes of Lipid Composition in Non-cultured and Cultured Porcine Embryos. Theriogenology 74(2): 265-276.
23. Betteridge JK (1998) An Historical Look at Embryo Transfer. Reproduction 62(1): 1-13.
24. Herrera C (2016) Clinical Applications of Preimplantation Genetic Testing in Equine, Bovine, and Human Embryos. Journal of Equine Veterinary Science 41: 29-34.
25. Söls ES, Goldschlag D, Levy DP, Davis OK, Rosenwaks Z (1999) Preimplantation Genetic Diagnosis: Considerations for Use in Elective Human Embryo Sex Selection. J Assist Reprod Genet 16(10): 509-511.
26. Seidel GE (2003) Economics of Selecting for Sex: The Most Important Genetic Trait. Theriogenology 59(2): 585-598.
27. Herrera C, Morikawa ML, Bello MB, Von Meyerlen M, Centeno J, et al. (2014) Setting up Equine Embryo Gender Determination by Preimplantation Genetic Diagnosis in a Commercial Embryo Transfer Program. Theriogenology 81(5): 758-763.
28. Seidel GE (2014) Update on Sexed Semen Technology in Cattle. Animal 8(51): 160-164.
29. Garcia JF (2001) Practical Considerations of Embryo Manipulation: Preimplantation Genetic Typing. Theriogenology 56(9): 1393-399.
30. (2016) Reproductive Health Technologies Project - Fertility - Pre-implantation Genetic Diagnosis (PGD).
31. Youngs CR (2011) Cryopreservation of Preimplantation Embryos of Cattle, Sheep, and Goats. J Vis Exp 54: pii: 2764.
32. Kasai M, Komi H, Takakamo A, Tsudera H, Sakurai T, et al. (1990) A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution without appreciable loss of viability. Reprod Fertil 89(1): 91-97.
33. Arav A (2014) Cryopreservation of oocytes and embryos. Theriogenology 81(1): 96-102.
34. Wessels CE, Penrose LL, Ahmad K, Prien SD (2017) Embryo Survival of Cryopreservation Can Be Detected Using a Noninvasive Embryo Assessment Technique and Establish Pregnancies. Theriogenology 103: 169-172.

35. Smith GD, Swain JE, Pool TB (2012) Embryo Culture: Methods and Protocols. New York, USA.

36. Krisher RL, Wheeler MB (2010) Towards the Use of Microfluidics for Individual Embryo Culture. Reprod Fertil Dev 22(1): 32-39.

37. Smith GD, Takayama S (2007) Gamete and Embryo Isolation and Culture with Microfluidics. Theriogenology 68(Suppl 1): 190-195.

38. Lai L, Takayama S, Smith GD (2015) Recent Microfluidic Devices for Studying Gamete and Embryo Biomechanics. J Biomech 48(9): 1671-1678.

39. Goovaerts I, Leroy J, Jorssen E, Bols P (2010) Noninvasive Bovine Oocyte Quality Assessment: Possibilities of a Single Oocyte Culture. Theriogenology 74(9): 1509-1520.

40. Kim MS, Bae CV, Wee G, Han YM, Park J (2009) A microfluidic in vitro cultivation system for mechanical stimulation of bovine embryos. Electrophoresis 30: 3276-3282.

41. Alegretti JR, Rocha AM, Barros BC, Serafini P, Motta E, et al. (2011) Microfluidic Dynamic Embryo Culture Increases the Production of Top Quality Human Embryos through Reduction in Embryo Fragmentation. Fertility and Sterility 96(3): 58-59.