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New Perspectives of Embryo Sexing Through Morphological Criteria and Sperm Fertilizing Parameters in Mammalian Species: A Review

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ABSTRACT

Predetermining embryo sexing before the time of conception could be controlled before fertilization of the oocyte or detected after fertilization. These methods are expensive, time-consuming and negatively affect the development of embryos. The methods used for embryo sexing are based on sperm separation before fertilizing the oocyte or molecular basis of nuclear or mitochondrial DNA. The selection of spermatozoon fertilizing controls the sex of the zygote *in vivo* or *in vitro* matured oocytes. In addition, the detection of embryo sexing is determined through aspiration of one cell from the eight-cell stage embryo and investigated through PCR. The oocyte fertilization by a spermatozoon bearing a Y-chromosome gives male offspring, although that fertilized by a spermatozoon bearing an X-chromosome gives female offspring. The embryonic cell aspiration negatively affected the further developmental competence of embryos. Therefore, establishing a new method for embryo sexing in mammals through measurable morphological criteria (zona pellucida, homogeneity of cytoplasm, previtelline space and stage of development) is a great boon in animals and humans. The success in embryo sexing through morphological criteria maximizes the advantages of assisted reproductive techniques. Therefore, the present review article addresses and discusses the preselection of embryo sexing at the time of conception and their limitation and effects in addition to sperm fertilizing parameters on mammals.

Key words: Embryos, Females, Males, Sexing, Transfer, Zygotes.

The functions' modulation of gonadal tissues are essential for the conservation of species, increased productivity and fertility treatments of mammalian species (Mohammed and Al-Hozab, 2020; Mohammed *et al.,* 2020, 2024; Mohammed and Al-Suweigh, 2023; Al-Mufarji *et al.*, 2023). Several assisted reproductive techniques (ARTs) have been recently developed for those purposes. The ARTs include several techniques as *in vitro* embryo production (IVP), embryo transfer, oocyte and embryo micromanipulation, sperm and embryo sexing, cryopreservation and transplantation of ovarian tissues, vitrification of oocytes and embryos (Aljubran *et al.,* 2023; Lanci *et al.,* 2024). The advantages of ARTs allow obtaining more offspring from superior animals for increasing the production of milk and meat, therapy of diseases and conservation of endangered species (Mohammed, 2019; Crafa *et al.,* 2023).

Animal production improvement through gonadal functions and embryo sexing in the world are required for the development of countries and economic prosperity (Senosy *et al.,* 2017; Sachan *et al.,* 2020). Therefore, such potential approaches are vital for animal production continuity and maintaining animal health and humans as well.

Significant development has been made in embryo sexing over the past couple of decades to increase reproductive performances or treatment of dysfunctions (Sachan *et al.*, 2020). Currently, the methods of embryo sexing could be carried out either before fertilizing the oocytes through sperm separation into X and y sperm or after fertilization. The methods of sperm sorting to control embryo sexing before fertilization are indicated in Fig 1. Such methods are expensive and carried out only in a few countries. Furthermore, the methods of embryo sexing are shown in Fig 2 (Mohammed and Al-Hozab, 2016).

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Those extensive methods are also time-consuming in addition to their side effects on further embryo development. Therefore, the probability of adapting morphological embryo sexing is a new perspective instead of sperm separation or PCR technique for embryo sexing. So, the present review article addresses and discusses the methods of preselection of offspring sex at the time of conception and their limitation and effects on mammalian species.

The current study was carried out according to the procedure approved by the Deanship of Scientific Research, King Faisal University, Saudi Arabia from August 2023 to January 2024. Ethical approval for this study was not required and the data were obtained from Science Direct, Google Scholar and PubMed databases in addition to our articles. The articles concerning sperm rheotaxis, embryo sexing through cytological methods, embryo sexing through morphological criteria, embryo biopsy and its applications, embryo sexing through separation of X and Y sperm, sex preselection through minerals supplementation to females were used to discuss the current approaches of embryo sexing.

Sperm rheotaxis

The unclear navigation mechanism of sperm in the female reproductive tract is indicated in mammals owing to its complex process (Hyakutake *et al.,* 2021). Sperm rheotaxis has recently an important topic in understanding the motility of mammalian sperm through the female genital tract. Positive rheotaxis is the tendency of a cell to orient and swim against the flow of the surrounding fluid oviduct (Miki *et al.*, 2013). To direct sperm toward the ovum in the oviduct, coitus drastically boosts oviductal fluid secretion and causes fluid flow. The oviductal fluid secretion in mice produces this flow within 4 hours after sexual arousal and coitus in female mice; it cleans the oviduct of debris, reduces viscosity and creates the stream that directs sperm migration in the oviduct (Miki *et al.*, 2013).

The physiological significance of positive rheotaxis for sperm transport *via* the genital system to the site of fertilization in mice and humans has recently been documented (Suarez 2010; El-Sherry *et al.*, 2014) and bull (Hyakutake *et al.,* 2021; Abdel-Ghani *et al.*, 2022) and ram (Abdel-Ghani *et al.*, 2020). To demonstrate that sperm might purposefully swim against the flow, rheotaxis was simulated in a microfluidic system to investigate the interaction of the sperm with various flows and the flow direction. According to the findings (Abdel-Ghani *et al.*, 2022), the sperms alter their orientation to go counter to the flow. In addition, when the flow's speed increased, the sperms' speed also increased.

The diverse reproductive tract environments have been modeled using microfluidic devices in assisted reproduction and the positive rheotaxis (PR) study of semen can help determine its reproductive performance in rams (Fokias and Bekaert 2022). Sperm quantity, progressive motility, pH, the proportion of viable cells and shape are typically measured by traditional methods of analyzing semen. Because only those samples with noticeably poor semen

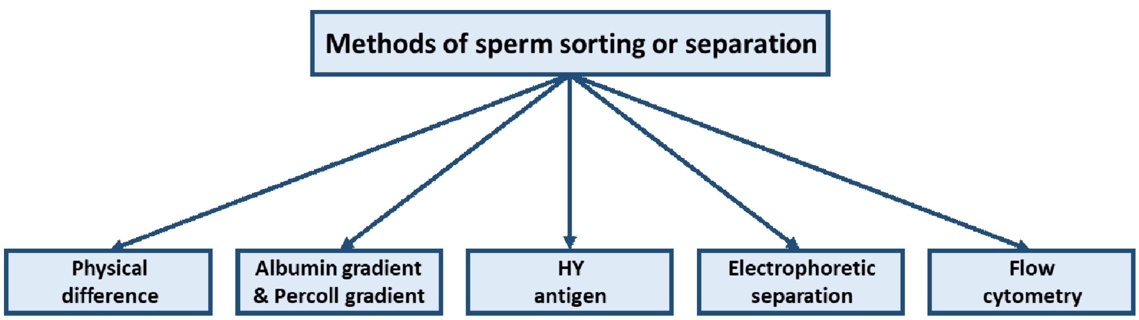

Fig 1: Methods of sperm sorting or separation (Mohammed and Al-Hozab, 2016).

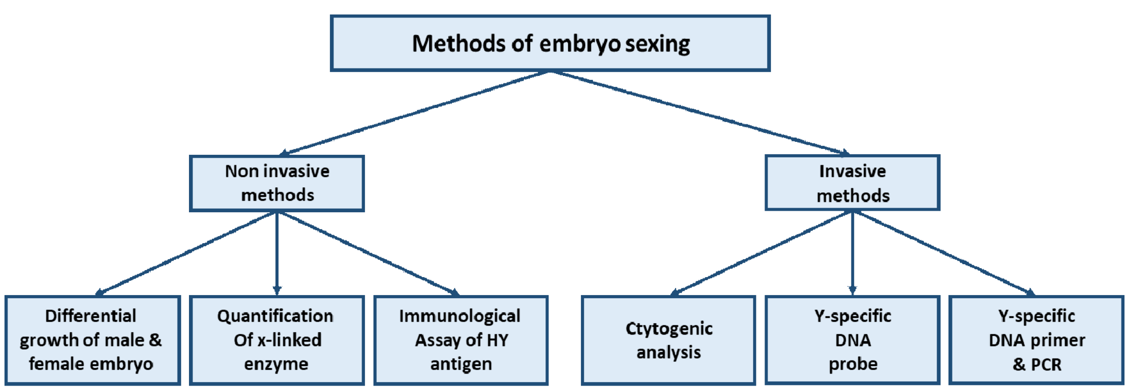

Fig 2: Methods of embryo sexing (Mohammed and Al-Hozab, 2016).

quality may be recognized, these assays might not be adequate in predicting the outcome of fertility. None, nevertheless, have so far produced reliable correlations with *in vivo* fertility because of their vast ranges of variance and low anticipated values. Therefore, the aims are to conduct sperm rheotaxis using microfluidic devices and second, to analyze the value of sperm rheotaxis about sperm kinetic parameters as potential indicators of *in vivo* fertility.

Embryo sexing through cytological methods

These methods are called karyotyping through the analysis of the genomic framework of a cell embryo based on the presence of the X or Y chromosome in the genome at the metaphase (Sachan *et al.,* 2020). Blastomere cells are biopsied from cleaved embryos and are checked at the metaphase through culturing with mitosis arresting agents as colchicines (Wakchaure *et al.,* 2015). In addition, colcemid is another mitosis-inhibitor agent, which also depolymerized microtubules and inactivates the spindle formation (Mohammed, 2006; Sharma *et al.,* 2017). The treated cells are lysed in a hypotonic solution to disperse chromosomes in addition to permanent DNA staining of two X chromosomes (female) and a single Y chromosome (male). This technique reduces the viability of embryos and low efficiency because of undispersed chromosomes in addition to time-consuming (Wakchaure *et al.,* 2015; Sharma *et al.,* 2017).

Embryo sexing through morphological criteria

The sex ratio is always of great interest and is influenced by sociocultural factors, environmental exposure and maternal and parental factors (Hesketh and Xing, 2006). This ratio is assumed to be slightly male-biased at birth (~51.3%) and to reach approximately 50% throughout life as a result of the survival advantage presented by females (Austad, 2015). Female and male preimplantation mammalian embryos differ not only in their chromosomal complement but also in their proteome and subsequent metabolome (Gardner *et al.,* 2010). Female and male embryos before implantation exhibit differences in their cellular phenotype. Roos Kulmann *et al.* (2021) found a male-skewed sex proportion in the offspring based on blastocyst morphology with a skewed sex proportion towards XY embryos. Several studies have noted a skewed sex ratio at birth towards males after blastocyst transfer, but not after cleavage stage embryo transfer (Maalouf *et al.,* 2014; Chen *et al.,* 2017). It has been observed from *in vitro* development of embryos of domestic species and humans that males were higher for embryos that reached earlier the blastocyst (Kawase *et al.,* 2021).

Embryo biopsy and its applications

Aspiration of embryonic cells (biopsy) of preimplantation embryos has been stated since a couple of decades ago using rabbit preimplantation embryos (Gardner and Edwards, 1968) (Fig 3). Embryo biopsies were used in veterinary and human medicine achievements. The biopsied embryo samples from humans and animals were successfully and commercially used for embryo sexing (Hasler *et al.,* 2002), preventing genetic diseases (Takeuchi, 2021), selecting specific traits (Hochman *et al.,* 1996), genotyping to select embryos with higher estimated breeding values (Mullaart and Wells, 2018). Although trophectoderm (TE) biopsy is still considered the gold standard test for detecting embryo chromosomal anomalies, cell-free DNA (cfDNA) from embryo culture medium analysis is promising for human embryo selection (Navarro-Sánchez *et al*., 2022). However, further studies are still required to understand the origin of cfDNA (TE or epiblast) in addition the technique needs a specific protocol in each laboratory to be validated (Oliveira *et al.,* 2023).

Embryo sexing through separation of X and Y sperm

Several studies have explored enrichment media to alter the sex ratio of mammalian spermatozoa. Several techniques were used to separate X from Y sperm spermatozoa including physical differences including size and shape and surface charge, albumin and Percoll gradients (Hadi and Al-Timimi, 2013), H-Y antigen (Sills *et al.*, 1998), electrophoretic separation (Ainsworth *et al.*, 2007) and flow cytometry (Flaherty *et al.*, 1997) (Fig 1). However, the separation of spermatozoa bearing Y or X chromosomes is debatable. Although the flow cytometry technique (Seidel and Gardner, 2002) is the most reliable method and it showed promising commercial potential to sort spermatozoa, it is not easily accessible and it requires expensive

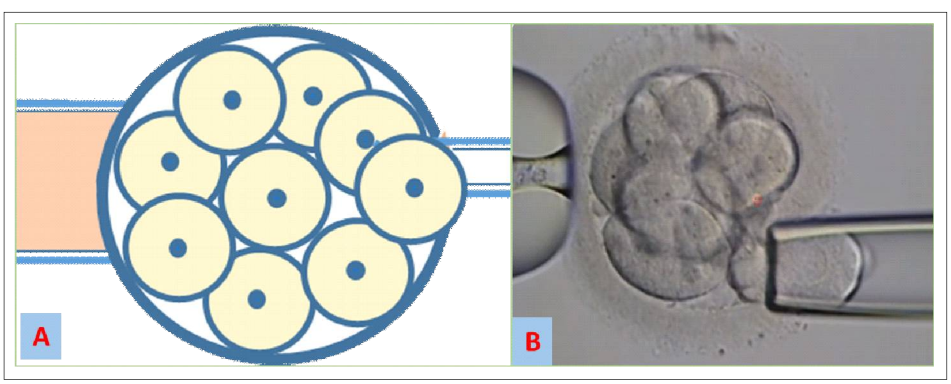

Fig 3: Embryonic blastomere biopsy or aspiration over opening zona pellucida.

equipment. The X and Y sperm are sorted based on their DNA content and charges. The X-chromosome has more DNA content than the Y-chromosome. The separated spermatozoa can be used in *in vitro* embryo production (IVEP) and artificial insemination (AI) programs to produce offspring of the desired sex. In addition, sperm sorting is crucial in the forensic investigation of alleged sexual assault cases (Fokias and Bekaert, 2022). In addition, we expect that the *in vitro* fertilization rate of oocytes with sorted sperms in addition to cleavage and embryo quality give comparable results with unsorted sperms.

The current approaches to embryo sexing

The methods used in the world for embryo sexing include non-invasive and invasive methods (Mohammed and Al-Hozab 2016) (Fig 2). The non-invasive and invasive methods include differential growth of male and female embryos (Dumoulin *et al.*, 2005), quantification of X-linked enzyme (Iwata *et al.* 2002) and Immunological assay of H-Y antigen (White *et al.*, 1987). Additionally, invasive methods for embryo sexing include cytogenetic analysis (Lyon, 2003), Y- Specific DNA probe (Cenariu *et al.*, 2011) and polymerase chain reaction (Cenariu *et al.*, 2011). The aforementioned methods of embryo sexing are time-consuming and in some cases require expensive equipment and are not commercially applicable. Therefore, the search for a new morphological way of embryo sexing to become precise, cheap and applicable is necessitated.

Sex preselection through minerals supplementation to females

Considerable controversy over the past decades for sex preselection of embryos before pregnancy has received great attention. Several studies and trials have been carried out with the advent of assisted reproductive techniques (Cramer and Lumey, 2010). Several experiments have investigated the factors that influence the rate of sex constitution, (Cramer and Lumey, 2010). Alhimaidi *et al.* (2021) have published unreliable and thought-provoking results concerning sex preselection through minerals supplementation to maternal ewes. They have got skewing of sex ratio to males and females upon minerals supplementation (Na^{+}, K^{+}, Ca^{++} and Mg^{++}). Simultaneously, Naidu *et al.* (2023) found that mineral supplementation to rabbits (Ca^{++} and Mg^{++}) was skewed sex ratio to females. Such effects might be due to changes in reproductives hormone values and functions of oviducts (Naidu *et al.*, 2023).

CONCLUSION

Preselection of embryo sexing either before fertilization or after fertilization of oocytes is required for agriculture and medical applications in animals and humans, respectively. The applications extend to produce cloned, transgenic, racing and sports animals. Therefore, embryo sexing is an outstanding approach over the world for sustainable meat and milk production from ruminant animals and avoiding the expression of diseases in humans. The adapted methods either before fertilization or after fertilization are still time-consuming and very expensive. In addition, aspiration of one cell from the eight-cell stage embryo for performing PCR negatively affected the embryo development thereafter. Hence, the development of such technology to become precise, cheap and applicable is necessitated.

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Conflict of interest

There is no conflict of interest for authors to declare.

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