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ARTICLE

Confirmed dioestrus in pseudopregnant mice using vaginal exfoliative cytology improves embryo transfer implantation rate

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Abstract Embryo transfer is a commonly performed surgical technique. In mice, protocols typically specify pairing recipient females with vasectomized males to induce a receptive uterine environment for embryo implantation. However, this induced receptive state is not always maintained until implantation occurs. The use of a well-characterized correlation between oestrous state and exfoliative vaginal cytology was therefore evaluated to assess uterine receptivity immediately before embryo transfer. Eight- to 12-week-old virgin female CD1 mice ($n = 22$) were paired overnight with vasectomized males and successfully mated, indicated by the presence of a vaginal plug. These dams underwent embryo transfer 3 days later with embryos obtained from superovulated 4-week-old F_1 (C57BL/6 × CBA) females. Non-invasive vaginal lavage was conducted immediately before transfer. Dams were killed 6 days after transfer and the uterus collected for histological analysis. Embryo implantation rate in mice was 96% when cytological analysis of the lavage samples signified dioestrus ($n = 6$), whereas the implantation rate was <15% ($n = 16$) when cytology signified other stages of oestrous. This simple, quick, non-invasive measure of receptivity was accurate and easily adopted and, when applied prospectively, will avoid unnecessary surgery and subsequent culling of non-suitable recipients, while maximizing the implantation potential of each recipient female.

KEYWORDS: blastocyst, mouse, optimization, pregnancy, recipient, smear

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Introduction

Successful embryo transfer requires a receptive uterine environment to support embryo implantation. In female laboratory animals inducing a receptive state is typically accomplished by mating with a sterile male (Behringer et al., 2014; Tarkowski, 1959). This process initiates a cascade of physiological changes termed 'pseudopregnancy', with the uterine decidua undergoing substantial remodelling in preparation for implantation of blastocysts (Paria et al., 2002; Wang and Dey, 2006). The successful induction of pseudopregnancy is typically assessed by the presence of a vaginal mucous plug the day after pairing (Bronson and McLaren, 1970; Hogan et al., 1986; Yang et al., 2009); however, this does not guarantee that the female will remain in the pseudopregnant state until implantation of embryos occurs, which is approximately 4 days after mating in mice, as pseudopregnant females have been found to re-enter the oestrous cycle in response to various stimuli (Whitten, 1956). Verifying that recipient mice have remained in the receptive 'dioestrus' state until the time of embryo transfer may provide a key measure of the suitability of the recipient to undergo the procedure and consequently increase the overall success of the transfer process.

The mouse oestrous cycle can be observed indirectly through changes in the reproductive tract. Cyclic changes in epithelial cell structure within the vaginal and uterine lumen have been characterized in many species, with cell type and relative prevalence correlating strongly with oestrous state (Byers et al., 2012; Gal et al., 2014; Nelson et al., 1982; Papanicolaou, 1933). This relationship was first documented in the guinea pig by Stockard and Papanicolaou (1917), and shortly after in the rat (Long and Evans, 1922) and mouse (Allen, 1922). The criteria used to determine oestrous state in these landmark papers has remained relatively unchanged (Bertolin and Murphy, 2013; Cora et al., 2015; Thung et al., 1956); however, its application in embryo transfer experiments has not been routine because vaginal cell sampling typically requires instrumental penetration of the vagina (Caligioni, 2009; Nelson et al., 1982), which can induce inflammatory processes, resulting in loss of the dioestrus state (Bertolin and Murphy, 2013; McLean et al., 2012). McLean and colleagues have recently addressed the 'negative' aspects of the sampling technique, proposing a protocol for non-invasive sampling of exfoliative epithelial cells in mice (McLean et al., 2012). Therefore, we hypothesized that embryo transfer done at the time of cytologically-proven dioestrus would result in an increased implantation rate, and application of this non-invasive protocol would allow accurate staging of the oestrous cycle while avoiding the unwanted effects of penetrative sampling via the vagina.

Materials and methods

Animals and treatments

All experiments were approved by the Monash Medical Centre Animal Ethics Committee on 6 June 2013 (reference number MMC A- 2011/84) and conducted in accordance with the 8th edition of the Australian Code of Practice for the care and use of animals for scientific purposes (2013). Mice were obtained from Monash Animal Services (Clayton, Victoria, Australia), and housed in high barrier specific pathogen free (SPF) housing ≤4 per cage, 12 h light cycle (lights on at 08:00 h) at 22–23°C, 40–55% relative humidity, and food and water were available *ad libitum*.

Inducing pseudopregnancy

Pseudopregnancy was induced by pairing 8- to 12-week-old CD1 females with vasectomized CD1 males. Oestrus was first induced in the females with the introduction of soiled bedding from a sexually mature male cage 3 days prior to mating (Whitten, 1956). CD1 males were housed one per cage, with a female introduced at 14:00 h and separated at 09:30 h the following morning. Females were independently examined for the presence of a vaginal plug and plug-positive mice were moved into conventional housing for 3 days prior to embryo transfer. The morning of plug detection was designated day 1 of pseudopregnancy, with embryo transfer conducted on the morning of day 4 (~72 h after plug detection). Non-plugged mice were returned to stock for re-pairing >2 weeks later.

Vaginal lavage

Avoiding contact with the vagina, 20 μl of sterile saline was repeatedly expelled (three to five times) onto the vaginal opening and re-drawn up into the pipette tip (McLean et al., 2012). The fluid was then expelled onto a glass slide and smeared using the pipette tip to facilitate evaporation at room temperature.

For the purposes of this study, vaginal lavage samples were not stained or examined until after embryo transfers had been performed to reduce potential experimenter bias.

Exfoliative vaginal cell staining

After lavage fluid had evaporated, cells were fixed with 10% neutral buffered formalin for 5 min. Smeared lavage fluid was stained with haematoxylin and eosin. Cells were rehydrated in deionized water for 15 s, stained with Harris haematoxylin (Amber Scientific, Victoria, Australia) for 6 min, differentiated with acid ethanol and submersed in basic ammonium for up to 10 s. Slides were counterstained with 1% aqueous eosin (Amber Scientific, Victoria, Australia) for 3 min, then dehydrated, cleared and coverslipped using DPX mounting medium. Slides were scanned using Aperio ScanScope (Leica Biosystems, NSW, Australia) and viewed using Aperio ImageScope software (Leica Biosystems; ver 11).

Cytological analysis

Oestrous state was determined from vaginal exfoliative cytology smears (as shown in **Figure 1**) following specific criteria based on Byers et al. (2012). Proestrus was defined as the presence of nucleated epithelial cells and leukocytes and the absence of cornified epithelial cells. Dioestrus was defined as the presence of leukocytes only, or with leukocytes