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A novel technique for oviduct occlusion to generate live births from cryopreserved rabbit oocytes after *in vivo* fertilisation

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ABSTRACT

Intraoviductal transfer technique in combination with *in vivo* fertilisation has arisen as an effective technique to assess live births after transfer of slow-frozen oocytes in the rabbit. Nevertheless, the great disadvantage of this method is the accumulation of tubal fluid in a large number of females after clamping the oviducts. In this study, we develop an alternative method to minimise damage to the oviduct and increase the birth rate. The aims of this study were (1) to evaluate the ability of cyanoacrylate tissue adhesive to occlude the oviduct for female sterilisation; (2) to evaluate the effect of oviduct occlusion immediately after transferring fresh oocytes on *in vivo* fertilisation; and (3) to assess this technique to generate live births from fresh and slow-frozen oocytes. In all the experiments, recipients were artificially inseminated 9 h prior to occluding the oviducts. In the first experiment, the left oviduct was blocked with cyanoacrylate tissue adhesive, while the right one was used as a control. Six days later, oviducts and uterine horns were flushed to assess embryo recovery rates. While the embryo recovery rate was 79.2% in the intact oviduct, no embryos were recovered in the blocked one. In the second experiment, fresh oocytes were transferred into both oviducts, which were immediately occluded. Six days later, the *in vivo* fertilisation success rate was 33.7%. Finally, in the last experiment, slow-frozen oocytes were transferred and the rate of live births was 13.2 ± 4.5%. The study shows that when using this method the generation of live births from slow-frozen oocytes increases significantly. In addition, our results suggest that *in vivo* environment could help improve the results of oocyte cryopreservation.

1. Introduction

Preservation of female genetics can be achieved through the preservation of oocytes and embryos (Saragusty and Arav, 2011). Since Whittingham (1971) successfully froze mouse embryos, cryopreservation methodology and materials have progressed to increase the number of lines, breeds and species that can be embryo cryostored in order to preserve biodiversity or improve the reproductive rate. Oocytes are very different from sperm or embryos with respect to cryopreservation (Saragusty and Arav, 2011). The first successful birth from a cryopreserved (slow-frozen) oocyte was reported in 1977 (Whittingham, 1977) and although several breakthroughs have been made since then, live offspring have only been obtained in a few species, such as mouse, human, rabbit, cattle, rat, horse and cat (Jiménez-Trigos et al., 2012). Specifically, in the rabbit live young had not been produced since 1989 and the rate

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of live births per oocyte transferred was reported to be 7.5% (4/53) (Al-Hasani et al., 1989), whereas Vincent et al. (1989) showed a figure of 8.6% (9/105), but in unborn offspring at day 25 of gestation. We recently obtained live young from slow-frozen oocytes showing an offspring survival rate of 3.3% (4/121) (Jiménez-Trigos et al., 2013c).

The rabbit has been used as an animal model organism to study mammalian reproduction for more than a century (Chang et al., 1970; Heape, 1981; Fischer et al., 2012). To this end, different technologies for *in vitro* production of embryos have been assayed, such as *in vitro* fertilisation (IVF) (Bedford and Chang, 1962; Brackett and Williams, 1968), intracytoplasmic sperm injection (ICSI) (Keefer, 1989; Deng and Yang, 2001; Li et al., 2001; Zheng et al., 2004; Cai et al., 2005; Jiménez-Trigos et al., 2013b) and parthenogenetic activation (Ozil, 1990; Salvetti et al., 2010; Naturil-Alfonso et al., 2011; Jiménez-Trigos et al., 2012, 2013a, 2013b). Although it seems possible, IVF has not been successful in the rabbit and a repeatable IVF technique has not yet been developed, possibly due to the lack of an efficient *in vitro* capacitation system for rabbit spermatozoa linked to the poor permeability of sperm plasma membrane (Curry et al., 2000). Similarly, ICSI has been widely used in the rabbit to study oocyte fertilisation and embryo development (Keefer, 1989; Zheng et al., 2004). However, this technique is difficult to carry out because rabbit oocytes have rough, dark granules in the plasma and easily lyse and die after the ICSI process (Cai et al., 2005). The success of ICSI in the rabbit is still very limited—in the range of 2–6% live births (Deng and Yang, 2001; Li et al., 2001). For this reason, in recent years parthenogenesis has appeared as an interesting, quick and efficient tool to assess the *in vitro* developmental rates into blastocysts of rabbit oocytes in preliminary studies, when pregnancy rates are not needed (Salvetti et al., 2010; Jiménez-Trigos et al., 2012, 2013a, 2013b).

As an alternative, oocyte transfer can be used as a method to induce pregnancies due to the minimal success of *in vitro* fertilisation in rabbits (Jiménez-Trigos et al., 2013c) and in mares (Carnevale et al., 2005; Deleuze et al., 2009). Specifically, in rabbits different surgical methods, such as laparotomy or laparoscopic procedures, are well established. On the one hand, intraoviductal transfer after laparotomy has been used to evaluate the ability of the sperm to fertilise transferred oocytes or to analyse the developmental ability of these transferred oocytes after fertilisation (Motlík and Fulka, 1974, 1981; Overstreet and Bedford, 1974; Bedford and Dobrenis, 1989). On the other hand, laparoscopic procedure has been employed for *in vivo* recovery of oocytes and embryos (Cortell et al., 2010) and has also been used to transfer embryos (Adams, 1962; Besenfelder and Brem, 1993; Vicente and Garcia-Ximénez, 1993; Mehaisen et al., 2004). Recently, laparoscopic intraoviductal oocyte transfer has emerged as an alternative technique to generate live births from cryopreserved oocytes (Jiménez-Trigos et al., 2013c). This technique consists of transferring fertilised oocytes into recipient females and clamping the oviducts with a clip immediately after the transfer procedure, avoiding entry of the recipient's own oocytes. Tubal ligation or tubal occlusion is the most widely used contraceptive method in the world (Lawrie et al., 2011). Clips block the fallopian tubes by clamping and cutting off the blood supply to a portion of the tubes, causing a small amount of scarring and fibrosis, which prevents passage of ovum or sperm and therefore fertilisation. The great disadvantage of this mechanical method in *in vivo* fertilisation is the accumulation of tubal fluid in a large number of females after clamping the oviducts (Jiménez-Trigos et al., 2013c). In this study, we develop an alternative chemical method to minimise the oviductal damage.

Thus, the aims of this study were (1) to evaluate the ability of cyanoacrylate tissue adhesive to occlude the oviduct for female sterilisation; (2) to evaluate the effect of occluding the oviduct immediately after transferring fresh oocytes on *in vivo* fertilisation; and (3) to assess this technique to generate live births from fresh and slow-frozen oocytes.

2. Materials and methods

All chemicals and reagents were purchased from the Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated.

2.1. Animals

All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette). Ethical approval for this study was obtained from the Universidad Politécnica de Valencia Ethics Committee. New Zealand white females ($n = 38$), of 5 months old, were used as oocyte donors and recipients. The animals used came from the experimental farm of the Universidad Politécnica de Valencia. The rabbits were kept in conventional housing (with light alternating cycle of 16 light hours and 8 dark hours, and under controlled environmental conditions: average daily minimum and maximum temperature of 17.5 and 25.5°C, respectively. All rabbits had free access to fresh food and water.

2.2. Oocyte collection

Cumulus oocyte complexes (COCs) at the metaphase II stage (MII) were collected from donor females induced to ovulate by an intramuscular dose of 1 μg of buserelin acetate (Suprefact, Hoechst Marion Roussel, S.A., Madrid, Spain). COCs were collected 14–15 h after ovulation induction by flushing each oviduct with Dulbecco's phosphate-buffered saline without calcium chloride (DPBS) and supplemented with 0.1% of bovine serum albumin (BSA). Finally, oocytes were treated for 15 min at room temperature with 0.1% (w/v) hyaluronidase in DPBS, and cumulus cells were removed by mechanical pipetting.

2.3. Slow-freezing of oocytes

The slow-freezing procedure was adapted from previously described methods (Siebzehnruebl et al., 1989). Briefly, oocytes were incubated for 15 min at room temperature in a solution containing 1.5 M 1,2-propanediol