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Vitrification of kidney precursors as a new source for organ transplantation

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Abstract

Kidney transplantation from deceased or living human donors has been limited by donor availability as opposed to the increasing demand, and by the risk of allograft loss rejection and immunosuppressive therapy toxicity. In recent years, xenotransplantation of developed kidney precursor cells has offered a novel solution for the unlimited supply of human donor organs. Specifically, transplantation of kidney precursors in adult hosts showed that intact embryonic kidneys underwent maturation, exhibiting functional properties, and averted humoural rejection post-transplantation from non-immunosuppressed hosts. Even if supply and demand could be balanced using xenotransplants or lab-grown organs from regenerative medicine, the future of these treatments would still be compromised by the ability to physically distribute the organs to patients in need and to produce these products in a way that allows adequate inventory control and quality assurance. Kidney precursors originating from fifteen-day old rabbit embryos were vitrified using Cryotop® as a device and VM3 as vitrification solution. After three months of storage in liquid nitrogen, 18 kidney precursors were transplanted into non-immunosuppressed adult hosts by laparoscopy surgery. Twenty-one days after allotransplantation, 9 new kidneys were recovered. All the new kidneys recovered exhibited significant growth and mature glomeruli. Having achieved these encouraging results, we report, for the first time, that it is possible to create a long-term biobank of kidney precursors as an unlimited source of organs for transplantation, facilitating the inventory control and distribution of organs.

Keywords: organogenesis, biobank, metanephros, laparoscopy, rabbit, Cryotop®

Introduction

Kidney transplantation from deceased or living human donors has been limited by donor availability as opposed to the increasing demand, and by the risks of allograft loss rejection and immunosuppressive therapy toxicity [4]. In recent years, xenotransplantation of developed kidney precursor cells has provided a novel solution for the unlimited supply of human donor organs [5,9]. Specifically, transplantation of kidney precursors in adult hosts showed that intact embryonic kidneys underwent maturation, exhibiting functional properties, and averted post-transplant cellular rejection from non-immunosuppressed hosts [9]. Even if supply and demand could be balanced using xenotransplants or lab-grown organs from regenerative medicine, the future of these treatments would still be compromised by the ability to physically distribute the organs to patients in need and produce these products in a way that allows adequate inventory control and quality assurance [2,7]. To this end, organ cryopreservation will be indispensable.

The long-term banking of human organs or their engineered substitutes [7] for subsequent transplantation is a long-sought [12, 21, 23] and important goal [1, 7, 8, 10, 11, 13, 22, 23, 26]. Storage below the critical temperature of -130 ºC allows the preservation of cells and tissues after a long storage in liquid nitrogen [15,20]. To date, small ovaries, blood vessels, heart valves, corneas and similar structures are the only macroscopic structures having the capacity to recover, at least in part, after vitrification [6]. Kidneys and hearts have been the most widely studied organs, but neither has been reproducibly recovered after cooling to temperatures lower than about -45ºC, evidently due at least in part to mechanical damage from ice itself, although in the case of kidneys at least, sporadic survival has sometimes been claimed after freezing to about -40º to -

80ºC [6,8]. Fahy et al. [6] reported a case history of one rabbit kidney that survived vitrification and supported the life of a recipient animal for an indefinite period of time. To our best knowledge, only Bottomley et al. [2] evaluated the cryopreservation of metanephroi immediately after thawing, but only under in vitro conditions.

In an effort to advance in organ cryopreservation, this study was conducted to evaluate the developed morphologically normal glomeruli of vitrified kidney precursors after their allotransplant in non immunosuppressive rabbits.

Materials and Methods

Chemicals

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

Animals and ethical clearances

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, 5 months old, were used as embryo donors and metanephroi 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 eight dark hours, and under controlled environmental conditions: average daily minimum and

maximum temperature of 17.5 and 25.5ºC, respectively). All rabbits had free access to fresh food and water.

Metanephroi recovery

Donor does were artificially inseminated with 0.5 mL of fresh heterospermic pool semen from fertile males at a rate of $40x10^6$ spermatozoa/mL in Tris-citric-glucose extender [25]. Immediately after insemination, ovulation was induced by an intramuscular injection of 1 μg buserelin acetate and the females were euthanised at day 15 post-insemination. Recovered 15 day old embryos (E15) were placed in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.2% of bovine serum albumin (BSA) at 38.5ºC. Metanephroi were surgically dissected under a dissecting microscope using previously described techniques [19]. Some of the recovered embryos were placed in Bouin's solution to fix. They were then dehydrated through ethanol series, cleaned with xylol and embedded in paraffin. Next, 5-7μm sections were cut for hematoxylin-eosin staining and the slides were studied by light microscopy in order to identify the position and the size of the metanephroi (Figure 1).

Vitrification procedure

Vitrification was performed within 1 h after recovery following the minimum essential volume (MEV) method, using Cryotop® as device [16] (Kitazato-dibimed, Valencia, Spain) and VM3 as vitrification solution [8] (21st Century Medicine, Fontana, CA, EEUU). Cryotop® is the special container, consisting of a fine thin film strip attached to a hard handle. This allows us to minimise the volume of vitrification easily. All manipulations were performed at room temperature (25 ± 1 °C) and all the media were

used at room temperature, except for the first warming solution, which was used at 37.5 °C.

Metanephroi were first submerged into 2.5 ml of equilibration solution that containing 1.7% w/v ethylene glycol (EG), 1.3% w/v formamide, 2.2% w/v dimethyl sulphoxide (DMSO), 0.7% w/v PVP K12 (polyvinylpyrrolidone of Mr 5000 Da) and 0.1% w/v final concentrations of commercially available SuperCool X-1000 and SuperCool Z-1000 (ice blockers) in base medium (BM: DPBS + 20% foetal bovine serum, FBS) for 3 min. Then, the metanephroi were submerged into 2.5 ml of solution containing 4.7% w/v EG, 3.6% w/v formamide, 6.2% w/v DMSO, 1.9% w/v PVP K12 and 0.3% w/v final concentrations of ice blockers in BM for 1 min. Finally, the metanephroi were submerged into 2.5 ml of vitrification solution consisting of 16.84% w/v EG, 12.86% w/v formamide, 22.3% w/v DMSO, 7% w/v PVP K12 and 1% w/v final concentrations of ice blockers in BM before being loaded into Cryotop® devices (Figure 1) and directly plunged into liquid nitrogen (LN2) within 1 min.

For warming, metanephroi were submerged into 2.5 ml of a solution containing 1.25 M sucrose in BM for 1 min and later transferred stepwise into decreasing sucrose solutions (0.6, 0.3 and 0.15 M sucrose in BM) for 30 s before and then washed twice in BM for 5 min.

Metanephroi transplantation

After three months of storage in liquid nitrogen, the metanephroi were transplanted into recipients. Metanephroi were transplanted within 45 minutes after warming or collected (fresh). Recipients were sedated by intramuscular injection of 5 mg/kg of xylazine (Rompun, Bayer AG, Leverkusen, Germany) and anaesthetised by intravenous injection

of 15 mg/kg ketamine hydrochloride (Imalgene®, Merial, S.A., Lyon, France) into the marginal ear vein. During laparoscopy, 3 mg/kg of morphine hydrochloride (Morfina, B.Braun, Barcelona, Spain) was administered intramuscularly. Abdominal laparoscopy was performed with two ports (one for the camera and one for dissecting forceps. Image). Metanephroi were aspirated in an epidural catheter (Vygon corporate, Paterna, Valencia, Spain), introduced into the inguinal region with an epidural needle and then transplanted into a pouch created by epidural needle in the retroperitoneal fat, adjacent to the renal vessels. Four metanephroi were transplanted into each host. After surgery, analgesia was administered for 3 days (0.03 mg/kg of buprenorphine hydrochloride, Buprex®, Esteve, Barcelona, Spain, each 12 hours, and 0.2 mg/kg of meloxicam, Metacam®, 5 mg/mL; Norvet; Barcelona, Spain, every 24 h). In addition, all the recipients were treated with antibiotics (4 mg/kg of gentamicin [10% Ganadexil, Invesa, Barcelona, Spain) every 24 h for 3 days]. No immunosuppression was given to recipients. Metanephroi transplantation was assessed in three sessions.

Histomorphometry of the renal corpuscle and growth of transplanted kidney precursors

Twenty-one days after transplantation, hosts having received an allograft were euthanised and the new kidneys were removed (Figure 2). The new kidneys were individually weighed, fixed in Bouin's solution and embedded in paraffin wax and stained, as previously described. The stained sections were examined with light microscopy for histological and histomorphometric analysis (Figure 3). In the histomorphometric measurements, 20 renal corpuscle and glomeruli on each sample were measured (area and perimeter) in each of the groups - control and experimental.

Photomicrographs were taken at total magnification of X1000. In addition, the glomerular tuft cellularity was estimated by counting the total number of nuclei of each glomerulus. Photomicrographs were measured using ImageJ analysis software (public domain http://rsb.info.nih.gov/ij/). Kidneys originating from a 5-week-old rabbit (coeval with the metanephroi age) were used as controls.

Statistics

The development rates after transplantation were analysed using the chi-square test. The weights of kidney precursors, renal corpuscle and glomeruli measured (area and perimeter) and the glomerular tuft cellularity were compared by analysis of variance ANOVA with sample type (fresh and vitrified) as a fixed factor and replicate as random factor. The replicate was non-significant and was removed from the model. Significance was attributed to analyses where P is less than 0.05. All statistical analyses were performed using the SPSS 21.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002). Data were expressed as means ± standard error of means.

Results

Two females were used as embryo donors of metanephroi. A total of 3 recovered embryos were fixed for histological examination (Figure 1) and 17 were surgically dissected. After obtaining the metanephroi, 16 were transplanted directly (fresh group) and 18 after vitrification procedure (vitrified group) into 9 recipient does. Twenty-one

196 days after transplant, all the new kidneys recovered exhibited significant growth (Figure
197 2). In total, 9 metanephroi (50%) were successfully grown after vitrification. Similar
198 rates were reached from fresh kidney precursors, as 7 metanephroi were obtained
199 (43.7%). Transplanted kidney precursors, 3 weeks post-transplant, weighed 0.25±0.04 g
200 and 0.37±0.05 g for vitrified and fresh kidney precursors, respectively, which was
201 significantly less than the kidneys of control animals (0.78±0.07 g, $P<0.001$).
202 Nevertheless, in all of them, new kidneys developed mature glomeruli (Figure 2). The
203 histomorphometry results as displayed in Table I show the significant increase in the
204 renal corpuscle area and perimeter ($p < 0.05$) of the fresh and vitrified new kidneys
205 when compared to the control group. Glomerular area showed a significant increase in
206 vitrified group when compared with the control group ($p < 0.05$). Vitrification has no
207 significant effect on glomerular perimeter, when compared to the corresponding values
208 in the control. Nevertheless, in all kidney graft explants, there was a significant
209 reduction in glomerular tuft cellularity when compared with the control group ($p <$
210 0.05).
211
212

213 **Discussion**

214 This is the first study reporting that metanephroi survived vitrification, underwent
215 differentiation and growth, became vascularised by blood vessels of host origin and
216 developed morphologically normal glomeruli. Only one previous study had examined
217 metanephroi cryopreservation, suggesting that vitrification yielded more promising
218 results, consistent with our findings [2]. Vitrification, in which the liquids in a living
219 system are converted into the glassy state at low temperatures, provides a potential
220 alternative to freezing that can in principle avoid ice formation altogether [6].
221 Specifically, tissues and organs are severely damaged by extracellular ice [24]. The

differences in methodology and evaluation methods (e.g. *in vitro* and *in vivo*) between Bottomley et al. [2] and our study make it difficult to compare. Briefly, Bottomley et al. [2] studied the effect of different cryopreservation procedures (slow freezing vs vitrification) directly on the metanephroi, using a vial as container and stored the samples at -135ºC for 48 hours.

To date, small ovaries, blood vessels, heart valves, corneas and similar structures are the only macroscopic structures with the capacity to recover, at least in part, after vitrification [6]. Presumably, the cause for our improvement was likely due to the combination of device and vitrification solution. Since its first reported application for embryo cryopreservation [18], the greatest improvement has been achieved by the use of newer vitrification containers that aimed to minimise the volume of vitrification solution and thereby increase the speed of cooling and warming (up to 20,000 ºC/min) by facilitating the rapid transfer of heat to liquid nitrogen [14]. Moreover, we used a vitrification solution specifically develop for kidney cryopreservation [8], whose critical cooling rate (the cooling rate above which ice formation is not observed) is <0.1ºC/min, and whose critical warming rate (the warming rate above which ice formation is not observed) is 3ºC/min. [27]. In addition, as E15 rabbit kidney precursors are < 0.1 mm in size, an adequate diffusion and equilibration of cryoprotective agents within the organ cells is not a serious issue and vitrification should be feasible [2]. Furthermore, the kidney precursor does not require immediate vascular anastomosis upon transplantation, as is the case in a vascularised organ [28]. Vitrified transplanted metanephroi developed a blood supply originating from the host vasculature similar to that of fresh metanephroi.

Our results on renal corpuscle histomorphometry further support earlier findings demonstrating that transplanted metanephroi have the ability to develop apparently normal glomeruli [3,5,17,19,29,30,31]. In this paper, we provide quantitative morphometric data that support these previous observations, but using vitrified metanephroi stored for 3 months. Although differences were observed in renal corpuscle area and perimeter between vitrified and control group, this can be explained by the fact that the metanephroi were not connected to the host's urinary system. Under this condition, unconnected metanephroi become hydronephrotic [31]. Furthermore, new kidneys originating from vitrified metanephroi exhibited similar renal glomerular morphometry, but with slightly less glomerular tuft cellularity than control. It may not be abnormal for a new kidney compared to kidneys from 5-weeks old animals, because the transplanted metanephroi have 30% of the organ mass compared to control. Moreover, such a small difference could be irrelevant. Although we recognise the potential limitations of this approach, different authors have already shown that transplants of fresh kidney precursors are able to filter blood and produce urine [3,5,17,19,29,30,31]. In further work, we shall explore whether transplantation of vitrified embryonic kidneys may become a viable approach to renal replacement therapy, evaluating the haemodynamic capacity of transplanted vitrified metanephroi.

Having achieved these encouraging results, we suggests for the first time that it may be possible to create a long-term biobank of kidney precursors as an unlimited source of organs for transplantation, facilitating most of the problems of matching organs to recipients to reduce rejection, transporting the organs to where they need to go, and scheduling surgery at a time and a place that is best for both the patient and the transplant surgeon.

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Figure 1. Histology of 15-day-old rabbit foetus and recovered metanephroi. (A) 15-day-old foetus. (B) Micrographs (H&E) showing 15-day-old foetus. Black arrow indicates metanephroi allocation. (C) Detail of 15-day-old metanephros. (D) Micrographs (H&E) showing 15-day-old metanephros. (E) Detail of 15-day-old metanephros loaded in a Cryotop® device. Detail of metanephros loaded into film strip of Cryotop®.

Figure 2. Successful development of new kidneys after allotransplantation of fresh and vitrified kidney precursors. (A) Macroscopic view of a fresh kidney precursor 3 weeks after transplantation. Black arrowheads indicate the new kidneys. Note massive growth and the blood vessels of a new kidney. White asterisk indicates the host kidney. Black arrowheads indicate the new kidneys. (B) Macroscopic view of a vitrified kidney precursor 3 weeks after transplantation. Black arrowhead indicates the new kidney and white asterisk indicates the host kidney. (C) Micrographs (H&E) showing glomeruli of the control kidney originating from a 5-week-old rabbit (coeval with the metanephroi age). (D) Micrograph (H&E) showing glomeruli of new kidney after allotransplant fresh kidney precursor. (E) Micrograph (H&E) showing glomeruli of new kidney after allotransplant vitrified kidney precursor. Scale bar: 0.1 mm (C, D and E).

Figure 3. Representative photomicrograph of the renal corpuscles (H&E). G; Glomerulus. RC; Renal corpuscle. BS; Bowman´s space. (A) Renal corpuscle of the control kidney originating from a 5-week-old rabbit (coeval with the metanephroi age). (B) Renal corpuscle of a fresh kidney precursor 3 weeks after transplantation. (C) Renal corpuscle of vitrified kidney precursor 3 weeks after transplantation. Scale bar: 0.01 mm