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# Embryonic Organ Transplantation: The New Era of Xenotransplantation

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Additional information is available at the end of the chapter

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## Abstract

Here, we review the recent advances towards the use of organs from embryonic donors, antecedent investigations, and the latest work from our own laboratory exploring the utility for transplantation of embryonic kidney as an organ replacement therapy. In addition, we have recently reported, for the first time, that it is possible to create a long-term biobank of kidney precursors as an unlimited source of organs for xenotransplantation, facilitating inventory control and the distribution of organs.

Kidney transplantation from deceased or living human donors has been limited by donor availability as opposed to the increasing demand. Simultaneously, the risk of loss of graft by rejection or toxicity of immunosuppressive therapy exacerbates this organ shortage. In recent years, xenotransplantation of developing pancreas and 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 humoral rejection post-transplantation from non-immunosuppressed hosts.

Organ primordia engraft, attract a host vasculature, and differentiate following transplantation to ectopic sites. Attempts have been made to exploit these characteristics to achieve clinically relevant endpoints for end-stage renal disease using animal models. We focused on two main points: (a) performing transplantation by a minimally invasive laparoscopic procedure and (b) creating a long-term biobank of kidney precursors, as an unlimited source of organs for transplantation, facilitating the inventory control and the distribution of organs. Because even if supply and demand could be balanced using xenotransplants or laboratory-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.

**Keywords:** chronic renal failure, cryopreservation, laparoscopy, organogenesis, organ primordia, vitrification, xenotransplantation

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## 1. Introduction

The functional failure of an organ has several origins, from malignancies to degenerative diseases. These latter ailments are non-infectious disorders characterised by progressive disability. Nowadays, more and more patients are suffering from degenerative processes that end in specific irreversible organ failure. Loss of function becomes irreversible once injury exceeds the inherent regenerative potential or redundancy of the affected organ system; in many instances, therapeutic options are limited to supportive measures and prevention of further damage [1]. Although substantial progress has been made in the minimisation of irreversible tissue loss in the acute phase of many disease processes, the restoration of lost tissue and organ function after critical damage has occurred has been less successful. In these cases, transplantation represents the ideal method of restoring full physiological organ function [2]. However, transplantation from deceased or living human donors has been limited by donor availability, as opposed to the increasing demand, by the risks of allograft loss rejection and immunosuppressive therapy toxicity [2,3]. These factors mean that many patients have to wait for long periods of time, entailing increased morbidity and mortality for tens of thousands of people each year [4], and a lot of patients die before receiving the desired organ. As proof, it is interesting to recall that, in April 2014 in the United States, approximately 122,000 patients were waiting for an organ transplant, but, a year later, less than 30,000 of them had received it [5].

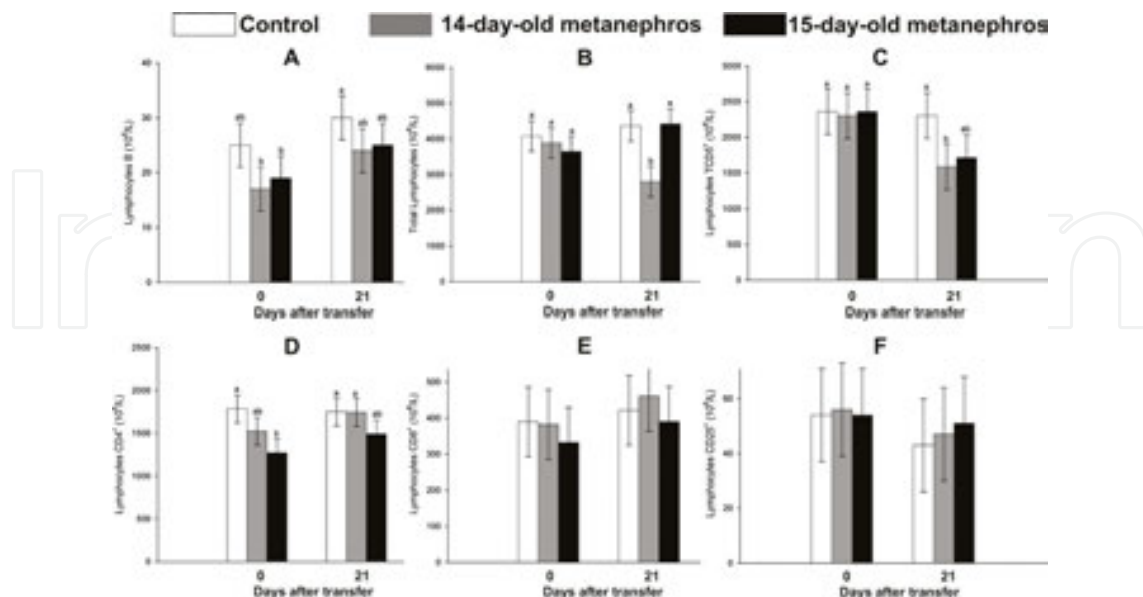
Currently, in the field of urology, the prevalence of chronic renal failure continues to outpace the development of effective treatment strategies. In the European Union, in late 2011, more than 42,000 patients were on waiting lists for kidney transplant [6]. During this year, 18,712 transplants were performed, of which 20.6% came from living donors. This means that patients with advanced renal disease are habitually obliged to resort to renal replacement therapies, such as haemodialysis or peritoneal dialysis. However, these techniques fail to meet the functional endocrine and reabsorption demands of normal kidney function [2], also affecting the quality of patient's life [7]. In the United States, approximately 100,000 individuals are waiting for a kidney transplant and more than 400,000 individuals are suffering some kind of end-stage kidney disease requiring haemodialysis [8,9]. Nevertheless, the issue is even more severe than in the United States, being a universal problem affecting approximately 5–7% of the world population [10]. Two decades ago in Spain, a leading country in the field of transplants, approximately 4400 patients were on the waiting list for kidney transplantation [11]. Today, approximately 129 (incidence) and 1039 (prevalence) patients per million habitants still require renal replacement therapies [12]. The therapeutic alternatives to transplant (haemodialysis or peritoneal dialysis) represent a cost of €1518 million to the country's public services. Whereas this is a problem in Spain, the issue is much more serious in other countries, such as

the United Kingdom, where organ donation rates are lower and the costs of renal replacement therapies amounted to £1.2 billion [13,14]. Therefore, seeking alternative solutions to this grave problem is indispensable.

## 2. Transplantation of embryonic organs as a novel solution to organ shortage

Even before obtaining an allogeneic organ, despite advances in renal transplant immunology, 20% of recipients will experience an episode of acute rejection within 5 years of transplantation, and approximately 40% of recipients will die or lose graft function within 10 years after transplantation [9]. Thus, the risk of graft rejection is still an obstacle in the field of kidney transplantation. Similarly, the use of xenotransplants has been considered for years as a possible solution to the organ shortage, but the risks of xenograft loss rejection and zoonosis have limited the clinical application of this kind of treatment [15,16]. The use of individual cells or groups of cells to repair damaged tissue (cellular therapies) offers an alternative for renal tissue replacement. However, the recapitulation of complex functions, such as glomerular filtration and reabsorption and secretion of solutes that are dependent on a three-dimensionally integrated kidney structure, is beyond the scope of most cellular replacement therapies [17].

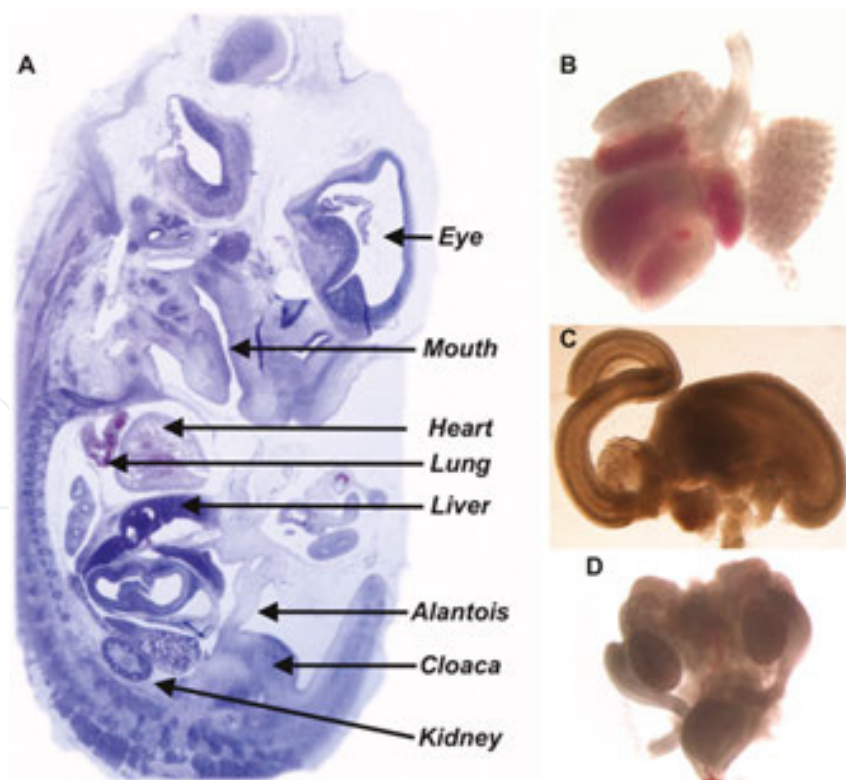
The field of renal transplantation is exploring new frontiers. Recently, following this line, and together with the production of specific pathogen-free animals [18], xenotransplantation of developing kidney precursors has provided a novel solution for these troubles [17,19]. Unlike embryonic stem (ES) cells or induced pluripotent stem cells, developing metanephric kidney



**Figure 1.** Effect of foetus metanephroi transplanted into 14- and 15-day-old rabbit on peripheral blood. (A) Total lymphocytes, (B) B lymphocytes (C), and T lymphocytes CD5<sup>+</sup> ( $\times 10^6/l$ ), (D) CD4<sup>+</sup> ( $\times 10^6/l$ ), (E) CD8<sup>+</sup> ( $\times 10^6/l$ ), and (F) CD25<sup>+</sup> cells ( $\times 10^6/l$ ) after 21 days in non-immunocompromised recipients. a,b significant differences ( $P < 0.05$ ).

cells are already committed to a genetic program of renal development and “knowing” its destination cell type and how it should be assembled [3], obviating the need to pre-program cell fate. Otherwise, transplantation of kidney precursors in adult hosts showed that intact embryonic kidneys underwent maturation, exhibiting functional properties and avoiding rejection from non-immunosuppressed hosts [17]. This happens because, in a developing kidney, antigen-presenting cells, which mediate direct host recognition of strange antigen, are absent because they would not have yet developed in the donor and migrated into the metanephroi [20,21]. Furthermore, metanephroi express fewer MHC class I and II antigens, which mediate host recognition, than the adult kidney [22,23] (**Figure 1**). In addition, the immunological response mediated by T helper lymphocytes is skewed when responding to a foetal organ compared to an adult organ [24].

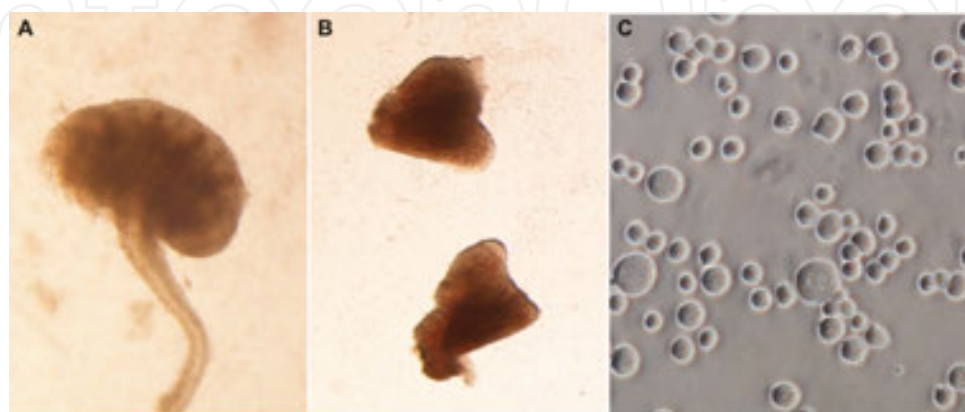
Moreover, it is important to recall that metanephroi trigger the formation of a vascular system directly from the host [25,26], attenuating rejection and encouraging their transplantation across the species barrier [19,26–28]. Additionally, renal primordials do not require immediate vascular anastomosis upon transplantation, as is the case in a vascularised organ [29]. Finally, into the bargain, the use of animal cells avoids ethical barriers to human ES cell use [3]. Therefore, the results achieved with embryonic organs (**Figure 2**) have returned the use of xenotransplantation as a possible solution to the shortage of vital organs, such as the kidney, to the front line of research [30].



**Figure 2.** Histology of 15-day-old rabbit foetus and recovery organs. (A) Foetus. (B) Lung and heart. (C) Stomach and intestines. (D) Gonads and cloaca.

### 3. Experiences in embryonic organ transplantation

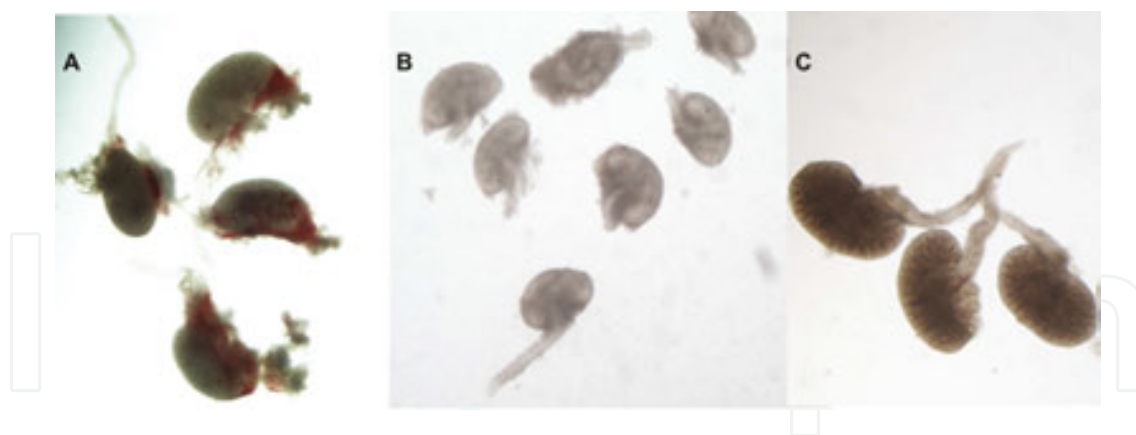
A major advantage inherent in the use of embryonic kidney or pancreas (**Figure 3**) for transplantation relative to more pluripotent undifferentiated cells is that the former differentiate spontaneously along defined organ-committed lines, albeit with a different outcome relative to what would occur if the primordia remained undisturbed within the embryo [31].



**Figure 3.** Detail of rabbit 15-day-old metanephros (A) and pancreas (B) and stem cells (C).

At present, experiences in this area revolve around the endocrine pancreas and kidney, the latter organ being the theme that will be the focus of this work. However, it is interesting to mention the major advances that have been achieved with embryonic pancreas to emphasise the potential of embryonic organ transplantation as a possible solution to solve many of today's diseases. Thus, it was reported that, when embryonic pancreas was transplanted through the species barrier (xenotransplantation), a selective development of endocrine tissue took place [32–38]. These developing  $\beta$  cells enter lymphatic vessels and engraft in mesenteric lymph nodes, secreting insulin in response to elevated blood glucose. Consequently, glucose intolerance can be corrected in formerly diabetic rats [32–35,37] and ameliorated in rhesus macaques [36,38] based on porcine insulin secreted in a glucose-dependent manner by  $\beta$  cells originating from transplants [31]. Furthermore, if embryonic pancreas were obtained in a specific time window of the embryonic development, these primordia were able to engraft in diabetic rats [32–35,37] and rhesus macaques [36,38] without immunosuppression treatment. However, although the results obtained in this field are promising, it is still too soon to predict the future of this line. This is because the experiences show that, depending on which xenogeneic barrier is crossed, the results will be different [31]: rat-to-mouse transplantation results in the formation of the new organ and requires host immunosuppression, whereas pig-to-rat or pig-to-rhesus macaque transplantation results in lymphatic dissemination of  $\beta$  cells and no immunosuppression is required. Therefore, further studies are required to clarify the matter and its therapeutic potential.

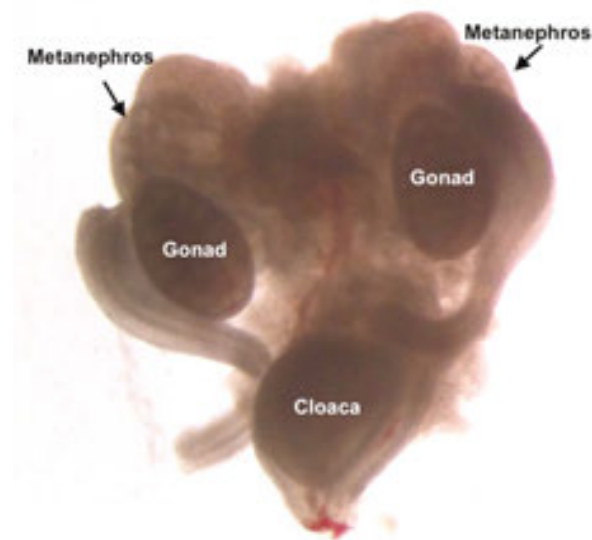
In the case of renal primordia, since Woolf et al. [39] reported a study of embryonic kidney tissue transplantation in 1990, several groups have investigated embryonic kidney transplantation, with surprising results [19,27,39–47] (**Figure 4**).



**Figure 4.** Image of metanephroi in different species. (A) Details of porcine 28-day-old metanephroi. (B) Details of rabbit 15-day-old metanephris. (C) Details of mouse 13.5-day-old metanephroi.

Woolf et al. [39] reported that mouse metanephroi continued to grow if transplanted into the renal cortex of a host mouse [39]. In this study, developed metanephroi showing vascularised glomeruli, mature proximal tubules, and extensions of metanephric tubules into the renal medulla were observed. This is a sufficiently encouraging result, which boosted research in this line. Later, Rogers et al. [27] reported in rats the first long-term survival (>10 days) after subcapsular transplantation of metanephroi into fully differentiated kidneys of animals in which nephron formation is no longer taking place as well as the first intraomental transplantation of metanephroi. Glomerular filtration in developed metanephroi transplanted was demonstrated both subcapsularily [39] and in the omentum [27]. Rogers et al. [40] demonstrated that pig metanephroi transplanted into pigs underwent growth and differentiation of nephrons over a 2-week period without the need for co-stimulatory blockade of hosts. Furthermore, pig metanephroi after 2 weeks of transplantation had enlarged, become vascularised, and formed mature tubules and glomeruli in host mice with the use of immunosuppressants. In the same year, Dekel et al. [19] transplanted metanephroi of both human and pig origins into mice, which differentiated into functional nephrons and their renal functionality, as evidenced by the dilute urine they produced. One year later, it was reported that the survival of rats with all native renal mass removed can be increased by prior metanephroi transplantation and ureteroureterostomy [41]. A couple of years later, Takeda et al. [42] confirmed experimentally that the predominant origin of endothelial cells after transplantation of embryonic pig metanephroi into rats is the host, whereas mesangial cells originate mainly from the donor. Recently, in 2012, the Hosoya et al. [43] group reported that transplantation of metanephroi produces plasma renin activity and contributes to raising arterial blood pressure in a rat model of acute hypotension and suppresses the progression of vascular calcification in rats with adenine-induced renal failure by significantly reducing vascular calcium and phosphorus content [44]. Thus, developed metanephroi in new renal tissue not only provide an excretion function but also an endocrine function, synthesising renal hormones such as renin and erythropoietin [43,45]. Interestingly, it is known that xenotransplanted embryonic kidney also provides a niche for endogenous mesenchymal stem cell differentiation into erythropoietin-producing tissue [46]. Furthermore, using metanephroi from transgenic ER-E2F1 suicide-inducible mice, the xenotissue component could be eliminated, leaving autolo-

gous EPO-producing tissue. These findings may alleviate adverse effects due to long-lasting immunosuppression and help mitigate ethical concerns [46]. One of the most important obstacles in this field of renal primordia transplantation is that, due to the growth and functionality of the nascent kidney, it ultimately developed hydronephrosis and did not grow in size because it lacked a urine excretion channel [47]. However, through the method described by Yokote et al. [47], it is possible to avoid this end. If metanephroi were transplanted beside bladders (developed from cloacas), the tubular lumina dilatation and interstitial fibrosis were reduced in comparison to single metanephroi transplant. In addition, if cloacal-developed bladder was connected to the host ureters, it avoided hydronephrosis and permitted the cloacas to differentiate well, producing and excreting urine through the recipient ureter and allowing the metanephroi to continue their growth (**Figure 5**).



**Figure 5.** Detail of cloaca structure. (A) Cloaca-gonads-metanephroi.

#### 4. Metanephroi transplant surgery and graft site

Initial studies have been performed using the renal subcapsular space and omentum, neither being an immunologically privileged site [48]. However, because the vasculature of the transplant is of host origin and the embryonic organ per se is less immunogenic [24], intense immunomodulation should not be required. Nonetheless, the influence of the insertion site of the kidney is not indifferent. Matsumoto et al. [45] reported that, when metanephroi were transplanted into the paraaortic area, where the developing kidney is exposed to hydrostatic pressure from the aorta, and in the omentum, where there is no hydrostatic pressure, renin production was greater in the metanephroi transplanted to the paraaortic area, although there were no site-specific differences in erythropoietin production. This result therefore suggests that renin production in our systems requires induction by vascular tension stimulus, whereas



erythropoietin production can be achieved by transplanting tissue into the omentum, where it is easily accessed by endoscopy.

Nevertheless, although to date metanephroi have been transplanted into different sites, such as the anterior eye chamber [49], intrarenally [27,39,49–51], intra-abdominally [52], or intra-mentally [27,53], all these experiments were performed through open surgery. To our best knowledge, our recent study [54] was the only experiment to tackle embryonic kidney transplantation through laparoscopic surgery.

## 5. Laparoscopic surgery for metanephroi transplantation

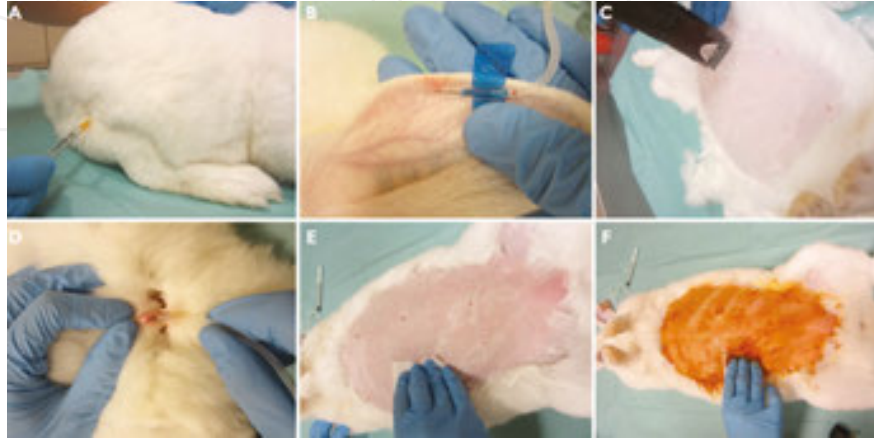
Taking into account all the information reported by some of the authors just mentioned, we have learnt that omentum is used mainly because it is not confined by a tight capsule, facilitating the growth of transplanted metanephroi [27,53] and the transplantation technique [45]. Until now, laparotomy was the sole method used to transfer metanephroi into recipients. In 2014, we developed a new minimally invasive laparoscopic procedure to transfer metanephroi into the retroperitoneal fat [54]. In addition, this new study was first conducted in rabbit as animal model, where our experience shows that size does play a crucial role. Choosing a healthy large animal more than 3–3.5 kg provides an experimental subject with a good capacity for the laparoscopic approach (**Figure 6**).



**Figure 6.** New Zealand rabbit.

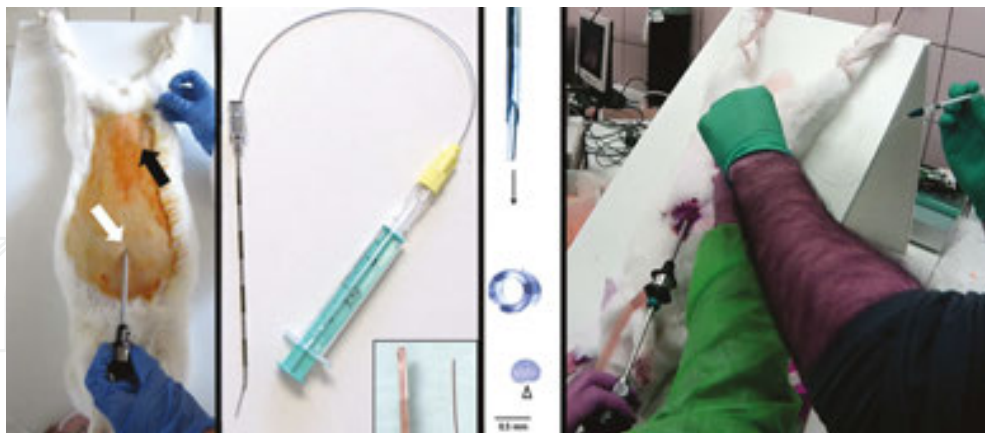
The rabbit (*Oryctolagus cuniculus*) is the third mammal most used as experimental animal in Europe after the mouse (59%) and the rat (17%) [56]. Moreover, the rabbit is phylogenetically closer to primates than rodents are [57], and disease aetiologies exhibited by humans are more similar to those in rabbits than in mice [58]. This animal is very docile and non-aggressive and hence easy to handle and observe. Widely bred and very economical compared to the expense of larger animals, rabbits also have short life cycles (gestation, lactation, and puberty). Taking these reasons into account, we consider that the rabbit is an excellent animal model for the first steps in this field. Nevertheless, we do not lose sight of the fact that further development of the art, in higher species more similar anatomically and physiologically to the human species in an attempt to finally reach clinical use, is crucial.

The effectiveness of the minimally invasive laparoscopic procedure was recently reviewed [6,54]. Briefly, recipient animals were sedated by intramuscular injection of xylazine (5 mg/kg) and morphine chloride (3 mg/kg). As surgical preparation, anaesthesia was performed by intravenous injection of ketamine hydrochloride (35 mg/kg) into the marginal ear vein (Figure 7).



**Figure 7.** Receptor preparation. (A) Sedation. (B) Anaesthesia by intravenous injection into the marginal ear vein. (C) Skin. (D) Evacuating the bladder using a urinary catheter. (E) Cleaning surgical area. (F) Disinfection of the surgical area.

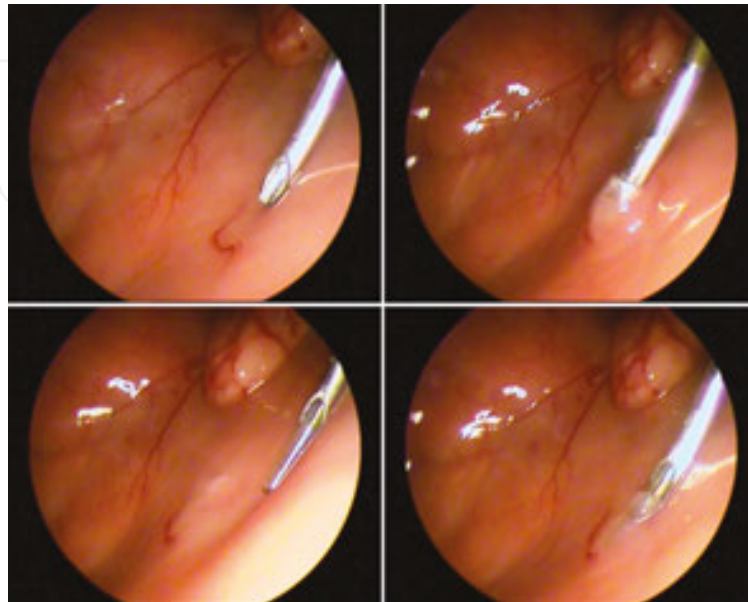
First, animals were placed in the stretcher in a vertical position (head down at 45° angle). Only one endoscope trocar was inserted into the abdominal cavity (Figure 8).



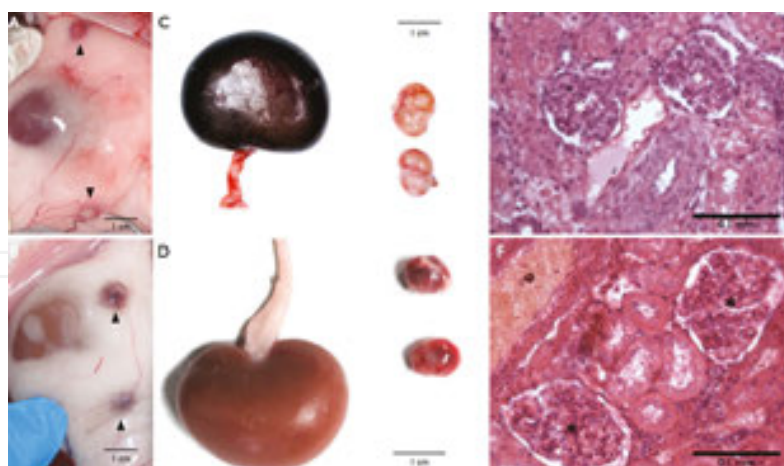
**Figure 8.** Laparoscopy procedure for metanephros transfers. (A) Allocation of the animal on the surgical table, trocar endoscope insertion position (white arrow) and epidural needle (black arrow). (B) Epidural needle (Perifix® 17 Ga × 4 in. (100 mm) and epidural catheter (17G, Vygon Corporate). (C) Epidural catheter, needle, and metanephros (white arrow). (D) Introduction of the catheter through the needle with the metanephros.

Then, a 17G epidural needle was inserted into the inguinal region (Figure 9). After identifying a vessel in the retroperitoneal fat, a hole was performed adjacent to the vessel (Figure 9). Then, kidney precursor was aspirated into an epidural catheter and the catheter was introduced

through the epidural needle and inserted into the performed hole (**Figure 9**). Four kidney precursors were transplanted in each host without immunosuppression (one metanephros per hole). After surgery, analgesia was administered every 12 h for 3 days [6,54].



**Figure 9.** Laparoscopy procedure for metanephroi transfers. (A) Epidural needle. (B) Performing a hole into the retro-peritoneal fat where the metanephros will be transferred. (C) Introduction of the catheter through the needle with the metanephros. (D) Insertion of the catheter with the metanephros into the hole and transfer.



**Figure 10.** Successful development of new kidneys after allotransplantation of metanephroi. (A) Macroscopic view of kidney precursor 3 weeks after transplantation. Note massive growth and the blood vessels of a new kidney. Black arrowheads indicate the new kidneys. White asterisk indicates the host kidney. (B) Macroscopic view of a vitrified kidney precursor 3 weeks after transplantation. Black arrowhead indicates the new kidney. White asterisk indicates the host kidney. (C) Micrographs [haematoxylin and eosin (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 fresh kidney precursor allotransplantation. (E) Micrograph (H&E) showing glomeruli of new kidney after allotransplantation of vitrified kidney precursor. Scale bar, 0.1 mm (C–E).

Following this protocol, we show that, 3 weeks after transplantation, 10 of 20 (50%) of 15-day-old and 12 of 26 (46.1%) of 16-day-old metanephroi grew and differentiated, presenting normally developed glomeruli, proximal and distal tubules, and collecting ducts [6,54,55] (Figure 10).

Thus, we describe, for the first time in the literature, laparoscopic allogeneic transplantation of metanephroi as a non-invasive and viable technique in receptors without immunosuppression [6,54]. In addition, our development of an appropriate research protocol reviewed by our institutional research ethics committee involving surgical procedures on white New Zealand rabbits has allowed us to carry out the project with good quality, control, and safety for both the researchers and the animals.

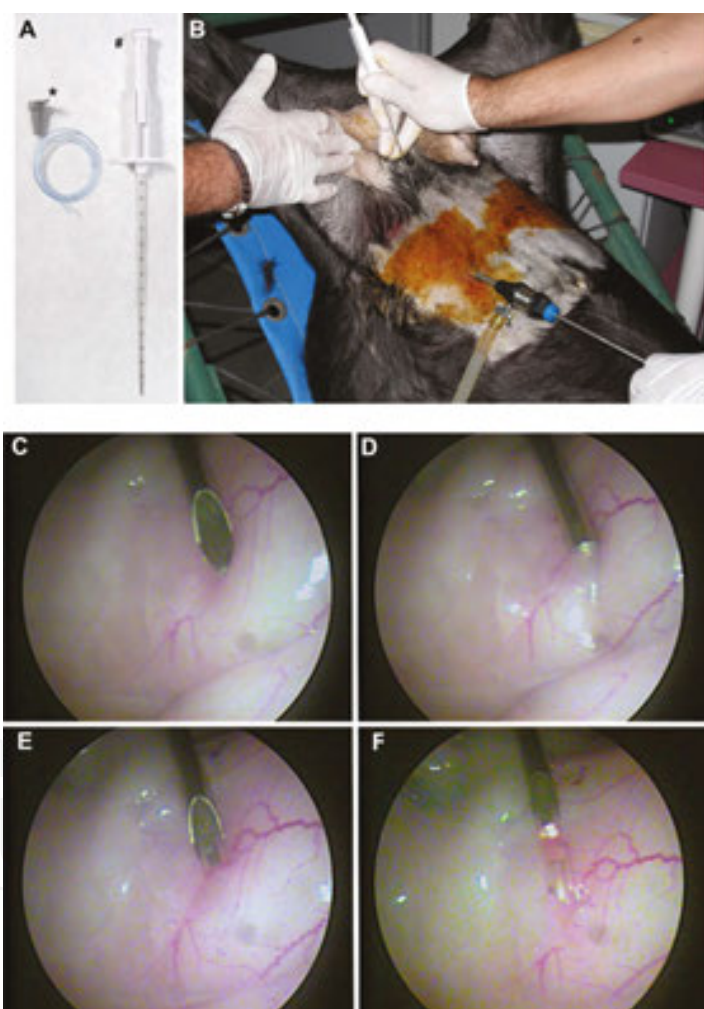


**Figure 11.** Laparoscopy procedure for metanephroi transfers. (A) Allocation of the animal on the stretcher. (B and C) Insertion of the trocar endoscope.

At the moment, successful embryo kidney transplantation tolerance has only been demonstrated previously in mice and rat. One attractive approach would be to apply this technology to large animals, whose nephron structure and size closely approximate human nephrons [3]. Larger animals, such as pigs, goats, sheep, and non-human primates, are ideal models. To the best of our knowledge, it has never been demonstrated in a large animal model. In this chapter, we develop a preliminary study in goat to provide a better test of the procedure feasibility for clinical application. As in the studies reported here in rabbit, we made use of our laparoscopy procedure adapted to this model. Briefly, recipient animals were sedated by intramuscular injection of xylazine (0.05 mg/kg) and butorphanol (0.1 mg/kg). As surgical preparation, anaesthesia was performed by intravenous injection of ketamine hydrochloride (0.5 mg/kg). First, animals were placed on an operating table in a vertical position (head down at 45° angle).

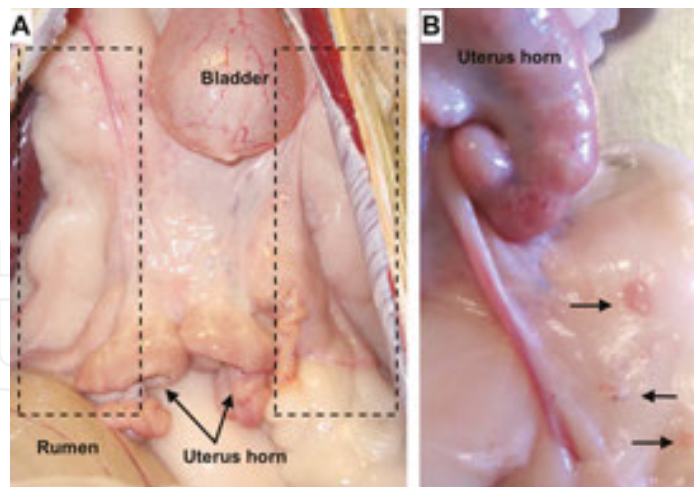
In Trendelenburg's position, rumen, stomach, and intestines do not cover the groin fat tissue into which metanephroi were transplanted. Only one endoscope trocar was inserted into the abdominal cavity (**Figure 11**).

Then, a 14G biopsy needle (Tru-Cut, 14G, 152 mm) was inserted into the inguinal region (**Figure 12**). After identifying a vessel in the retroperitoneal fat, a hole was performed adjacent to the vessel (**Figure 12**). Then, kidney precursor was aspirated in an adapted orogastric feeding catheter and the catheter was introduced through the biopsy needle and inserted into the aperture (**Figure 12**). Kidney precursors were transplanted in each host without immunosuppression (one metanephros per hole). After surgery, analgesia was administered every 12 h for 3 days [6,54].



**Figure 12.** Laparoscopy procedure for metanephroi transfers. (A) Biopsy needle (Tru-Cut®, 14G, 152 mm) and orogastric feeding catheter. (B and C) Insertion of the biopsy needle. (D) Perform a hole into the fat where the metanephros will be transferred. (E) Introduction of the catheter through the needle with the metanephros. (F) Insertion of the catheter with the metanephros into the hole. (F) Metanephros transfer.

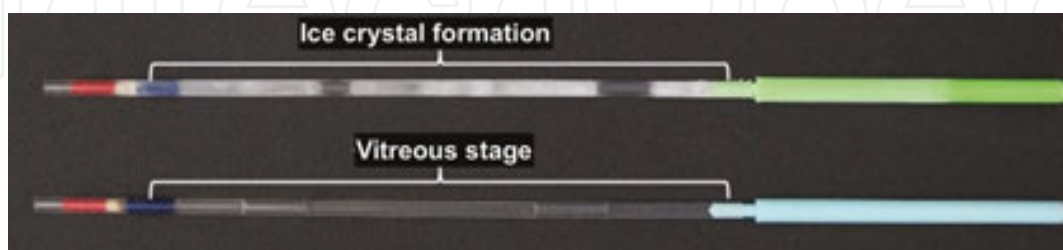
Following this protocol, we show that 6 weeks after transplantation of 15-day-old rabbit, metanephroi grew (**Figure 13**).



**Figure 13.** Successful development of new kidneys after xenotransplantation of rabbit metanephroi to goat. (A) Transplant area. (B) Growth.

## 6. Cryoconservation of embryonic kidney

Even if in a most favourable future situation the organ supply and demand could be balanced using xenotransplants or laboratory-grown organs from regenerative medicine, without proper cryopreservation procedures, 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 [59]. To this end, organ cryopreservation will be indispensable. Cryobiology is the study of the effects of low temperatures on living organisms. The aim of this discipline is to shift the pendulum from cell death to immortality at low temperatures. To achieve this, it is necessary to eliminate the two main causes of cell death associated with cryopreservation, ice crystal formation and lethal concentration of solutes, while maintaining the functional capacity of intracellular organelles [60–62] (**Figure 14**).



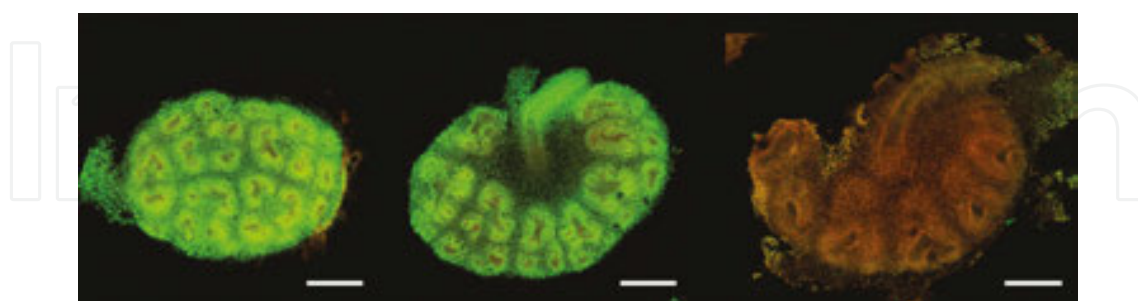
**Figure 14.** Successful development of new kidneys after xenotransplantation of rabbit metanephroi.

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 [63]. Fahy et al. [63] 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. Based on this knowledge, we recently described a method to cryopreserve metanephroi whole organs and generate kidneys after transplantation into a syngeneic non-immunosuppressed host [59]. Previously, to our best knowledge, only Bottomley et al. [64] evaluated the cryopreservation of metanephroi immediately after thawing, but only under *in vitro* conditions. Briefly, vitrification was performed following the minimum essential volume method using Cryotop® as device and VM3 as vitrification solution (Figure 15). Kidney precursors were first exposed for 3 min to equilibration solution containing 1.7% (w/v) ethylene glycol (EG), 1.3% (w/v) formamide, 2.2% (w/v) dimethyl sulfoxide (DMSO), 0.7% (w/v) PVP K12 (polyvinylpyrrolidone of Mr 5000 Da), and 0.1% (w/v) SuperCool X-1000 and SuperCool Z-1000 (ice blockers) in base medium [BM: Dulbecco's PBS + 20% foetal bovine serum (FBS)]. Then, the kidney precursors were exposed for 1 min to 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) ice blockers in BM. Finally, the kidney precursors were transferred to 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) ice blockers in BM before being loaded onto Cryotop® devices and directly plunged into liquid nitrogen within 1 min.



**Figure 15.** Details of 15-day-old metanephros loaded in a Cryotop® device. Details of metanephros loaded into film strip of Cryotop®.

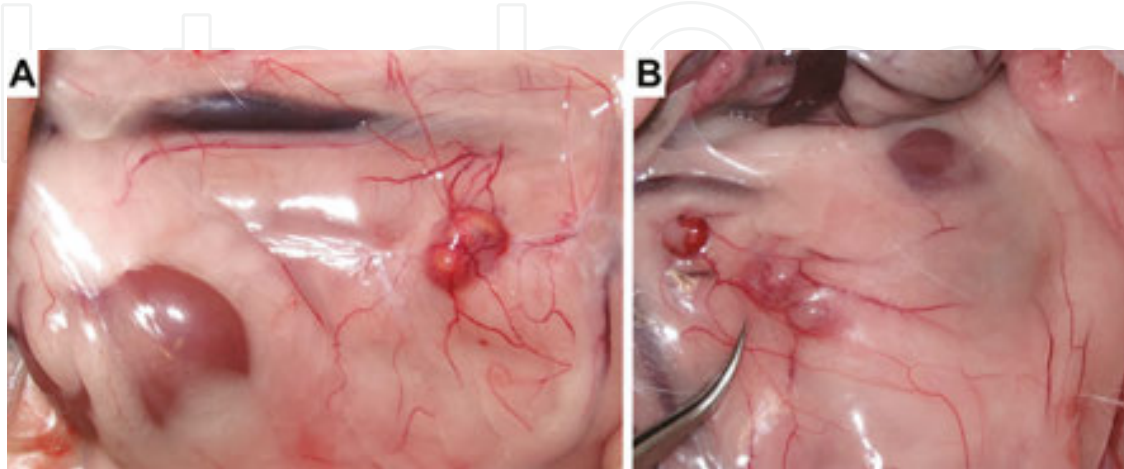


**Figure 16.** Kidney precursor viability analysis evaluated by a confocal microscope. Viability cells were evaluated by SYBR-14 (live) and propidium iodide (dead) fluorescence. (A) Fresh metanephros. (B) Live vitrified metanephros. (C) Dead vitrified metanephros. Scale bar, 1 mm.

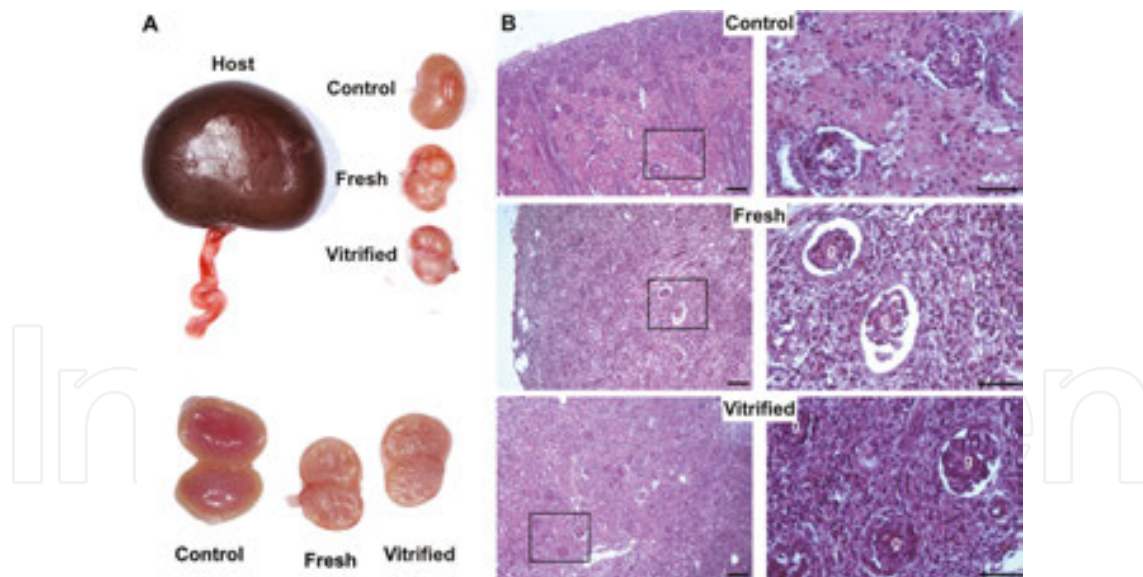
For warming, kidney precursors were placed in a solution composed of 1.25 M sucrose in BM for 1 min and later transferred stepwise into decreasing sucrose solutions (0.6, 0.3, and 0.15 M sucrose in BM) for 30 s before and then washed twice in BM for 5 min. When the kidney

precursors were thawed and processed without further culture, a high percentage of kidney precursors was considered as viable (80%; **Figure 16**).

In our study, 14 metanephroi were transplanted after 3 months of vitrification (storage). Twenty-one days after transplant, the capacity for angiogenesis of the metanephros after laparoscopic transplantation was observed (**Figure 17**).



**Figure 17.** Generation of new kidneys using fresh (A) and vitrified (B) metanephroi after allotransplantation in rabbits. Note massive growth and blood vessels of the new kidney.



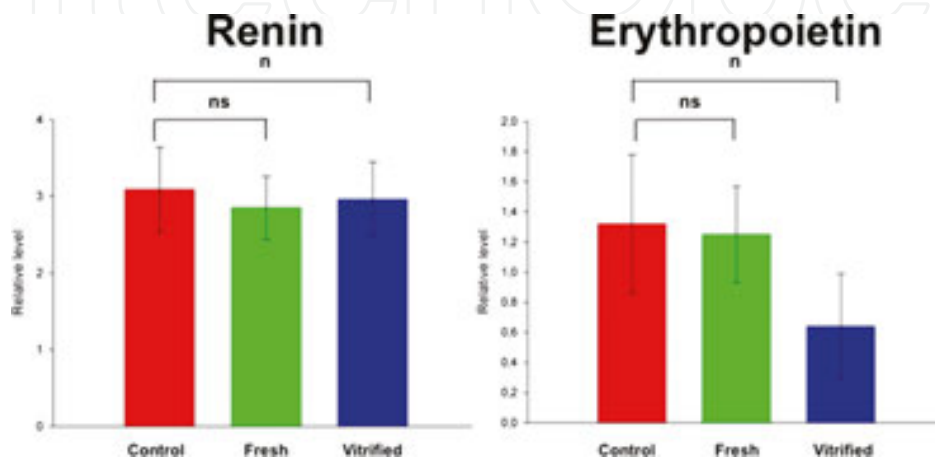
**Figure 18.** Generation of a kidney using vitrified kidney precursors after allotransplantation in rabbits. (A) Image showing the growth and shape of kidneys as well as the appearance of the renal cortex and renal medulla. Control kidney originating from a 5-week-old rabbit. (B) Histological analysis, by H&E staining, of the vitrified and fresh kidney precursors under in vivo culture for 3 weeks after transplantation. Control kidney originating from a 5-week-old rabbit. g, glomeruli. Scale bar, 0.1 mm.

In all of the recipients, new kidneys were recovered and examined. In total, 7 (50.0%) vitrified metanephroi were successfully grown. Similar rates were reached from fresh kidney precur-



sors (43.7%; **Figure 18**). In all of them, new kidneys developed mature glomeruli whose histomorphometry analysis showed that vitrification has no significant effect on glomerular perimeter compared to the corresponding values in the control (**Figure 18**).

Finally, we examined whether kidneys had normal endocrine functionality. We analysed the expression profile of the renin and erythropoietin transcript by quantitative real-time PCR (RT-PCR). The expression of renin and erythropoietin was similar in vitrified new kidneys, consistent with previous reports (**Figure 19**).



**Figure 19.** RT-PCR analysis of renin and erythropoietin transcript expression in kidneys from vitrified and fresh meta-nephroi and control kidney. Gapdh was used as reference gene.  $n=6$ ; mean $\pm$ SD. Student's  $t$  test:  $P$  value not significant (ns).

## 7. Conclusion

Our study essentially makes two innovative contributions in the field of transplantation of embryonic organs. First, we provide the first evidence of a successful long-term storage of an entire vital organ, enabling the generation of new kidneys after transplantation into a syngeneic non-immunosuppressed host. Our results therefore make a substantial contribution to the development of a long-term biobank of kidney precursors as an unlimited source of kidneys, facilitating sanitary and inventory control and the distribution of organs. Second, we show that a translational future application to transplant patients is possible using a very simple and minimally invasive laparoscopic procedure. Our present findings should also encourage the future development of bioengineering technologies to reconstitute primordial organs as an alternative approach to regenerative medicine.

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