Amelioration of renal ischaemia-reperfusion injury by synthetic oligopeptides related to human chorionic gonadotropin

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Abstract

Background. We have previously reported that small synthetic oligopeptides related to human β-chorionic gonadotropin (β-hCG) can reduce inflammation. Here we investigated whether such oligopeptides can reduce renal ischaemia-reperfusion injury in the mouse.

Methods. Ten different oligopeptides were administered 1 min before induction of renal ischaemia and 1 min before reperfusion.

Results. Survival at 72 h post-reperfusion was significantly higher in mice treated with oligopeptides MTRV, LQG, VLP ALPQ or AQGV as compared to placebo-treated mice. Some oligopeptides were more effective than others. AQGV completely prevented mortality and best preserved kidney function. Next, AQGV was tested in a dose-escalating study in a range of 0.3–30 mg/kg. A survival gain was observed with all doses. Improvement of kidney function was observed from 1 mg/kg. Highest survival and best preserved kidney function were observed at 3 and 10 mg/kg. Upon treatment with AQGV, a significantly lower influx of neutrophils was found, apoptosis was decreased, whereas tubular epithelial cell proliferation was significantly increased at 24 h post-reperfusion. Serum levels of TNF-α, INF-γ, IL-6 and IL-10 were significantly decreased at 24 h post-reperfusion. E-selectin mRNA levels in kidneys were significantly decreased at 6 h post-reperfusion. AQGV did not reduce mortality when treatment was started after reperfusion.

Conclusions. This study shows that small oligopeptides related to the primary structure of β-hCG, especially AQGV, are promising potential drugs for preventing the development of renal ischaemia-reperfusion injury.

Keywords: ischaemia reperfusion; kidney; peptide; transplantation

Introduction

Inflammation plays a major role in the pathophysiology of renal ischaemic injury [1]. The initial ischaemic injury results in upregulation of adhesion molecules on acti-

Materials and methods

Experimental design

The experimental protocol was approved by the Animal Experiments Committee under the Dutch Experiments on Animals Act and adhered to the
rules laid down in this national law that serves the implementation of ‘Guidelines on the protection of experimental animals’ by the Council of Europe (1986), Directive 86/609/EC.

Ten different hCG-related oligopeptides (MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ, AQG, AQGV, LAG and LAGV) were evaluated for their capacity to reduce ischaemia-reperfusion-induced renal injury as compared to mice treated with phosphate buffered saline (PBS). A total of 5 mg/kg body weight of oligopeptide or PBS in a volume of 0.1 mL was administered intravenously (i.v.) 1 min before clamping, and 1 min before releasing the clamp.

Subsequently, a dose-escalating study was performed with AQGV. The AQGV was given in doses of 0.3, 1, 3, 10 and 30 mg/kg in a volume of 0.1 mL and was administered i.v. 1 min before clamping, and 1 min before releasing the clamp. Possible toxic side effects were studied by careful observation of control and peptide-treated mice for signs of discomfort.

Contra-lateral kidney samples were obtained for further analysis. At 24 and 72 h post-reperfusion, mice were sacrificed and clamped kidneys were harvested and snap frozen for further analysis. Serum urea levels were measured to determine kidney function. Infiltrating cells were analysed using immunohistochemistry. In all groups, survival was assessed and analysed by Kaplan–Meier analysis.

In an additional experiment, AQGV was given in a dose of 5 mg/kg BW in a volume of 0.1 mL and administered i.v. 1 min before clamping, and 1 min before releasing the clamp. At 6 and 24 h post-reperfusion, mice were sacrificed and blood was obtained for cytokine measurements in serum. From the 6 h post-reperfusion group, the clamped kidney was harvested for determination of mRNA expression levels.

Furthermore, survival experiments were performed in which mice received PBS or AQGV (5 mg/kg BW) at 12 and 24 h, or at 6 and 12 h post-reperfusion.

Mice
Male C57BL/6JOlAwHsd mice of 12–16 weeks of age were obtained from Harlan (Horst, The Netherlands). They were kept under standard labora-

tory conditions (temperature 20–24°C, relative humidity 50–60%, 12 h light/12 h dark) and were allowed free access to food (Hope Farms, Woerden, The Netherlands) and water.

Ischaemia model
Mice were anaesthetized by isoflurane inhalation. Anaesthesia was maintained using a mixture of N2O/O2/isoflurane. Blood was collected by retro-orbital puncture. Body temperature was maintained by placing the mice on heating pads. Following a midline abdominal incision, the left renal pedicle was localized and clamped for 25 min using anatraumatic micro-vascular clamp. After inspection for signs of ischaemia, the wound was covered with PBS-soaked cotton and the animal was covered with a tin foil insulation sheet. After release of the clamp, restoration of blood flow was inspected visually and a contra-lateral nephrectomy was performed. The abdominal wound was closed in two layers, and mice were given 0.5 mL PBS subcutaneously.

Oligopeptides
Selection was based on either the known preferential cleavage sites or known in vivo nick sites of the sequence MTRVLQGVLPALPQ (aa41–54) of loop 2 of the β-subunit of hCG [5,9–12]. Selected oligopeptides were MTR (aa41–43), MTRV (aa41–44), LQG (aa45–47), AQG and LAG (alanine replaced oligopeptides of LQG), LQGV (aa45–48), AQGV and LAGV (alanine replaced oligopeptide of LQGV), VLPALP (aa48–53), VLPALPQ (aa48–54). Oligopeptides were synthesized (Ansynth BV, Roosendaal, The Netherlands) using the fluorenylmethoxycarbonyl (Fmoc)/tert-butyl-based methodology with a 2-chlorotritylchloride resin as the solid support. Oligopeptides were dissolved in PBS at a concentration of 1 mg/mL and stored at −20°C in small aliquots.

Functional measurements
Serum urea and creatinine values were measured using a kinetic urease method where the decrease in NADH absorbance is measured photometrically, using an ELAN multi analyser (Eppendorf-Merck, Germany).

Immunohistochemistry
Primary antibodies used were rat-anti-mouse CD4, CD8, CD45, neutrophils, macrophages, CD54 (Serotec, Oxford, UK). The antibodies were diluted in a PBS:5% BSA solution. The primary antibodies were applied for 30 min at RT and slides were subsequently incubated with a mixture of goat-anti-rat IgG+IgM (H+L) alkaline-phosphate conjugated antibody (Southern Biotech, Birmingham, AL, USA) for 30 min at RT. Enzyme detection was performed using a Naphthol AS-MX, New Fuchsine, sodium nitrite and levamisol mixture in Tris–HCl pH 8 as a substrate for 30 min at RT in the dark.

Formalin-fixed paraffin sections (3 µm) were used for Ki-67 staining. Slides were deparaffinized and rehydrated and boiled for antigen retrieval in a 0.1 M sodium citrate solution for 30 min in a microwave oven. Endogenous peroxidase was blocked with a 0.03% H2O2 solution. The sections were incubated overnight at 4°C with a rat-anti-mouse Ki-67 primary antibody (Dako Cytomation, Glostrup, Denmark) and subsequently incubated for 30 min at RT with rabbit-anti-rat IgG conjugated with a HRP secondary antibody (Dako Cytomation, Glostrup, Denmark). Enzyme detection was performed using DAB as a substrate. Slides were rinsed in tap water, counterstained with haematoxylin and rinsed with tap water again. As a negative control, the primary antibody was omitted. Positive cells were counted in 10 high-power fields (400×) using a semi-quantitative scoring system as follows—0: no positive cells, 1: 1–10 cells, 2: 11–30 cells, 3: 30–60 cells, 4: >60 cells.

Measurement of apoptotic cells
Formalin-fixed paraffin sections were stained for apoptotic cells by TUNEL staining using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, CA, USA) according to the manufacturer’s instructions. Positive cells were counted in 10 fields at a magnification of 400×.

Cytokine measurements
TNF-α, IFN-γ, IL-6, IL-10, IL-12 and MCP-1 were measured using a commercially available cytometric bead array (CBA) (BD Biosciences,
San Jose, CA, USA) and a BD FACSAArrayTM Bioanalyzer (BD Biosciences). Analysis of the data was performed using the FCAP ArrayTM software (BD Biosciences). The assay sensitivity was 2.5 pg/mL.

Real-time quantitative (RQ) PCR analysis
Sections of kidney were homogenized, and RNA was isolated using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). In total, 1 µg of RNA was reverse transcribed and RQ-PCR using an Applied Biosystems 7700 PCR machine (Foster City, CA, USA) was performed as previously described [13]. In all 6-h samples, the mRNA transcript levels of TNF-α, IFN-γ, IL-6, IL-10, IL-12, MCP-1 and the adhesion molecules E-selectin and ICAM-1 were determined. Transcript levels of these genes were quantified by normalization against ABL.

Statistical analysis
Survival data were compared by log-rank analysis. Other data were analysed using ANOVA, followed by a Mann–Whitney U-test. Calculations were performed using SPSS v11.0 for Windows. A P-value <0.05 was considered statistically significant. Data were presented as mean values ± standard error of the mean.

Results

Effect of hCG-related oligopeptide treatment on survival
Twenty-five minutes of warm renal ischaemia and contralateral nephrectomy resulted in a survival of 50% in the control group at 3 days post-reperfusion (Table 1). The groups treated with oligopeptides MTR, LQGV, VLPALP, AQG, LAG and LAGV (5 mg/kg) had survival rates not significantly different from controls. Treatment with LQG led to a significantly better survival (90%), while treatment with oligopeptides MTRV, VLPALPQ or AQGV totally prevented mortality.

Effect of hCG-related oligopeptide treatment on kidney function
Treatment of mice with oligopeptide MTRV, AQGV or LAGV provided significant (P < 0.05) functional protection against renal I/R injury at both 24 h and 72 h, as measured by serum urea levels (Figure 2). Although treatment with LQG resulted in significantly decreased serum urea at 24 h post-reperfusion (P < 0.05), at 72 h no significant beneficial effect was found. While treatment with VLPALPQ did not cause a significant decrease in serum urea at 24 h, at 72 h it was significantly decreased as compared to the control group (P < 0.05). Treatment with AQGV provided the most powerful protection against renal ischaemia-reperfusion injury at both 24 h (P < 0.01) and 72 h (P < 0.01).

Effect of different doses of AQGV (0.3–30 mg/kg) on survival
Because AQGV showed the most powerful protection against warm renal I/R injury, we determined the optimal dose of this oligopeptide in a dose-escalating study. Therefore, AQGV was administered in doses ranging from 0.3 to 30 mg/kg, and compared to mice treated with PBS. A survival rate of 60% was seen in the control group (Table 2). Although treatment with 0.3, 1 and 30 appeared to result in a survival benefit, no significant difference could be measured (80%, 90% and 80%, respectively). The doses of 3 and 10 mg/kg totally prevented mortality (P < 0.05).

Effect of different doses of AQGV (0.3–30 mg/kg) on kidney function
Treatment of mice subjected to renal I/R damage with 1, 3, 10 and 30 mg/kg AQGV resulted in significant reduction of serum urea levels at 24 h (P < 0.05). A dose of 3 mg/kg resulted in best preservation of kidney function, with return to normal function already observed at 72 h (P < 0.01). With 0.3 mg/kg no significant benefit was observed (Figure 3).
Creatinine values confirmed these data but in our model did not show the same level of responsiveness to the injury as urea.

**Effects of AQGV on cellular infiltration, apoptosis and proliferation**

To study the mechanism underlying the protective effect of AQGV, we investigated the cellular infiltrate and proliferation in the kidneys of mice treated with 5 mg/kg AQGV. At both 24 and 72 h post-reperfusion the neutrophil influx was significantly decreased in the AQGV-treated group ($P = 0.03$ and $P = 0.022$, respectively) (Figure 4A). Additional staining for CD4+, CD8+ cells and macrophages revealed no differences between the two groups (data not shown). TUNEL staining identified apoptotic cells that were localized mainly in the tubular epithelium (Figure 4B, middle and lower panels). The number of TUNEL-positive cells was significantly lower in AQGV-treated animals 24 h after reperfusion (Figure 4B). Ki-67 staining showed a significantly higher proliferative activity of renal tubular epithelial cells in AQGV-treated mice at 24 h (Figure 4C). At 72 h, this difference had disappeared.

**Effects of AQGV on serum cytokine levels and renal mRNA transcript levels**

Using the bead array, we determined serum cytokine levels at 6 and 24 h post-reperfusion. MCP-1 was below the detection limit in all samples. No differences in serum TNF-$\alpha$, IFN-$\gamma$, IL-6, IL-10 and IL-12 levels were observed at 6 h post-reperfusion. At 24 h post-reperfusion, the levels for all cytokines were decreased upon AQGV treatment, with IL-6, IL-10, IFN-$\gamma$ ($P < 0.05$) and TNF-$\alpha$ ($P < 0.01$) being significantly lower (Figure 5A).

AQGV treatment showed no effect on inflammatory cytokine mRNA levels 6 h post-reperfusion (data not shown). AQGV treatment did result in a significant ($P < 0.05$) down-regulation of renal E-selectin, but not ICAM-1 mRNA expression at 6 h post-reperfusion as compared to PBS-treated mice (Figure 5B,C).

**Effect of post-reperfusion AQGV treatment on survival**

AQGV treatment given either at 12 and 24 h post-reperfusion or at 6 and 12 h post-reperfusion did not
Amelioration of renal ischaemia-reperfusion injury by synthetic oligopeptides related to hCG

**Fig. 4.** (A) Renal neutrophil influx as assessed by immunohistochemical staining. AQGV treatment reduced neutrophil infiltration after 25 min of renal warm ischaemia as assessed at 24 and 72 h post-reperfusion. Data are expressed in a semi-quantitative way as described in the Material and Methods section. *P < 0.05 (n = 10 animals/group). (B) AQGV treatment significantly reduced the number of apoptotic cells in the kidney 24 h after renal ischaemia-reperfusion injury. *P = 0.01 versus PBS-treated controls at 24 h (n = 6–10 animals/group, upper panel). Middle and lower panels: representative photomicrographs of TUNEL stained control- and AQGV-treated kidneys respectively, 24 h after reperfusion (200×). (C) Proliferation as assessed by Ki-67 immunohistochemistry. AQGV treatment significantly enhanced cellular proliferation at 24 h after renal ischaemia-reperfusion injury. Although a higher trend of proliferation was seen at 72 h as well, no statistically significant difference was found. Data are expressed in a semi-quantitative way as described in the Material and Methods section. *P < 0.05 (n = 10 animals/group).

Both natural hCG and commercial hCG preparations have been investigated for their role in the immune system, because of their putative immunomodulating role during pregnancy in protecting the fetus from rejection [14].

Our previous work [6] shows that short-term treatment of female NOD mice, with a hCG preparation purified from first trimester pregnancy urine, starting prior to the onset of hyperglycaemic symptoms, inhibits the development of type I diabetes. Interestingly, however, the anti-diabetic activity of the used hCG preparation did not reside in the heterodimeric hCG molecule, or its subunits, but in a 400–2000 Dalton fraction.

Subsequently, we showed in a model of LPS-induced systemic inflammatory response syndrome in mice that treatment with this low weight molecular fraction was capable of inhibiting the septic shock morbidity as well as mortality [7]. The same beneficial effect was obtained with the synthetic oligopeptide VLPALP, whose sequence is part of loop 2 of the β-chain of hCG [7]. Recently, we showed that hCG-related oligopeptides reduce inflammation and liver injury in a rat model of haemorrhagic shock and resuscitation [15].

During pregnancy, hCG occurs in a variety of forms and breakdown products in serum and urine, including intact

**Discussion**

We investigated whether treatment with synthetic oligopeptides, consisting of three to seven amino acids, based on the primary structure of hCG, was able to reduce warm ischaemia-reperfusion injury of the kidney. We demonstrate for the first time that oligopeptides as small as three or four amino acids can significantly reduce mortality seen after severe renal I/R injury and improves kidney function as measured by serum urea levels. Especially AQGV showed superior results in enhancing survival and preservation of kidney function after 25 min of renal ischaemia. A dose of 3–10 mg/kg proved to be the most potent with regard to reducing mortality as well as preserving kidney function. Furthermore, up to 30 mg/kg, no toxicity was observed. Also in rats, dogs and a human phase I study, no harmful side effects of single and repeated AQGV administration were found. Data of these studies will be published elsewhere (manuscript in preparation).

improve survival (∼50%) as compared to the control group (data not shown).
hCG, α- and β-subunits, nicked hCG, hCG β-core fragment and smaller peptide fragments. Both nicked hCG and the β-core subunit consist of a β-chain with a defective loop 2. This loop, consisting of the amino acid residues 41–54, is absent in the β-core subunit, and is cleaved in nicked hCG [9–12]. Since the immunomodulatory activity of hCG resided in the low molecular weight fraction, we hypothesized that in vivo liberated breakdown products, such as those originating from the proteolytic cleavage of peptide bonds between amino acid residues 41–54, may have significant biological activity [8]. Based on known preferential cleavage sites [5,9,10,12], we tested synthetic oligopeptides MTR, MTRV, LQG, LQGV, VLPALP, VLPALPQ and, based on alanine-replacement mapping, the LQG and LQGV analogues AQG, LAG, AQGV and LAGV (Figure 1). Of these oligopeptides, MTRV, LQG, VLPALPQ and AQGV appeared able to reduce mortality and decline in kidney function induced by warm renal ischaemia-reperfusion injury, AQGV being the most effective (Table 1 and Figure 1).

Cell migration plays an important role during the initial phase of renal I/R injury. Upregulation of adhesion molecules on endothelial cells, induced by locally produced pro-inflammatory mediators, is amongst the first changes observed after renal I/R injury and is central to the pathogenesis of ischaemic acute kidney injury [1,16]. Subsequently, leucocytes become activated by local pro-inflammatory factors, thereby facilitating adherence to endothelial cells and subsequent renal tissue infiltration [1]. Sequestered neutrophils induce parenchymal damage, followed by cytokine production by resident renal cells and infiltrating cells, which promotes further tissue damage [1,17]. It has been demonstrated that renal mRNA expression of the early adhesion molecule E-selectin peaks within 6 h post-reperfusion, with neutrophils infiltrating in parallel. E-selectin blockade with the selectin-specific ligand sPSGL has been shown to inhibit renal neutrophil infiltration after I/R and to preserve kidney function [18]. In mice treated with AQGV, we observed decreased E-selectin mRNA levels 6 h post-reperfusion and decreased renal neutrophil infiltration at 24 h post-reperfusion. Apoptotic cell death, an important determinant of cellular damage in ischaemic kidneys [19], was also significantly reduced in AQGV-treated mice. Additionally, serum levels of the inflammatory cytokines TNF-α, INF-γ, IL-6 and IL-10 were significantly decreased 24 h post-reperfusion upon AQGV treatment. These data are indicative of decreased renal injury and fit with the preservation of kidney function we observed upon AQGV treatment. The lower levels of systemic cytokines observed upon AQGV treatment may be a reflection of reduced formation as well as better renal clearance of these cytokines [20]. The lower serum cytokine levels likely contribute to the decreased mortality by preventing systemic inflammation and subsequent complications in these animals [21].

Our data indicate that AQGV treatment protects against renal I/R injury by interfering with early E-selectin upregulation, thereby reducing neutrophil influx, parenchymal damage and possibly cytokine production. So far, it is unclear what the molecular mechanism of action is by which AQGV exerts its effects. It is possible that AQGV mediates its effect by an as yet unidentified receptor. However, we cannot exclude the possibility that, due to the small size and molecular weight, AQGV penetrates the cell membrane [22] and exerts its action either by interfering with signalling cascades or the transcriptional machinery. E-selectin is expressed de novo on endothelial cells after transcriptional induction by pro-inflammatory agents [23]. Whether AQGV inhibits the local production of pro-inflammatory mediators that induce E-selectin or directly

![Graph A](https://example.com/graph_a.png)

**Graph A.** (A) Treatment with AQGV reduces serum cytokine levels at 24 h after renal ischaemia-reperfusion injury. *P < 0.05, †P < 0.01 (n = 5 per group). (B) Treatment with AQGV reduces renal E-selectin but not ICAM-1 (C) mRNA levels at 6 h after renal ischaemia-reperfusion injury. Data are presented as mean value ± standard error of the mean. *P < 0.05 (n = 6 animals/group).
interferes with the intracellular signalling cascade involved in activating E-selectin transcription is not clear so far. In contrast to E-selectin, ICAM-1 expression was not altered by peptide treatment. The transcription factor HMGA1 is required for optimal activation of E-selectin gene transcription, while it has no role in activating ICAM-1 transcription [24,25]. Therefore, it is possible that AQGV interferes specifically with pathways required for E-selectin transcriptional activation.

Although previous work revealed a pathophysiologic role of the T-cell as mediator of ischaemic acute renal failure [26,27], we did not find a significant difference between the AQGV and placebo-treated mice in numbers of CD4+ and CD8+ T-cells or macrophages. Our data fit with the observation that RAG-1-deficient mice (lacking both T- and B-cells) are not protected from renal I/R injury [28].

Ki-67, a marker for cellular proliferation, is part of a nuclear protein complex expressed in the G1, S, G2 and M phases of the cell cycle in proliferating cells [29,30]. Mice treated with AQGV showed significantly increased numbers of Ki-67-positive renal tubular epithelial cells at 24 h post-reperfusion, reflecting enhancement of the regenerative process [31]. The increase in proliferation is likely facilitated by a reduction in inflammation-induced tissue injury, since high levels of pro-inflammatory cytokines have been shown to suppress regeneration of ischaemically damaged kidneys [32].

AQGV treatment, at a dose of 5 mg/kg BW, given at either 12 and 24 h post-reperfusion or at 6 and 12 h post-reperfusion, was not associated with improved survival. Although we cannot formally exclude that these post-reperfusion treatment regimens improved kidney function, it appears that in the currently used model AQGV only prevents the onset of renal ischaemia-reperfusion injury. This may indicate that AQGV inhibits the activation of pathophysiologic pathways involved in renal ischaemia-reperfusion injury, but is unable to reverse these pathways once activated. However, since we do not exclude that higher doses of AQGV given post-reperfusion do reverse renal-ischaemia-reperfusion injury, detailed dose-response studies are warranted to gain full insight into the renoprotective effect of AQGV, and other hCG-related oligopeptides.

In conclusion, this study shows that treatment of mice with 5 mg/kg of either one of the hCG-related oligopeptides MTRV, LGQ, VLPALPQ or AQGV shortly before and immediately after renal pedicle clamping can significantly reduce mortality and ameliorate kidney injury in a model of warm ischaemia-reperfusion injury. Of the various oligopeptides evaluated, AQGV appeared to be the most potent one. The renoprotective effect of AQGV was associated with decreased renal E-selectin transcripts, decreased renal neutrophil infiltration, reduced numbers of apoptotic tubular epithelial cells and a reduction of systemic levels of TNF-α, IFN-γ, IL-6 and IL-10. These data imply that AQGV interferes with the early renal inflammatory response induced by I/R and as such prevents parenchymal damage and organ dysfunction. These new renoprotective oligopeptides show great promise for preventing the development of renal ischaemia-reperfusion injury and may well be used in clinical situations where renal I/R is foreseeable, such as semi-therapeutic surgeries including kidney transplantation, cardiac surgery and abdominal aorta surgery. So far, phase I A and phase IB studies with AQGV (EA-230) have been successfully completed and phase II studies are underway [33,34].

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Conflict of interest statement. None declared.

References

Cervical heterotopic kidney transplantation in rats using non-suturing and preserving-bag techniques

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Abstract

Background. This study describes a simple and stable cervical heterotopic kidney transplantation method in rats that uses artery sleeve anastomosis, vein cuff anastomosis and preserving-bag techniques.

Methods. The donor graft, consisting of kidney, renal vein (RV), renal artery (RA) and a ureterocystic flap, was removed en bloc and perfused in situ. The donor RA was end-to-end anastomosed to the recipient left common carotid artery (CCA) using a sleeve anastomosis, and the donor RV was connected to the recipient right external jugular vein (EJV) using a cuff technique. During the vascular anastomosis, the kidney graft was placed in a lactated Ringer’s solution ice-water preserving bag. The donor bladder patch was exteriorized to form cervical cutaneous stoma.

Results. A total of 104 heterotopic renal transplantations were performed, which included pre-experimental (62 operations) and experimental stages (42 operations). The success rates of the two stages were 80.6% and 95.2%, respectively. The time for surgery was 40 ± 6 min, the average time for donor surgery was 20 ± 5 min, the preparation time for the graft was 8 ± 2 min, the operative time for the recipient was 18 ± 3 min that included the time for the arterial anastomosis (5 ± 2 min) and venous anastomosis (2 ± 1 min), the cold ischaemic time of the graft was 15 ± 3 min and the warm ischaemic time of the graft was 2 ± 1 min.