Specific therapy of digoxin intoxication in dogs by hybrid kidney overexpressing multidrug resistance protein

SHUICHI TSURUOKA, KENTA NISHIKI, KOHICHI SUGIMOTO, MAKOTO SUZUKI, MASASHI IMAI, and AKIO FUJIMURA

Departments of Clinical Pharmacology and Pharmacology, Jichi Medical School, Tochigi, Japan

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Background. We have recently developed a unique hybrid artificial kidney, where the proximal tubular cell line, overexpressing multidrug resistance protein, MDR-1 (PCTL-MDR), was cultured on hollow fibers. While this module efficiently removed digoxin in vitro, its efficacy in vivo remained to be determined.

Methods. The system was scaled up by connecting 10 similar modules in parallel, with the MDR-1 (PCTL-MDR) overexpressed proximal tubular cell line cultured as in our previous study. The system was connected to dogs intoxicated with digoxin, a representative substrate of MDR-1. Blood was circulated for 90 minutes through the system. Arterial and venous blood concentrations of digoxin and inulin were monitored. Complete blood cell count and granulocyte elastase were measured before and at the end of the study.

Results. By using the system with PCTL-MDR, the arterial digoxin concentration was dramatically decreased from 2.89 ± 0.10 to 0.92 ± 0.11 ng/mL, but not by the system with PCTL alone. The clearance was 22.4 ± 2.1 and 1.5 ± 0.2 mL/min for the PCTL-MDR and PCTL equipment, respectively. Inulin was not transported in either system. White blood cell and platelet counts were slightly reduced by the treatment while hematocrit was unchanged; the granulocyte elastase concentration was slightly increased.

Conclusion. These data suggest that our new type of hybrid kidney can selectively remove digoxin sufficiently to reduce its systemic blood concentration in dogs with digoxin intoxication. Taking previous studies into consideration, this system may be a more powerful tool for the treatment of intoxication.

METHODS
Animal preparation

To establish an animal model of digoxin intoxication, the drug was injected (625 µg/kg, intramuscularly) in healthy dogs (body weight; 10 to 16 kg) every 12 hours for three days before the experiment [4]. Twelve hours after the final injection, the animals were anesthetized with an intravenous injection of pentobarbital-sodium (1 mg/kg). An additional injection was given when the animals awoke. Respiration was supported by a ventilator (350 mL tidal volume, 14 times/min; Respirator R-60; Ichikawa-Shinseido Co. Ltd., Tokyo, Japan). Electrocardiography and rectal temperature were monitored throughout the study. The cubital vein and femoral artery were cannulated and served as the blood access. Arterial blood pressure was continuously monitored during the study by connecting the arterial cannula to a pressure transducer.
Preparation of the hybrid kidney

The artificial hybrid kidney, designed especially for digoxin removal, has been described previously [3]. In brief, PCTL, a renal proximal tubule cell line, was originally made by adding SV40 large T antigen to a primary culture of rabbit S1 cells. cDNA of human MDR-1 was introduced to the PCTL by electroporation [5]. Cells that were resistant to the highest concentration of colchicine, a substrate of MDR-1, was cloned and named PCTL-MDR. This clone possesses 10 times greater amount of MDR-1 protein and about 100 times larger \( K_m \) and \( V_{max} \) values for digoxin than PCTL, while the transport capacity of paraaminohippurate (PAH) was similar between the two cell lines. Transcellular inulin transport was negligible in both.

To make a hybrid kidney, hollow-fiber module for immunoisolation (Culture flo\textsuperscript{TM}; Asahi Medical Co. Ltd., Tokyo, Japan) was used for culture of these cells [3]. In short, the fiber was made by polyethylene, with surface area being 200 cm\(^2\). About \( 1 \times 10^8 \) cells were injected to each module with acid soluble type I collagen (Sigma, St. Louis, MO, USA) at 8 days before the experiment. Once 1/8 of total cells were injected, culture medium was started to circulate at 0.1 mL/min for 30 minutes following a 15-minute equilibration period. Next, the module was rotated 45\(^\circ\) around the long axis and the same procedure was repeated. These procedures were performed to seed the cells equally in the module. After the cell injection for eight times, capillary side of the module was perfused with culture medium [Dulbecco’s modified Eagle’s medium (DME)/Ham’s F-12 with 10% fetal calf serum (FCS), 1 mL/min] in a CO\(_2\)-incubator that was maintained at 37\(^\circ\)C and 95% O\(_2\)/5% CO\(_2\) for eight days [3]. A part of the connected tubing was made of silicon to enhance gas exchange in the incubator.

Evaluation of the hybrid kidney in digoxin-intoxicated dogs

For each experiment, ten modules were used that were connected in parallel just before the treatment of digoxin intoxication (Fig. 1). Comparison of the efficacy between the systems treated with PCTL-MDR and PCTL was evaluated by a randomized open cross-over design with a two-week interval. The treatment with PCTL served as a control. On the day of the treatment, these modules were transferred from the incubator to a temperature-controlled warm bath at 37\(^\circ\)C with 95% O\(_2\)-oxygenization after the parallel connection. The culture medium inside of the modules was replaced once with warm saline and then arterial blood of the dog was flowed in the system. During the experiment, the capillary lumen was perfused with blood (10 mL/min) while the pericapillary side was counter-currently perfused with an artificial solution for hemofiltration (Sublood; Fuso Pharmaceutical Co. Ltd., Osaka, Japan; composition in mmol/L, 140 Na\(^+\), 2K\(^+\), 3.5 Ca\(^{2+}\), 1 mg\(^+\), 110 Cl\(^-\), 3.5 acetate, 25 HCO\(_3\), 0.52 glucose, 10 mL/min). Inulin was continuously infused (20 mg/dL) from the arterial side of the module. Nafamostat mesilate (15 mg/h; Futhan; Torii Pharmaceutical Co. Ltd., Tokyo, Japan) was continuously infused from the arterial side for anticoagulation during the procedure.
During the treatment, arterial blood was drawn just before and at 30, 60 and 90 minutes after the initiation of the study to measure serum concentrations of digoxin, inulin and granulocyte elastase, and complete the blood cell count. Venous blood (just at the post-hybrid kidney) was also obtained at 30, 60 and 90 minutes after the initiation to measure same parameters. The digoxin and inulin concentrations of the artificial solution in the pericapillary side were determined also. Serum digoxin and inulin concentrations were measured by a monoclonal polarization immunoassay technique (TDx autoanalyzer; Dinabot Co.) [6] and the modified Jaffe’s method with an autoanalyzer [7]. Granulocyte elastase was measured by an enzyme immunoassay (PMN elastase kit; Merck Immunossay, Darmstadt, Germany) [8].

The effects of hemodialysis with a machine not using cultured cells also were evaluated in digoxin-intoxicated dogs under a similar protocol.

**Statistics**

All data are shown as the mean ± SE. Statistical evaluation was performed by one-way ANOVA and t test as appropriate. A P value less than 0.05 was regarded as significant.

**RESULTS**

After three days of the digoxin injection, all animals suffered from anorexia and lost body weight. Mean serum digoxin concentration just before the treatment was 2.89 ± 0.10 ng/mL (N = 14). Effects on the serum digoxin concentration of the hybrid kidney with PCTL-MDR was shown in Figure 2A. The concentration of arterial side was dramatically reduced at 30 minutes after the initiation and continued to decrease until the end of the treatment. Digoxin concentration at 90 minutes after the initiation of the treatment was 0.92 ± 0.11 ng/mL (N = 7). The concentrations of venous side were significantly lower than those of arterial side at each observation point. Although treatment with PCTL alone also slightly but significantly reduced serum digoxin concentration, the decrement was significantly smaller than that with PCTL-MDR (Fig. 2B). In order to compare the efficacy of this equipment with that of an ordinary artificial kidney, complementary studies were conducted in which hemodialysis was performed in the absence of cultured cells (Fig. 2C). As can be seen, the slope of the decrease in the arterial digitalis concentration was steeper than that of the hybrid kidney with PCTL alone, but less steep than that of the hybrid kidney with PCTL-MDR. The apparent elimination half-life of digoxin in the experiment with each type of module was 47.5 ± 3.4, 212.1 ± 10.3 and 173.2 ± 7.7 minutes in the PCTL-MDR, PCTL and control groups, respectively. Digoxin removal estimated by the amount of the drug in the artificial solution that flowed out from the pericapillary side of the modules is shown in Figure 3A. It was significantly higher by PCTL-MDR than by PCTL (3.4 ± 0.5 and 0.3 ± 0.1 mg/90 min for PCTL-MDR and PCTL, respectively). In the absence of cell culture, it was slightly higher (1.0 ± 0.3 mg/90 min) than PCTL alone, but less than the PCTL-MDR group. The calculated digoxin clearance (that is, amount of drug removed/mean arterial concentration) is shown in Figure 3B. It was about 15-fold greater in PCTL-MDR than in PCTL alone, and still about fourfold higher than the cell culture-free system.

Changes of serum inulin concentration at arterial and venous sides were monitored to verify the absence of leakage of the modules. This parameter was not changed during the treatment with PCTL-MDR or PCTL (Fig. 4). The serum inulin concentration in the artificial solution drained from pericapillary side was lower than the detection threshold (0.5 mg/dL) in the PCTL-MDR and PCTL groups. Clearance of digoxin and inulin in the PCTL alone group was lower than those in the control group. These results are compatible with our previous in vitro data [3], and we believe that it may be because these solutes can pass more easily through the dialyzer membrane alone than through the membrane with PCTL.

Several parameters were measured to evaluate possible adverse reactions by this procedure. The change of
hematocrit is shown in Figure 5A. Although a little clotting was observed in the module during the treatment, the hematocrit value did not significantly decrease after the treatment in either the PCTL-MDR or PCTL group.

DISCUSSION
This study extends our previous in vitro finding of a selective removal of digoxin, a substrate of MDR-1, by
a tissue engineering system applied for the treatment of
digoxin-intoxicated dogs. As expected, the application of
this device in vivo removed a larger amount of digoxin,
which was sufficient to reduce its serum concentration.
To our knowledge, this is the first report showing the
usefulness of the hybrid kidney with the introduction of
a responsible gene to renal cells for the treatment of
drug intoxication.

Hemoperfusion with activated charcoal is frequently
used to treat digoxin intoxication, but it has some disad-
vantages [1, 2]. First, removal of the substance is not
specific, and physiologically important materials in the
body, such as protein and platelets, also are removed.
Thrombocytopenia is frequently observed in clinical sit-
uations after the procedure. We have previously reported
that thrombocytopenia was seen in dogs with digoxin
intoxication that had been hemoperfused with charcoal
[4]. Although the blood flow rates were not identical and
therefore impossible to compare precisely, the platelet
count was reduced from 205,600 ± 7842 to 32,340 ±
6570/μL (83.5% reduction) using the three-hour treat-
ment with charcoal. These data suggest that the severity
of thrombocytopenia was mild and also that the removal
of digoxin is more specific during the treatment with
the hybrid kidney. Our finding that inulin transport was
negligible by this system also supports this hypothesis.
The second disadvantage of the charcoal is its low trans-
port capacity. Our previous study showed that the esti-
mated capacity to remove digoxin by charcoal is about
8.69 mg/3 h at a 50 mL/min of blood flow rate [4], which
is only about 26% of capacity of the hybrid kidney ob-
tained in our current study. Although the blood flow rate
was not same with this study and thus a precise compari-
on is impossible, we speculate that our novel system has
a higher capacity, and may be superior to conventional
hemoperfusion with respect to both efficacy and safety.
Indeed, with this device digoxin clearance is almost equiv-
alent to its renal clearance and about half of total body
clearance in normal dogs [9]. For the treatment of human
subjects, this system should be up-scaled more than five-
fold because digoxin clearance in humans is about 100 mL/
min [10]. Further evaluation is needed before applying
this system to human subjects. The priming volume of
the whole system in our current study is about 110 mL;
the volume for each mini-module is about 5 mL and the
volume of tubing to connect the whole system is about
60 mL. Because this system requires that it be scaled up
to about fivefold for humans and it is complicated, it
needs to be modified and simplified for clinical situations.

Treatment of digoxin intoxication should be required
for emergency situations. As described in our earlier
article, we need to wait about a week to use this column
after the culture is started [3]. In addition, the maximum
capacity of drug removal in this device can be obtained
on days 7 to 10 after the cells are cultured [3]. Thus, we
have to start the cell culture before the intoxication is
detected, which is a disadvantage of this system. For future
clinical situations, we believe that the cells could be con-
tinuously cultured in the device and thus be available in
case of intoxication. If we can establish such a system,
this device may be useful even in the clinical situation.

We also measured serum granulocyte elastase to evalu-
ate the biocompatibility of this system [11], and with
the treatment found it increased. As the increment was
much lower than that by cardiopulmonary bypass in dogs
[8], we think that such a change is acceptable. Nafamostat
mesilate, which we used as the anticoagulant, is reported
to protect neutrophil activation during hemodialysis and
cardiopulmonary bypass [12, 13] and hybrid liver [14].
This agent might lead to the better biocompatibility of
our system.

We have recently reported that a β2-microglobulin
adsorption column for the treatment of amyloidosis asso-
associated with renal failure also possesses a higher capacity
for digoxin clearance [4]. Although the apparent clear-
ance seems to be greater than observed in the present
study, precise comparison is impossible because of differ-
ent blood flow rates between the two devices. To achieve
a higher efficacy, the device must be improved to endure
the higher blood flow rate. On the other hand, the ad-
sorption column has some disadvantages such as the
larger reduction of red blood cells, platelets and albumin
than those observed in the present device.

Hybrid organs with tissue engineering have been re-
ported mainly in primary cultures of hepatocytes for the
treatment of liver failure [15, 16]. Recently, a hybrid organ
with renal cells was reported for the treatment of acute
renal failure in dogs [17]. Although that report used
primary culture of renal cells, it would be better to intro-
duce specific gene(s) to the cells if the modified cells
have the enhanced transport capacity of the substrates.
If we obtain gene-introduced cells that possess specific
physiological/pharmacological functions, such cells may
apply to this new cell culture system and thus provide
specific functions to patients. Thus, our system may be
useful not only for the treatment of digoxin intoxication,
but also for compensation of physiological function. If
the elimination mechanisms of uremic toxin(s) from the
kidney are fully understood, a hybrid kidney with an
introduction of the genes to renal cells may be a more
powerful tool for the treatment of renal failure. We
seeded the cells on the outside of the hollow fiber, while
Humes et al seeded on the inside [17]. We believe that
there are two advantages in our system. (1) We are afraid
that because the inner diameter of the module is very
small, cells cultured on the inner surfaces of the fibers
may obstruct blood flow during the treatment. (2) There
is a concern about the contact of non-human cells with
human blood during the treatment. Because of specific
difference, unexpected immunological problems might
occur in clinical situations. By seeding cells to outside of the fiber, such problems might be avoided.

In summary, we evaluated the efficacy of a novel artificial hybrid kidney with the introduction of MDR-1, by parallel connection of 10 hollow-fiber systems in dogs with digoxin intoxication. As expected from our previous in vitro study, this system can selectively remove the drug from circulating blood to the extent that is sufficient to reduce its serum concentration to a therapeutic range by 90 minutes of the treatment. Compared to activated charcoal, the capacity of drug removal with this system was higher. The biocompatibility of this device in dogs was relatively acceptable. While our results are encouraging, further studies are needed to apply this device to renal patients.

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Reprint requests to Shuichi Tsuruoka, M.D., Department of Clinical Pharmacology, Jichi Medical School, 3311 Yakushiji, Minamikawachi, Kawachi, Tochigi 329-0498, Japan.
E-mail: tsuru@jichi.ac.jp

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