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A newly developed hydroxyl radical scavenger, EPC-K1 can improve the survival of swine warm ischemia-damaged transplanted liver grafts

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Abstract. Using a swine orthotopic liver transplantation (SOLTx) model, we assessed the effect of a new hydroxyl radical scavenger EPC-K1 on warm ischemic damage of the liver graft and recipient survival. Animals were divided into 5 groups. The first group (control group 1) consisted of 5 pigs which were not operated on but served as controls for the indocianine green disappearance rate (K-ICG) determinations. In the second group (control group 2), 10 livers were transplanted without warm ischemia (WI) and the K-ICG values were measured. The third group (control group 3) was the main control group for the study groups and consisted of 5 liver transplants with 30 min of WI without any special treatment. The fourth and fifth groups served as study groups 1 and 2. Five transplants were carried out in each group, as in control group 3. In study group 1 recipients were treated with an additional 5 mg/kg i.v. EPC-K1 and in study group 2 with 20 mg/kg i.v. EPC-K1. Significant improvement in glutamic oxaloacetic transaminase (GOT) and lactate dehydrogenase (LDH) levels, K-ICG values and histological findings were observed in the EPC-K1 treated groups. The intravenous administration of this agent had a strong protective effect on warm ischemic damage after 30 min of WI and could significantly prolong the graft and recipient survival.

Key words: EPC-K1 – Hydroxyl radical – Liver transplantation – Warm ischemia – Reperfusion injury – K-ICG

Warm ischemic damage is one of the most troublesome and important aspects of organ transplantation which can contribute to the occurrence of pirmary nonfunction (PNF) [10]. Recently, many authors have reported that oxygen-free radicals, derived from warm ischemia damaged tissue, play a major role in reperfusion injury [3, 5]. Therefore, many kinds of radical scavengers, for example, allopurinol and superoxide dismutase (SOD) have been investigated carefully in an effort to reduce warm ischemic damage.

A new compound, EPC-K1 L-ascorbic acid 2- [3, 4-dihydro-2, 5, 7, 8-tetramethyl-2-(4, 8, 12-trimethyltridecyl)-2 H-1-benzopyran-6yl hydrogen phosphate] potassium salt, was developed by Senju Pharmaceutical Co., Ltd., Hyogo, Japan in 1989. EPC-K1 has a unique phosphodiester bond between Vitamin E and Vitamin C (Fig. 1). This compound has several properties that are of benefit in liver transplantation such as hydroxyl radical scavenging, human phospholipase A_2 blocking [4] and high affinity to liver tissue. In this study, we assessed whether this agent could prevent warm ischemic damage after liver transplantation and improve survival, using a swine orthotopic liver transplantation (SOLTx) model.

Materials and methods

We used 55 large white pigs weighing 20–25 kg (mean, 22.3 ± 1.26 kg) in this study and divided them into five groups. In control group 1, 5 pigs were used for measurement of the normal indocianine green disappearance rate (K-ICG). These animals were not operated on and only the K-ICG values were evaluated as described below. In control group 2, 20 pigs were paired and hepatic transplantation was carried out without warm ischemia (WI); warm ischemic time (WIT) was zero. The K-ICG values were evaluated. In control group 3, 10 other pigs were paired and hepatic transplantation was carried out with 30 min of WI. In study group 1, 10 pigs were paired and hepatic transplantation was carried out after 30 min of WI. Thy were given 5 mg/kg EPC-K1 as described below. In study group 2, 10 pigs underwent the same procedure as the pigs in study group 1 but the dose of EPC-K1 was increased to 25 mg/kg.

Liver transplantation protocol. After the donor liver was prepared for retrieval, in all groups except control group 2, warm ischemia was commenced by cross clamping and the intraperitoneal temperature was maintained at 37 °C by the continuous pouring of hot saline for 30 min. Then the graft was preserved in Euro-Collins solution until the recipient hepatectomy was performed and the graft was transplanted orthotopically into the recipient pig. The details of the whole transplantation procedure have been reported previously [9]. There were no statistically significant differences in body weights, total ischemic time and anhepatic time between the groups.

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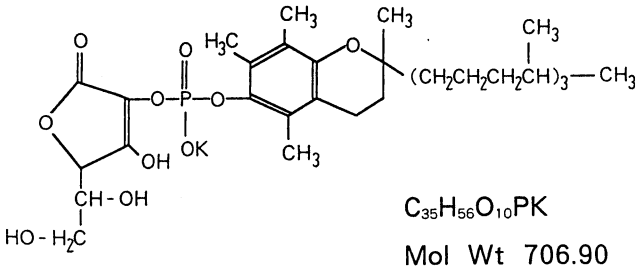

Fig. 1. Chemical structure of EPC-K1

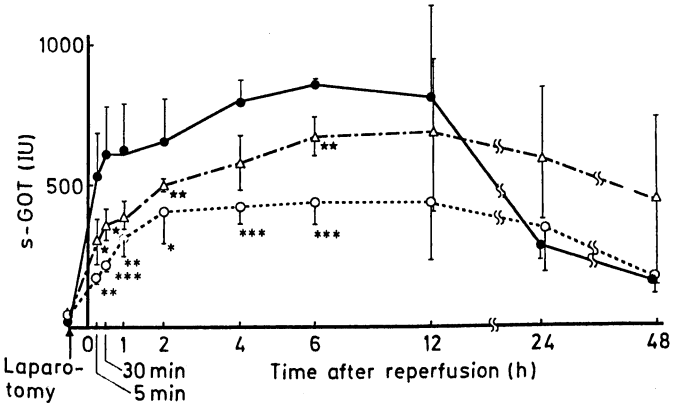

Fig. 2. Changes in serum GOT levels up to 48 h after reperfusion in three groups (mean ± SD). The serial serum GOT levels in treated groups were significantly lower than that in untreated group up to 6 h after reperfusion. * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$, study group 1 vs. control group 3; ★ $P < 0.05$, ★★ $P < 0.02$, study group 2 vs. control group 3). (Key ●—●, control group 3; ○---○, study group 1; △---△, study group 2)

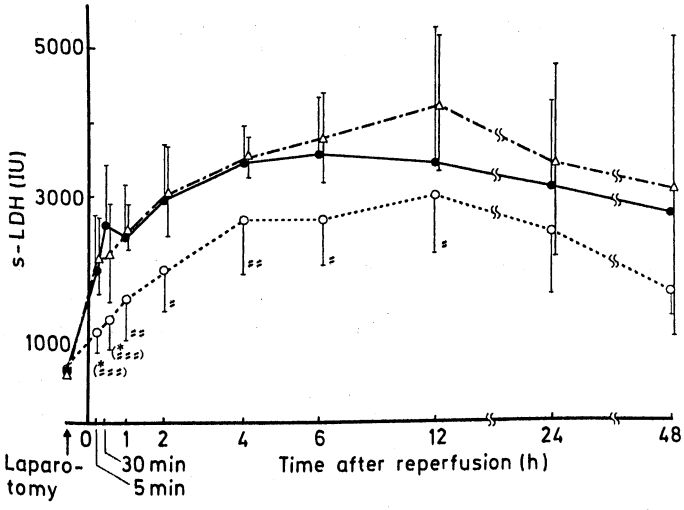

Fig. 3. Changes in serum LDH levels up to 48 h after reperfusion in three groups (mean ± SD). In serum LDH levels, significant suppression of s-LDH level was found only in study group 1. * $P < 0.05$ study group 1 vs. control group 3; $P < 0.05$, # # $P < 0.02$ study group 1 vs. 2). Key ●—●, control group 3; ○---○, study group 1; △---△, study group 2)

EPC-K1 protocol. The animals were divided into three groups (control group 3, study groups 1 and 2). Recipient pigs in control group 3 ($n = 5$) served as controls and were not pre-treated prior to reperfusion. Pigs in study group 1 ($n = 5$) and study group 2 ($n = 5$) were pre-treated with a low dose of EPC-K1 (5 mg/kg) and a high dose

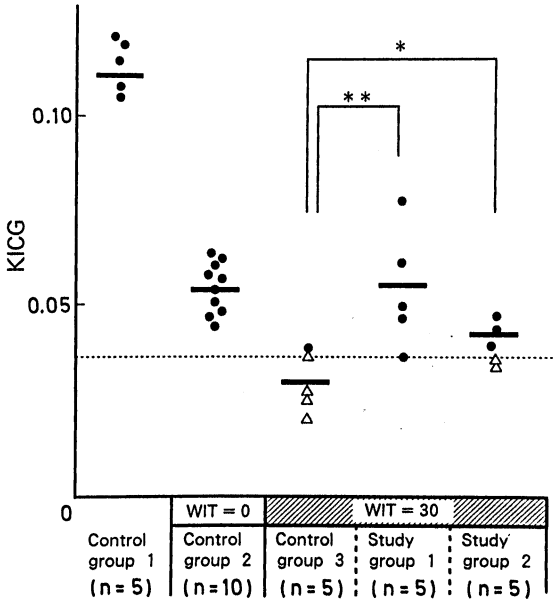

Fig. 4. The K-ICG values in normal pigs and in recipient pigs of SOLTx (mean ± SD). The first and second columns indicate K-ICG values in normal pigs (control 1 group, mean K-ICG value, 0.113 ± 0.006) and in the SOLTxed pigs without warm ischemic stress respectively (control group 2, 0.055 ± 0.006). The third to fifth columns show K-ICG values in recipients after 30 min of warm ischemia (0.030 ± 0.007, 0.055 ± 0.014, 0.040 ± 0.005). The *broken line* demonstrates the K-ICG value above which recipients survived for more than 1 week ($P < 0.01$). * $P < 0.05$, ** $P < 0.01$ (Key ●, cases surviving more than 1 week; △, cases dead within a week)

(20 mg/kg) respectively. EPC-K1 was administered 10 min prior to hepatectomy and 10 min prior to reperfusion in two equal doses.

Biochemical analysis and K-ICG. Analysis of graft function included serial serum glutamic oxaloacetic transaminase (GOT) and serum lactate dehydrogenase (LDH) measurements, and the determination of K-ICG values. K-ICG was calculated from three samples obtained at 5 min intervals after the bolus injection of indocianine green (0.5 mg/kg i.v.) which was administered just 30 min after reperfusion.

Histology. Biopsy specimens in control group 3, and study groups 1 and 2 were obtained 30 min after reperfusion from each transplanted liver. Blocks of tissue were fixed in 10% neutral-buffered formalin and were stained with hematoxylin and eosin for light microscopic examination.

Statistical analysis. Data are expressed as mean ± SD. Significance was tested using Student's *t*-test and the Chi-square test, taking $P = 0.05$ as the limit of significance.

Results

Biochemical analysis and K-ICG

Up to 6 h after reperfusion, significant suppression of elevation of serum GOT levels was observed in study groups 1 and 2 ($P < 0.05$) compared to control group 3 (Fig. 2). As shown in Fig. 3, the serum LDH level was suppressed in study group 1 up to 12 h after reperfusion but

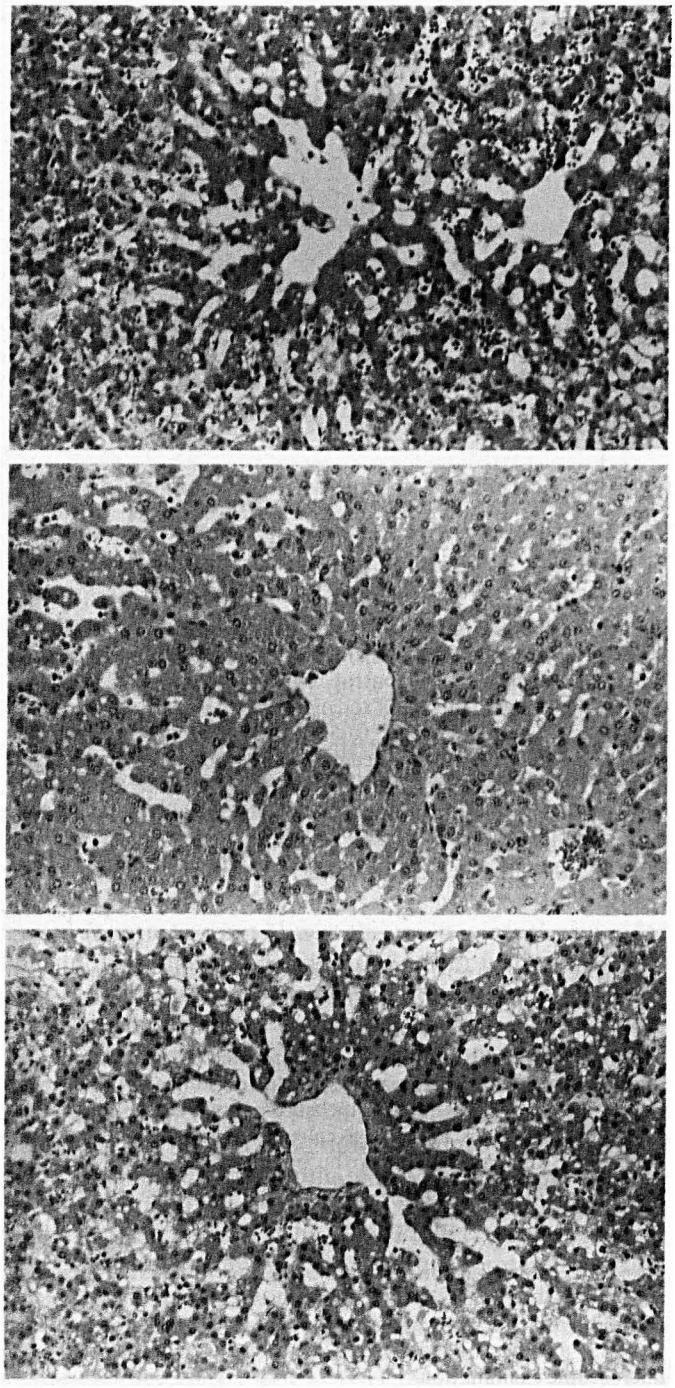

Fig. 5. Light micrographs of biopsy specimens 30 min after reperfusion (× 168, H.E. staining). **A** Untreated group: marked disruption of hepatic architecture was observed in the centrilobular zone. Vacuolar degeneration of hepatocytes (HCs) and detachment of sinusoidal lining cells (SLCs) were seen. **B** EPC-K1 5 mg/kg i.v. group: almost normal structure was maintained. **C** EPC-K1 20 mg/kg i.v. group: the hepatic architecture was not disrupted, but vacuolar change of HCs and dissection of SLCs were observed in moderation

statistical significance was observed only between the two study groups ($P < 0.05$).

In control group 2 (WIT = 0), the mean K-ICG value following SOLTx surgery was decreased to almost half the value calculated in non-operated controls (Fig. 4). Furthermore, this decrease in mean K-ICG value was greater after 30 min of WIT as can be seen in Fig. 4. The mean K-ICG values in the study groups were significantly higher than in control group 3 (0.055 ± 0.014, and 0.040 ± 0.005 vs. 0.030 ± 0.007, $P < 0.01$, $P < 0.05$, Fig. 4).

Histology. As shown in Fig. 5, warm ischemic damage was marked in the centrilobular zone. Severe vacuolar degeneration of hepatocytes, congestion, dissection of the sinusoidal lining cells and disruption of hepatic architecture was observed in control group 3. In study group 1, the hepatic architecture remained almost normal. In study group 2, there were moderate vacuolar changes in the hepatocytes.

Survival. The survival rates for each group are presented in Table 1.

Discussion

It is well known that molecular oxygen generated in reperfused tissue can cause severe damage in previously ischemic tissue during organ transplantation, and the hydroxyl radical ($\cdot OH$) appears to be the most harmful oxygen-free radical because of its affinity for organic molecules and its low specificity. This effect may lead to primary non-function of the transplanted organ. It is suggested that the reperfused tissue itself and the neutrophils which flow into the graft after reperfusion are the sources of these oxygen-free radicals during transplant surgery [1]. Therefore, potent radical scavengers may facilitate the improvement of graft viability. EPC-K1 was synthesized as a specific hydroxyl radical scavenger, and it catalyzes reactions in vitro in a dose-dependent manner [7]. Furthermore, the latest study revealed that this agent could also scavenge the superoxide radical (O_2^-) derived from endotoxin-induced migrating neutrophils in the intraperitoneal space of the rat (unpublished data). The advantages of EPC-K1 in clinical use are, (1) it is a soluble and stable material suitable for injection, (2) it has high a affinity for liver tissue, and (3) it has strong binding properties to cell membranes owing to its molecular similarity to lipid membrane molecules.

The released enzymes, represented by GOT and LDH, reflected the degree of oxidative cell injury. Elevation of GOT in the early phase was suppressed significantly by EPC-K1 regardless of dose. However, significant differences in LDH values were found only in recipients treated with 5 mg/kg EPC-K1 after 30 min of WI damage. This discrepancy between dose and effect was also noted in the K-ICG values, histological findings and survival rates.

The K-ICG value reflects liver function and hepatic blood flow and was regarded as a sensitive indicator of graft function in this study because recipients were jaundice-free. Most of the subjects who lived longer than 1 week showed a value of over 0.035, and the K-ICG value above which recipients survived for more than 1 week was 0.035 ($P < 0.01$). Both mean K-ICG values in the study groups were significantly better than that in control

Table 1. One-week survival rate in three experimental groups with 30 min of WI. Significant improvement of mean survival time (MST) and 1-week survival rate in EPC 5 mg/kg treated group (100%) was demonstrated

Groups	Treatment		WIT (min)	Survival (day)	MST (day)	1 week-survival rate (%)
Control group 3 (n = 5)		–	30	1, 1, 1, 2, 18	4.7 ± 6.7	20
Study group 1 (n = 5)	EPC-K1	5 mg/kg (i.v.)	30	7, 14, 16, 20, 48	24.3 ± 13.8*	100
Study group 2 (n = 5)	EPC-K1	20 mg/kg (i.v.)	30	3, 3, 7, 7, 9	6.4 ± 2.0	60

*; $P < 0.05$ (vs control group 3)

group 3, but the effect of low dose administration was somewhat superior to high dose administration.

Caldwell-Kenkel suggests that oxygen-free radical injury could explain the extensive cell death observed in the oxygen-rich periportal area compared to the oxygen-poor pericentral regions in the reperfused rat liver model after cold storage [2]. However in our histological study, the centrilobular zone was damaged more severely than the periportal zone after 30 min of WI. Some investigators have suggested that nonparenchymal cells may play an important role in the pathogenesis of oxygen-free radical injury [2, 6]. Marked detachment of sinusoidal lining cells and congestion increase the intrasinusoidal pressure, causing poor local circulation and creating anaerobic conditions. If vacuolation of hepatocytes, which is prominent in the centrilobular zone of the warm ischemia damaged graft, contributes to secondary changes in an effort to overcome the increased sinusoidal pressure, as Trowell has suggested [11], vacuolation may not be due to the anaerobic condition of hepatocytes during WIT, but may be the result of reperfusion injury that is mainly expressed in sinusoidal lining cells.

EPC-K1 effectively protected the grafts from warm ischemic damage and significantly improved clinical outcome. Increasing the dose of EPC-K1 from 5 mg/kg to 25 mg/kg surprisingly did not improve this effect (Table 1). This discrepancy may be explained by the detergent-like characteristics of EPC-K1, which may contribute to cytotoxity in higher doses. The only recognized side effect of EPC-K1 was a decrease in blood pressure at a dose above 25 mg/kg (i.v.) in a rat model (unpublished data). We did not observe any significant decrease in blood pressure following EPC-K1 injections in our model.

Three striking findings emerged from our present study: (1) EPC-K1 prevented warm ischemic damage after 30 min of WI in SOLTx and could significantly prolong the survival of the graft and recipient, (2) the intravenous administration of EPC-K1 suppressed significantly the increase in released enzymes, in line with the improvement of K-ICG values and histological findings and, (3) the protective effects of the agent were maximized at the dose of 5 mg/kg given intravenously. Further studies are required to confirm our findings and to establish a side effect profile of EPC-K1. The agent may prove to be a powerful oxygen-free radical scavenger in successful human liver transplantation.

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