Transpl Int (1993) 6: 8–13

Transplant International

An in vitro method for comparing the efficacy of two preservation solutions in one canine liver using the 5'-nucleotidase assay

T. M. van Gulik[1], C. R. Nio[1], W. M. Frederiks[2], P. J. Klopper[1], M. N. van der Heyde[1]

[1] Department of Surgery and [2] Laboratory of Cell Biology and Histology, University of Amsterdam, Meibergdreef 9, NL-1105 AZ Amsterdam, The Netherlands

Received November 25, 1991/Received after revision March 18, 1992/Accepted April 9, 1992

Abstract. The activity and localization of the plasma membrane-bound enzyme 5'-nucleotidase (5'-NT) in liver tissue are sensitive parameters of ischemic damage. The value of 5'-NT as a marker of liver graft viability was studied in relation to liver preservation. In six mongrel dogs, the main right and left branches of the portal vein were cannulated and flushed separately in situ with cold University of Wisconsin (UW) solution and Euro-Collins (EC) solution, respectively. After hepatectomy, the right and left liver lobes were split and stored at 5 °C in either of the two solutions. 5'-NT activity was demonstrated in cryostat sections of liver tissue using the lead salt method. After 48 h of storage in EC solution, the 5'-NT score had decreased to 31 % ± 16 % ($n = 6$), whereas in UW solution the 5'-NT score was 76 % ± 10 % ($n = 6$). Significantly ($P < 0.05$) higher 5'-NT scores were also found after 24-h and 72-h preservation times in UW versus EC solutions. This result is in keeping with the higher preservation tolerance of liver grafts preserved in UW solution. The 5'-NT assay was studied in relation to graft function in orthotopic liver transplantation experiments in dogs. All dogs with liver grafts preserved in UW solution for 24 h ($n = 4$) and 48 h ($n = 3$) survived (> 5 days). Pretransplant 5'-NT scores ranged from 61 % to 100 %. The 72-h-preserved livers ($n = 5$) did not show life-supporting function. Pretransplant 5'-NT scores (33 % ± 12 %, $n = 5$) were significantly ($P < 0.05$) decreased. The 5'-NT score pretransplantation was a more reliable indicator of graft function than peak SGOT values post-transplantation. In conclusion, the 5'-NT assay, in conjunction with the double flush method through the portal vein, provides a simple and rapid "in vitro" method to test solutions for liver preservation.

Key words: Preservation, liver – 5'-Nucleotidase, liver preservation – Liver preservation, 5'-nucleotidase

Correspondence to: T. M. van Gulik

The ultimate test of organ preservation quality is graft function and survival after transplantation in large animal experiments. Liver transplantation in large animals, such as dogs or pigs, is, however, costly and time-consuming. In addition, a variety of systemic responses that are difficult to anticipate in these animals may influence the outcome of the experiment and give rise to inconsistent results. Therefore, parameters that can indicate the viability of the graft without the direct proof of post-transplant function are of help in assessing the efficacy of preservation solutions and can be used to define optimal preservation conditions. We report here on the use of the 5'-nucleotidase (5'-NT) assay in liver tissue to determine its viability. Applying this viability assay, an in vitro method was devised to test the efficacy of two preservation solutions in one canine liver.

5'-NT is an enzyme present in the plasma membranes of bile canaliculi and sinusoidal endothelium. Its activity and localization have been shown to be sensitive markers of ischemic injury in the rat liver [2, 4]. A reliable, enzyme-histochemical method has been developed in our laboratory that selectively demonstrates 5'-NT activity in cryostat sections of liver tissue and that allows direct assessment of the enzyme by light microscopy. The value of 5'-NT as a marker of liver viability was assessed in three sets of experiments.

First, canine liver tissue was exposed in vitro to warm and cold ischemia and assessed for localization and intensity of 5'-NT. The purpose of this experiment was to examine the staining characteristics of 5'-NT in canine liver tissue under normothermic and hypothermic ischemic conditions. In the second series of experiments, the right and left lobes of canine livers were separately flushed in situ with either chilled University of Wisconsin (UW) solution [11] or Euro-Collins (EC) solution and subsequently stored at 5 °C. Biopsies of both right and left liver lobes were assayed for 5'-NT for up to 72 h of cold ischemia. This method allowed us to study the preservation quality of two solutions under similar test conditions. Finally, the 5'-NT assay was applied in conjunction with orthotopic liver transplantation experiments perfor-

med in the dog. The results of the 5'-NT assay were related to functional outcome of the graft as determined by survival of the recipient dog and peak enzyme values in the blood.

Materials and methods

5'-Nucleotidase assay

5'-NT activity was demonstrated using the metal salt method of Wachstein and Meisel (1957) as previously described [2, 4]. Briefly, cryostat sections of unfixed liver specimens were cut (thickness 8 μm) and incubated for 20 min at 37°C in a medium containing 17% (w/v) polyvinyl alcohol (hot water-soluble, average molecular weight 40000 Da; Sigma, St. Louis, Mo., USA), 100 mM TRIS-maleate buffer (pH 7.2), 5 mM adenosine 5'-monophosphate (AMP; Merck, Darmstadt, FRG), 10 mM $MgCl_2$, and 7.2 mmM lead nitrate. At this point, the white lead phosphate precipitates at sites where the enzyme is present. After rinsing the sections with water at 60°C, the sections were incubated for 1 min in a solution of 1% (v/v) ammonium sulfide in water. In this way, lead phosphate is transformed into brown lead sulfide crystals. The sections were subsequently washed in water and mounted in glycerol jelly.

A semiquantitative method was devised to score the localization and intensity of stained, bile canalicular membranes. All tissue sections were analyzed in a blinded fashion. Five portal areas were judged in a tissue section. At a magnification of ×320, a portal area was divided into four quadrants. Each quadrant was scored by judging the sharpness and staining intensity of bile canalicular membranes. Staining with a definite brown color and sharply delineated bile canaliculi was graded as 10. A weak stain with faint and broad outlines of bile canaliculi was graded as 0. Staining of moderate intensity with moderately widened borders was graded as 5. Consequently, the highest score that could be considered was 200, as found in fresh liver tissue, and the lowest score was 0, corresponding to prolonged ischemia of the liver. The score was expressed as a percentage of the highest possible value, 200.

Assessment of cold and warm ischemic injury in canine liver tissue

Slices of fresh canine liver were wrapped in aluminium foil and immediately stored at either 37°C or 5°C. Biopsies (approximately 5 × 5 × 5 mm) of the liver tissue were taken after 60 min and 120 min. Each biopsy was instantly placed in liquid nitrogen and kept at –80°C until the time of processing for the 5'-NT assay. Control biopsies of fresh canine liver tissue were obtained and processed similarly. All biopsies in this experiment were assayed at the same time, under the same conditions, and with the same test solutions.

Double flush method of canine livers

Six female mongrel dogs weighing approximately 25 kg were used. Anesthesia was induced with 6 mg/kg ketamine (Aescoket-plus, Aesculaap, The Netherlands) and 3 ml Rompun (Bayer, FRG) intravenously. Maintenance of anesthesia was by nitrous oxide/oxygen (1:1) while on mechanical ventilation. All dogs were pretreated with chlorpromazine (2 mg/kg IV) and methylprednisolone (7.5 mg/kg IV). A cannula was passed through the splenic vein into the portal vein and placed in the main right branch of the portal vein. A second cannula was then passed through the coronary vein into the portal vein and placed in the main left portal branch. After the dog was given heparin (10000 IU), the main right and left portal branches were tied around the cannulas and cold perfusion (5°C) was immediately started through each cannula with UW solution (DuPont Pharmaceuticals) and EC solution, respectively (Fig. 1). After hepatectomy, the right and left liver lobes were split and stored separately at 5°C in either solution for up to 72 h. 5'-NT activity and localization were scored in biopsies of the liver taken immediately after laparotomy (To), after the initial cold flush of the liver, and after 24 h, 48 h, and 72 h of preservation.

Orthotopic transplantation of canine livers

Female mongrel dogs (weight 20–25 kg) were used as donors and matched by weight to female recipients (weight 25–30 kg). The dogs were anesthesized as described above. Methods for harvesting and orthotopic transplantation of the canine liver were as described else-

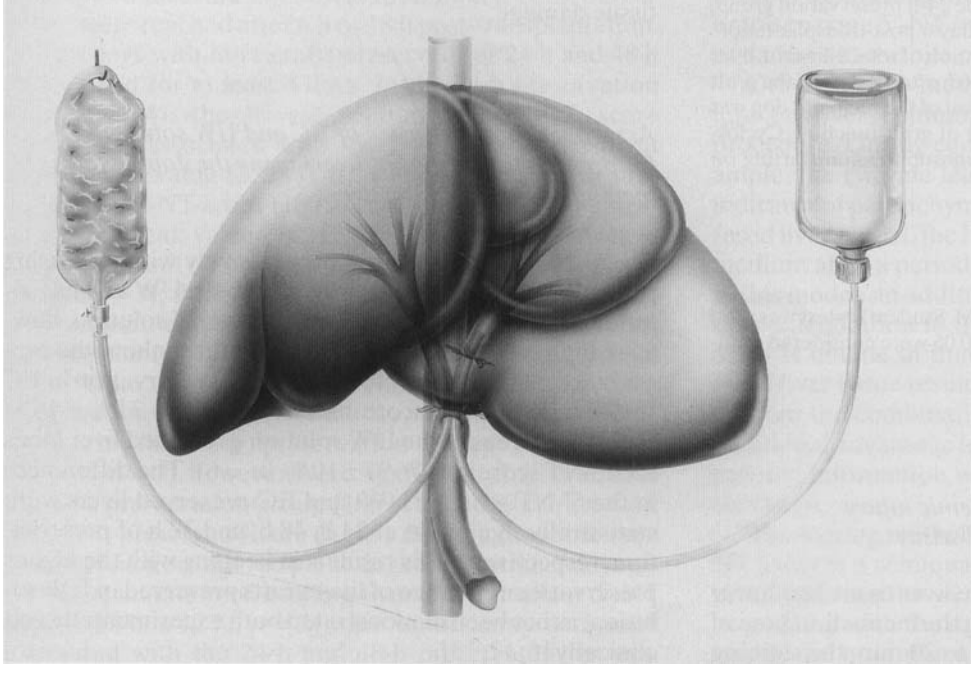

Fig. 1. Double flush method in a canine liver. The main right and left branches of the portal vein are cannulated and flushed separately in situ with two different, chilled preservation solutions. After splitting the liver, the right and left liver lobes are stored at 5°C. Assessment of the 5'-nucleotidase (5'-NT) score in serial biopsies of both parts of the liver shows the progression of preservation injury

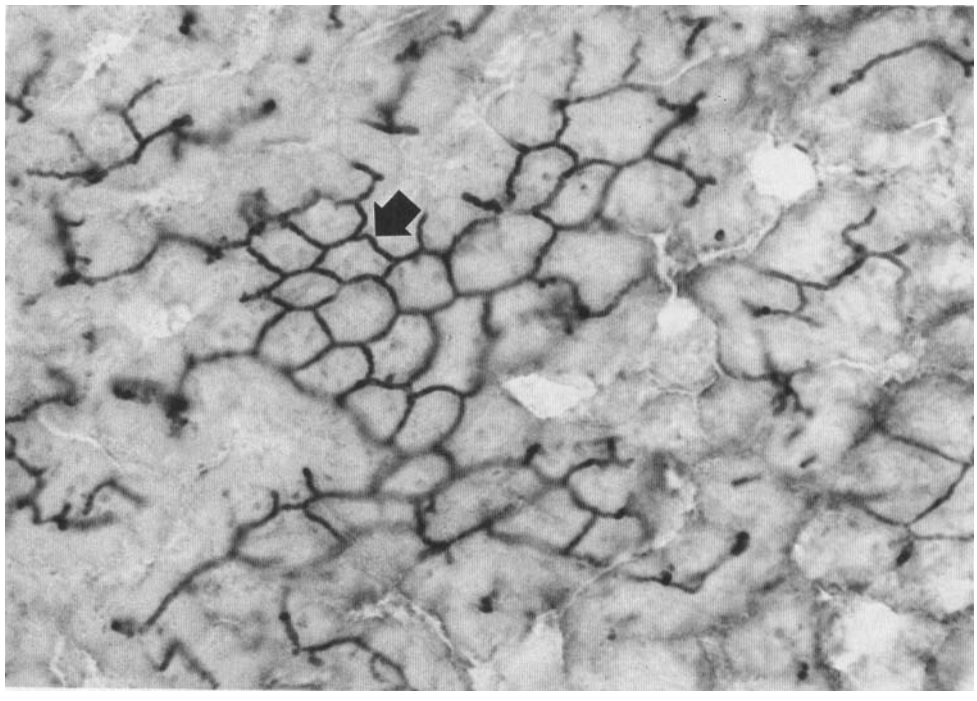

Fig. 2. Micrograph (× 320) showing the activity and localization of 5'-nucleotidase (5'-NT) in bile canalicular membranes of fresh, canine liver tissue. The enzyme-histochemical reaction was based on the metal salt method. Note the sharply delineated and dark stained bile canaliculi *(arrow)*. (Any lack of clarity in this photomicrograph is due to a difference in depth of focus)

where [5, 9], with some minor modifications. Donor dogs were pretreated with chlorpromazine (2 mg/kg IV) and methylprednisolone (7.5 mg/kg IV). The liver was flushed out with 2 l UW solution (DuPont Pharmaceuticals) through the portal vein and stored at 5 °C. To each 1-l bag of UW solution, 3 mM reduced glutathione (GSH) was freshly added [1]. Livers were cold-stored for 24 h ($n = 4$), 48 h ($n = 3$), and 72 h ($n = 5$). These livers were preserved with hyperbaric oxygen as part of an experimental protocol (manuscript in preparation). Briefly, after hepatectomy, the liver was placed in a pressure chamber and pressurized with 100% oxygen (1.5 ATA). The pressure chamber containing the liver immersed in UW solution was subsequently transferred to a cold room (5 °C) in which it remained during the preservation period. The 5'-NT assay was performed in biopsies of the graft taken after initial cold flush of the liver in the donor dog, at implantation of the graft before reflow, and 1 h after reflow in the recipient dog. In the 24-h preservation group, 5'-NT was also assessed in the graft at day 6 post-transplantation. Graft function was assessed by determination of total bilirubin, liver enzymes, and coagulation parameters at 6 h after reflow of the graft and, subsequently, on a daily basis. Survival of the recipient dog was considered the most important criterion of graft function. Cyclosporin A (20 mg/kg) was given for immunosuppression starting on day 1 post-transplantation.

Statistical analysis

All results are given as mean values ± SEM. Student's *t*-test was used for statistical analysis. *P* values less than 0.05 were considered significant.

Results

Comparison of cold and warm ischemic injury in canine liver tissue using the 5'-NT assay

The activity of 5'-NT in fresh canine liver tissue was lower than in rat liver tissue. By extending the incubation time of the sections of liver tissue from 10 to 20 min, the staining intensity could be increased in the canine liver. Moreover, the activity of 5'-NT in canine liver tissue, as opposed to rat liver tissue, was far more apparent in bile canaliculi than in sinusoidal endothelium (Fig. 2).

After 60 min and 120 min of normothermic (37 °C) ischemia, the 5'-NT score had decreased to 7% ± 3% ($n = 6$) and 0% ($n = 6$), respectively. Under hypothermia (5 °C), 60 min and 120 min of ischemia resulted in a decrease in the 5'-NT score to 81 ± 4% and 79% ± 6% ($n = 6$), respectively. These results show that warm ischemia causes a total loss of 5'-NT activity within 2 h, whereas under hypothermia 5'-NT activity persists, in accordance with the protective effect of hypothermia against ischemic tissue damage.

Assessment of the efficacy of EC and UW solutions for preservation of canine livers using the double flush method and the 5'-NT assay

The 5'-NT score decreased progressively with increasing preservation time of canine livers in both UW and EC solutions. In the liver lobes preserved in UW solution, however, higher 5'-NT scores were found throughout the preservation period (Fig. 3). After 48 h of preservation in EC solution, the 5'-NT score had decreased to 31% ± 16% ($n = 6$), whereas in the UW solution-preserved liver lobes the 5'-NT score was 76% ± 10% ($n = 6$). The differences in the 5'-NT score of UW- and EC-preserved livers were statistically significant at 24 h, 48 h, and 72 h of preservation, respectively. This result is in keeping with the higher preservation tolerance of liver grafts preserved in UW solution, as has been demonstrated both experimentally and clinically [6, 12, 13].

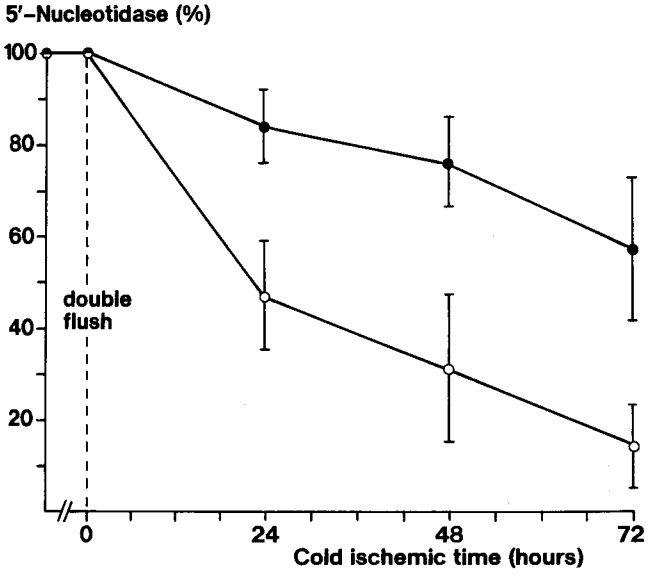

Fig. 3. Graphic representation of the 5'-nucleotidase (5'-NT) scores determined in canine liver lobes stored in UW solution (●—●) and Euro-Collins solution (○—○), respectively, according to the double flush method. Significantly higher 5'-NT scores were found in liver tissue preserved in UW solution after 24 h, 48 h, and 72 h storage times (mean ± SEM, $n = 6$)

5'-NT score compared with graft function after orthotopic transplantation in the dog

The results of 24-h, 48-h, and 72-h liver preservation experiments are shown in Table 1. A transplanted liver graft was considered to function when the recipient dog survived for more than 5 days with serum glutamic oxaloacetic transaminase (SGOT) levels having returned to near-normal. The dogs were sacrificed after 6–13 days, depending on their response to immunosuppressive therapy. The peak SGOT value after transplantation was recorded as a measure of preservation injury. Peak SGOT values were reached after 6 h or 18 h post-transplantation.

The dogs with liver grafts preserved for 24 h and 48 h all survived for at least 5 days. In the 24-h preservation group ($n = 4$), the lowest pretransplant 5'-NT score (61%) was associated with the highest post-transplant SGOT peak value (3388 IU/l), whereas the highest pretransplant 5'-NT score (100%) corresponded to the lowest SGOT peak value (715 IU/l; normal SGOT values in 12 dogs: mean 11.7 IU/l, SD 8.6). In the 48-h preservation group ($n = 3$), the biopsies of one graft were lost to analysis. Assessment of the two remaining grafts revealed pretransplant 5'-NT scores (83% and 71%) that did not differ from pretransplant 5'-NT scores of the 24-h preservation group (75% ± 9%, $n = 4$). Peak SGOT values in the 48-h preservation group (4967 IU/l ± 701 IU/l, $n = 3$), however, were significantly higher than in the 24-h preservation group (1839 IU/l ± 559 IU/l, $n = 4$). The 72-h-preserved livers ($n = 5$) did not show life-supporting function. All recipient dogs died within 5 days after transplantation of a failing liver. Pretransplant 5'-NT scores (33% ± 12%, $n = 5$) were significantly decreased compared with the 24-h and 48-h preservation groups. Peak SGOT values (4817 IU/l ± 577 IU/l, $n = 5$) did not differ from the 48-h preservation group but were significantly higher in comparison with the 24-h preservation group. In this series of liver transplant experiments, the 5'-NT score pretransplantation appeared to be a more reliable indicator of graft function than peak SGOT values post-transplantation.

A remarkable finding in all three groups was that 1 h after reflow, the 5'-NT score was considerably decreased, confirming that on reperfusion the graft is subjected to an additional trauma. In the 48-h and 72-h preservation experiments, this reperfusion phenomenon was most striking. The activity of 5'-NT in these grafts was hardly detectable after reperfusion (5'-NT score 0). Interestingly, biopsies of the graft on day 6 post-transplantation showed a restoration of 5'-NT in the membranes of the bile canaliculi.

Discussion

The 5'-NT assay provides a simple, enzyme-histochemical method for rapid assessment of ischemic injury in canine liver tissue. It does not require special instruments and can be performed on a routine basis in 30 min. Polyvinyl alcohol was added to the incubation medium used in the assay to prevent leakage of soluble enzymes from the tissue section into the medium and to enable the use of unfixed sections without enzyme denaturation [2]. This is important since one cannot rule out the possibility of the localization of 5'-NT changing from a plasma membrane-bound form into a soluble form.

The significance of the changes in localization and activity of 5'-NT in ischemic liver has not been fully understood. It has been shown that changes in the plasma membranes of rat liver parenchymal cells, as studied at the ultrastructural level, occur early during ischemia. In the rat, these changes were observed after 30 min of ischemia under normothermia (37°C) [3, 8]. Therefore, the alterations in the localization and activity of the plasma membrane enzyme 5'-NT may represent an indirect parameter of damage to the bile canalicular membranes.

Other in vitro methods for studying the preservation injury of livers commonly involve a perfusion phase in the procedure. During continuous machine perfusion, for example, the enzyme leakage into the perfusate is a useful indicator of parenchymal damage [10]. In the isolated perfused liver model, the liver is perfused with an oxygenated medium after a period of cold storage [7]. It is likely that, in this model, an additional "reperfusion" trauma is introduced in addition to the storage injury per se. The 5'-NT assay is unique in that it allows direct assessment of injured liver tissue resulting from either static storage alone or from the combination of storage and reperfusion. An additional advantage is that serial biopsies of one liver can provide information with increasing time periods of preservation.

The scoring method applied in conjunction with the 5'-NT assay is a semiquantitative one. Comparable and reproducible results were obtained by different observers acquainted with the method. The site chosen for the liver biopsy did not influence the outcome of the 5'-NT assay

Table 1. 5'-Nucleotidase (5'-NT) scores assessed in canine liver grafts after flushing with UW solution (cold flush), before reflow (end preservation time), and 1 h following reflow. Maximum serum transaminase (peak SGOT) values are shown in conjunction with the corresponding time after reflow (post-transplantation time)

Dog	Preservation time	5'-NT Score			Peak SGOT		Survival time[a] (days)
		After cold flush	End preservation time	1 h after reflow	U/l	Post-Tx time	
1	24 h	100%	70%	57%	1665	18 h	6
2	24 h	100%	61%	33%	3388	6 h	6
3	24 h	100%	70%	55%	1584	6 h	6
4	24 h	100%	100%	37%	715	6 h	6
5	48 h	100%	83%	0%	5615	6 h	13
6	48 h	100%	71%	0%	3566	18 h	8
7	48 h	100%	–	–	5720	18 h	9
8	72 h	100%	10%	0%	5334	48 h	4
9	72 h	100%	0%	0%	4893	18 h	2
10	72 h	100%	60%	0%	4224	18 h	1
11	72 h	100%	57%	0%	3080	18 h	2
12	72 h	100%	37%	0%	6556	18 h	3

[a] Dogs 1–4 were sacrificed on day 6 post-transplantation

either. Preliminary experiments (results not shown) did not reveal significant differences in 5'-NT scores between biopsies from central or peripheral regions of cold-stored canine livers.

The double flush method of canine livers, as elaborated in this study, offers a new perspective in connection with the 5'-NT assay. Although the liver harvesting procedure in the experimental setting is standardized, the response to anesthesia and drugs of individual large animals is subject to considerable variation, in particular when mongrel dogs are used. Flushing out the right and left liver lobes at the same time, but separately, via the main right and left portal branches, ensures that the starting point of preservation takes place under the same "donor" conditions. In this model, the liver lobes are flushed out through the main portal branches only, and not through the arterial system. This is not considered to affect the quality of preservation in the canine liver since in a successful application of an orthotopic liver transplantation method in the dog, flushing out the liver in the donor animal was commonly undertaken solely via the portal vein [5, 9]. The same method was used in the transplantation experiments described here. In the dog, the right and left liver lobes are connected by a parenchymal bridge of little thickness, thus limiting the overflow of preservation solution from the right portal system to the left liver lobes, and vice versa. Also, the biopsies were taken at a distance from the cut surface of the split liver. The EC and UW preservation solutions – two solutions whose efficacy in liver preservation has been well established, both experimentally and clinically [6, 12, 13] – were chosen to test the double flush model. The difference in preservation quality in favor of the UW solution convincingly showed up in the results of the 5'-NT assay.

To assess the reliability of the 5'-NT score in terms of a true viability marker, the 5'-NT assay was used in relation to liver transplantation experiments in the dog. In the transplantation experiments the variation was such that a real cut-off point in the 5'-NT values corresponding to viable and nonviable liver grafts was not evident. The 5'-NT scores of the 72-h-preserved, nonviable liver grafts, regarded as one group, were, however, significantly decreased compared with the scores of the 24-h and 48-h-preserved viable liver grafts. Peak SGOT and SGPT levels in the early postoperative period after liver transplantation are generally associated with preservation injury to the graft. In these studies, the 5'-NT score proved to be a better indicator of graft performance than postoperative, peak SGOT levels in the blood.

There is a discrepancy between the 5'-NT scores of the livers used in the transplantation experiments and the scores resulting from the partial livers preserved with UW solution in the double flush experiment. On the whole, the biopsies of the liver halves flushed with UW solution yielded slightly higher 5'-NT scores than those of the livers used for transplantation, following similar preservation times. The biopsies taken during the double flush experiment were, in fact, of better quality. To account for this difference in quality, several factors need to be considered. In the double flush experiment, one liver half was biopsied after 24 h, 48 h, and 72 h, respectively. The bag containing the liver half was simply opened while remaining in the cold room and tied again after the biopsy was taken. In the transplantation experiments, different livers were preserved for either 24 h, 48 h, or 72 h and directly transplanted after taking the biopsy. Also, the procedure before taking the biopsy was less controlled than in the double flush experiment since livers for transplantation were first prepared on the back table, remained in the bowl during hepatectomy and construction of the portal-systemic shunt in the recipient dog, and were biopsied just before reflow, after the liver was washed out to remove the UW solution.

In conclusion, the 5'-NT assay provides a simple and rapid "in vitro" method to assess the preservation tolerance of a cold-preserved canine liver without the need to reperfuse the organ. In conjunction with the double flush method through the portal vein, the assay can be used to efficiently test and improve solutions for liver preservation. Liver transplantation experiments in the dog, for the pur-

pose of preservation studies, may then be carried out effectively, using a limited number of animals.

References

1. Boudjema K, Gulik TM van, Lindell SL, Vreugdenhil PK, Southard JH, Belzer FO (1990) Effect of oxidized and reduced glutathione in liver preservation. Transplantation 50: 948–951
2. Frederiks WM, Marx F (1988) A quantitative histochemical study of 5'-nucleotidase activity in rat liver using the lead salt method and polyvinyl alcohol. Histochem J 20: 207–214
3. Frederiks WM, Myagkaya GL, Veen HA van, Vogels IMC (1984) Biochemical and ultrastructural changes in rat liver plasma membranes after temporary ischemia. Virchows Arch [B] 46: 269–282
4. Frederiks WM, Marx F, Myagkaya GL (1988) A quantitative histochemical study of 5'-nucleotidase activity in rat liver after ischemia. J Pathol 154: 277–286
5. Gulik TM van, Lindell SL, Boudjema K, Pienaar BH, Vreugdenhil PK, Southard JH, Belzer FO (1990) Combined cold storage and perfusion preservation of the canine liver. Transplant Proc 22: 520–522
6. Jamieson NV, Sundberg R, Lindell S, Claesson K, Moen J, Vreugdenhil PK, Wight DGD, Southard JH, Belzer FO (1988) Preservation of the canine liver for 24–48 hours using simple cold storage with UW solution. Transplantation 46: 517–522
7. Jamieson NV, Sundberg R, Lindell S, Southard JH, Belzer FO (1988) A comparison of cold storage solutions for hepatic preservation using the isolated perfused rabbit liver. Cryobiology 25: 300–310
8. Myagkaya GL, Veen HA van, James J (1984) Ultrastructural changes in rat liver sinusoids during prolonged normothermic and hypothermic ischemia in vitro. Virchows Arch [B] 47: 361–373
9. Pienaar BH, Lindell SL, Gulik TM van, Southard JH, Belzer FO (1989) Revised method of orthotopic hepatic transplantation in canines. Surg Gynecol Obstet 169: 341–346
10. Pienaar BH, Lindell SL, Gulik TM van, Southard JH, Belzer FO (1990) Seventy-two-hour preservation of the canine liver by machine perfusion. Transplantation 49: 258–260
11. Southard JH, Gulik TM van, Ametani MS, Vreugdenhil PK, Lindell SL, Pienaar BH, Belzer FO (1990) Important components of the UW solution. Transplantation 49: 251–257
12. Todo S, Nery J, Yanaga K, Podesta L, Gordon RD, Starzl TE (1989) Extended preservation of human liver grafts with UW solution. JAMA 261: 711–714
13. Todo S, Podesta L, Ueda Y, Imventarza O, Casavilla A, Oks A, Okuda K, Nalesnik M, Venkataramanan R, Starzl TE (1989) Comparison of UW with other solutions for liver preservation in dogs. Clin Transplant 3: 253–259