

Hyaluronate levels in donor organ washout effluents: a simple and predictive parameter of graft viability

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Abstract: The principal cause of primary non-function in orthotopic liver transplantation is thought to be preservation injury to the microvasculature. We, therefore, evaluated if effluent levels of hyaluronate, whose uptake is an endothelial cell marker, could predict early graft function and ultimate graft outcome in orthotopic liver transplantation. A total of 102 cases were studied in two phases. In the first phase, we attempted to determine if a correlation existed between effluent hyaluronate levels, early graft function and ultimate graft outcome. This phase of the study was also used to determine hypothetical cut-off values for hyaluronate which could discriminate between good and bad livers. Thirty-two livers orthotopically transplanted to randomly selected primary recipients were studied. After varying periods of static cold storage (4°C) in University of Wisconsin solution, the livers were reinfused with cold (4°C) lactated Ringer's solution. The first 50 ml of the reperfusion effluent was collected from the infrahepatic vena cava. Effluent samples were analyzed for hyaluronate. Linear regression analysis demonstrated a significant correlation between effluent hyaluronate levels and post-operative aspartate and alanine aminotransferase levels ($p < 0.001$ for both). Logistic regression demonstrated a highly significant correlation ($p = 0.0056$) between effluent hyaluronate levels and ultimate graft outcome. Generation of Receiver Characteristics Curves indicated that a level between 400 and 430 $\mu\text{g} \cdot \text{l}^{-1}$ could possibly discriminate between good livers and those at risk of early graft failure. The authenticity of this hyaluronate cut-off level was further confirmed in the second phase of the study where 70 consecutive primary crossmatch-negative transplants were performed. A highly significant difference was observed in peak aspartate and alanine aminotransferase levels in the first week ($p < 0.0006$ and $p < 0.0005$, respectively) between livers with effluent hyaluronate levels $\leq 400 \mu\text{g} \cdot \text{l}^{-1}$ and livers with hyaluronate levels higher than $400 \mu\text{g} \cdot \text{l}^{-1}$. Logistic regression revealed a highly significant correlation between effluent hyaluronate levels and graft success ($p = 0.0001$). Since hyaluronate uptake by the microvascular endothelial cell is significantly greater than production, high hyaluronate effluent levels in failed livers would be due to decreased hyaluronate uptake by the injured microvascular endothelial cell. We therefore conclude that effluent hyaluronate levels may prove to be a reliable preoperative test to assess early graft function and outcome in clinical orthotopic liver transplantation.

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Hyaluronate levels and graft function

It is estimated that between 2–23% of human liver allografts never function, with consequent death or retransplantation (1–17). When no apparent technical or immunological causes can be identified for the early graft failure (between 1 and 14 days) and the inability to sustain life, necessitating a retransplant, the syndrome is defined as primary nonfunction (PNF). The principal cause of PNF is thought to be preservation injury to the microvascular endothelial cell (MVEC) (7, 8).

Since the critical target of preservation injury is the MVEC (9–11), we attempted to correlate effluent levels of hyaluronate (HA), an endothelial cell specific marker (12–22) with early graft function in a clinical study.

In the liver, the major sources of HA are the Ito cells (23) and the extracellular matrix (24). HA produced by these cells is taken up by the MVEC and catabolized by the action of hyaluronidase and N-acetylglucosamine-6-phosphate deacetylase (25). The rate of HA uptake by the MVEC is significantly greater than the rate of HA production by the Ito cell (23). HA uptake studies have been extensively used as a measure of MVEC integrity and function (26–30). We therefore hypothesize that preservation injury to the MVEC would reduce HA uptake by the MVEC, resulting in elevated HA levels in livers which had been injured during preservation. This hypothesis was tested in a clinical study of 102 cases.

Materials and methods

A total of 102 cases were studied in two phases. In the first phase, 32 livers were transplanted orthotopically to consecutive adult primary recipients with negative lymphocytotoxic crossmatches; 29 were treated with FK-506 and low dose steroids (20 mg/day) and three were treated with cyclosporine and 200 mg/day methylprednisone with taper. During and following procurement, the livers were infused with University of Wisconsin (UW) solution at 4°C. After varying periods of static cold storage (13.3 ± 2.3 SD hours), 150 cc of cold Ringer's lactate was infused through the portal vein. The suprahepatic cava was clamped and the first 50 ml effluent was collected through the infrahepatic vena cava. These livers were then reflushed with 500 cc of cold UW solution. These perfusions were performed on the backtable while the livers were being prepared for implantation. The 50 ml effluent collections were centrifuged at approximately $1000 \times g$ for 10 min to remove erythrocytes and aliquots were analyzed for HA levels. All the livers that were used in this phase of the study satisfied established preoperative criteria (4, 6, 31).

In the second phase of the study, 70 consecutive

primary crossmatch-negative adult orthotopic livers were transplanted in 70 adult recipients. All the preservation, flushing and effluent collection techniques were identical to those used in phase I. All the patients received 1 g of methylprednisone in the operating room and were then maintained on FK-506 and 20 mg/day of methylprednisone. All 102 recipients were monitored daily for liver function. Ultrasound, biopsy and other invasive procedures were performed as clinically indicated.

Donor organ washout with UW solution

A separate group of 14 adult primary recipients with negative lymphocytotoxic crossmatches were studied. In this group of patients the donor organ was flushed with 150 cc of cold UW solution instead of the Ringer's lactate used in the remaining 102 cases. As before, the suprahepatic cava was clamped and the first 50 ml effluent was collected through the infrahepatic vena cava. The livers were subsequently reflushed with 500 cc of cold UW solution.

HA assay

The effluent was assayed for HA values with a radiometric assay, using kits marketed by Pharmacia Diagnostics (Uppsala, Sweden) (12). All samples, including standards, were run in duplicate. Percent variation of results never exceeded 2.2%. The study was conducted in a double-blind fashion with the clinical care physicians in the intensive care unit or wards being unaware of the HA levels, and the laboratory personnel (PNR, JS) not being involved in or cognizant of the clinical monitoring, diagnostic tests, including biopsies, or treatment of the patients.

Stratification of post-operative graft function

Graft function after transplantation was stratified by the criteria of Makowka et al. Based on a retrospective statistical analysis of 219 human liver transplants, the study defines peak serum aspartate aminotransferase (AST) levels of <1500 IU/l and peak serum alanine aminotransferase (ALT) levels <1000 IU/l at any time during the first post-operative week as indications of good grafts versus poor grafts if the levels were higher. These criteria are now extensively used by workers in this field (1,31,32).

Statistical analysis

Linear regression analysis was used to study the correlation between preoperative effluent levels of

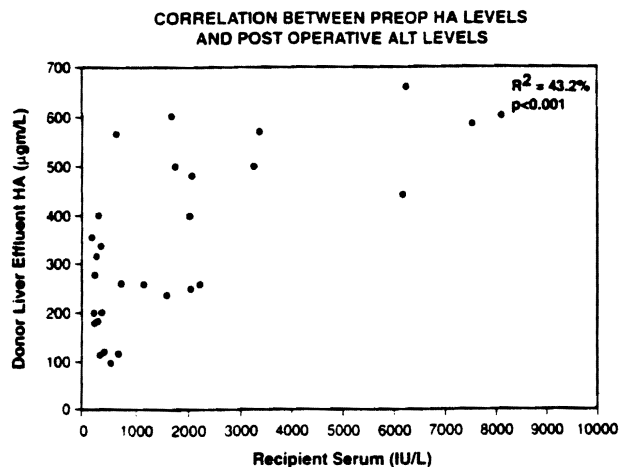


Fig. 1. Correlation between effluent hyaluronate (HA) levels and peak ALT levels in the first postoperative week. A highly significant correlation was observed between donor effluent HA levels and peak recipient serum ALT levels ($R^2=43.20\%$, $p<0.001$).

HA and post-operative AST and ALT levels. The correlation between effluent HA levels and graft survival was studied by logistic regression (33). Hypothetical pre-operative cut-off levels of HA which could discriminate between grafts exhibiting either good or poor post-operative function were determined by the generation of Receiver Characteristic Curves (ROC) (34). The accuracy of these cut-off levels was confirmed in the second phase of the study. The Mann-Whitney U-test, a nonparametric equivalent to the standard two sample *t*-test, was used to compare HA and liver function between groups. Univariate logistic regression was used to predict PNF based on HA levels.

Results

Phase I: To study the correlation between effluent HA levels, early graft function, ultimate graft outcome and the establishment of hypothetical HA cut-off levels

Linear regression. A strong positive correlation was observed between effluent HA levels and post-operative AST ($R^2=37.30\%$, $p<0.001$) and ALT levels ($R^2=43.20\%$, $p<0.001$). (Fig. 1).

Stratification of HA levels. Stratification of the 30 grafts with available AST and ALT levels by the criteria of Makowka et al. (1, 31, 32) resulted in 19 grafts being classified as good and 11 as poor grafts on the basis of AST levels. The mean effluent HA level in good grafts was $256.0 \pm 132.0 \mu\text{g} \cdot \text{l}^{-1}$ and was significantly lower ($p=0.0003$) than the mean HA levels in the poor grafts ($405.0 \pm 139.0 \mu\text{g} \cdot \text{l}^{-1}$).

When the data were stratified on the basis of

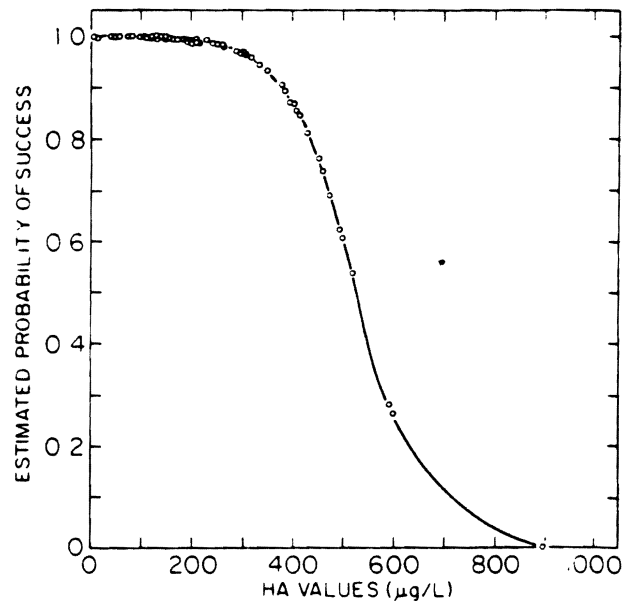


Fig. 2. Correlation between effluent hyaluronate (HA) levels and graft outcome. Logistic regression analysis demonstrated a highly significant correlation ($p=0.0056$) between effluent HA levels and graft survival up to 90 days.

post-operative ALT levels, 17 grafts were classified as good and 13 as poor. Mean HA levels in the good grafts were $256.0 \pm 140.0 \mu\text{g} \cdot \text{l}^{-1}$ and were significantly lower ($p=0.0017$) than the mean effluent HA levels of $485.0 \pm 139.0 \mu\text{g} \cdot \text{l}^{-1}$ in the poor grafts

Correlation between effluent HA levels and graft outcome. Logistic regression analysis of the data demonstrated a highly significant ($p=0.0056$) correlation between effluent HA levels and graft outcome up to 90 days (Fig. 2).

Hypothetical cut-off levels of HA which could be used to potentially discriminate between good livers and livers at risk of early graft failure. Generation of ROC indicated that an HA level between 400 and $430 \mu\text{g} \cdot \text{l}^{-1}$ could possibly discriminate between good livers and those at risk of early graft failure (Table 1)

Effluent HA levels in livers that failed from primary non-function (PNF). Table 2 shows the HA levels in the five livers that failed from PNF in this phase. All livers had HA levels higher than the $400\text{--}430 \mu\text{g} \cdot \text{l}^{-1}$ cut-off. However, two of the six livers had an HA level close to the $430 \mu\text{g} \cdot \text{l}^{-1}$ cut-off level. We, therefore, hypothesized that an HA level of $400 \mu\text{g} \cdot \text{l}^{-1}$ would be a more appropriate value at which to discriminate between good livers and those at risk of early graft failure. The authenticity

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Table 1. Hypothetical cut-off levels for effluent hyaluronate(HA)

Parameter	Value	Hypothetical cut-off % false-positive ^a	% true-positive ^b
HA $\mu\text{g} \cdot \text{l}^{-1}$	250.0	55.0	100.0
	350.0	33.0	100.0
	370.0	29.0	100.0
	400.0	22.0	100.0
	410.0	22.0	100.0
	430.0	22.0	100.0
	450.0	22.0	80.0
	550.0	11.0	60.0

^a False-positive: % good livers which would have HA levels above hypothetical cut-off (i.e., % of good livers which would be "discarded" as poor livers if we used this hypothetical HA cut-off).

^b True-positive: % of bad livers which would be evaluated as bad using hypothetical HA cut-off (i.e., % of bad livers, which would be "discarded" if we used this HA cut-off).

Table 2. List of cases with high preoperative hyaluronate (HA) levels that failed from primary non-function

Patient	HA $\mu\text{g} \cdot \text{l}^{-1}$	Peak ^a AST (IU/l)	Peak ^a ALT (IU/l)	Most significant post-op. biopsy results (90 min to 10 days)
DM	440.0	6965	6164	Preservation injury
JD	480.0	1602	2088	Preservation injury
SC	568.0	4844	3402	Low flow infarction
PU	600.0	2121	1740	Ischemic injury
MB	585.0	6755	7517	Ischemic injury

^a In first week.

Table 3. AST and ALT levels following orthotopic liver transplantation

Group	AST (IU/l)	ALT (IU/l)
HA $\leq 400.0 \mu\text{g} \cdot \text{l}^{-1}$	1287 \pm 1326 (n=57)	983 \pm 1041 (n=57)
HA $> 400.0 \mu\text{g} \cdot \text{l}^{-1}$	4687 \pm 3439 (n=12)	3774 \pm 2438 (n=12)
p-value ^a	<0.0006	<0.0005

^a Wilcoxon sign-rank test. HA=hyaluronate.

of this effluent HA cut-off level was confirmed in Phase II of the study.

Phase II: Confirmation of the effluent HA cut-off level: comparison of early graft function following OLTX in livers with effluent HA ≤ 400 and those with effluent levels $> 400 \mu\text{g}/\text{l}$

Early graft function following OLTX was monitored by AST and ALT. AST and ALT levels in the first 7 post-operative days were both significantly elevated in the group with high effluent levels ($> 400 \mu\text{g} \cdot \text{l}^{-1}$) as compared to the group with HA levels $\leq 400 \mu\text{g} \cdot \text{l}^{-1}$ (p<0.0006 for AST, p<0.0005 for ALT, Table 3).

Table 4. Graft survival

Group	Total no of cases	Unequivocal Graft survival	Failure from PNF	Failure other causes
HA $\leq 400.0 \mu\text{g} \cdot \text{l}^{-1}$	58	55 (94.8%)	0	3
HA $> 400.0 \mu\text{g} \cdot \text{l}^{-1}$	12	6 (50%)	6 (50%)	0

^a For the entire study: 90-day graft survival=87/102 (85.29%) (HA $\leq 400 \mu\text{g} \cdot \text{l}^{-1}$)
Incidence of PNF=11/102 (10.78%) (HA $> 400 \mu\text{g} \cdot \text{l}^{-1}$)
HA=hyaluronate. PNF=Primary non-function

Table 5. Analysis of false-positive livers

Group	Clinical group	AST (IU/l)	ALT (IU/l)
1	≤ 400.0 (n=58)	1287 \pm 1326	983 \pm 1041
2	PNF (n=6)	5137 \pm 4110	4046 \pm 3545
3	False-positive (n=6)	4237 \pm 2940	3502 \pm 2230

AST: GR1 vs GR3 - p<0.003
GR1 vs GR2 - p<0.05
GR2 vs GR3 - p=NS

ALT: GR1 vs GR3 - p<0.002
GR1 vs GR2 - p<0.05
GR2 vs GR3 - p=NS

PNF=primary non-function

Comparison of graft survival. Table 4 shows an overall 90-day graft survival of 87.1% (61 of 70). PNF was only noted in livers with effluent HA levels $> 400 \mu\text{g} \cdot \text{l}^{-1}$. None of the 58 livers with effluent levels $\leq 400 \mu\text{g} \cdot \text{l}^{-1}$ developed PNF, while 6 of 12 livers with effluent levels $> 400 \mu\text{g} \cdot \text{l}^{-1}$ failed from PNF (p<0.005). Graft survival beyond 90 days was achieved in 55 of 58 livers with low effluent HA levels as compared to 6 of 12 livers with effluent HA values $> 400 \mu\text{g} \cdot \text{l}^{-1}$ (p<0.001). Three livers with low HA levels failed between the seventh and ninth days post-operatively. These livers were lost to hepatic artery thrombosis in two cases and death from pneumonia in the third.

Comparison of effluent HA levels between successful grafts and those that failed from PNF. Mean HA levels of successful grafts were $218.4 \pm 128.0 \mu\text{g} \cdot \text{l}^{-1}$ and were significantly lower than the effluent HA levels in the six grafts that failed from PNF, of $549.3 \pm 187.3 \mu\text{g} \cdot \text{l}^{-1}$ (p=0.0002). As in the first phase, all the PNF grafts had effluent HA levels $> 400 \mu\text{g} \cdot \text{l}^{-1}$.

Analysis of false-positive livers. False-positive livers were defined as those that were successfully transplanted despite effluent HA levels $> 400 \mu\text{g} \cdot \text{l}^{-1}$. The livers, however, had an enzyme release pattern

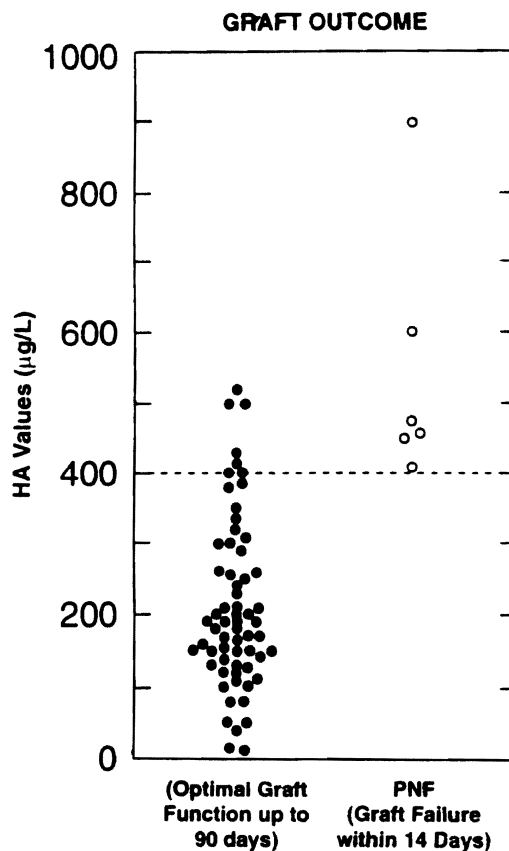


Fig. 3. Correlation between effluent hyaluronate (HA) levels and graft outcome and incidence of primary non-function. Logistic regression demonstrated a highly significant correlation between effluent HA levels, incidence of PNF and ultimate graft outcome ($p=0.0001$, improved Chi-square=22.13).

similar to the six livers which experienced PNF (Table 5).

Correlation between effluent HA levels, graft outcome and incidence of PNF. Logistic regression demonstrated a highly significant correlation between effluent HA levels, incidence of PNF and ultimate graft outcome ($p=0.0001$, improved Chi-square=22.13) (Fig. 3).

HA levels in livers with UW washout. Two of the 14 livers studied failed from PNF. HA levels in these two livers were $692.50 \pm 576.25 \mu\text{g} \cdot \text{l}^{-1}$. Mean HA levels in the remaining 12 livers were $524.25 \pm 334.60 \mu\text{g} \cdot \text{l}^{-1}$. The HA levels in the two groups were similar, although no statistical inference can be drawn because of the limited number of cases.

Discussion

The results of this study suggest that effluent levels of HA reflect HA uptake by the MVEC

and can be used to evaluate graft viability with a significant degree of accuracy. The washout has to be with lactated Ringer solution. In pilot experiments with animal and human livers, the HA test lost all discrimination if the reperfusion flush was performed with UW solution. Similar results of the non-discriminating properties of the HA test, if performed with non-Ringers lactate washout, have also been reported in a recent study (35) where the backtable flush was performed with 5% dextrose. The reason for this selective requirement of a lactated Ringer's flush solution is currently not known. The Ringer's lactate flush did not have a deleterious effect on ultimate graft outcome since the incidence of PNF in this study (11/102, 10.78%) was not significantly different from that reported earlier (1, 4, 6).

All 102 livers used in the study satisfied established preoperative criteria (4, 6, 31) regarding their suitability as donor organs and should not have failed from PNF. All the 11 livers (5 in the first phase and 6 in the second) which failed from PNF (Table 3 and Fig. 3), had effluent levels $>400 \mu\text{g} \cdot \text{l}^{-1}$. Despite satisfying preoperative criteria, all these livers subsequently demonstrated histological evidence of severe preservation injury. Conversely, none of the 79 livers (21 in phase 1, 58 in phase 2) with effluent HA levels $\leq 400 \mu\text{g} \cdot \text{l}^{-1}$ failed from PNF.

Twelve (6 in each phase, 11.76%) of the 102 livers are classified as false-positive since they ultimately survived despite effluent HA levels which were $>400 \mu\text{g} \cdot \text{l}^{-1}$. However, 9 of these 12 livers (3 in the first phase+6 in the second phase) exhibited a severe injury pattern which was just short of catastrophic. The remaining three livers were unremarkable.

PNF is the leading cause of early graft loss and is responsible for enormous morbidity, mortality and expense. We believe that systematic measurement of effluent HA levels will permit detection of livers that have been severely injured and are, therefore, at high risk of developing PNF, or at the very least exhibit a severely troubled post-operative course (false positive). In the presence of a shrinking donor pool, this may not be sufficient to prompt a transplant surgeon to discard a liver. The utility of the test, we believe, lies in the fact that a low HA level guarantees a good liver, while the presence of a high HA level should forewarn the surgeon of the increased risk of PNF or a significantly deranged early graft function. The HA test is easy to perform and can be rapidly assayed within 2 h.

We, therefore, conclude that:

1. There is a strong correlation between effluent

HA levels and post-operative graft function as reflected by serum AST and ALT levels.

2. There is a strong correlation between effluent HA levels and graft outcome.
3. An effluent HA level of $\leq 400 \mu\text{g} \cdot \text{l}^{-1}$ appears to indicate minimal, if any injury to the microvascular endothelial cells and, therefore, minimal or no risk of the graft failing from PNF.
4. Conversely, an effluent HA level $> 400 \mu\text{g} \cdot \text{l}^{-1}$ appears to indicate severe injury to the microvasculature with an increased risk of the graft developing PNF or at the very least a very troubled post-operative course.

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