

Published in final edited form as:

*Transplant Proc.* 2011 June ; 43(5): 1484–1488. doi:10.1016/j.transproceed.2010.12.066.

## Hepatocyte viability and ATP content decrease linearly over time during conventional cold storage of rat liver grafts

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### Abstract

**Introduction**—The gold standard in organ preservation is static cold storage (SCS) using University of Wisconsin solution (UW). While it's known that there is a finite limit to SCS preservation, and that there is a correlation between the ATP levels and organ function post preservation, a quantitative relationship has not been established, which is important in understanding the fundamental limitations to preservation, minimizing cold ischemic injury and hence maximizing utilization of the donor organ pool.

**Aim**—To determine the time limits of cellular viability and metabolic function during SCS, and to characterize the relationship between cellular viability and energetic state, using clinically relevant techniques in organ preservation.

**Methods**—Rat livers were procured and stored using conventional storage in UW solution at 4°C. Viability was assessed by determining the amount of viable hepatocytes and intracellular ATP content after 0, 24, 48, 72 and 120 hours of storage.

**Results**—Numbers of viable hepatocytes that were isolated from these livers decreased steadily during SCS. After 5 days, viable hepatocytes decreased from  $25.95 \times 10^6$  to  $0.87 \times 10^6$  cells/gram tissue. Intracellular ATP content decreased from 9.63 to 0.93  $\mu$ moles/gram tissue. Statistical analysis (ANOVA) established a linear relation for both parameters as a function of time ( $P < 0.05$ ).

**Conclusion**—The linear correlation between hepatocyte viability, ATP content, and storage time suggests a shared physiological foundation. These findings confirm ATP as direct predictor for organ quality in the context of liver preservation, which will aid quantitative assessment of donor organs for various applications.

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## Introduction

The shortage of donor organs for transplantation is a major crisis in treatment of organ failure, with the gap between demand and supply growing rapidly. Attempts to address this gap include development of superior storage solutions and machine perfusion [1–3], enabling longer organ storage times and recovery of discarded donor livers [4]. Through these progressions, transplantation surgery will advance from an urgent and precarious practice towards being an elective procedure. However, evaluation of the organ post-storage and prior to transplantation remains subjective, with assessment generally limited to gross morphology and donor criteria [5]. Histology is performed occasionally, although reports in kidney transplantation indicate its use does not significantly increase the likelihood of graft survival [6–8]. Even machine perfusion has yet to provide definitive quantitative criteria of organ viability, despite the availability of multiple dynamic measurements [9]. The establishment of markers for organ viability would assuage uncertainty for the surgical team, and reduce the number of organs discarded due to poor and often conservative assessment of organ viability. Furthermore, such a method would be of immediate use in the procurement of hepatocytes from discarded organs, which serve as a finite resource for cell transplantation and bio-artificial livers.

Obvious parameters to consider as being correlated with graft survival are those associated with the energy state of the organ. It is well known that as the duration of organ storage increases, the levels of metabolic substrates ATP, adenosine and ADP, decreases [10]. However, the dynamics of energy level depletion during storage have not been quantified in detail; hence it is unknown whether this correlation can be leveraged for accurate viability assessment. Therefore, in this preliminary study we analyzed the transient changes in ATP content during static cold storage. In order to gauge the quality of the organ, we measured the amount of viable hepatocytes in a liver graft after cell isolation, which is a known measure of function for cell transplantation [11, 12].

## Experimental Procedures

### Procurement of rat livers

Experiments were performed using female Lewis rats ( $n=30$ ) weighing 150–200 g (Charles River Labs, Wilmington, MA). The animals were maintained in accordance with National Research Council guidelines and the experimental protocols were approved by the Subcommittee on Research Animal Care, Massachusetts General Hospital. Subjects were anesthetized with isoflurane (Forane, Baxter, Deerfield, IL) using a Tech 4 vaporizer (Surgivet, Waukesha, WI). The liver was harvested using the technique previously described by Delrivière and Kamada [13, 14]. During the last stage of surgery, livers were flushed with 5ml of cold UW using an 18G intravenous catheter introduced into the portal vein and secured with a 6-0 silk ligation, marking the start of cold storage time. Livers were washed and weighed (average weight  $7.87 \pm 2.3$ g), and then flushed with an additional 5ml of UW immediately after procurement.

### Static Cold Storage of rat livers

Livers were stored in UW solution at 4°C for 0, 24, 48, 72, and 120 hours ( $n=6$  livers per time point). Literature indicates that rat livers can be stored and transplanted after an average SCS time of 18–24 hours [15–21] with one report extending the storage time to 48 hours [22], hence this design incorporates two storage durations that would very likely result in successful transplantation (0 and 24 hours), two with expected negative outcomes (72 and 120 hours), and one with marginal results (48 hours), providing a relevant time span to assess the value of ATP in evaluation of graft viability.

## Hepatocyte isolation

The protocol used is adapted from the enzyme-based hepatocyte isolation protocol developed by Kreamer et al [23]. The livers were perfused with Krebs Ringer's Lactate with a flow rate of 17ml/min. Digestion was achieved with collagenase (Type IV, Sigma, St Louis, MO). The hepatocytes were isolated from the non-parenchymal fraction by filtration and centrifugation steps and live cells were segregated through Percoll gradient purification. Viability was >90% in all experiments, as assessed by Trypan blue staining using a hemocytometer.

## ATP assay

After 0, 24, 48, 72, and 120 hours, tissue biopsies (20–40mg each) were taken from each specimen (threefold to decrease sampling error) and flash-frozen using liquid nitrogen. After thawing, samples were kept at 4°C. The tissue samples were weighed and homogenized in perchloric acid (Biovision, Mountain View, CA) in order to neutralize phosphatases present in the tissue. Samples were centrifuged, resuspended and deproteinized using a Deproteinizing Sample Prep Kit (Biovision, Mountain View, CA). Concentration of ATP in each sample was determined using an ATP colorimetric/Fluorometric Assay Kit (Biovision, Mountain View, CA) and fluorescence was detected using an *f*max microplate reader (Molecular Devices, Sunnyvale, California).

## Statistical analysis

Pearson correlation coefficient (R) and linear regression were used to analyze the relationship of cell yield, ATP level, and storage time. Analysis of variance and post-hoc Tukey test were performed for comparisons between groups with different storage times.  $P < 0.05$  was considered to be significant.

## Results

### Cell yield during SCS

In order to assess the viability of each graft, the live hepatocyte fraction was determined at various SCS storage times. Figure 1a shows the results of the hepatocyte isolations for each time point. Cell yields declined consistently with the increase in storage time; the 120 hour control time point showed a yield of  $0.87 \pm 0.42 \times 10^6$  hepatocytes per gram of liver tissue, or 3.3% of the amount of freshly isolated hepatocytes ( $25.95 \times 10^6$  cells/gram liver). Linear regression analysis revealed a correlation between the viable cell numbers and storage time in UW (R 0.96 with a significance of  $P=0.0081$ ). We analyzed independent differences between the individual groups ( $P < 0.05$ ) using analysis of variance and post-hoc Tukey test. There was no significant difference between any two groups stored for less than 24 hours. However, significant variation existed in the comparison of 0 hours vs.  $\geq 48$  hours, 24 hours vs. 120 hours s, and 48 hours vs. 120 hours of SCS.

### ATP levels during SCS

ATP was measured at each storage time. Figure 1b depicts the intracellular content of ATP per gram of liver tissue. A linear relationship between the amount of ATP isolated from each sample and the amount of time the sample had been stored was determined by regression analysis (R calculated at 0.95 with a significance of  $P=0.011$ ). Individual group differences were significant in the comparison of 0 hours vs.  $\geq 48$  hours and 24 hours vs. 120 hours of SCS. As was the case for cell yield, no differences were significant between groups stored less than 24 hours. The 120 hour control group showed ATP levels averaging  $0.93 \mu\text{moles/gram tissue}$  (<10% of fresh ATP levels).

## Relationship of cell yield and ATP

A scatter plot of cell yields against ATP levels is shown in Figure 1c. The cell yields and ATP level had a linear relationship with each other across all storage times ( $R=0.98$ ;  $P=0.004$ ). It's therefore possible to predict the amount of hepatocytes in a rat liver from the ATP content, at the ratio of  $26.94 \times 10^6$  hepatocytes /  $\mu\text{moles ATP} \cdot \text{gr liver}^{-1}$ .

## Discussion

In this study we have demonstrated that when a healthy liver is stored *ex vivo* there is a strong association between the amount of viable hepatocytes and the ATP level, both of which decline linearly with time. The dynamic correlation of this process supports further exploration into the use of cellular energy levels as a quantitative criterion of graft viability. For example, previous studies have demonstrated that rat livers can be stored and transplanted after an average SCS time of 18–24 hours [15–21] with one report extending the storage time to 48 hours [22], while no success has been shown at 72 and 120 hours. Our results indicate that during a storage time of 24 hours, both the viable cell yield and ATP level decline. It is notable that there is no statistical difference in ATP content or cell viability during the first 24 hours of SCS, which is in accordance with the established preservation periods in rat liver transplantation. Significant differences between individual groups surfaced when storage time was extended beyond 24 hours. After 24 hours of SCS, ATP levels decline to 70% of initial levels, and at 72 hours, this value is only 30%. These results suggest then that 70% of fresh (or  $0.59/ \mu\text{moles ATP} \cdot \text{g liver}^{-1}$ ) might be a relevant value to indicate the limit of viability, independent of preservation time. Furthermore, we propose that an ATP-based criterion can serve in early detection of pathologies such as ischemic injury, steatosis and other donor factors, although experimental confirmation will be necessary. The one potential disadvantage of this concept is that it requires a biopsy, however in the case of machine perfusion a non-invasive indicator of energy could be developed.

Confirmation of these results in an animal transplantation model is necessary to verify this approach, and should include the viability analysis of non-parenchymal cell types such as biliary epithelial cells, which are crucial for long-term graft function. For applications where primary hepatocyte isolation is required, such as bio-artificial livers, the ATP status can be a direct predictor for the expected viable cell yield per gram of liver tissue [24, 25], and the values determined in this work can provide an initial guideline for studies with human organs.

## Acknowledgments

Funding: Professor Michael – van Vloten foundation, National Institutes of Health (R01 DK59766, R01 EB 008678, R00 DK080942), Shriners Hospitals for Children

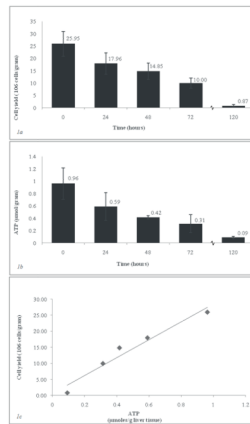
Tim Berendsen was supported by the Prof. Michaël-van Vloten foundation. Funding from the National Institutes of Health (R01 DK59766, R01 EB 008678, R00 DK080942), and the Shriners Hospitals for Children are gratefully acknowledged.

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**Fig. 1. Hepatocyte isolations and ATP assay for various periods of SCS. Error bars reflect standard deviation**

Fig. 1a) Cell yield after percoll-purification (>90% viability, n=3 per time point). Depicted are numbers of viable hepatocytes (>90% viability) per time point (10<sup>6</sup> cells/g).

Fig. 1b) Average ATP per gram of liver tissue (n=3 livers per time point, 3 sections per liver).

Fig. 1c) Cell yield in 10<sup>6</sup> cells/gram vs. Average ATP per gram of liver tissue.