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Subnormothermic Preservation Maintains Viability and Function in a Porcine Hepatocyte Culture Model Simulating Bioreactor Transport

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Bioartificial liver (BAL) systems have been developed to bridge patients with acute liver failure (ALF) to liver transplantation or liver regeneration. Clinical application of BAL systems is dependent on the supportive quality of cells used and direct availability of the whole system. Reliable transport of BAL systems from the laboratory to remote treatment centers is therefore inevitable. Subsequently, preservation conditions play a crucial role during transport of a BAL, with temperature being one of the most determining factors. In this study, we assessed the effect of subnormothermic preservation on freshly isolated porcine hepatocytes cultured in monolayer under oxygenation. Additionally, the effect of the University of Wisconsin (UW) preservation solution was compared with Williams' E (WE) culture medium at 4°C. The control group was cultured for 3 days at 37°C, whereas the transport groups were cultured at 4°C, 15°C, 21°C, or 28°C for 24 h at day 2. All groups were tested each day for cell damage and hepatic functions. Subnormothermic culture (i.e., 15°C to 28°C) for a period of 24 h did not reduce any hepatic function and did not increase cellular damage. In contrast, culture of hepatocytes in WE medium and preservation in UW solution at 4°C significantly reduced hepatic function. In conclusion, freshly isolated porcine hepatocytes can be preserved for 24 h at subnormothermic temperatures as low as 15°C. Future research will focus on the implementation of the AMC-BAL in an oxygenated culture medium perfusion system for transport between the laboratory and the hospital.

Key words: Bioartificial liver; Hepatocyte; Transport; Temperature; Cell function

INTRODUCTION

In this era of organ donor shortage, many acute liver failure (ALF) patients die on the liver transplantation waiting list (1,3). To bridge ALF patients to liver transplantation (OLT) or liver regeneration, several bioartificial liver (BAL) systems have been developed (40). A BAL is an extracorporeal system consisting of a bioreactor loaded with hepatocytes from either animal or human origin. One of these systems has been developed at the Academic Medical Center of Amsterdam, the AMC-BAL. The unique features of the AMC-BAL are: 1) direct contact between the plasma of the patient and small aggregates of hepatocytes, which are attached to a nonwoven polyester matrix; 2) on-site oxygenation of the hepatocytes provided by gas capillaries situated between the spirally wound layers of the polyester matrix (11,12). The AMC-BAL has been extensively tested in vitro (1,11, 12), showed significant improvement of survival time in ALF animal models (13,14,33), and proved to be safe in a phase I clinical trial conducted in Italy (38–40).

Logistical problems may, however, affect the outcome of BAL treatment. These include limited availability of sufficient functional hepatocytes and undefined conditions for transport of a loaded bioreactor to the center of treatment. At least 10×10^9 to 15×10^9 viable and well-functioning hepatocytes are thought to be needed in a BAL to effectively treat ALF patients (22,29,41). At present, 9 of 11 BAL systems clinically tested worldwide use primary porcine hepatocytes (40). These cells are the most suitable alternative for primary human hepatocytes, as human hepatoma and hepatocyte cell lines display impaired functionality.

Transport of hepatocytes for BAL treatment from the BAL-preparing laboratory to the center of treatment, either enclosed in or separate from the bioreactor, should

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be carried out with preservation of function. For clinical application of the HepatAssist system, developed by Demetriou and coworkers, cryopreserved hepatocytes are sent separately from the bioreactor (23,27,28). Hepatocytes are thawed in the treatment center and loaded in the bioreactor, usually under uncertain conditions in terms of safety management and experience of the team involved. No data are available of the function of the HepatAssist bioreactor charged with thawed, cryopreserved hepatocytes prior to treatment. A second option to preserve cell-loaded bioreactors during transport is by using a preservation solution at 4°C, which can be flushed out before connecting the BAL system to the patient (38, 39). We used this strategy for the AMC-BAL, but observed a rapid decrease in hepatocyte viability and liver-specific functions after 12-h transport with Celsior at 4°C. Ammonia elimination and urea synthesis capacity decreased below 40% of normothermic (37°C) cultured controls (7). In addition, a recent study concluded that the effect of 8-h hypothermic (4°C) storage of bioreactors loaded with primary human hepatocytes isolated from discarded donor livers had a drastic negative effect on cell integrity (31).

Transport of hepatocytes at normothermic or subnormothermic (15°C–35°C) temperatures can be an attractive alternative to reduce ATP depletion and prevent accumulation of reactive oxygen species caused by cold preservation as well as cryopreservation. Imber et al. (16) showed that livers preserved by continuous normothermic, oxygenated sanguineous perfusion were significantly superior to cold-stored livers in terms of function and damage parameters. In vitro studies from our own laboratory showed stable BAL function during several days of oxygenated culture in culture medium at 37°C (41). A stable temperature of 37°C is, however, difficult to maintain under transport conditions as an energy-depending heating system is required. It may therefore be preferable to transport a BAL system without active heating control and allow temperatures to drop to subnormal levels. One of the scarce data on subnormothermic culture or preservation has been published by Wigg et al. They showed that cell integrity and function of isolated rat hepatocytes could be preserved at a temperature of 25°C for at least 24 h during suspension culture (43). Although the temperature range in which cellular functions of porcine hepatocytes are still preserved has not been defined yet, we hypothesized that subnormothermic temperatures can be used to adequately preserve porcine hepatocytes for BAL transport purposes. We therefore explored, in this study, the possibilities of subnormothermic preservation of freshly isolated porcine hepatocytes in a two-dimensional culture model by assessing the effects of a range of different temperatures (15°C–35°C) on cellular viability and hepatocyte function.

MATERIALS AND METHODS

Hepatectomy and Hepatocyte Isolation

Livers were obtained by hepatectomy from young female pigs weighing 20–25 kg under sterile conditions. All procedures were approved by the institutional guidelines of the Animal Ethical Committee of the University of Amsterdam. The hepatectomy procedure has been described previously (41).

Porcine hepatocytes were isolated according to the protocol described by Seglen (32), which was revised for AMC-BAL purposes (41). In brief, hepatocytes were isolated with a two-step isolation procedure using a flush of Ca^{2+}-free medium followed by recirculation of collagenase P (Roche) buffer for 25 min at 37°C. After perfusion, the liver capsula was opened manually and liver cells were flushed out of the liver. Hepatocytes were collected after filtration through a surgical gauze. The hepatocyte suspension was washed three times using $50 \times g$ centrifugation in culture medium based on Williams' E medium (Bio-Whittaker) supplemented with 10% (v/v) heat-inactivated FBS (Bio-Whittaker), 2 mM glutamine (Bio-Whittaker), 1 μM dexamethasone (Centrafarm), 20 mU/ml insulin (Novo Nordisk), 2 mM ornithine (Sigma-Aldrich), and penicilline/streptomycine/fungizone (Bio-Whittaker).

Total number of isolated hepatocytes was estimated by determination of the volume of cell pellet after centrifugation for 3 min at $50 \times g$. It was assumed that 1 ml of cell pellet represents approximately 92×10^6 hepatocytes (41). Cell viability was determined using the trypan blue exclusion assay with a Bürker Bright line cytometer (Optik Labor).

Hepatocyte Culture Under Different Temperature Conditions

Hepatocytes were seeded in six-well Primaria plates (Falcon) at a concentration of 3.6×10^6 cells per well in 2.0 ml culture medium and cultured at 37°C in culture gas (5% CO_2/95% air). Four hours after seeding, culture plates were washed twice with phosphate-buffered saline (PBS) to remove nonattached, dead hepatocytes. Hepatocytes were then cultured in 2.0 ml culture medium. After overnight incubation at 37°C, culture medium was replaced by 2.0 ml test medium, which is composed of the previously described culture medium supplemented with 500 mg/L lidocaine (Sigma Chemical), 2.75 mM D-galactose (Sigma Chemical), 2 mM L-lactate (Sigma Chemical), and 5 mM NH_4Cl (Merck). Each six-well plate represented a temperature/condition group and was subsequently tested during 3 consecutive days. Samples were taken at 0, 4, 8, and 24 h. The first test day represented initial hepatocyte function before transport, whereas the second day corresponded with the

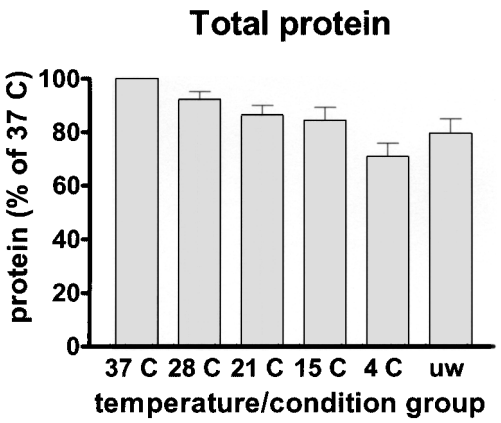

Figure 1. Total protein content determined at the end of day 3; values are given as percentage of 37°C, mean ± SEM. UW, UW preservation at 4°C.

transport period of the bioreactor. The second day, new test medium was added after washing the wells twice with PBS. Hepatocytes were then cultured under different conditions and temperatures: 37°C, 28°C, 21°C, 15°C, 4°C in test medium and 4°C in University of Wisconsin (UW) solution (ViaSpan DuPont Pharma), respectively. All plates, except for the UW cold-preserved plate, were oxygenated with culture gas. The third day, medium or preservation solution was replaced by fresh test medium and the plates were again cultured at 37°C, simulating the conditions of hepatocytes in the BAL system prior to patient treatment.

Determination of Cell Number in Culture

Total protein content was determined at the end of day 3 after finishing the last function test to determine the number of hepatocytes cultured in each well. Cell proliferation during the 3-day culture period at 37°C was determined by measuring total protein of one six-well plate at the end of each culture day. Protein was quantified by spectrometry using Coomassie blue (Bio-Rad).

Biochemical Analyses

General. Lactate-dehydrogenase (LDH) and aspartate aminotransferase (AST) leakage, ammonia and lidocaine clearance, and urea and albumin synthesis rates were determined by calculating the changes in concentration in test medium over 24 h.

LDH and AST Leakage. Both LDH and AST activity assays were analyzed spectrophotometrically using a P800 Roche Diagnostics analyzer. LDH activity was measured by conversion of lactate into pyruvate at 37°C. AST activity was determined with pyridoxalphospate at 37°C.

Ammonia Clearance. Ammonia concentrations were determined by an enzymatic kinetic colorimetric assay, using glutamate dehydrogenase and NADPH performed in a P800 Roche Diagnostics analyzer.

Urea Synthesis. Urea concentrations were determined using the blood urea nitrogen test of Sigma Chemical Co. The assay was performed according to the instructions of the manufacturer.

Albumin Synthesis. Albumin concentrations were determined by using the Pig Albumin ELISA Quantitation Kit (Bethyl Laboratories, Inc).

Statistical Analysis

Data were analyzed using GraphPad Prism software (San Diego, CA). Results are reported as means ± SEM. Paired Mann-Whitney *U*-tests were used to compare outcome between different groups. Significance was reached if $p < 0.05$.

RESULTS

To determine the effects of a 24-h period of different subnormothermic culture conditions or cold preserva-

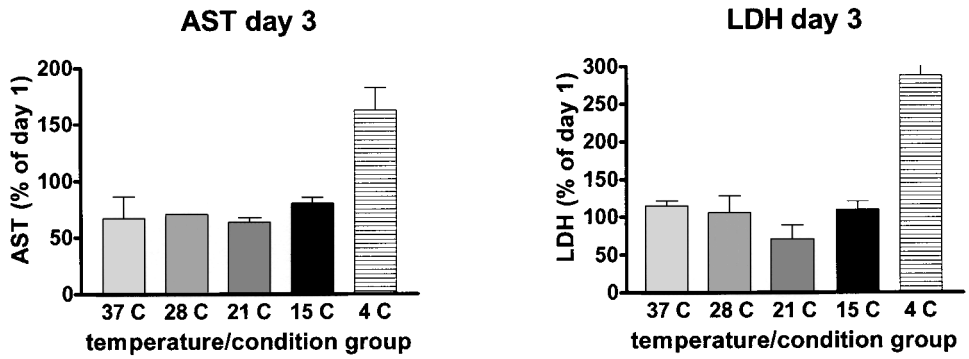

Figure 2. LDH and AST release at day 3 as percentage of day 1 per temperature group.

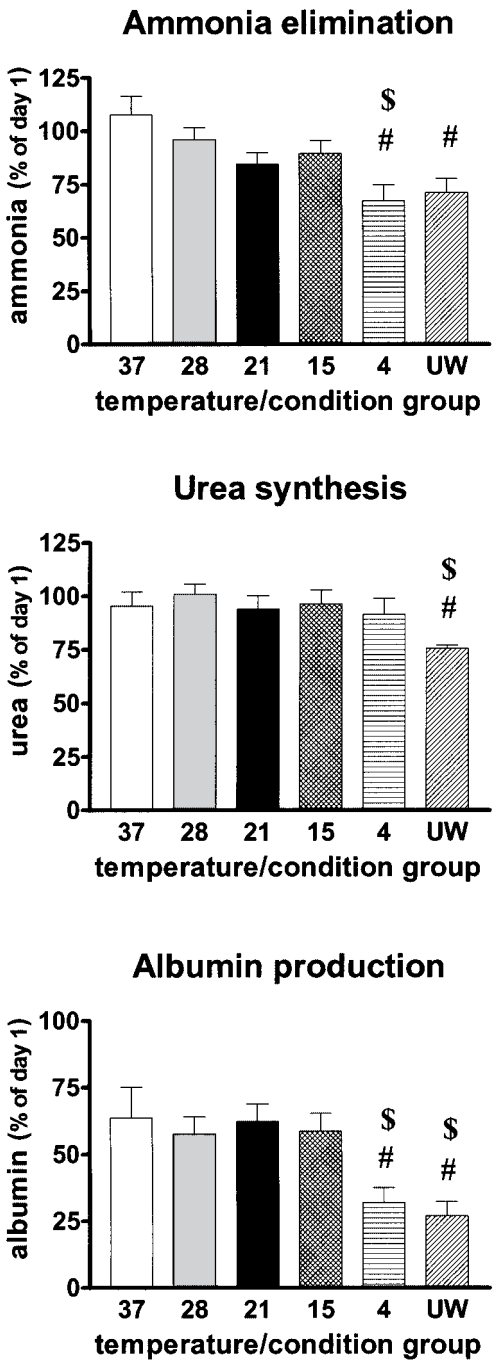

Figure 3. Liver-specific functions of hepatocytes at day 3 as percentage of day 1 per temperature/condition group. #$p <$ 0.05 compared to 37°C; \$$p < 0.05$ compared to 15°C.

tion, primary porcine hepatocytes were tested for liver functions and liver enzyme leakage.

Number of Hepatocytes During Testing

To verify the number of hepatocytes per well, total protein of all wells at the end of day 3 was determined and found to vary less than 10% within each temperature/condition group. The total protein content per well at day 3, however, increased significantly with increased culture temperatures at day 2 (Fig. 1). To assess whether temperature-dependent protein content might be associated with cell proliferation rate, we determined the proliferation rate by total protein content after three successive culture days at 37°C ($n = 3$). Total protein content increased with $19 \pm 0.3\%$ and $49 \pm 2.6\%$ at days 2 and 3, respectively. Because of two washing steps with PBS, most of the extracellular protein was excluded from the analysis. Moreover, cell proliferation could be mainly attributed to hepatocyte proliferation since >98% of isolated cells were hepatocytes (32). These findings suggested that cell proliferation rate was affected by a decrease in culture temperature. However, an increase in proliferation rate had no effect on the presented data as the function of all groups is expressed as a percentage of day 3 versus day 1 within the same group (e.g., same wells).

Hepatocyte Damage at Day 3 Versus Day 1

LDH and AST release of the subnormothermic temperature groups at day 3 did not differ significantly compared to the 37°C group. However, culture at 4°C at day 2 resulted in a twofold increase in LDH and AST release ($n = 2$) measured at day 3 (Fig. 2), indicating increased cell damage rate after 24-h preservation at 4°C compared to normothermic and subnormothermic temperatures. LDH and AST release in UW preservation at 4°C was not measured.

Liver-Specific Functions at Day 3 Versus Day 1

Liver-specific functions of porcine hepatocytes (e.g., ammonia elimination, urea synthesis, and albumin production) were not significantly affected after 24-h subnormothermic culturing. Ammonia elimination capacity did not decrease below 80% compared to normothermic culture. In contrast, ammonia elimination in the 4°C culture medium group decreased to 60%, which is comparable to the 4°C UW group, both significantly lower than the 37°C culture group (Fig. 3). Data for urea synthesis showed no differences between temperature groups except for the 4°C UW group, which showed a significant 25% decrease compared to the other groups (Fig. 3). Albumin production showed significant differences between both the 4°C culture and 4°C UW group compared to the 37°C and subnormothermic groups (Fig. 3). Albumin production at day 3 decreased to 60% compared to day 1.

Kinetics of Liver Functions

Temperature-dependent kinetics of ammonia elimination and urea synthesis at three consecutive test days

of three temperature groups (37°C, 15°C, and 4°C) are presented in Figure 4. These graphs show that ammonia elimination and urea synthesis were linear in time at all 3 days, indicating an immediate temperature effect on hepatocyte-specific function. A decrease in temperature per 1°C resulted in a decrease of ammonia elimination and urea and albumin synthesis at day 2 of 4.0%, 3.5%, and 2.9%, respectively. Between 15°C and 4°C, the decrease in metabolism was less than 1% per 1°C temperature decrease (Fig. 5).

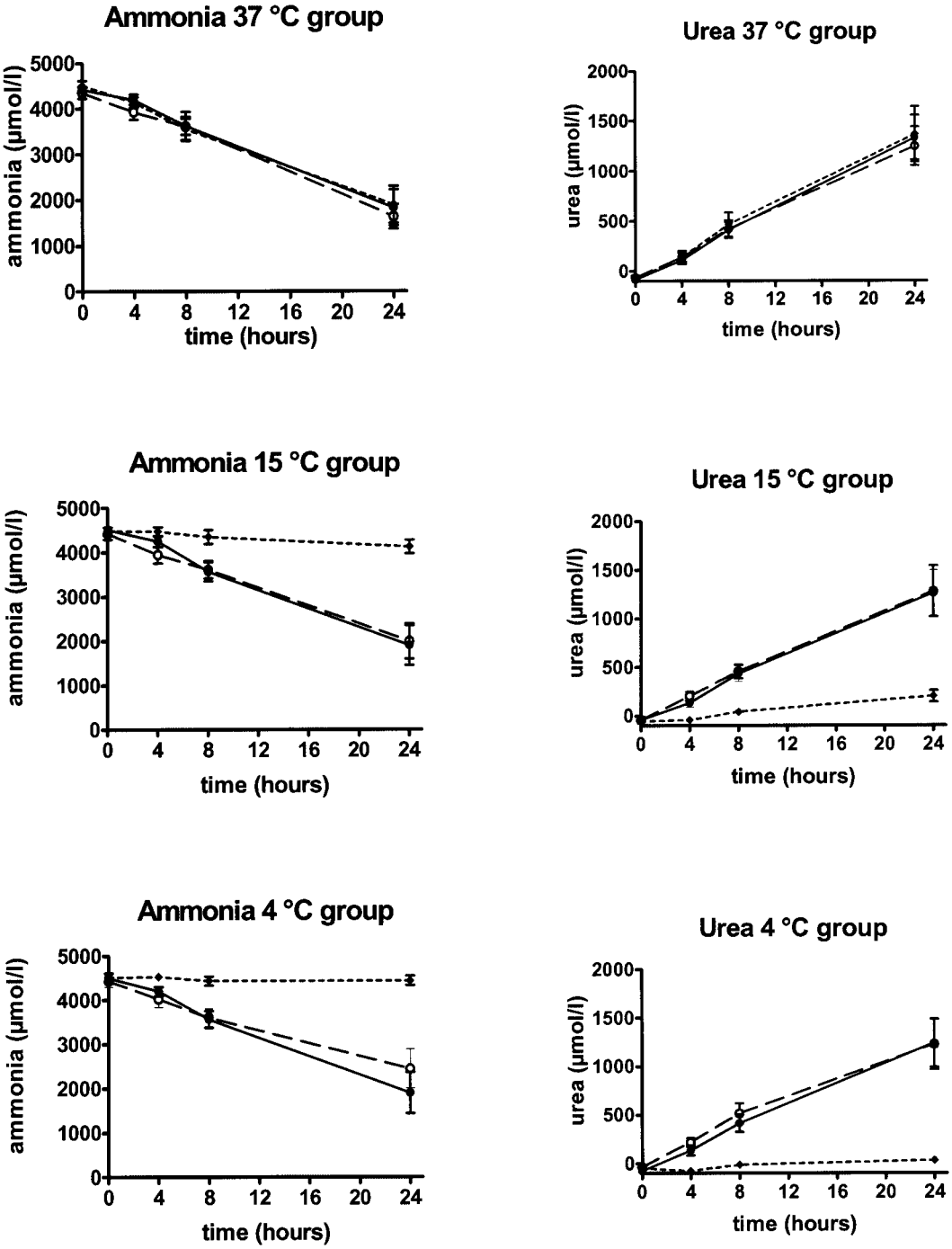

Figure 4. The kinetics of ammonia elimination and urea synthesis during the three consecutive test days with culture groups at 37°C, 15°C, and 4°C. Day 1: culture at 37°C (filled circles, solid line); day 2: culture at variable temperatures depending on temperature group (filled diamonds, dotted line); and day 3: culture at 37°C (open circles, dashed line).

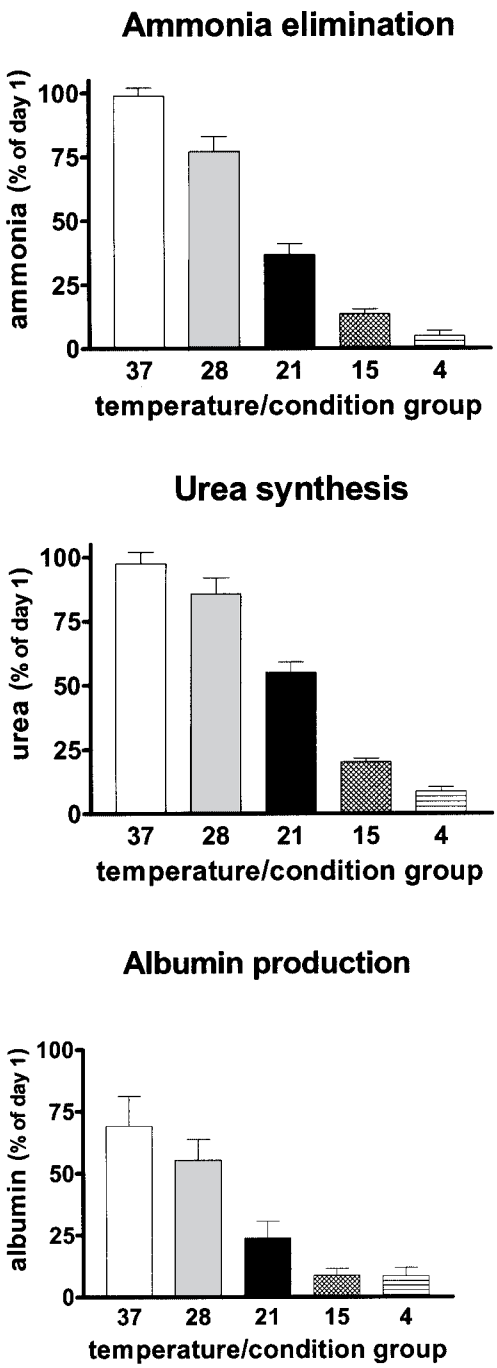

Figure 5. Ammonia elimination, urea synthesis, and albumin production at day 2 as percentage of day 1.

DISCUSSION

Transport of hepatocytes or hepatocyte-loaded bioreactors for clinical BAL application is currently managed by the use of cold preservation and cryopreservation. Postthawing viability and function of cryopreserved hepatocytes is generally low (2,5,8,36). However, successful reports of cold preservation of rat hepatocytes were also published (15,21,25,42). But, in general, cold-preserved and cryopreserved porcine hepatocytes have a reduced survival attachment capacity and diminished liver-specific function after rewarming [(3,9,10,18,22, 30), own experience (not published)]. It should be taken into account that the majority of hepatocyte preservation studies were performed with small cell numbers. Outcome of preservation with large cell numbers (e.g., suitable for BAL treatment) is even worse (44). Therefore, cryopreservation and cold preservation of large amounts of porcine hepatocytes or complete hepatocyte-loaded bioreactors are not realistic options for clinical BAL application. In contrast to cryopreservation and cold preservation, almost no data are available concerning subnormal temperature preservation of (porcine) hepatocytes. One of the scarce data on subnormothermic culture or preservation has been published by Wigg et al. They showed the maintenance of integrity and function of isolated rat hepatocytes for at least 24 h during suspension culture at 25°C (43).

In this study, the experimental set-up was based on the assumption that a 24-h transport period should be more than sufficient to transport a cell-loaded BAL from the laboratory to all possible treatment centers on one continent. To avoid logistical problems of a guaranteed normothermic transport, we investigated the possibility of oxygenated subnormothermic hepatocyte preservation. In this study, we showed with a two-dimensional oxygenated porcine hepatocyte culture model that liver-specific functions were well preserved for a period of 24 h at subnormothermic temperatures as low as 15°C. In contrast, culture at 4°C and preservation in UW solution at 4°C showed a decrease of liver-specific functions, which can be ascribed partly to an increased in cell death.

We assume that these findings are applicable to a three-dimensional bioreactor culture as well. However, the effects may be different to some extent as hepatocytes grow in cell aggregates inside the BAL, which can enhance their functionality and possibly decrease their sensitivity to preservation or culture conditions (6,17, 30). In addition, perfusion of medium through the bioreactor during transport may improve functionality by allowing effective supply of nutrients and cofactors and disposal of toxic waste products, in contrast to static culture, as has been observed for perfusion of donor livers during cold preservation (34).

Our study shows that (sub)normothermic transport at a temperature of 15°C or higher will assure a therapeutically more effective BAL for patient treatment, compared to the current transport situation at 4°C where a significant loss of liver-specific function was observed (7). As also shown in this study, a significant metabolic activity (i.e., 5–10%) (Fig. 5), is still present in the hepatocytes at 4°C, requiring ATP. At hypothermia, ATP

levels decrease deeply in liver (4). In addition to ATP depletion, hypothermia induces the accumulation of reactive oxygen species, which leads to calcium influx, membrane damage, and eventually to cell death by ischemia and apoptosis (19,20,26). Subsequent reperfusion or reoxygenation at 37°C of cells induces even more cell death due to produced reactive oxygen species (19,24,26,35). These events may have taken place in cold-preserved hepatocytes, when these cells were oxygenated and cultured in medium or preserved in UW, considering their loss of function and release of liver enzymes. To prevent ATP loss and damage by cold preservation and ischemia and reperfusion injury, subnormothermic oxygenated perfusion of a BAL during transport is recommended.

The kinetics of the hepatocyte metabolism at all temperatures showed linear functions at all 3 days, indicating that the temperature changes immediately affect the functionality of the cells. Consequently, the AMC-BAL can be connected to the patient within the first hours after subnormothermic transport and rewarming in the treatment center, despite the decrease in function during transport.

At this moment, we are developing a disposable (sub)normothermic transport system, based on a gas pressure-driven perfusion pump (Airdrive®, Doorzand Medical Innovations, Amsterdam, the Netherlands), in which pressurized culture gas provides oxygenation and perfusion of medium through the AMC-BAL. This system will eventually be applied to assure delivery of a ready-to-use AMC-BAL to the bedside of the ALF patient.

In conclusion, the results of this study suggest that the AMC-BAL can be preserved and therefore can be transported for 24 h at subnormothermic temperatures, even at 15°C, in an oxygenated culture medium perfusion system.

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