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Cryoprotective potential of urea in bat cells *in vitro*

Monika Němcová[1], Šimon Adam[1], Šárka Bednaříková[1], Vladimír Piaček[1], Jiří Pikula[1]

[1]University of Veterinary Sciences Brno, Faculty of Veterinary Hygiene and Ecology,
Department of Ecology and Diseases of Zoo Animals, Game, Fish and Bees, Brno, Czech Republic

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Abstract

Hibernating bats potentially risk cold-induced cell damage. However, the cells of hibernating bats have previously been shown to be able to cope with sub-zero temperatures, either alone or due to glucose cryoprotection. The aim of our study was to determine the survival rate of bat-derived cells *in vitro* after 24 h at −20 °C in a medium supplemented with urea at concentrations ranging from 0 to 320 mM or with a urea-glucose combination. Urea showed limited cryoprotective potential, with cell survival rates not exceeding 3% at urea concentrations of 20 to 40 mM, which corresponds to reported levels for blood urea in bats. Higher urea concentrations tended to be toxic rather than protective. Importantly, urea appeared to relieve delayed-onset cell death after cell rewarming, and also improved the cryoprotective effect of glucose up to three-fold. Thus, supplementation of cell cultures with a cryoprotectant mix containing urea could increase cell survival rates.

Cell culture, cryoprotectant, heterothermy, hibernation, Myotis myotis, Nyctalus noctula

Insectivorous bats of the temperate zone are heterothermic mammals that endure the cooler part of the year in hibernation (Davis 2012; Perry 2013). To reduce the risk of death due to cold-induced cell and tissue damage, bats optimise their hibernation strategies (Davis 2012). Following exposure to extremely low temperatures, normal cells undergo apoptosis (Kizkai et al. 2001) and, when body fluids freeze, ice crystals may cause extensive damage to cells, resulting in necrotic death (Baust et al. 2001). To combat this, bats choose a suitable hibernation site with optimal microclimatic conditions (Perry 2013), undertake occasional arousals (Davis 2012), and use metabolic compensation (Davis 2012) to prevent total freezing of body fluids when their body temperature drops close to freezing point during deep hibernation (Davis and Reite 1967).

However, it also appears that bats may have other protection mechanisms against the destructive impacts of ice crystals at sub-zero temperatures. There have been documented cases of bats having rectal temperatures lower than 0 °C during supercooled events (Davis and Reite 1967), along with observations of cell survival in bat-derived cultures where the extracellular medium was completely frozen, though with considerable loss of cell viability (Nemcova et al. 2022, 2023). In addition to external factors such as the cooling rate or final temperature (Gao and Critser 2000), intracellular and extracellular mechanisms such as presence of specific organic compounds, proteins, amino-acids or saccharides (Davies and Hew 1990; Costanzo and Lee 2005, 2008) can have a significant impact on the percentage of surviving cells. Along with substances synthetized and/or stored purposefully as a cryoprotectant, e.g. glycoprotein anti-freeze in fish (Davies and Hew 1990), organic compounds naturally present in most organisms, such as low-molecular weight permeable osmolytes, can reduce freezable body water and protect cells against injuries. Likewise, substances such as glucose or urea may contribute to the winter survival of freeze-tolerant amphibians (Costanzo and Lee 2005, 2008).

Address for correspondence:
Jiří Pikula
Department of Ecology and Diseases of Zoo Animals, Game, Fish and Bees
University of Veterinary Sciences Brno
Palackého tř. 1946/1, 612 42 Brno, Czech Republic

Phone: +420 541 562 655
E-mail: pikulaj@vfu.cz
http://actavet.vfu.cz/

In our previous study on bat-derived cell cultures (Nemcova et al. 2023), we confirmed that bat cells were able to cope with extremely low temperatures using the cryoprotective effects of glucose at high concentrations. Here, we extend this study by assessing the cryoprotective potential of urea. To replace experimental animals (Kaňová et al. 2022), we used bat-derived cell culture models.

Materials and Methods

For the purposes of this study, we cultured cells derived from the liver and kidney of the common noctule bat (*Nyctalus noctula*) and nervus olfactorius-derived cells and peritoneal macrophages of the greater mouse-eared bat (*Myotis myotis*). The methods for cell culture preparation, immortalisation, and cell type identification have previously been described in He et al. (2014) and Nemcova et al. (2023). In this study, no live animals were used.

To obtain enough cells of the same passage for individual experiments, cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum (BCS) and penicillin 100 IU/ml and streptomycin 100 µg/ml antibiotics (ATB) at 37 °C under a humidified atmosphere of 5% CO_2, with the culture medium being replaced every three days. Before each experiment (see below), the culture medium was removed, the cells washed with 1× phosphate-buffered saline (PBS) and detached from the cultivation surface using 0.5% trypsin solution. The cells were then washed with a medium supplemented by 10% BCS and centrifuged at 160 *g* for 6 min, after which the supernatant was removed. The cell pellets were then resuspended in test solutions (see below).

In the present study, cultures were exposed to −20 °C for 24 h, with the medium freezing completely 25 min after insertion into the freezer (−20 °C). Subsequent thawing of the extracellular medium was completed at room temperature within approximately 18 min, after which the cell suspension was mixed by pipette flow, dyed with Trypan blue and enumerated in a Fast-Read 102® plastic counting chamber (Kisker Biotech GmbH & Co.KG, Steinfurt, Germany). All experiments were performed as three replicates with three repetitions.

Experiment 1

Cells (10^5 cells/100 µl/well in 96-well plates) were resuspended in PBS supplemented with urea to obtain final concentrations of 0, 10, 20, 40, 80, 160 and 320 mM urea and exposed to −20 °C for 24 h. After thawing, the number of cells were determined.

Experiment 2

Cells (10^5 cells/100 µl/well in 6-well plates) were resuspended in PBS or PBS with 20 mM urea and exposed to −20 °C for 24 h. The cultures were thawed and all surviving cells were then cultured under optimal conditions (37 °C, humidified atmosphere, 5% CO_2, DMEM medium supplemented with 10% BCS and 1% ATB). Subsequently, the number of adherent cells (i.e. healthy cells, Nemcova et al. 2022) were determined after culturing for three and six days in 2 cm and 6 cm dishes, respectively.

Experiment 3

Cells (10^5 cells/100 µl/well in 96-well culture plates) were resuspended in PBS or PBS supplemented with 20 mM urea and/or 40 mM glucose and exposed to −20 °C for 24 h. After thawing, the number of cells were determined.

Statistical analysis

All statistical analyses were performed using software GraphPad Prism 8.4.3 (GraphPad Software, Inc., San Diego, California, US). For all experiments, data normality was first tested using the Kolmogorov-Smirnov and Shapiro-Wilk tests. For Experiment 1, as cell survival rate values were non-normally distributed, the data were subjected to logarithmic transformation and tested using Dunnett's multiple comparisons test for comparing cell survival rates (proportion of cells entering the experiment) between cell types at different urea concentrations against 0% survival. For Experiment 2, cell regrowth rate was calculated as change against the previous measurement. As the values were non-normally distributed, the data were subjected to logarithmic transformation and tested using Dunnett's multiple comparisons test for comparing regrowth rate after exposure to −20 °C for 24 h vs growth rate of control cells not exposed to −20 °C. For Experiment 3, differences in survival rate of cell types in different media exposed to −20 °C were compared using the non-parametric Kruskal-Wallis test.

Results

Low cryoprotective potential of urea

The survival rate is characterised by the proportion of viable cells after exposure to −20 °C for 24 h and subsequent thawing of the medium compared to pre-freezing cell

counts. Dunnett's multiple comparison confirmed a significant positive influence of urea concentration and cell type (both $P < 0.01$) on survival after exposure to −20 °C. Survival rates peaked at 20 to 40 mM urea in all cell types, with survival rates at these concentrations significantly higher than other urea concentrations ($P < 0.01$ for all comparisons; no significant difference between concentrations at 20 mM and 40 mM, $P > 0.99$). Macrophages showed the highest survival rate peak (1.6% on average), followed by liver-derived cells. Survival peaks for nerve- and kidney-derived cells were non-significant ($P = 0.13$ and $P = 0.12$, respectively) compared to 0% survival (Fig. 1).

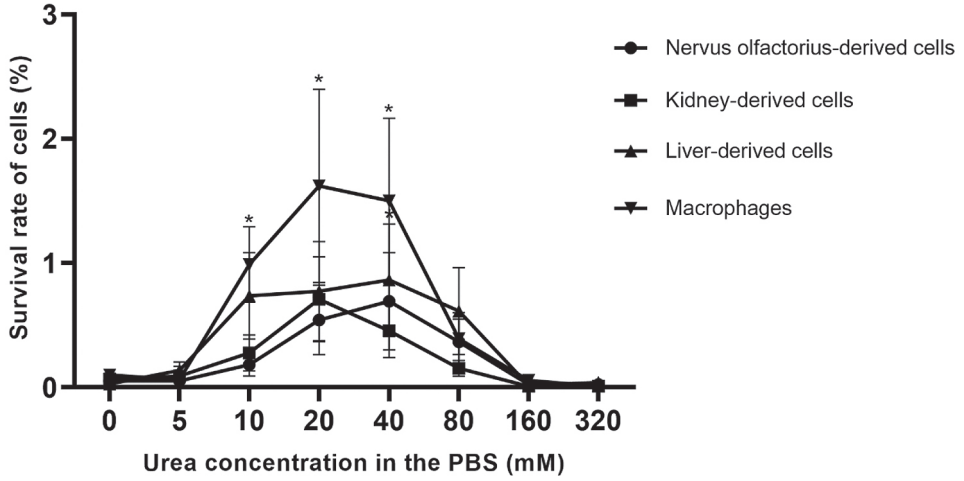

Fig. 1. Survival rates of different bat organ-derived cells

* - Difference in cell survival vs 0% survival; individual points - average of three runs (replicates) with three repetitions; error bars - standard error of the mean; PBS - phosphate buffered saline

Urea-induced decrease in delayed-onset cell death after exposure to −20 °C and subsequent thawing

The regrowth rate is characterised by a change in the cell count compared with the previous measurement. All cell types showed a similar regrowth pattern, with a significant drop in cell number within the first three days in cells exposed to −20 °C (with or without urea).

A higher drop in cell numbers was observed in PBS without urea after exposure to −20 °C, compared to PBS with 20 mM urea. In all cell types exposed to −20 °C (with or without urea), re-growth rates on day 6 were the same ($P > 0.05$ in all cases) as those in cells not exposed to −20 °C.

The highest decrease in cell numbers was observed for all cell types ($P < 0.01$), except kidney-derived cells ($P = 0.06$), after exposure to −20 °C in PBS without urea (compared to PBS + 20 mM urea). A significant difference ($P < 0.01$) in the regrowth rate was observed between cells exposed to −20 °C for 24 h (PBS + 0 and + 20 mM urea) vs control cells not exposed to −20 °C after three culturing days under optimal conditions. Likewise, with the exception of liver-derived cells at 20 mM urea ($P = 0.32$), regrowth rates of cells exposed to −20 °C differed significantly ($P < 0.01$) between culturing days 3 and 6. Regrowth rates for all cell types (with or without urea) exposed to −20 °C were the same ($P > 0.05$ in all cases) as cells not exposed to −20 °C on day 6 (Fig. 2).

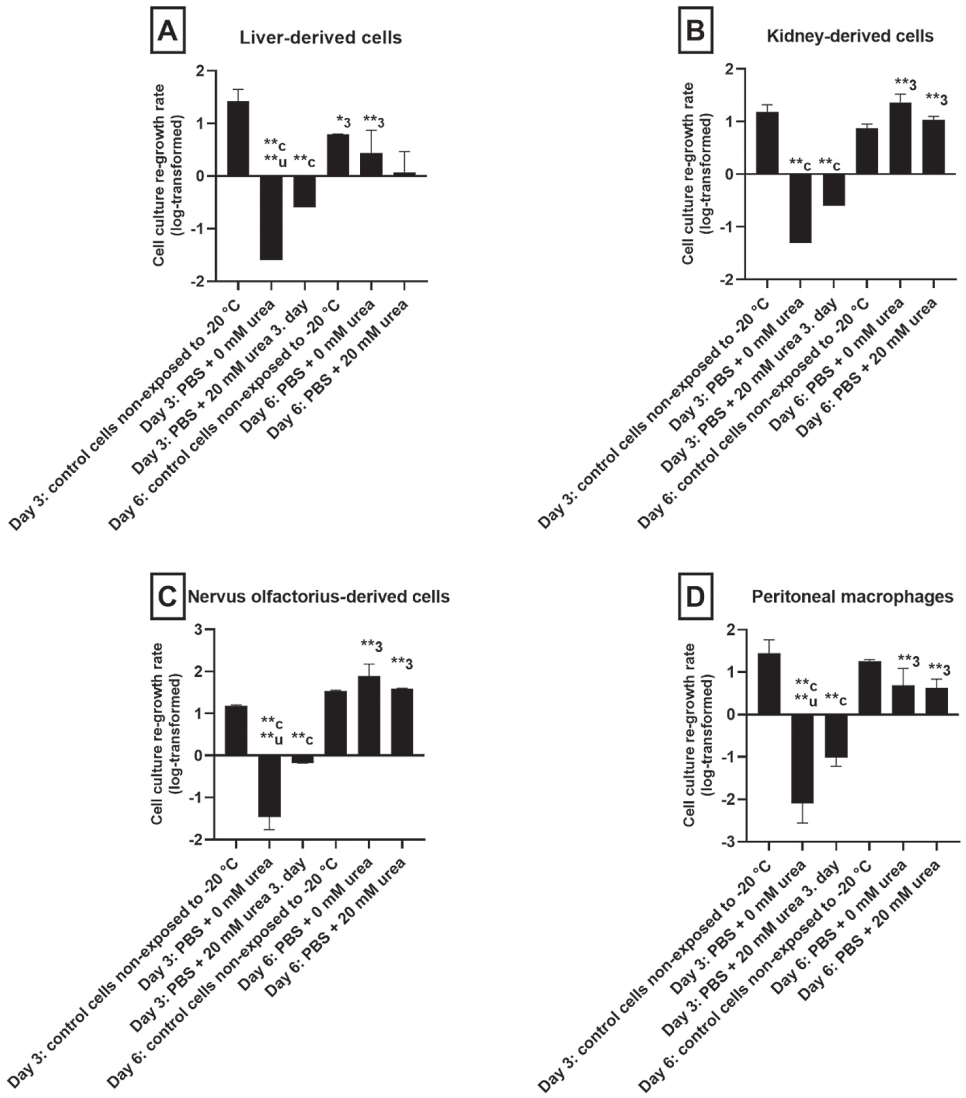

Fig. 2. Regrowth rates of cell cultures derived from different bat organs. A) *Nyctalus noctula* liver; B) *N. noctula* kidney; C) *Myotis myotis* nervus olfactorius; and D) *M. myotis* peritoneal macrophage under optimal conditions (37 °C, 5% CO_2) after exposure at −20 °C for 24 h in phosphate buffered saline (PBS) with 0- or 20-mM urea.

Error bars - standard error of the mean; *c - difference between cells exposed to −20 °C vs control cells not exposed to −20 °C on the same day of measurement; *3 - difference in comparison with the same treatment measured on day 3; *u - difference between cells treated with 0 vs 20 mM urea; * - $P < 0.05$; ** - $P < 0.01$.

Improvement in cell survival rate after freeze-thawing using a urea-glucose mixture

Cell survival was significantly influenced by supplementing the freezing medium with urea and/or glucose ($P < 0.01$; Fig. 3), with the highest average survival rates for

the combination glucose + urea ($P < 0.001$) and second highest for glucose ($P < 0.01$). Urea alone had a non-significant influence on survival ($P = 0.08$) as did PBS without supplementation ($P = 0.25$; Fig. 3).

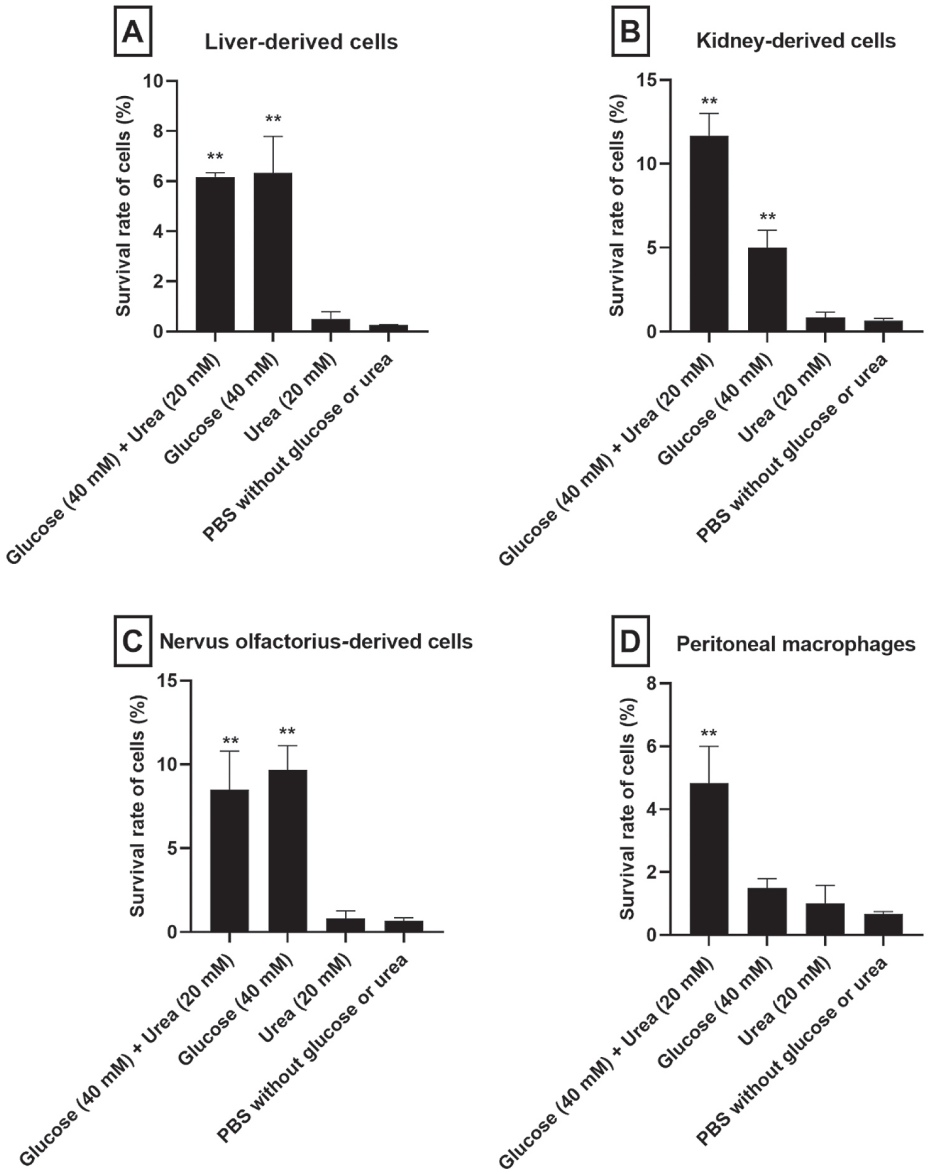

Fig. 3. Survival rates of different bat cell types. A) *Nyctalus noctula* liver; B) *N. noctula* kidney; C) *Myotis myotis* nervus olfactorius; and D) *M. myotis* peritoneal macrophage after exposure to −20 °C for 24 h in different media (phosphate buffered saline [PBS] supplemented with 20 mM urea and/or 40 mM glucose or PBS with no supplementation).

Error bars - standard error of the mean; ** - significant differences ($P < 0.01$) compared to 0% survival.

Discussion

Blood urea concentrations remain relatively high in bats throughout the year owing to their protein-rich insectivorous diet (Bandouchova et al. 2020). Urea, a waste by-product of nitrogen metabolism, has been mostly neglected regarding its possible role as a cryoprotective agent (Costanzo and Lee 2005), despite its known ability to balance fluctuations in osmotic pressure arising during the freezing-thawing cycle, which can lead to cell stress and deformation of the cellular membrane (Costanzo and Lee 2005). This would suggest that urea could contribute to cell protection at freezing temperatures (Costanzo et al. 1993). Our results confirmed that urea did indeed slightly improve cell survival rates after exposure to −20 °C, with peak survival at urea concentrations of 20 to 40 mM.

In this study, urea-treated nerve- and kidney-derived cells after freeze-thawing had survival rates comparable to those of cells cultured in PBS without urea. This corresponds with the results of Costanzo and Lee (2005), who found that urea had no significant beneficial cryoprotective effects on the survival of kidney cells of the wood frog (*Rana sylvatica*) after freeze-thawing. On the other hand, macrophages treated with urea exhibit the highest survival rate, suggesting different responses by different cell types to some cryoprotectants (Nemcova et al. 2023).

Many potentially cryoprotective substances are naturally present in organisms (Costanzo and Lee 2005, 2008; Nemcova et al. 2023) and, while the action of such substances on their own may seem insignificant, they may enhance cell survival after freezing in combination with other cryoprotectants (Costanzo and Lee 2005, 2008; Nemcova et al. 2023). In the present study, for example, survival rates were significantly higher using a glucose-urea mixture than when using glucose alone, with a two-fold increase in kidney cell survival and up to a three-fold increase using macrophages. Some other mechanisms at cellular, organ, and organismal levels may also be involved (Gao and Critser 2000). Alongside the synergistic effect of individual cryoprotectants, cell survival rates will also be affected by differences in their availability. For example, relatively high concentrations of urea are always circulating in blood, being available for all tissues at any time (Bandouchova et al. 2020) and thus able to provide virtually immediate protection in contrast to glucose, which, while more effective as a protectant, needs time to synthesise and distribute (Costanzo et al. 1993).

Cryoprotectants also play an important role in the prevention of delayed-onset cell death, which can occur within hours or a few days after thawing (Baust et al. 2001; Nemcova et al. 2023). In most of the cells tested here, urea reduced the number of cells undergoing cell death within the first three days of culturing under optimal conditions. After six cultivation days, cells exposed to freezing proliferated as rapidly as those not exposed to −20 °C. Though the highest survival rates were observed for all cell types at physiological concentrations of urea (i.e. 20–40 mM), the difference between the effects of the different concentrations was relatively low, even approaching absence of concentration-dependence (Costanzo and Lee 2005). On the other hand, high concentrations of urea, significantly exceeding physiological levels, are known to be toxic (EPA 2011).

To conclude, our *in vitro* experiments with bat-derived cell cultures exposed to urea showed that urea reduced delayed-onset cell death after cell rewarming and improved the cryoprotective effect of glucose up to three-fold. Urea can thus enhance cryoprotective mechanisms that make the survival of heterothermic mammals under severe hibernation conditions possible. Urea seems to be a readily available cryoprotective osmolyte reaching physiologically high blood concentrations in bats specialised for protein-rich insectivorous diet, although the direct cryoprotective effect is low. Therefore, cryoprotective effects may also be worth studying in other osmolytes (and their combinations) naturally present

in organisms, synthetized and/or concentrated in acral parts, in particular, and other tissues prone to significant temperature fluctuations and/or cold-induced damage during winter hibernation periods. Furthermore, our results could have broader implications in cryobiology, when supplementation of cryoprotectant mixtures by urea and glucose or other osmolytes could increase the cell survival rates without major toxicity risks such as changes in gene expression associated with the frequently used chemical cryoprotectant dimethyl-sulphoxide (Verheijen et al. 2019).

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