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Differential function of protective agents at each stage of the hypothermic preservation of hepatocytes

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Hypothermic preservation of bioartificial liver (BAL) has long been appreciated in BAL storage and transportation. However, the deterioration of cell activity during hypothermia/rewarming limits its clinical use and the complete prevention of hypothermia-induced hepatocyte injury has not been achieved. In this article, a miniaturized BAL that underwent three preservation stages (*i.e.* pre-incubation, hypothermia and rewarming) was applied as a hypothermic preservation model to locate the protection of several protective agents against hypothermia-induced cell injury. The agents, including vitamin E, schisandrin B, glycyrrhizic acid, *N*-acetyl-cysteine, ruthenium red, trehalose, anisodamine, fructose-1, 6-diphosphate, cyclosporin A and matrine (Mat), were found to exert their functions at different preservation stages, which were speculated to associate with the specific protection of each agent as well as the corresponding cell injuries at each stage. Such hypothesis was further strengthened by focusing on Mat, which only suppressed the hypothermia-induced injury through the inhibition of Ca^{2+} overload at the rewarming stage, whereas its presence at the hypothermic stage excessively down-regulated the cytosolic free Ca^{2+} and then aggravated cell death. The results indicate that the specific cell injury at each preservation stage requires a corresponding protective agent. However, the untimely misuse of the agents may inversely aggravate cell injury.

Keywords: hypothermic preservation/matrine/miniaturized bioartificial liver/primary rat hepatocyte/protective agent.

Abbreviations: Ani, anisodamine; ATP, adenosine triphosphate; BAL, bioartificial liver; CsA, cyclosporin A; DHR123, dihydrorhodamine 123; FDP, fructose-1, 6-diphosphate; GA, glycyrrhizic acid; GSH, intracellular glutathione; Mat, matrine; MMP, mitochondrial membrane potential; MPT, mitochondrial permeability transition; MTT, methyl thiazolyl tetrazolium; NAC, *N*-acetyl-cysteine; PBS, phosphate buffer solution; RFU, relative fluorescence units; ROS, reactive oxygen species; RH123, rhodamine 123; RR, ruthenium red; Sch B, schisandrin B; Tre, trehalose; Van, sodium orthovanadate; VE, vitamin E.

Bioartificial liver (BAL) devices have been extensively considered as a 'clinical bridge' for liver transplantation or regeneration (*1*). As a commodity, the hypothermic treatment of BAL is so far an ideal method to reduce cell metabolism for its transportation and storage (*2*). However, hypothermia-induced hepatocyte injury during the preservation causes adverse effects on the therapeutic efficacy of BAL (*3*).

Based on long-term studies on the hypothermic preservation of hepatocytes, multiple injuries have been proposed to be involved in the hypothermic procedure. Despite the lack of direct evidence, the collapse of plasma membrane and cytoskeleton (*4*), as well as adenosine triphosphate (ATP) shortage (*5*) during the early phase of hypothermia were deemed the earliest evens in hypothermia-induced injury. The subsequent disturbance of Na^{+}, Ca^{2+} and Fe^{2+} homoeostasis (*6–8*), as well as the generation of reactive oxygen species (ROS) (*2*) upon both hypothermia and rewarming, followed by an onset of the mitochondrial permeability transition (MPT) (*9*) and the release of cytochrome c from the mitochondria (*10*) in the late phase of preservation, were considered to aggravate further cellular injury and finally activate the intracellular apoptosis and necrosis pathways (*11*).

The use of diverse protective agents, including antioxidants (*2*), ion chelators (*6*, *7*), membrane stabilizers (*4*), etc., was the most convenient strategy to attenuate hypothermia-induced cell injury. But these protective agents were randomly added into culture medium. Some groups of protective agents with similar functions, or even the same agents, were added at different preservation stages or different combinational stages (*12*, *13*). For example, vitamin E (VE), as an antioxidant, was used at pre-incubation to reduce LDH release of rat hepatocytes, whereas VE analogue of Trolox was used during hypothermia to improve the viability of human hepatocytes (*12*, *13*). m-Iodobenzylguanidine, an MPT inhibitor, was used during the whole process of preservation to protect rat hepatocytes (*14*), whereas another MPT inhibitor, cyclosporin A (CsA), was added at rewarming to present protection (*15*).

Regarding the different cellular injuries among different preservation stages (*4*, *5*, *9*, *10*), protective agents should be particularly screened for each stage.

However, the selection of appropriate protective agents specific for each stage has never been reported, limiting the significant improvement of prevention against hypothermia-induced hepatocyte injury. Moreover, the corresponding defined protective agents could facilitate to understand the mechanism of cell injury involved in specific stage and, in turn, greatly suppress the relevant hypothermia/rewarming cell injury.

In this respect, a miniaturized BAL was used as a facilitative tool in the current study to evaluate the protection of several agents at each preservation stage as well as to illustrate the protective mechanism involved. The hepatoprotective agents selected in this research were accordance with the hypothermia/rewarming cell injury mentioned in previous reports, including the antioxidants of VE, schisandrin B (Sch B), glycyrrhizic acid (GA) and *N*-acetyl-cysteine (NAC); the Ca^{2+} uniporter blocker of ruthenium red (RR); the membrane stabilizers of trehalose (Tre) and anisodamine (Ani); the energy substrate of fructose-1, 6-diphosphate (FDP); the MPT inhibitor of CsA and matrine (Mat), an active compound from Chinese herb with undefined protective mechanism.

Experimental procedures

Chemicals

Williams' E basal medium, bovine serum albumin and collagenase (type IV) were purchased from Gibco (Gaithersburg, USA). L-Glutamine, penicillin and streptomycin were purchased from Amresco Inc. (Solon, Ohio, USA). Insulin, dexamethasone, glucagons, Fura-2/AM and NAC were purchased from Sigma (St Louis, MO, USA). Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Eng. Material Co., Ltd. (Hangzhou, China). Methyl thiazolyl tetrazolium (MTT) was purchased from Huadong Medicine Group Company (Hangzhou, China). Rhodamine 123 (RH123) and dihydrorhodamine 123 (DHR123) were purchased from Invitrogen (Karlsruhe, Germany). The rat albumin ELISA quantitation kit was purchased from Bethyl Laboratories, Inc. (Montgomery, TX, USA). VE was the gift from Zhejiang NHU Co., Ltd. (Zhejiang, China). Sch B was purchased from Sikehua biotechnology Co., LTD (Chendu, China). GA and Ani were gifts from Hangzhou Minsheng Pharmaceutical Group Co., Ltd. (Hangzhou, China). RR was purchased from Fluka (Buchs, Switzerland). Tre was purchased from Yuanye biotechnology Co., Ltd. (Shanghai, China). FDP and CsA were purchased from Bio Basic Inc. (Toronto, Canada). Mat was purchased from Shanghai Winherb Medical S & T Development Co. Ltd. (Shanghai, China). Sodium Orthovanadate (Van) was purchased from Applichem (Darmstadt, Germany). All the other reagents were purchased from local chemical suppliers and were of analytical grade.

Hepatocyte cultures

All animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals by the United States National Institutes of Health. Hepatocytes were isolated from Sprague-Dawley rats (weighing 200–250 g) by the two-step collagenase perfusion method as previously described (*16*). Cells with a viability of >85% by trypan blue exclusion were used. Freshly isolated hepatocytes were mixed with the collagen solution and loaded into hollow fibers (polysulfone, 100 kDa molecular weight cut-off, Yuandong Pharmaceutical Machinery Corporation, Shanghai, China) by injection as described previously (*16*). Then, the hollow fibers containing gel-entrapped hepatocytes were immersed in a culture dish full of prewarmed culture medium (10.78 g/l Williams' E basal medium, 3.575 g/l HEPES, 2.2 g/l $NaHCO_3$, supplemented with L-Glutamine 2 mmol/l, 392 mg/l dexamethasone, 4 mg/l glucagon, 200 U/l insulin, albumin 500 μg/ml, EGF 2 ng/ml, penicillin 100 U/ml, streptomycin 100 g/ml) and incubated in a 37°C, 5% CO_2 incubator for hepatocyte culture.

Hypothermic preservation of hepatocytes

The detailed scheme was shown in Fig. 1. Briefly, hepatocytes were pre-incubated in a 37°C, 5% CO_2 incubator for 18 h, followed by culturing under normal atmosphere for 24 h at 4°C with the cold preservation medium (10.78 g/l Williams' E basal medium, 3.575 g/l HEPES, 0.168 g/l $NaHCO_3$, 1.39 g/l NaCl, supplemented with L-Glutamine 2 mmol/l, 392 mg/l dexamethasone, 4 mg/l glucagon, 200 U/l insulin, albumin 500 μg/ml, EGF 2 ng/ml, penicillin 100 U/ml, streptomycin 100 g/ml). Afterwards, hepatocytes were recultured in pre-warmed culture medium and incubated in a 37°C, 5% CO_2 incubator for rewarming.

Due to the entrapment culture of hepatocytes, the protective agents could not rapidly penetrate into cells and were, therefore, added to the culture medium 1 h before the next preservation stage. This kind of manipulation ensured that the cells could obtain enough protective agents before temperature was changed, according to the pre-experiment. On the other hand, for periodically observing the impact of protective agents on cell activity, gel-entrapped hepatocytes treated with or without protective agents were all cultured without hollow fibers and at the end of rewarming 3 h, cell viability were assayed for evaluating the protection of these agents on cells. To address the protective mechanism of Mat, cells treated with or without Mat were all cultured within hollow fibers for the prolonged rewarming time for 48 h. The cells undergoing the normal culture at 37°C and those undergoing the hypothermic preservation at 4°C without exposure to any protective agents were treated as negative and positive controls, respectively.

As protective agents, VE, Sch B, CsA and Mat were dissolved in ethanol as 600×, 2000×, 1000×, 2000× stock solution and used as the final concentration of 200 μM, 25 mM, 0.2 μM and 24 μM separately. GA, RR and Ani were dissolved in phosphate buffer solution (PBS) as 200×, 1000×, 200× stock solution and used as the final concentration of 58, 10 and 32.7 μM, separately. NAC, Tre and FDP were dissolved in culture medium directly as the final concentration of 5 mM, 0.2 M and 10 mM, separately and pH was adjusted if needed. No effect of the solvent control on cell viability was observed (data not shown).

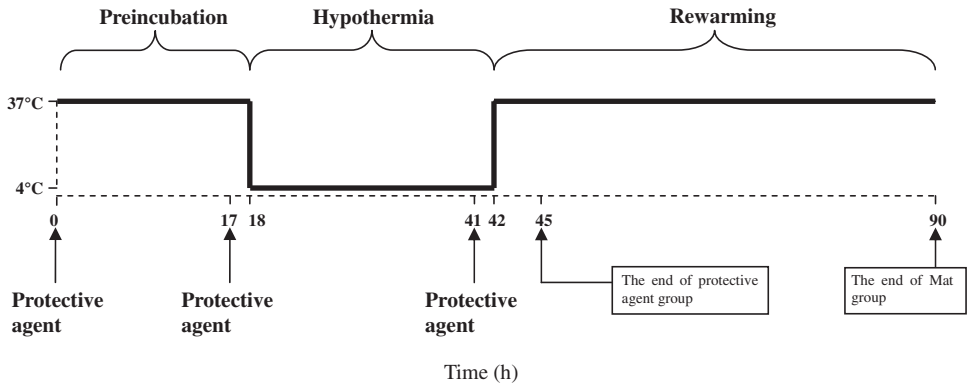

Fig. 1 Detailed scheme of the hypothermic preservation procedure.

Van was dissolved in PBS as a 200× stock solution, and then diluted into culture medium to a final concentration of 100 μM.

Cell viability assay

Cell viability was assayed by MTT reduction as previously described (*17*). Briefly, gel-entrapped hepatocytes were extruded from the hollow fibers using a syringe and immersed in 0.65 ml of the MTT–PBS (1.15 mg/ml) solution in 24-well plates followed by incubation at 37°C for 3 h. After the MTT–PBS solution was removed, 1.5 ml of isopropanol was added to cells and the solution was agitated for 1 h at room temperature. The absorbance of the solution containing the extracts was recorded at 570 nm on a spectrophotometer. Cell viability was presented as the percentage of the absorbance normalized to the negative control at the same time.

Albumin accumulation assay

Samples were taken from culture medium at different time point. Rat albumin concentrations were determined by an immunometric method (ELISA), as previously described (*18*).

Mitochondrial membrane potential assay

Mitochondrial membrane potential (MMP) of hepatocytes was determined by fluorescence dye Rh123. The gel-entrapped hepatocytes were extruded from the hollow fibers and loaded in PBS with 0.5 μM RH123 for 30 min at 37°C or 1 h at 4°C and then washed with dye-free PBS. The fluorescence was monitored using a fluorescence microplate reader (Tecan, Germany) with excitation at 485 nm and emission at 535 nm (*19*).

ROS assay

Accumulation of intracellular ROS was assayed by the fluorescence emission of DHR123. Briefly, gel-entrapped hepatocytes were firstly extruded from the hollow fibers and incubated with 1 μM DHR123 for 30 min at 37°C or 1 h at 4°C and washed with dye-free PBS twice after incubation. Then, the fluorescence was monitored using a fluorescence microplate reader (Tecan, Germany) with excitation at 485 nm and emission at 535 nm (*18*).

Intracellular glutathione assay

The gel-entrapped cells were extruded from the hollow fibers and suspended in 100 μl of PBS. After collection, hepatocytes were sonicated and centrifuged at 16,000 *g* to precipitate cellular fragments. The intracellular glutathione (GSH) content in cell supernatants was determined by DTNB assay as previously described (*20*) and then presented as a percentage of the absorbance normalized to the negative control at the same time.

Assessment of cytosolic free Ca^{2+}

At the detection time, gel-entrapped cells were extruded from the hollow fibers and cultured with Ca^{2+} buffer solution (6.435 g/l NaCl, 0.3725 g/l KCl, 2.385 g/l HEPES, 1.8 g/l D-Glucose, 0.19 g/l $MgCl_2$, 0.294 g/l $CaCl_2{\cdot}2H_2O$, adjust pH to 7.4) containing 1 μM fluorescence dye Fura-2/AM for 30 min at 37°C or 1 h at 4°C. And then cells were washed three times with Ca^{2+} buffer solution to remove the Fura-2/AM. The fluorescence was monitored using a fluorescence microplate reader (Tecan, Germany) with excitation at 340 nm/380 nm and emission at 510 nm.

Data analysis

Data in the figures were expressed as mean + SD for clarity. Comparisons between multiple groups were performed with the one-way ANOVA test. $P<0.05$ were considered statistically significant.

Results

Differential function of the protective agent at each preservation stage

The known hepatoprotective agents of VE, Sch B, GA, NAC, RR, Tre, Ani, FDP, CsA and Mat were, respectively, used at each preservation stage. Cell viabilities were all examined at 3 h of rewarming to evaluate the effect of the agents on hypothermically preserved hepatocytes (Fig. 2). For comparison, the normal culture at 37°C and the hypothermic preservation at 4°C without any exposure to protective agents were conducted as negative and positive controls, respectively. Relative to the positive control at 4°C without protection, hepatocytes treated by antioxidants of VE, Sch B, GA or NAC either in the pre-incubation or rewarming presented a better tolerance of the hypothermia-induced injury. During rewarming, the exposure to RR, a Ca^{2+} uniporter blocker, also maintained cell activity well, but the same treatment at either pre-incubation or hypothermic stage caused severe cell death. Hepatocytes with exposure to the membrane stabilizers of Tre as well as Ani at the hypothermic stage showed better survival. However, the same treatment at either pre-incubation or rewarming process aggravated the cellular injury. The energy substrate of FDP only showed its protection on cells at pre-incubation, whereas its presence during hypothermia displayed significant toxicity to cells. In contrast, CsA, the MPT inhibitor, exerted its protection on cells when it was

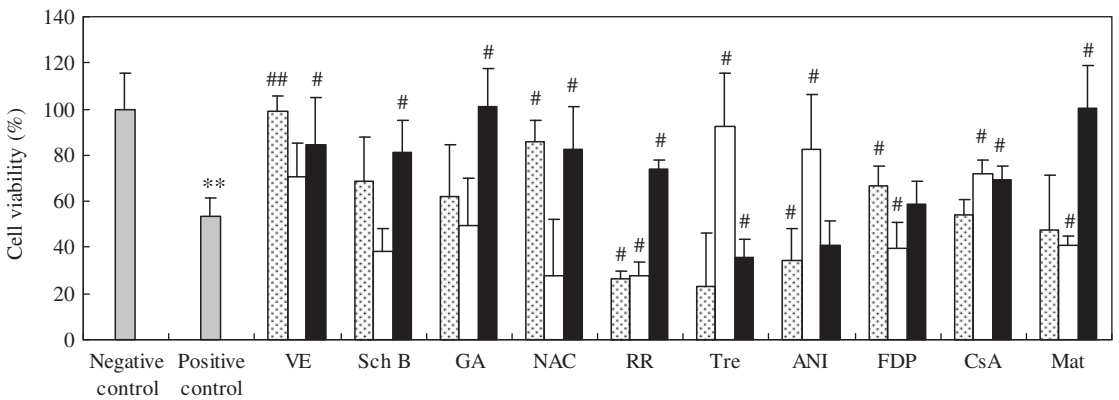

Fig. 2 Hepatocyte viability after 24 h of hypothermia at 4°C and 3 h of rewarming at 37°C with diverse protective agents added into culture medium at each preservation stage (dashed box: pre-incubation, open box: hypothermia, filled box: rewarming), respectively. Hepatocytes undergoing normal culture at 37°C or hypothermic preservation at 4°C without exposure to protective agent were taken as negative and positive controls, respectively. The data were represented as the mean + SD of three experiments. $^{**}P<0.001$ compared with negative control, $^{\#}P<0.05$, $^{\#\#}P<0.001$ compared with positive control.

used at either hypothermia or rewarming. Finally, the presence of Mat during rewarming could significantly improve cell viability but showed no function at pre-incubation and even causing a side effect during hypothermia.

Protective mechanism of Mat against hypothermia/rewarming-induced cell injury

Mat, with undefined protective mechanism, was selected as a representative to discuss the appropriate stage of exerting its protection based on an illustration of its potential protective mechanism on hepatocytes.

The presence of Mat during the rewarming stage against the hypothermia-induced cell injury was firstly evaluated by detecting the cell viability and albumin synthetic function at each preservation stage. Compared with the negative control, the positive control showed no distinction in cell morphology as well as cell viability at the end of the hypothermic stage by trypan blue exclusion (data not shown), whereas a slight decline in cell viability by MTT method (Fig. 3A) was largely due to the post-hypothermic cell injury in assay of cell viability at 37°C for 3 h. Upon rewarming without the presence of Mat, the cell viability declined to ~70% of the negative control after 5 h of rewarming, followed by a continuous decrease to 38% at 48 h. In contrast, hepatocytes exposed to Mat during rewarming could improve cell viability to ~91 and 69% of the negative control at rewarming for 24 and 48 h, respectively. A similar trend was found in the albumin synthetic function of hepatocytes by Mat protection (Fig. 3B).

The other cellular indices were detected subsequently. As demonstrated in Fig. 4A, in the positive control, MMP decreased rapidly to 19–26% of the

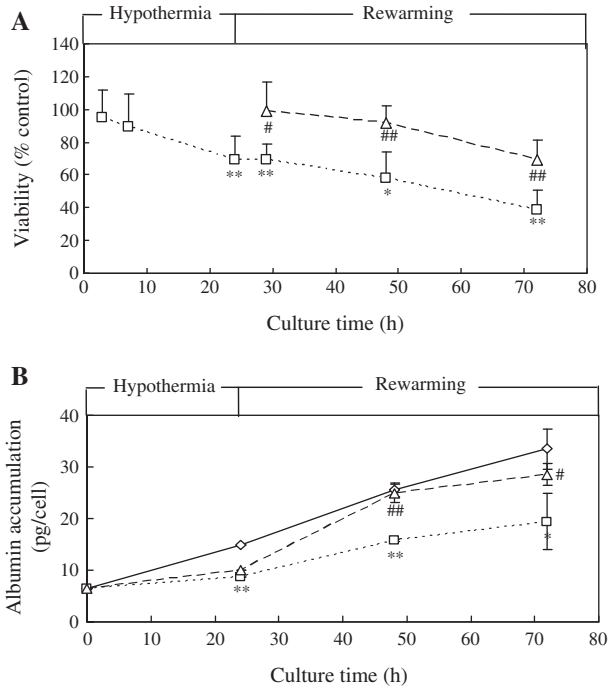

Fig. 3 Time course of viability and albumin secretion of hepatocytes during hypothermia and rewarming. (A) cell viability. (B) albumin secretion. Open diamond: negative control, open square: positive control, open triangle: hepatocytes that underwent hypothermic preservation with the presence of Mat during rewarming. The data were represented as the mean + SD of three experiments. $^{*}P<0.05$, $^{**}P<0.001$ compared with negative control; $^{\#}P<0.05$, $^{\#\#}P<0.001$ compared with positive control.

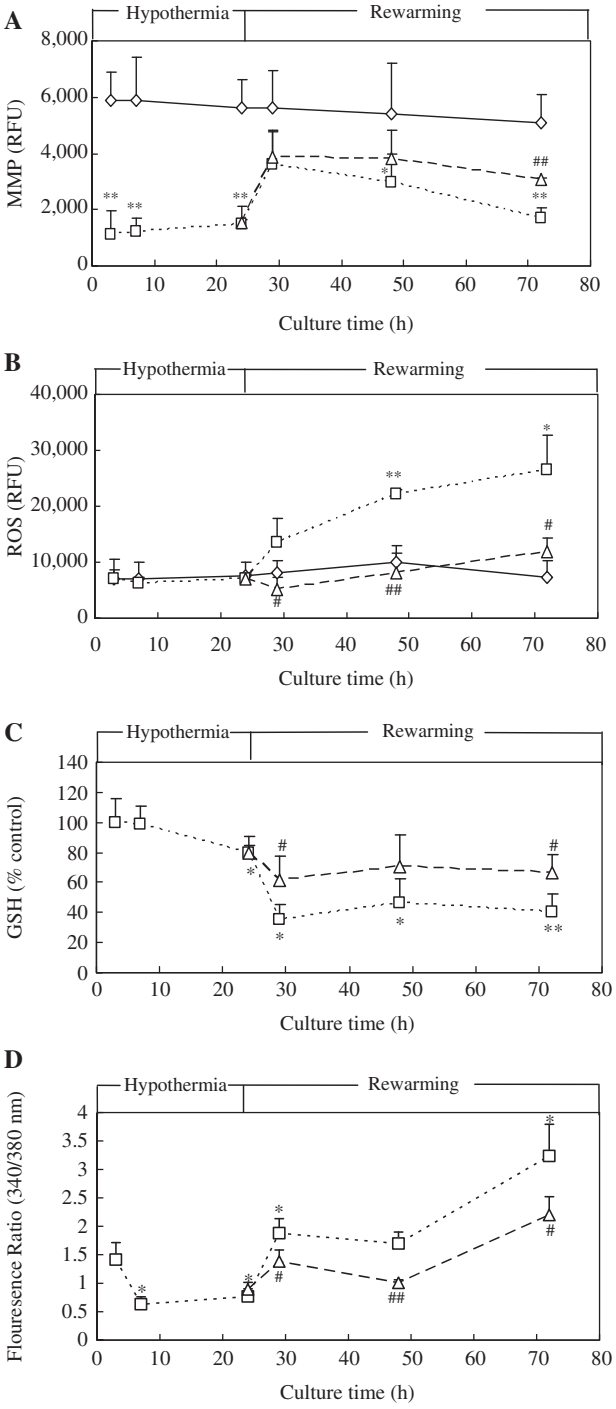

Fig. 4 Time course of MMP, ROS, GSH and cytosolic free Ca^{2+} of hepatocytes during hypothermia and rewarming. (A) MMP, (B) ROS, (C) GSH and (D) cytosolic free Ca^{2+}. Open diamond: negative control, open square: positive control, open triangle: hepatocytes that underwent hypothermic preservation with the presence of Mat during rewarming. The data were represented as the mean + SD of three experiments. $^{*}P<0.05$, $^{**}P<0.001$ compared with negative control; $^{\#}P<0.05$, $^{\#\#}P<0.001$ compared with positive control.

negative control during hypothermia and recovered to 72% of its original level as soon as rewarmed. After rewarming, MMP continuously decreased in both the positive control and Mat group within the first 5 h of rewarming but was gradually alleviated in the Mat group. As indicated in Fig. 4B and C, no obvious accumulation of intracellular ROS was detected during hypothermia, whereas a significant depletion (20%) of GSH was observed especially at that late phase. Upon rewarming, both ROS accumulation and GSH depletion became severe in the positive control while the situation was much better in the Mat group.

The cytosolic free Ca^{2+} concentration was detected during hypothermia and rewarming. As indicated in Fig. 4D, the concentration of Ca^{2+} in the positive control decreased 50% during hypothermia and then rapidly increased to >200% of its initial level at 48 h of rewarming. But in the presence of Mat during rewarming, the cytosolic free Ca^{2+} was maintained well at the first 5 h of rewarming and only slightly increased 38% after 48 h.

As the cell injury was aggravated by the presence of Mat at the hypothermic stage (Fig. 2), the cytosolic free Ca^{2+} concentration, ROS accumulation and GSH depletion of hepatocytes were assayed at 3, 7 and 24 h of hypothermia, respectively, to confirm the effect of Mat on cells. Between the two hypothermically treated groups with or without Mat in the incubation medium, no marked difference in intracellular ROS accumulation or GSH depletion was observed (Fig. 5B and C), except for a lower cytosolic free Ca^{2+} concentration in the Mat group (Fig. 5A).

Inhibition of Mat protection by Van during rewarming

Van was a potent inhibitor of plasma membrane Ca^{2+}-ATPase and thus could enhance the cytosolic free Ca^{2+} (*21*, *22*). In this study, Van was used during rewarming for the further identification of the regulation of cytosolic free Ca^{2+} by Mat. As illustrated in Fig. 6, Van not only increased the cytosolic free Ca^{2+}, but also obviously blocked the suppression of cytosolic free Ca^{2+} by Mat during rewarming. Moreover, the increase of cytosolic free Ca^{2+} by Van during rewarming, as in Van and Mat + Van group, was accompanied by aggravated cell death as assessed by MTT method (Fig. 6).

Discussion

Different kinds of protective agents have been attempted to prevent the hypothermia/rewarming-induced multiple injuries of hepatocyte during hypothermic preservation. However, the exact efficacy of these protective agents added at different preservation stages has rarely been considered. Thus, in the current article, the differential function of several protective agents on hepatocytes at each preservation stage was addressed to improve the quality of post-preservation hepatocytes for potential application in BAL.

In this research, five classifications of protective agents (*i.e.* antioxidant, Ca^{2+} uniporter blocker, membrane stabilizer, energy substrate and MPT inhibitor) were found to provide protection at specific stages of hypothermic preservation. The antioxidants exerted high protection against cellular damage only during pre-incubation and rewarming. Their ineffectiveness at the hypothermic stage might be associated with

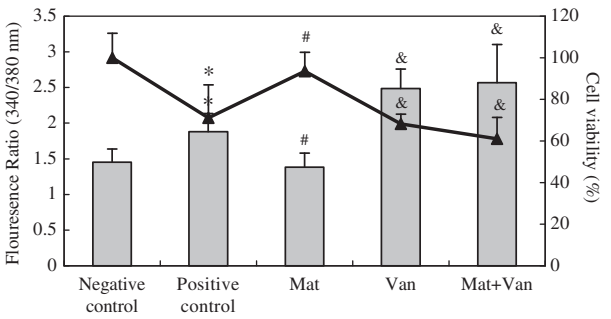

Fig. 6 Cytosolic free Ca^{2+} (column) and cell viability (filled triangle) of hepatocytes after 5 h of rewarming treated with Mat and Van alone or together during rewarming. The data were represented as the mean + SD of three experiments. $^{*}P<0.05$, compared with negative control; $^{\#}P<0.05$, compared with positive control and $^{\&}P<0.05$, compared with Mat group.

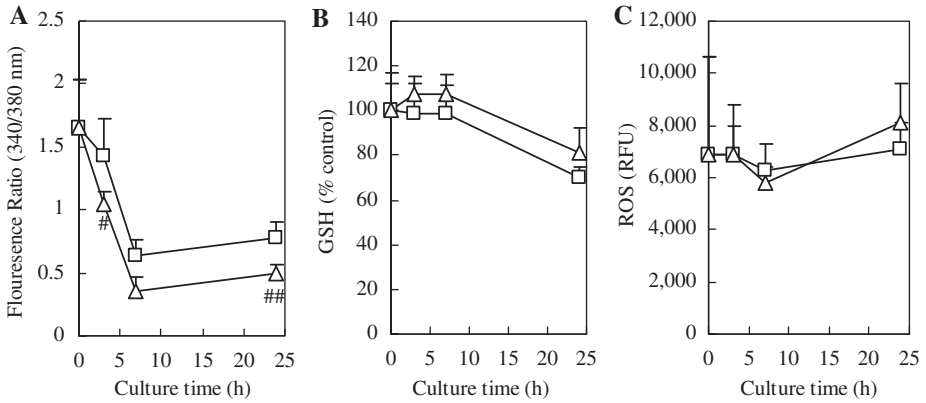

Fig. 5 Time course of cytosolic free Ca^{2+}, GSH depletion and ROS accumulation of hepatocytes treated with or without Mat during hypothermic stage at 4°C for 3, 7 and 24 h without rewarming. (A) Ca^{2+}, (B) GSH depletion and (C) ROS accumulation. Open square: positive control, open triangle: hepatocytes that underwent hypothermic stage with the presence of Mat. The data were represented as the mean + SD of three experiments. $^{\#}P<0.05$, $^{\#\#}P<0.001$ compared with positive control.

the low generated ROS at 4°C during the hypothermic stage (*23*), corresponding with the detected low ROS level at this stage in Fig. 4B. As for the Ca^{2+} uniporter blocker (RR) and the membrane stabilizers (Tre and Ani), their protections were proposed to respectively involve the prevention of the influx of the overloaded cytosolic Ca^{2+} into the mitochondria occurred during rewarming and suppression of hypothermia-induced cellular membrane and cytoskeleton damage occurred at the hypothermic stage (*4*). The energy substrate of FDP only exerted protection during pre-incubation, suggesting that the energy accumulation prior to hypothermia could support hepatocytes to survive hypothermia through the restoration of more cellular ATP (*24*). CsA, an inhibitor against the formation of permeability transition pore (*25*), triggered protection during hypothermia and rewarming. This suggested the involvement of MPT at these two stages, as confirmed in Fig. 4A as well as in a previous study (*26*). Taken together, each classification of protective agents was distinguished by the appropriate adding stage and, in turn, could facilitate to understand the cellular injury occurred at different stages of hypothermic preservation.

As an herbal lupine alkaloid from *Sophora flavescenes* Ait. (*27*), Mat has been reported to provide diverse protection in scavenging ROS, maintaining intracellular GSH in rat hepatocyte (*28*, *29*) and regulation of cytosolic free Ca^{2+} in smooth muscles and ventricular myocytes of guinea pigs (*30*, *31*). Mat has been previously demonstrated to protect hepatocytes under its continuous presence during both hypothermia and rewarming (*19*), whereas in the current study, the function of Mat during either hypothermia or rewarming was separately addressed. As shown in Fig. 2, the addition of Mat during rewarming enhanced its protection on cell activity, but the presence of Mat during hypothermia adversely aggravated cell injury. The investigation on the protective mechanism of Mat indicated that Mat preferably regulated intracellular ROS, GSH content and cytosolic free Ca^{2+} at the early phase of rewarming (Fig. 4), but could exclusively down-regulate the cytosolic free Ca^{2+} during hypothermia (Fig. 5). Thus, the down-regulation of cytosolic free Ca^{2+} by Mat during rewarming was speculated to be the earliest event by the protection of Mat against hypothermia-induced cell death. To strengthen this hypothesis, Van was used to block the regulation of cytosolic free Ca^{2+} by Mat during rewarming and thus to impair the protection of Mat against hypothermia-induced cell death (Fig. 6). It indicated that Mat might exert its protection by binding to specific sites on the plasma membrane Ca^{2+}-ATPase, and thus reducing cytosolic free Ca^{2+}, in a manner similar to calmodulin (*22*) and ethanol (*32*). Taken together, the protective mechanism of Mat was mainly related to the down-regulation of cytosolic free Ca^{2+} during rewarming, subsequently suppressing the deterioration of intracellular ROS and GSH, preventing the release of MMP, and finally influencing the synthesis function and viability of hepatocytes. In contrast, the excessive regulation of cytosolic free Ca^{2+} by Mat at the hypothermic stage might disrupt the intracellular ion homoeostasis and contribute to additional damage to cells.

As shown above, Mat as well as the other nine agents could function as a protective agent, if added at the appropriate preservation stage, corresponding well with their mechanism-based protection against the cell injury at each stage. In turn, the identified appropriate stage for adding protective agents could provide valuable information to clarify the protective mechanisms of the agents. This benefited the alleviation of hypothermia/rewarming-induced cellular injuries, but more in-depth work was required. In view of the multiple injuries involved in hypothermic preservation, the combinational use of these agents in different stages was recommended here because of their capability in providing various protections at specific stage.

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Conflict of interest

None declared.

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