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Full Length Research Paper

Effect of vitrifying solutions, ethylene glycol and 1, 2-propandiol, on intracellular K+ and lactate dehydrogenase (LDH) of hepatocytes in primary culture

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Shortage of donor liver tissue for the isolation of hepatocytes requires the development of improved cryopreservation techniques for long-term storage of these cells for more efficient use. The aim of this study is to compare two cryoprotectants for preserving living cells in culture by vitrification. The viability of hepatocytes after cryopreservation was determined by the intracellular concentration of potassium (K^+) and lactate dehydrogenase (LDH). Cryomicroscopy was used to check the state of vitrification and morphology of these cells subjected to the process of freezing and thawing. This experiment made it possible to observe an early vitrification with 40% of Ethylene glycol (EG) or 1,2-propanedio (1, 2-PD) to reach the final vitrified state with 60%. The treatment of these cells in culture with solutions containing EG or 1, 2-PD revealed a failure in functional properties of the plasma membrane. Thus, a decrease of intracellular concentration of LDH and K^+ was noticeable. This decrease was more significant when it came with 60% EG or 1, 2 - PD. The use of a cryopreservation solution additionally containing NaCl, KCl, Ca^{2+} and sucrose and the treatment of hepatocytes in culture at +4°C greatly minimized the functional impairment of the plasma membrane. These results suggest that the osmo-diffusional mechanism is at the basis of membrane damage letting out LDH and K^+ into the extracellular compartment. However, this mechanism can be controlled by a selection of treatment solution parameters such as temperature, osmolarity and electrolytes concentration while taking the cryoprotectants coefficient of permeability into consideration.

Key words: Hepatocyte, cryopreservation, cryoprotectant, vitrified state, vitrification.

INTRODUCTION

At present penetrating cryoprotectants (intracellular) glycerol, dimethyl sulphoxide, ethylene glycol and 1, 2-propanediol and non-penetrating cryoprotectants (extracellular) polyglycol, polyvinyl pyrrolidine and dextran are used. All cryoprotectants when used in concentrations adequate to vitrify have some toxicity (as measured by their negative effect on cell survival and function) particularly when they are used at the high concentrations (of more than 20%). The damage is the result of their toxicity and their high osmotic activity. However, a partial damage (5 to 20%) could significantly limit the use of biological material for biomedical research. For best preservation it is necessary not only to eliminate ice formation by using cryoprotectants, but to eliminate toxicity from cryoprotectants (Wusteman et al.,

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Abbreviations: EG, Ethylene glycol; **1, 2-PD,** 1,2-propanediol; **K^+,** potassium; **LDH,** lactate dehydrogenase; **NaCl,** sodium chloride; **KCl,** potassium chloride; **Ca^{2+},** calcium.

2002; Fuller et al., 2004; Pegg, 2007; Lawson et al., 2011).

Long term culture of primary rat hepatocytes is a widely used experimental model to estimate drug metabolism and toxicity. However, standard monolayer culture of primary hepatocytes on tissue culture dish - either uncoated or coated with a biological material such as collagen, fibronectin and laminin - is problematic. Although animal experiments and human clinical trials for drug metabolism studies are available, these models are expensive and ethically controversial. However, *in vitro* investigation of the metabolic profile of a drug is more economical, easier to carry out, and less controversial. Therefore, toxicology studies using isolated hepatocytes are recognized as among of the most relevant and practical models (Berry et al., 1992; Hewitt et al., 2007; Brophy et al., 2009, 2010; Bierwolf et al., 2011).

In view of cryopreservation of the liver, which appears as a complex structure, any partial injury necessarily involves a dysfunction of this organ in general (Volk et al., 2006; Gerlach et al., 1997; Valdes et al., 1990; Fahy et al., 1990; Armitage, 1988). The study of the origin of the cellular damage in each stage of cryopreservation would determine the best methods of long-term preservation of hepatocytes in suspension and culture. Similarly, it would be possible to improve the methods of cryopreservation of the liver.

This approach makes it possible to understand the adaptative mechanisms of biological systems at critical temperatures and osmotic conditions. To achieve these goals, it has been found useful to use high concentrations (up to 60%) of EG or 1, 2-DP penetrating cryoprotectants to prevent cellular damage from the formation of ice crystals and to allow vitrification for cryopreservation.

MATERIALS AND METHODS

Perfusion methods and hepatocytes culture

Perfusions for obtaining hepatocytes were made between 8 and 10 am on ninety *ad libitum* Wistar strain rats of either sex with weights ranging from 180 to 200 g. A two-stage perfusion method was used. This is an improvement from Seglen (1976), Naik et al., (1996), LeCluyse et al. (2005) and Fiegel et al. (2006).

The liver was first perfused via the portal vein with Ca^{2+} free (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7. 65) at +37°C for 25 min The composition of the solution was as follows: 160.8 mM NaCl, 3.15 mM KCl, 0.7 mM $NaHPO_4 12H_2O$, 2.05 mM EDTA, 0.33 mM HEPES. The second stage of the infusion was made using the same solution in the presence of 0.025% collagenase and 0.075 mM $CaCl_2$ for 15 min.

At the end of this two-step perfusion, the liver was slightly crushed in the HEPES buffer. Using the Percoll centrifugation, hepatocytes were purified and suspended in the Dulbecco Modified Eagle Medium (DMEM) (GIBCO, Gaithersburg MD USA) at a concentration of 106 cells/ml. After a methylene blue test, the viability of hepatocytes was 90 ± 5%. DMEM in the presence of 8% (v/v) fetal calf serum (FCS) and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml gentamicin) used to new surroundings suspension before being transferred to Petri dishes, 60 mm in diameter, previously coated with collagen. The culture was carried out for 2 h of incubation at 37°C with humidity CO2/Air 1:19. Then the culture medium was replaced by Hank's Balanced Salt Solution (HBSS). Culture of hepatocytes thus obtained is used in the series of planned experiments. An identical culture of hepatocytes was performed on coverslips for experimental cryomicroscopy.

Preparation of cryoprotectant solutions

Cryoprotectant solutions used contain different concentrations of NaCl: A (75 mM), B (150 mM), C (450 mM), in 5 mM phosphate buffer (pH 7.4) but the solution of S cryoprotectant contains 150 mM NaCl, 5 mM phosphate buffer, 150 mM KCl, 0.6 mM $CaCl_2$ and 5% sucrose (pH 7.4). The cryoprotectants are directly added as per the volume concentration just before experiments. Treatments of cell cultures to different solutions of cryoprotectants are made by levels of concentration 10, 20, 40 and 60% with an exposure time of 10 min at +20 or +4°C.

Freezing and thawing method

The freezing was achieved using a programmable cooling YOP-6 unit to cool the cells at 10°C per min to -100°C (Simione, 1998). Thawing took place in a water bath at +42°C.

Biological material production method

The biological material was obtained by proper destruction of hepatocytes in culture by cell lysis (Xia, 2005). Total destruction is obtained by adding 5 ml of bidistillat in boxes of dishes, used for culture at +20°C for 3 h. In this way the intracellular substances are dissolved and thus constitute the biological material to be used to determine levels of LDH and K^+. The volume of dissolution bidistillat (5 ml), dissolution time 3 h and dissolution temperature (+20°C) are strictly respected in these experiments.

Determining the LDH concentration method

100 mM Tris/HCl (pH 8.8), 65 mM lactate (pH 7.4) and 7 mg/ml NAD solution used to determine the concentration of LDH. This was determined by sequential addition of 2 ml of Tris/HCl, 40 µl in the spectrophotometer cell to determine the control and 500 µl of biological material, thereby determining the concentration of intracellular LDH. The concentration of LDH was determined by spectrophotometer MPF-2A (Hitachi, Japan) at wavelengths of excitation and emission respectively of 340 and 460 nm (Meyer, 2010).

Determining the K^+ concentration method

The biological material obtained by proper destruction of hepatocytes in culture is directly used to determine the K^+ concentration (Graf et al., 1988; Dickman and Goldstein, 1990; Penney, 2008). This concentration is determined using a spectrophotometer MPF-2A (Hitachi, Japan) at wavelengths of excitation and emission respectively of 450 and 600 nm. The experimental protocol was the same used for LDH determination.

Statistics

The values are expressed as mean ± S.D. The S.D. has a $0.37 \leq \pm$ S.D ≤ 0.52 fluctuation. The results were analyzed statistically by

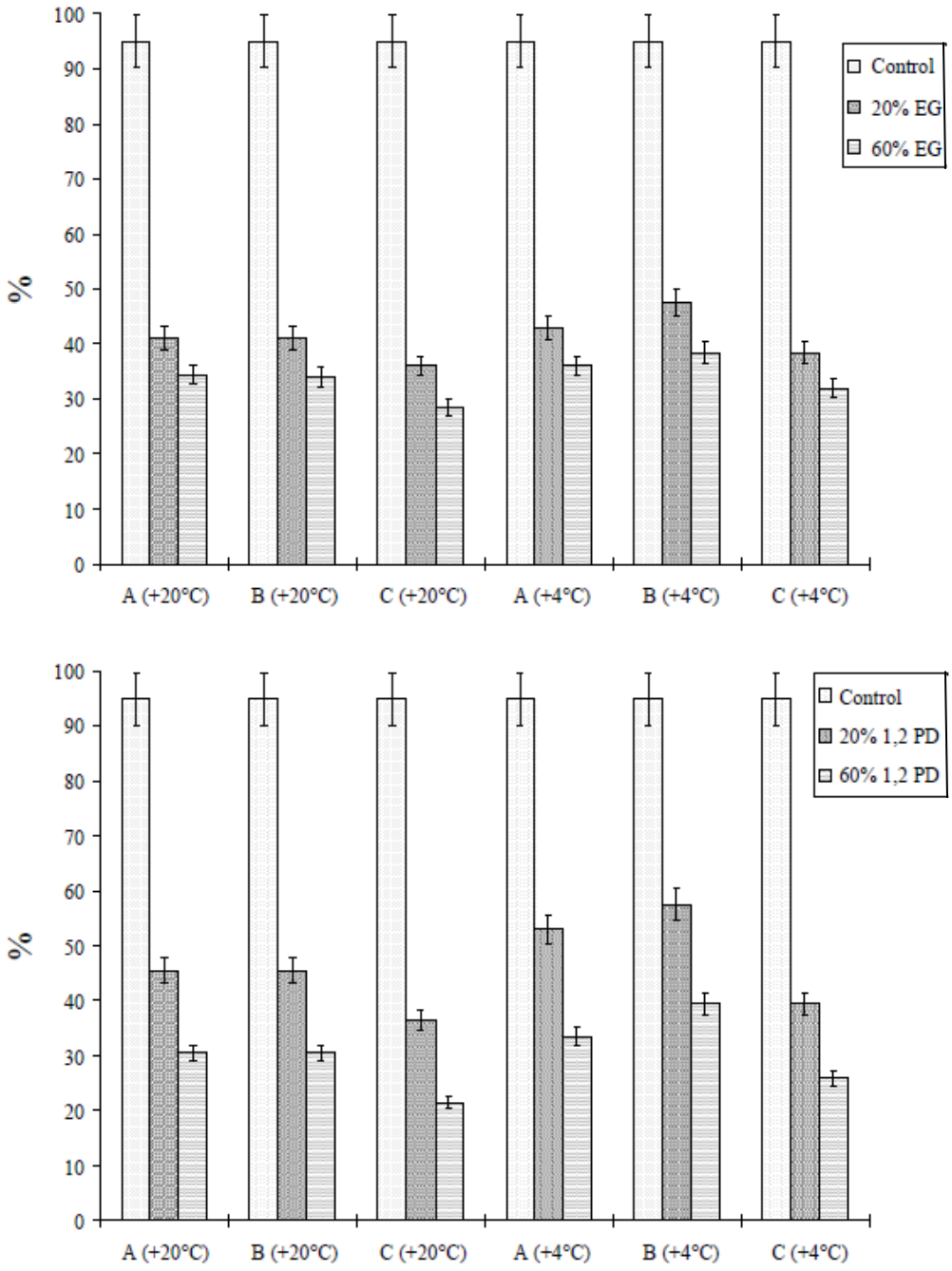

Figure 1. Effect of incubation solution NaCl concentration on intracellular K^+ concentration: A (75 mM NaCl), B (150 mM NaCl), C (450 mM NaCl).

using Student-Fisher method.

RESULTS

The obtained results suggested that treatment of hepatocytes to solutions of EG or 1, 2-PD led to a decrease of intracellular concentration of LDH and K+. This decrease was observed independently of temperature and concentration of NaCl initially present in the treatment solution. The maximum of this decrease was achieved at 60% EG or 60% 1, 2-PD.

Analysis of viability showed that treatment of hepatocytes in culture with solutions containing 60% cryoprotectant and 150 mM NaCl had a greater retention of intracellular LDH and K+ compared to 75 and 450 Mn NaCl (Figures 1 and 2).

However, cryomicroscopic investigations showed a

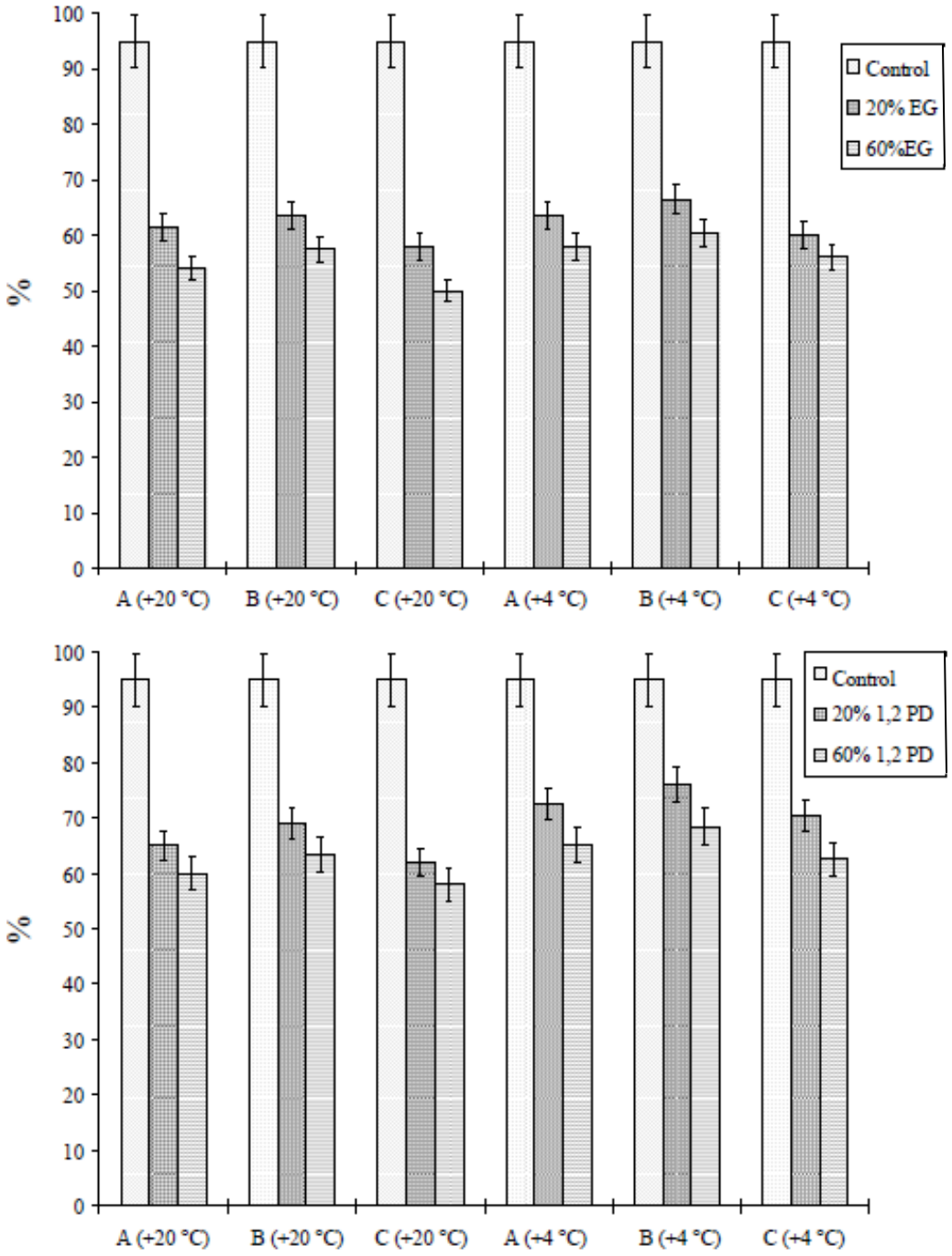

Figure 2. Effect of incubation solution NaCl concentration on intracellular LDH concentration: A (75 mM NaCl), B (150 mM NaCl), C (450 mM NaCl).

beginning of hepatocytes vesiculation only 30 minutes after treatment with EG or 1, 2-PD solutions at +20°C (Figure 3).

Treatment of hepatocytes in culture with solutions of EG or 1, 2-PD carried out in hypothermia (0 to 4°C) made it possible to delay the beginning of vesicles development (Figure 3). Isotonic solutions of EG and 1, 2-PD may achieve the highest level of intracellular concentration of LDH and K^+ regardless of temperature and concentration of cryoprotectants. The results obtained with supplemented solution containing KCl, Ca^{2+} and sucrose made it possible to improve the results, that is, to keep

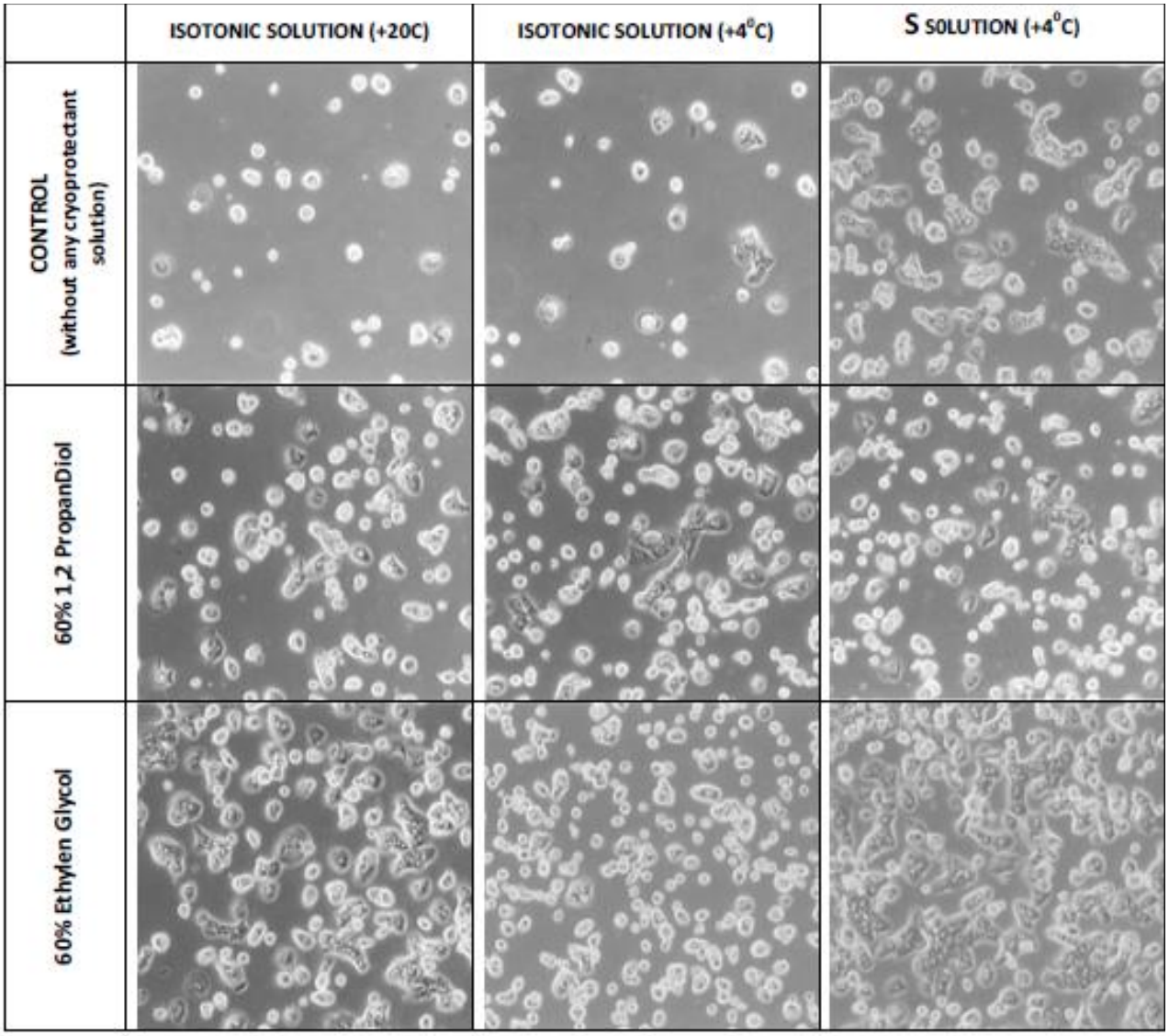

Figure 3. Hepatocyte culture cryomicroscopy. Control: without any cryoprotectant solution (pH 7.4). Isotonic solution B: 150 mM, 5 mM buffer-phosphate and 60% 1, 2-PD or EG (pH 7.4). S solution: 150 mM NaCl, 5 mM buffer-phosphate, 150 mM KCl, 0.6 mM $CaCl_2$, 5% sucrose and 60% 1, 2-PD or EG (pH 7.4).

intracellular concentration of LDH and K+ to 25 to 35% higher level according to the cases (Figures 4 and 5). In these conditions, cryomicroscopy investigations showed a beginning of vesiculation to be observed only after 6 days for 60% solution of 1, 2-PD and 5 days for 60% solution of EG following hypothermic preservation (0 to 4°C) (Figure 3).

DISCUSSION

A comparison of two cryoprotectants shows that the solution of 1, 2-PD provides better conservation of intracellular concentration of LDH and K^+ compared to EG (Figures 1 and 2). This fact can be explained by the physicochemical properties of these two cryoprotectants, namely their toxicity and their coefficient of permeability. This is justified not only by the difference in intracellular concentration of LDH and K^+ after treatment but also by the different time of onset of vesicles using the same concentration of cryoprotectant (60%) at 20°C after 30 min for EG and 45 min for 1, 2-PD, at 4°C after 60 to 65 min for EG and 90 min for 1, 2-PD (Figures 3).

These results suggest that the interaction of these cells having complex metabolic functions with high concentrations of penetrating cryoprotectants EG or 1, 2-PD is the cause of the disturbance of diffusion properties of these cells plasma membranes and, therefore, the progressive loss of intracellular substances such as LDH and K^+ (Innes et al., 1992; Crenesse et al., 1994).

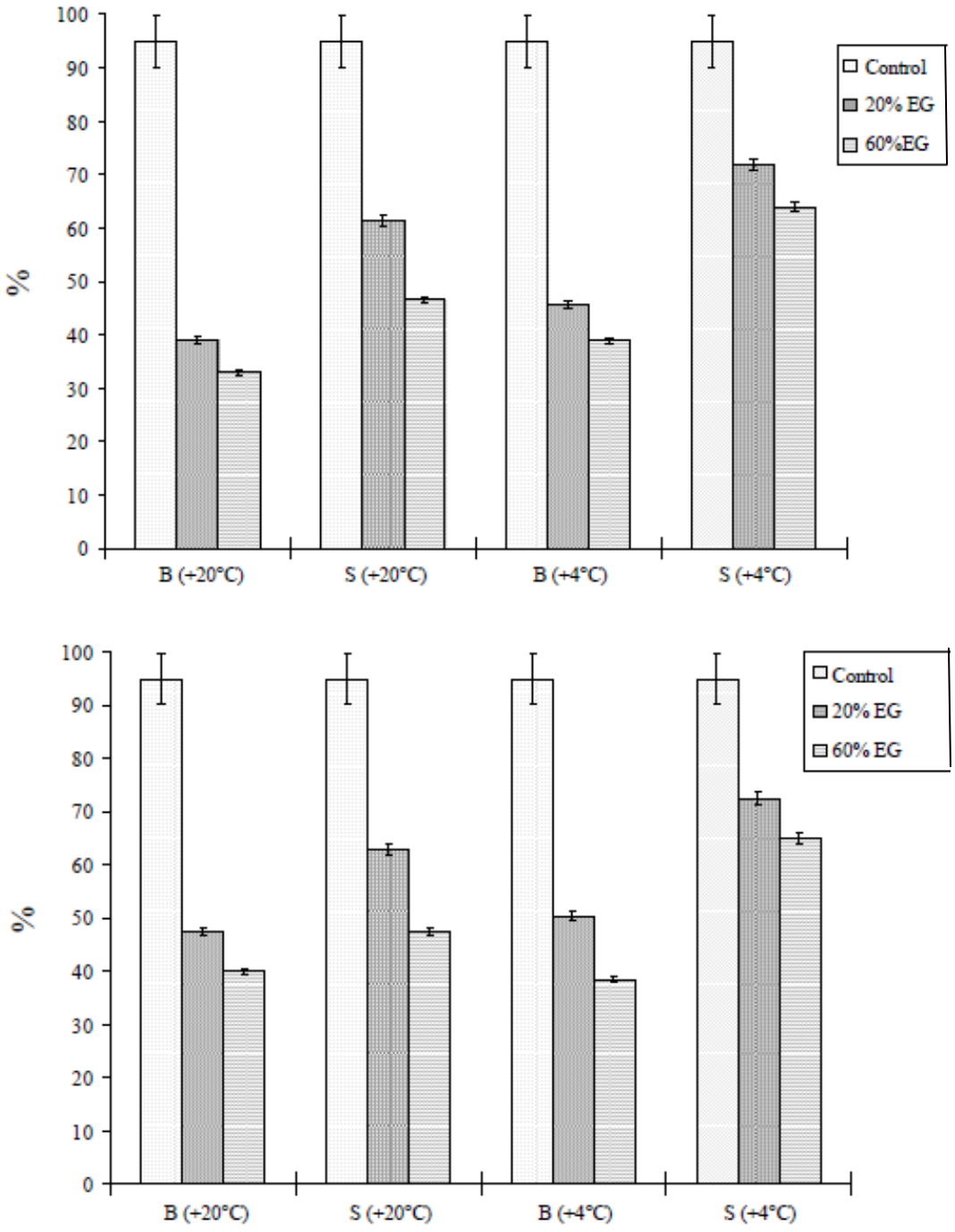

Figure 4. Effects of incubation temperature and quality of cryoprotectants on intracellular K+ concentration: B (150 mM NaCl in 5 mM buffer-phosphate) and S (150 mM NaCl, 150 mM KCl, 0.6 mM CaCl2, 5% sucrose).

A reduction in cell volume may cause folds of the plasma membrane so that the vesicles thus formed will be separated from the cell just as in a bud. Such an irreversible loss of membrane portions constitutes a stress.

Previous studies by Armitage et al. (1988), Innes et al. (1992), and Hamilton et al. (2001) showed that the distribution coefficient Q of the cryoprotectant solution, on both sides of the plasma membrane under these conditions tends to 1. This fact is surely related to the phase homogeneity which can allow better conditions for vitrification.

The vitrifying properties of cryoprotectants depend on hepatocytes in culture initial conditions of treatment and on cryoprotectants physicochemical characteristics (Meng, 2003; Son et al., 2004). A variation in the amount

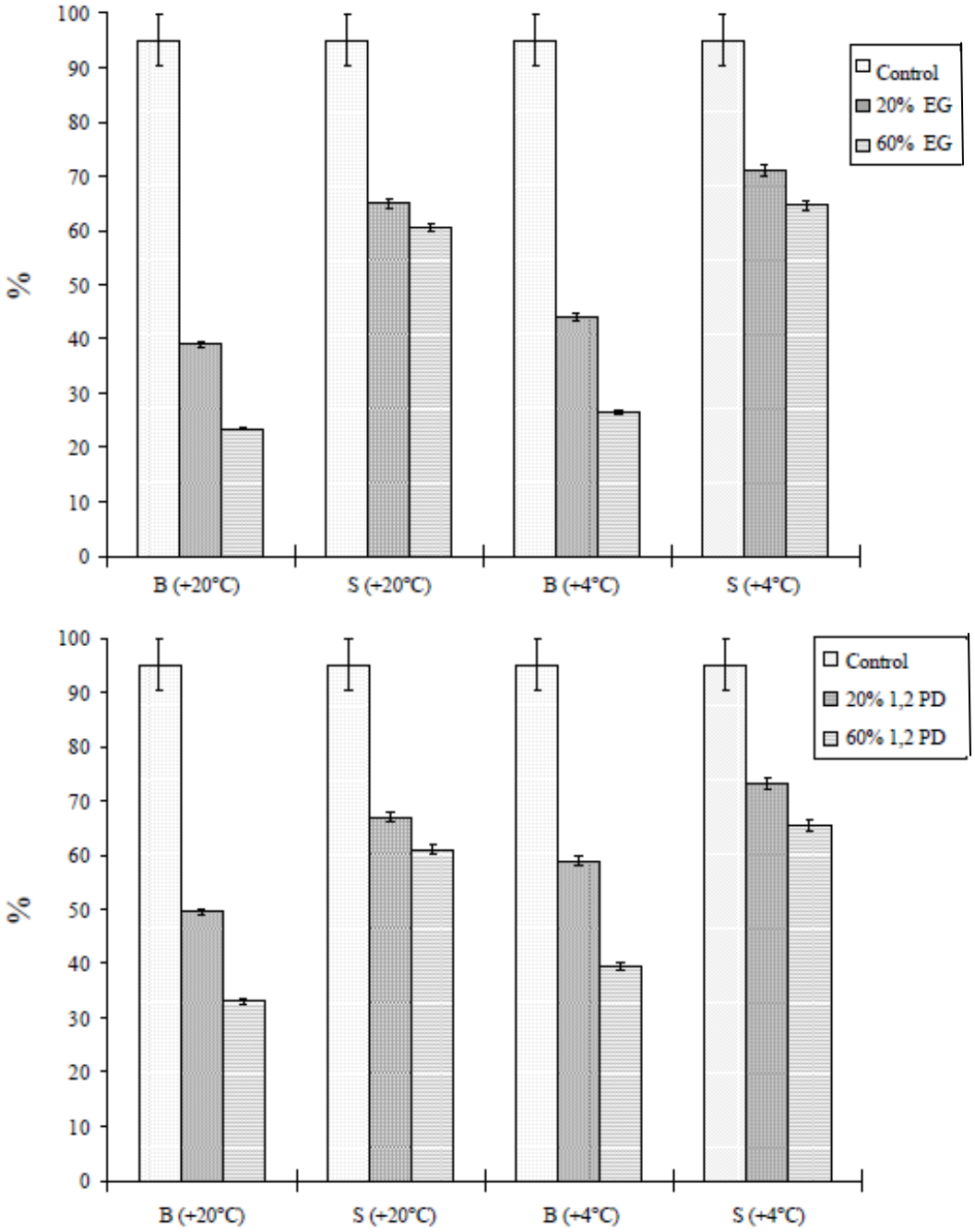

Figure 5. Effects of incubation temperature and quality of cryoprotectants on intracellular LDH concentration: B (150 mM NaCl in 5 mM buffer-phosphate) and S (150 mM NaCl, 150 mM KCl, 0.6 mM $CaCl_2$, 5% sucrose).

of unfrozen water in conditions of variation of the cryoprotectants concentration in the treatment solution requires a variation in the cell water balance. Therefore, there is a variation of the hepatocytes volume and morphological characteristics.

A variation of processing conditions in order to lower toxicity, including preparation of processing solutions in conditions of hypothermia should enhance the vitality of hepatocytes.

To improve these results, we thought it necessary to add KCl in order to prevent the increasing loss of K^+, Ca^{2+} for maintaining cellular homeostasis and finally sucrose for its osmostabilising properties (Kuleshova et al., 1999; Smith et al., 1991; Umeshita et al., 1988).

The size and extent of the cell volume variation observed under the effect of the concentration variation

of treatment solutions depend on the osmotic pressure applied on the plasma membrane and on the permeability of this membrane (Figure 3). Osmotic stress can be reduced by processing in steps of 10, 20, 40 and 60% successively by maintaining cells for 10 min at each stage or by adding sucrose. However, the contracted form of hepatocytes in culture under the effect of sucrose does not constitute signs of damage (Glicklis et al., 2004).

A comparison of the two parameters studied here (LDH and K^+) makes it possible to understand that intracellular concentration of K^+ is the most sensitive criterion enabling to determine the damage of the hepatocytes plasma membrane and to define cellular integrity compared successively to LDH and methylene blue (Figures 4 and 5). The decrease in intracellular K^+ concentration and Na^+/K^+ ratio begins well before the observation of the decreased concentration of LDH and before staining with methylene blue.

In the other hands, chicken hepatocytes in suspension obtained from different methods (some obtained from a perfusion, the others from an intracellular disintegration) showed different concentrations of K^+ (193 and 139 mM/mg) while they had exactly the same code with methylene blue (Fladmark et al., 1997).

The decrease in intracellular K^+ concentration over several hours of treatment of hepatocytes with 40% EG or 1, 2-PD solution approximately, occurred while an invariable quantity of liver cells stained with methyl blue (6 to 8%) (Fuller and De Loecker, 1995; Fuller et al., 1982).

Recently, it was shown that short-term storage of hepatocytes (up to 4 days) can be achieved by maintaining the cells in modified University of Wisconsin solution at 4°C (Kunieda et al., 2003; Spinelli et al., 2002).

Cryopreservation of hepatocytes is essential for the emergency treatment of acute liver failure (Strom et al., 1997; Bilir et al., 2000; Soriano et al., 2001). The ultimate goal of any improved cryopreservation protocol is to minimize sudden intracellular formation of ice crystals that could result in ultrastructural damage, and thus maintains cell viability, attachment, and metabolic activity on thawing (Wu et al., 1996; Tostões et al., 2011).

The results achieved using high concentrations (up to 60%) of EG or 1, 2-PD penetrating cryoprotectants show that it is possible to prevent cellular damage from the formation of ice crystals and to allow vitrification for cryopreservation.

Storage time of cryopreserved hepatocytes at temperatures well below −100°C (for example, liquid nitrogen, and −140°C freezers) may play an important role in the quality of thawed cells.

These results reveal a progress made to date in cell isolation, short and long-term cryopreservation permitting preservation of viability and handling of hepatocytes. It will hopefully lead to a wider use of cellular therapies in liver diseases.

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REFERENCES

Armitage WJ, Moss SJ, Easty DL (1988). Effects of osmotic stress on rabbit corneal endothelium. Cryobiology, 25: 425-439.

Berry MN, Halls HJ, Grivell MB (1992). Techniques for pharmacological and toxicological studies with isolated hepatocyte suspensions. Life Sci., 51: 1-16.

Bierwolf J, Lutgehetmann M, Feng K, Erbes J, Deichmann S, Toronyi E, Stieglitz C, Nashan B, Ma PX, Pollok JM (2011). Primary rat hepatocyte culture on 3D nanofibrous polymer scaffolds for toxicology and pharmaceutical research. Biotechnol. Bioeng., 108(1): 141-150.

Bilir BM, Guinette D, Karrer F, Kumpe DA, Krysl J, Stephens J, McGavran L, Ostrowska A, Durham J (2000). Hepatocyte transplantation in acute liver failure. Liver Transplant, 6: 32-40

Brophy CM, Luebke-Wheeler JL, Amiot BP, Khan H, Remmel RP, Rinaldo P, Nyberg SL (2009). Rat hepatocyte spheroids formed by rocked technique maintain differentiated hepatocyte gene expression and function. Hepatology, 49(2): 578-586.

Brophy CM, Luebke-Wheeler JL, Amiot BP, Remmel RP, Rinaldo P, Nyberg SL (2010). Gene expression and functional analyses of primary rat hepatocytes on nanofiber matrices. Cells Tissues Organs, 191(2): 129-140.

Crenesse D, Fossat B, Craffa F, Chaland P, Porthe-Nibelle J, Poiree JC, Gugenheim J (1994). Potassium uptake and water content in hepatoctes isolated from rat livers preserved in Euro-Collins and UW solutions and after transplantation. Cryobiology, 31(6): 540-548.

Dickman KG, Goldstein L (1990). Cell volume regulation by skate erythrocytes: role of potassium. Am. J. Physiol., 258: R1217-R1223.

Fahy GM, Saur J, William's RJ (1990). Physical problems with vitrification of large biological system. Cryobiology, 27(5): 492-510.

Fiegel HC, Bruns H, Höper C, Lioznov MV, Kluth D (2006). Cell growth and differentiation of different hepatic cells isolated from fetal rat livers *in vitro*. Tissue Eng., 12(1): 123-130.

Fladmark KE, Gjertsen BT, Molven A, Mellgren G, Vintermyr OK, Doskeland SO (1997). Gap junctions and growth control in liver regeneration and in isolated cat hepatocytes. Hepatology, 25: 847-855.

Fuller BJ, Grout BW, Woods RJ (1982). Biochemical and ultrastructural examination of cryopreserved hepatocytes in rats. Cryobiology, 19(5): 493-502.

Fuller BJ, De Loecker W (1995). Changes in permeability characteristics of isolated hepatocytes durant slow freezing. Cryo Letters, 6: 361-370.

Fuller BJ (2004). Cryoprotectants: the essential antifreezes to protect life in the frozen state. Cryo Letters, 25(6): 375-388.

Gerlach JC, Ziemer RA, Spatkowski G, Zeilinger K, Neuhaus P (1997). Vizualisation of liver sinusoïdal endothelial cell repair behavior after preservation by *in vitro* time-laps video microscopy. Transplantation, 63(3): 455-459.

Glicklis R, Merchuk JC, Cohen S (2004). Modeling mass transfer in hepatocyte spheroids via cell viability, spheroid size, and hepatocellular functions. Biotechnol. Bioeng., 86(6): 672-680.

Graf J, Haddad P, Haeussinger D, Lang F (1988). Cell volume regulation in liver. Ren. Physiol. Biochem., 11: 202-220.

Hamilton GA, Westmoreland C, George E (2001). Effects of medium composition on the morphology and function of rat hepatocytes cultured as spheroids and monolayers. *In vitro* cell and Developmental Biology - Animal (Springer). 37(10): 656-667.

Hewitt NJ, Gómez-Lechón MJ, Houston JB, Hallifax D, Brown HS,

Innes HM, Borel R, Toner M, Ezzell RM, Tompkins RG, Yarmush ML (1992). Effects of dimethyl sulfoxide on cultured rate hepatocytes in sandwich configuration. Cryobiology, 29: 443-453.
Kuleshova LL, MacFarlane DR, Trounson AO, Shaw JM (1999). Sugars exert a major influence on the vitrification properties of ethylene glycol-based solutions and have low toxicity to embryos and oocytes. Cryobiology, 38(2): 119-130.
Kunieda T, Maruyama M, Okitsu T, Shibata N, Takesue M, Totsugawa T, Kosaka Y, Arata T, Kobayashi K, Ikeda H, Oshita M, Nakaji S, Ohmoto K, Yamamoto S, Kurabayashi Y, Kodama M, Tanaka N, Kobayashi N (2003). Cryopreservation of primarily isolated porcine hepatocytes with UW solution. Cell Transplant, 12(6): 607-616.
Lawson A, Ahmad H, Sambanis A (2011). Cytotoxicity effects of cryoprotectants as single-component and cocktail vitrification solutions. Cryobiology, 62(2): 115-122.
LeCluyse EL, Alexander E, Hamilton GA, Viollon-Abadie C, Coon DJ, Jolley S, Richert L (2005). Isolation and culture of primary human hepatocytes. Methods Mol. Biol., 290: 207-229.
Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GMM, Hengstler JG (2007). Primary hepatocytes: Current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab. Rev., 39(1): 159-234.
Meng Q (2003). Hypothermic preservation of hepatocytes. Biotechnol. Prog., 19: 1118-1127.
Meyer G (2010). Pollution in urban-type Carbon Monoxide and sensitivity to myocardial ischemia-reperfusion syndrome: Cardioprotective Role of Exercise, PhD Thesis, University of Avignon and Pays de Vaucluse, Marseille, France. p. 94.
Naik S, Trenkler D, Santangini H, Pan J (1996). Isolation and culture of porcine hepatocytes for artificial liver support. Cell Transplant, 5: 107-115.
Pegg DE (2007). Principles of cryopreservation. Methods Mol. Biol., 368: 39-57.
Penney MD (2008). Sodium, water, and potassium. In: Marshall WJ, Bangert SK, editors. Clinical Biochemistry: Metabolic and Clinical Aspects. 2nd ed. Philadelphia: Churchill, Livingstone, Elsevier, p. 32.
Seglen PO (1976). Preparation of isolated rat liver cells. Meth. Biol., 13: 29-83.
Simione FP (1998). Cryopreservation Manual. M.S. of the American Type Culture Collection (ATCC) in cooperation with Nalge Nunc International Corp. pp. 1-15.
Smith MJ, Thor H, Orrenius S (1991). Toxic injury to isolated hepatocytes is not dependent on extracellular calcium. Sciences, 213: 1257-1259.
Son JH, Kim KH, Nam YK, Park JK, Kim SK (2004). Optimisation of cryoprotectants for cryopreservation of rat hepatocyte. Biotechnol. Lett., 26: 829-833.
Soriano HE, Superina R, Ferry GD, Wang LJ, Alonso E, Almond S, Wood RP, Whitington PF (2001). Hepatocyte transplantation (HTx) in children with liver failure. Hepatology, 34(11; Part 2; Suppl S): 306.
Spinelli SV, Rodriguez JV, Quintana AB, Mediavilla MG, Guibert EE (2002). Engraftment and function of intrasplenically transplanted cold stored rat hepatocytes. Cell Transplant, 11: 161-168.
Strom SC, Fisher RA, Thompson MT, Sanyal AJ, Cole PE, Ham JM, Posner MP (1997). Hepatocyte transplantation as a bridge to orthotopic liver transplantation in terminal liver failure. Transplantation, 63: 559-569.
Tostões RM, Leite SB, Miranda JP, Sousa M, Wang DIC, Carrondo MJT, Alves PM (2011). Perfusion of 3D encapsulated hepatocytes-a synergistic effect enhancing long-term functionality in bioreactors. Biotechnol. Bioeng., 108(1): 41-49.
Umeshita K, Monden M, Fujimori T, Sakai H, Gotoh M, Okamura J, Mori T (1988). Extracellular calcium protects cultured rat hepatocytes from injury caused by hypothermic preservation. Cryobiology, 25: 102-109.
Valdes CA, Abas Mazni O, Kanadawa H, Fujikawa S (1990). Configuration by cryoelectron, microscopy of the absence of cristallization using a vitrification solution. Cryo Letters, 11: 351-360.
Volk GM, Harris JL, Rotindo KE (2006). Survival of mint shoots tips after exposure to cryoprotectant solution components. Cryobiology, 52(2): 305-308.
Wu FJ, Friend JR, Hsiao CC, Zilliox MJ, Ko WJ, Cerra FB, Hu WS (1996). Efficient assembly of rat hepatocyte spheroids for tissue engineering applications. Biotechnol. Bioeng., 50(4): 404-415.
Wusteman MC, Pegg DE, Robinson MP, Wang LH, Fitch P (2002). Vitrification media: Toxicity, permeability, and dielectric properties. Cryobiology, (1): 24-37.
Xia P (2005). The effects of electrical stimulation at high frequency in a cellular model *in vitro*, p. 29. PhD Thesis, University Joseph Fourier, Grenoble, France.