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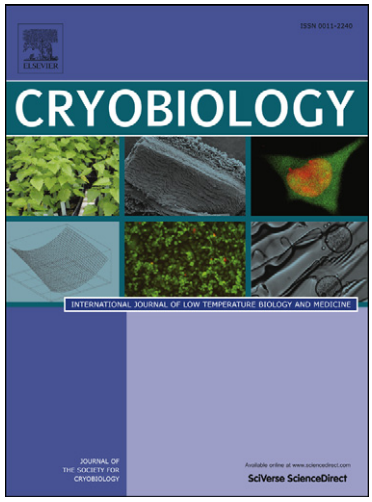

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EXTENDED COLD STORAGE OF CULTURED HEPATOCYTES IMPAIRS ENDOCYTIC UPTAKE DURING NORMOTHERMIC REWARMING

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Abstract

During hypothermic preservation of cells (0-4°C), metabolism is diminished and energy-dependent transport processes are arrested. The effect of hypothermic preservation of hepatocytes in endocytic transport following rewarming has not been previously reported. We evaluated the uptake of EGF (Epidermal Growth Factor) ligand conjugated to fluorescent Quantum Dots (QDs) probes in rat hepatocytes after 24 and 72 h cold storage in University of Wisconsin (UW) solution at 4°C. QDs uptake was visualized during rewarming to 37°C under air or, in a second approach, at the end of rewarming under 5% CO_2. After 24 h in UW solution, QDs were internalized under both rewarming conditions similar to non-preserved hepatocytes and cells maintained a normal cytoskeleton distribution. However, in hepatocytes preserved 72 h none of the cells internalized QDs, which remained bound to the membranes. After rewarming, this group showed diminished actin staining and 60% reduction in ATP levels, while viability was maintained at ~70%. Our results present evidence that, hypothermic preservation for 72 h in UW solution at 4°C does not prevent EGFR (Epidermal Growth Factor Receptor) activation but irreversibly impairs endocytic uptake upon EGF stimulation; presumably due to actin cytoskeleton disassembling besides reduced ATP pool. Our approach can be applied on other membrane receptor systems and with other hypothermic preservation solutions to understand the effect of cooling in endocytic transport and to determine the optimal cold storage period.

Key words: Hypothermic preservation; receptor-mediated endocytosis; cultured rat hepatocytes; Epidermal Growth Factor Receptor; Quantum Dots

Abbreviations: WE: Williams' E medium; UW solution: University of Wisconsin solution, EGFR: Epidermal Growth Factor Receptor; QDs: Quantum Dots; ATP: adenosine-5´-triphosphate; LDH: Lactate Dehydrogenase; HP: hypothermic preservation; NR: normothermic rewarming; DIC: differential interference contrast

Introduction

Cold storage of mammalian cells in preservation solutions is a well-known methodology to maintain and provide a regular source of viable and metabolically competent hepatocytes for cell banking, hepatocellular therapies or bioartificial liver devices [8; 31]. Hypothermic preservation slows down all non-enzymatic and enzymatic processes usually by a factor of 1.5 to 3 per 10 ºC of temperature decrease and leads to structural membrane damage [7] and to reduction in ATP intracellular pool [25; 39]. University of Wisconsin (UW) solution was designed to prevent cell swelling, intracellular acidosis, injury from oxygen-free radicals and to maintain ATP levels [40]. Nevertheless, when the cells are rewarmed to 37 ºC, a natural situation that occurs when the organ or the cells are transplanted, they may undergo structural and functional damage as the result of metabolic changes occurred during the cold storage period [22].

Low temperatures cause reorganization of the membrane microstructure, e.g. the lipid-lipid and lipid-protein interactions [19] as well as cytoskeleton distribution [42] affecting the global integrity of the bilayer and the dynamic of transport processes. Furthermore, the increased viscosity diminishes rate of lateral diffusion, clustering and distribution of membrane embedded tyrosine kinase receptors as well as the assembling of the endocytic and signalling machinery [3; 28].

A thoroughly studied tyrosine kinase receptor is the epidermal growth factor receptor (EGFR), for which ligand-induced receptor dimerization, stimulates its intrinsic protein tyrosine kinase activity, leading to auto-phosphorylation and activation [34]. Following receptor activation several endocytic proteins are efficiently recruited [28] and the EGF signal is down regulated through internalization of the receptor-ligand complex [4; 34]. During this process, actin polymerization provides the force for generating membrane invaginations and for the scission of the endocytic vesicles from the plasma membrane [32].

Previous studies showed that at 4°C lateral mobility of EGFR is reduced but not abolished [10; 12]. In addition, stimulation with EGF at 4°C (ice cooled condition) results in phosphorylation of the receptor with similar kinetics to the phosphorylation induced at 37°C [11; 23]. Furthermore, in the case of EGFR recruitment of both effectors signalling molecules in nascent clathrin coated pits and endocytic machinery was observed at 0°C although receptor internalization was inhibited [28]. All these studies were carried out for short periods at 4°C (max 60 min), during which membrane and cytoskeleton integrity were not compromised and endocytosis inhibition could be reversed [12; 24; 44]. Therefore, it is possible that hypothermic preservation periods beyond 24 h, impair the EGF stimulated endocytic uptake, due to loss of membrane structural integrity, cytoskeleton alteration [42] and/or as the result of time dependent ATP reduction induced by cold storage [7; 25; 36]. How hypothermic preservation may affect the initial endocytic uptake of EGFR stimulated immediately after normothermic rewarming has not been previously studied.

Fluorescence imaging techniques has been dramatically improved with the introduction of quantum dots (QDs), colloidal nanocrystals with unique optical properties for long-term and multicolour imaging [1]. Complexes of streptavidin-conjugated quantum dots (QDs) with biotinylated EGF are biochemically competent ligands for EGFR [15] and has been employed to monitor EGFR dimerization, activation and endocytosis [14].

In the present study we evaluated the effect of hypothermic preservation on Epidermal Growth Factor (EGF) receptor mediated endocytosis in cultured and cold preserved rat hepatocytes after normothermic rewarming (NR).

Materials and Methods

Culture medium and rewarming solutions

Cell-attachment culture medium: Williams' E medium (MP Biomedicals, Cleveland, OH, USA), supplemented with 5 % fetal bovine serum (FBS, Sigma F7524) plus 1 g/L BSA (Sigma), 2.2 g/L $NaHCO_3$, 133 IU/L penicillin (ICN), 0.1 mg/L streptomycin (Sigma), pH 7.2.

Post cell-attachment culture medium (serum free): William's E basal medium, plus 1 g/L BSA, 2.2 g/L $NaHCO_3$, 5 mg/L insulin, 133 IU/L penicillin, 0.1 mg/L streptomycin, 5 mg/L insulin (Betasint-U40) and 50 µM prednisolone 21-hemisuccinate (MP Biomedicals, Solon, USA), pH 7.20.

Tyrode´s buffer: 135 mM NaCl, 10 mM KCl, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 1 g/L BSA, 20 mM glucose, pH 7.20.

University of Wisconsin (UW) solution

We employed a modified UW solution previously described [30]. In this solution hydroxyl-ethyl starch was replaced by Polyethylene glycol (PEG) due to its beneficial effect in preventing hypothermic cell swelling and in maintaining cytoskeleton integrity [18; 42]**.**

Composition of modified UW solution : 100 mM lactobionic acid, 25 mM K_2HPO_4, 5 mM $MgSO_4$, 30 mM raffinose, 2.5 mM adenosine, 3 mM reduced glutathione GSH, 1 mM allopurinol, 5 % PEG 8000, 15 mM glycine, 0.25 mg/mL streptomycin, and 10 IU/mL penicillin G, pH 7.40, 340-380 mOsm/Kg. The solution was bubbled with 100% N_2 for 20 min at 4°C before use in order to minimize aerobic metabolism and thus the accumulation of ROS (reactive oxygen species) during preservation [17]. All reagents were of analytical grade.

Biotin-EGF-Streptavidin-QDs complexes

Biotinylated-EGF (Invitrogen, Eugene, Oregon, USA) was conjugated to Qdot®$_{655}$-Streptavidin conjugate (Invitrogen, Eugene, Oregon, USA) molar ratio 4:1. QDs emitting at 655 nm were chosen to minimize the bleed through of hepatocyte autofluorescence in the QD channel. Stocks solutions of preformed complexes were prepared incubating biotin-EGF and streptavidin-QD_{655} for 30 min at room temperature in PBS+ 20 g/L BSA. Stock solutions were stored at 4°C and used within 5 days. Complexes were 10-fold diluted in Tyrode´s buffer to 2 nM QD_{655} final concentration prior to incubation with hepatocytes.

Animals

Adult male Wistar rats weighting 290-340 g were obtained from the Central Animal Building of the School of Biochemistry and Pharmaceutical Sciences, National University of Rosario. Rats were maintained on standard food pellets and water ad-libitum and protocols used were approved by the National Council of Research in Argentina and the Local Ethics Committee from the School of Biochemistry and Pharmaceutical Sciences from the National University of Rosario, which are in accordance with international regulations.

Hepatocyte isolation and culture

The animals were anesthetized by i.p. injection with 300 mg/kg body weight chloral hydrate (Parafarm, Buenos Aires, Argentina). Hepatocytes were isolated by collagenase (from Clostridium h*istolyticum*, Sigma, Lot. 089K8623) perfusion without recirculation using the procedure originally described by Seglen [37] and adapted in our laboratory [30]. Cell viability of freshly isolated cells was tested by the exclusion of Trypan Blue (TBE) dye (0.2 % in PBS). Preparations with a TBE higher than 80 % were considered suitable for cell culture. Hepatocytes were seeded at a density of $7x10^5$ cells/cm2 in collagen-coated culture dishes (Orange Scientific, Braine-l'Alleud, Belgium) with collagen coated 18x18 mm glass coverslips and incubated in William's E medium supplemented with 5% FBS at 37°C in a gas-controlled incubator under 5% CO_2 atmosphere. Three hours after cells seeding on collagen, medium was replaced by *post cell-attachment (*serum free) *culture medium* and cells were cultured for up to 24 h at 37°C before hypothermic preservation.

Hypothermic preservation (HP) and Normothermic Rewarming (NR)

After 24 h, cultured hepatocytes were washed twice with cold PBS and preserved at 4°C for 24 and 72 h in the culture dishes with 1 mL UW solution/$1x10^6$ plated hepatocytes saturated with N_2. Culture dishes were kept in a tight sealed container at 4°C and left undisturbed until analysis.

After hypothermic preservation cells were washed twice with cold PBS and immediately rewarmed to 37°C (Table 1).

Table 1

Experimental groups

Hepatocytes cultured 24 h as non-preserved controls (HC); hepatocytes preserved 24 h and 72 h in UW solution without further rewarming (HP24-t_0) and (HP72-t_0) respectively; hepatocytes preserved 24 or 72 h in UW solution followed by 30 min normothermic rewarming (HP24-t_{30}) and (HP72-t_{30}) respectively; hepatocytes preserved 24 h and 72h in UW solution followed by 120 min rewarming (HP24-t_{120}) and (HP72-t_{120}), respectively (see Suppl. Mat. Fig. S1.). Hepatocyte morphology was observed by phase contrast microscopy immediately after hypothermic preservation and after rewarming in WE serum free medium.

Incubation with EGF-QDs complexes

After each cold storage period hepatocytes on coverslips were thoroughly rinsed with cold Tyrode´s buffer to eliminate residual UW before rewarming. Then, cells were incubated with 100 µL EGF:QDs (8 nM EGF:2 nM QD_{655}) complexes for 10 min on ice-water bath (8-10°C) to maximize binding to EGFR without internalization, followed by 5 min at RT to stimulate receptor activation [24]. Excess complexes were washed with Tyrode´s buffer and cells were rewarmed as described above. Under both rewarming conditions, controls for non-specific binding of the QDs were carried out by adding non tagged QDs (without EGF) at the same concentration as the employed in preformed complexes.

Fluorescence confocal microscopy

In live cells during NR in Tyrode's buffer: Confocal microscopy of live cells was carried out at controlled temperature in a modular perfused chamber (MPC) designed in our lab [38]. Before imaging the complete chamber was thermostated at 4°C without the sample. After recording a stable temperature the coverslip; with cells preincubated with the EGF-QDs complexes, was placed on the chamber and immediately covered with 500 µL chilled Tyrode´s buffer. Then, temperature was increased to 37°C, and after thermal stabilization (approx. 2 min.), single confocal planes or stacks were acquired every 5 min during 30 min. A control for unspecific binding of QDs was performed by incubating cultured and preserved hepatocytes with 2 nM QDs in the absence of ligand and monitored under the same conditions as described above.

In fixed cells after NR in WE medium: After 24 and 72 h of cold storage hepatocytes were incubated with EGF-QDs complexes as described above, and immediately fixed in 2% PFA in PBS. Additional two coverslips for each rewarming period were transferred to a sterile culture dish and rewarmed for 30 min and 120 min in serum free WE medium under 5 % CO_2 atmosphere and then fixed in 2% paraformaldehyde (PFA) in PBS. Individual confocal planes or stacks were acquired for each preservation and rewarming condition (more details in section *Image acquisition and processing*).

Actin staining

After 24 and 72 h cold storage and after 0, 30 min rewarming, F-actin was stained with Alexa Fluor® 633 phalloidin (A22284, Molecular Probes) following the protocol of the manufacturer. Briefly, cells were fixed in 2% PFA in PBS for 10 minutes at room temperature and were permeabilized with 0.1% Triton X-100 in PBS, 3 to 5 minutes. Each coverslip was then incubated with 100 µL of a ten-fold dilution in PBS + 10 g/L BSA of the stock solution 6.6 µM Phalloidin Alexa 633 (in methanol), for 20 min in the dark at RT. Cells were rinsed with PBS and imaged in the same buffer.

Image acquisition and processing

Imaging was performed in a Nikon C1 plus confocal microscope mounted on Eclipse TE-2000-E2 inverted microscope (Panel D) equipped with a 40X dry, numerical aperture, 0.95 Plan Apo-Chromat objective (Nikon, Melville, NY, USA). QD_{655} was excited at λ=488 nm and detected with a long pass filter LP650. Gain and laser power were set in label free cells to minimize bleed through of hepatocyte autofluorescence in the QDs channel. Image processing was performed using NIH Image J free software. Images were background corrected and two dimensional (2D) representations of 3D cells were created from maximum intensity projections of five slices in the z-dimension excluding (when possible) the top and bottom planes of all cells in the microscopic field. Actin staining was visualized by exciting Phalloidin Alexa 633 with laser line at λ 633 nm and fluorescence was detected with LP650 filter with fully open pinhole.

Lactate Dehydrogenase (LDH) retention

Membrane integrity was assessed by measuring the intracellular enzyme activity of LDH in all experimental groups. LDH activity was determined in 500 µL media supernatants or UW solution and in cell lysates after lysis with 0.1% Triton X-100 in PBS in cultured and in cold stored and rewarmed cells as previously described [9]. Briefly, LDH activity was determined by measuring NADH oxidation at λ=340 nm, ΔAbs/min was monitored for 3 min at 37°C. Results were expressed as the percentage of retention of LDH enzyme, (intracellular enzyme activity relative to total enzyme activity measured per well).

ATP assay

ATP content was determined in all experimental groups from at least two hepatocyte isolation procedures. Cultured and preserved hepatocytes were detached from culture dishes in 1 mL PBS. Cell were counted in Neubauer chamber (between 1.0 to 5.0 $x10^5$ cells/well) and pelleted by centrifugation (13 000 g - 30 s), the cell pellet was deproteinized by addition of 500 µL of cold 3%

$HClO_4$. After centrifugation, the protein free supernatant was neutralized with K_2CO_3 and immediately stored in liquid N_2. ATP was quantified by luciferase-catalyzed oxidation of luciferin, employing the Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Product Number FL-AA, Saint Louis, USA) as described by the manufacturer. Luminescence was counted using a microplate reader (Biotek, Synergy HT). The instrument was set to integrate the amount of light produced over a 10 second interval without an initial delay at 25°C. ATP was determined in samples (duplicates) as nmoles ATP/10^6 cells by comparison to a standard ATP curve (duplicate). ATP concentrations were assessed before and after 30 min rewarming. The results were expressed as mean % of ATP ± standard error, relative to the amount of ATP before rewarming.

Data analysis and statistics

All data were obtained from three to eight independent isolation procedures. For LDH retention samples were obtained from two dishes per preservation and rewarming condition, per hepatocyte isolation. Results were presented as mean ±SD. Statistical significance of differences between LDH percentage values was assessed by analysis of variance (ANOVA) followed by multiple comparisons according to Tukey and $p < 0.05$ values were considered statistically significant. Statistical significance of the differences between ATP content in HP24 t0 and t30 NR and HP72 t0 and t30 NR was assessed by analysis of variance (ANOVA) from two independent hepatocytes isolations in HP24 and three independent isolations in HP72.

Results

Hypothermic preservation periods of 24 and 72 h were selected based on previous experience of our group [20; 27; 41] and others with hepatocyte in suspensions [21; 25; 26] or in culture [29; 42]. First, we evaluated morphological alterations after HP in UW solution followed by 120 min normothermic rewarming in WE medium (Fig.1). After 24 h in culture, restoration of cell-cell contacts and polygonal hepatocyte-like cell shape were clearly visible as well as the formation of

bile canaliculi-like structures as indicated by the light areas between cells (Panel A). Polygonal morphology was maintained after 24 h (Panel B) and 72 h (Panel D) in UW solution. After 120 min. rewarming, cell-cell contacts and shapes were maintained in HP24-t_{120} (Panel C). However, visible deterioration appeared in HP72-t_{120} (Panel E) revealed by poorly conserved polygonal shape, less defined cellular and nuclear membranes and a more granulated cytoplasm.

Figure 1

Endocytic uptake of targeted QDs nanoparticles

Live cell imaging was performed during a rewarming period of 30 min at 37°C in Tyrode´s buffer under air. Figure 2 shows that after 5 min. at 37°C, QDs uptake is readily visible in HC (Panel A) revealed by the typical dot pattern distribution of endosomes but not in HP24-t_0 (Panel D), where QDs were mainly concentrated on the membranes. Only after 30 min rewarming, QDs were internalized in HP24 (Panel E) visible as a more intense fluorescence dots comparable to non-preserved controls HC after NR (Panel B). In HP72-t_{30}, QDs remained bound during the whole rewarming period (Panels G, H). In several hepatocytes perinuclear distribution of internalized red fluorescent QDs is visualized beside the green cytoplasmic autofluorescence, characteristic of hepatocytes (Figure S2, Suppl. Info.).

Figure 2

QDs distribution after normothermic rewarming in WE medium

Williams E culture medium is more appropriate as physiological solution for hepatocytes rewarming than Tyrode´s buffer, allowing for extended incubation periods. However, it is not suitable for live cells imaging in the employed thermostated chamber, as this medium still requires a controlled CO_2 atmosphere for its pH buffering capacity. Consequently, in the second rewarming

approach (see Table 1), images were acquired in cells fixed after NR in serum free WE medium during 30 min (Figure 3). QDs distribution was similar to the one obtained by live cell imaging in all experimental groups (Panels A, C, E), but after NR in WE medium, HP72-t_{30} showed a better preserved morphology by DIC (Panel F). In this case, QDs formed visible clusters on the membrane but were not internalized. Panel G shows that, untargeted QDs added after preservation did not bind during NR in HP72-t_{30}. Overall, these results showed that endocytic uptake of the EGF-QDs complexes during NR are sensitive to the period of cold storage.

Figure 3

Actin distribution, membrane integrity and energy status after hypothermic preservation and rewarming

F-actin distribution was visualized in each experimental group by fluorescence microscopy after staining with Phalloidin-Alexa633 (Figure 4). In HP24-t_0 and HP72-t_0 actin was concentrated under the plasma membrane in regions of contact with neighbouring cells, showing higher intensity spots corresponding to biliary canaliculi-like structures, similar to non-preserved controls HC. After 30 min NR, a continuous subcortical distribution is clearly visible in HP24-t_{30}. However, in HP72-t_{30}, cells tended to round up, and detach and biliary canaliculi-like structures were barely detected in hepatocytes in contact. Additionally, in HP72-t_{30} a global decrease in the fluorescence intensity was observed suggesting depolymerization of actin during NR.

Figure 4

Due to the compromised structural integrity of the membranes during cold storage [7] the intracellular LDH activity was measured at the end point of preservation and after 30 and 120 min NR in serum free WE medium and compared to the values in non-preserved controls. As shown in

Figure 5 cells preserved 24 h and rewarmed up to 120 min retained LDH at percentages above 85% comparable to control cells for the same NR periods. Whereas cells preserved 72 h showed a significant decrease in LDH retention at 30 and 120 min compared to the HC for the same rewarming periods and a significant decrease compare to HP72-t_0, without NR. For hepatocytes preserved 72 h and rewarmed 30 min, LDH activity was also measured under the conditions employed for live cell microscopy including the incubation time with EGF-QDs complexes, and the retention percentage was approx. 80% similar to HP72 after NR in WE medium.

Figure 5

Changes in energy status during rewarming was assessed by measuring ATP intracellular content in HP24 and HP72, before and after rewarming in WE medium (Table 2). Following 30 min rewarming, mean ATP content showed a 10 % decrease in HP24 and ~60 % decrease in HP72 relative to the corresponding values obtained immediately after cold storage.

Table 2

Discussion

Substantial amounts of epidermal growth factor (EGF) are cleared from the circulation by hepatocytes via receptor-mediated endocytosis and subsequently degraded within lysosomes. Since receptor-mediated endocytosis by the liver represents a process by which levels of various hormones, growth factors and other ligands are regulated, changes in this mechanism could disrupt numerous metabolic and homeostatic events in the liver and total organism [6]. How hypothermic preservation of hepatocytes between 4-8°C may affect energy dependent endocytic transport has not been studied within storage periods consistent with clinical applications. In the present work, we target the tyrosine kinase receptor EGFR for which the ligand stimulated clustering of dimers, activation and endocytosis has been thoroughly documented at low (< 10°C) and normal temperatures (37°C) [24; 28; 34; 35]. Although hepatocytes suspensions are regularly used to determine how hypothermic storage affects liver cell metabolism and viability [21], cultured cells proved more suitable for cell by cell microscopic analysis of transport processes. In the present work, live cells imaging performed in our designed thermostated chamber allowed monitoring Quantum Dots uptake during 30 min rewarming in Tyrode´s buffer under air. Although suitable for imaging, this Hepes-based buffer is still basic to support cell survival for longer rewarming periods. Therefore, in a second approach, hepatocytes were rewarmed in Williams E medium without serum and 5% CO_2 mimicking cell culture conditions and QDs uptake was analysed at the end of rewarming. In addition, rewarming in WE medium allowed to extent rewarming period up to 120 min to evaluate morphology and viability. However, for fluorescence imaging of QD_{655} and Phalloidin-$Alexa_{633}$ cell fixation was unavoidable due to insufficient buffering capacity of WE medium to perform live cells microscopy under air.

We demonstrated that after 24 h hypothermic preservation in UW solution at 4°C, rat hepatocytes are able to reassume endocytic uptake during rewarming. However, after 72 h none of the cells internalized the QDs, which remained bound to membranes under the two different rewarming conditions explored.

Previous studies on A431 cell line overexpressing the EGFR; showed that aggregation of the receptors during short term cooling (4°C) is reversible indicating that lipid phase transitions induced by lowering the temperature do not trap EGF receptors permanently into particular membrane domains [12]. Stimulation of isolated hepatocytes with epidermal growth factor (EGF) causes rapid tyrosine phosphorylation of the EGF receptor (EGFR) and adapter/target proteins at 4 °C clustering the receptors at the membrane [24]. Consistent with these observations, we showed in a previous work that EGF-QD complexes directly added in UW solution at 4°C bind to the cell membranes during cold storage [38]. Furthermore, upon rewarming to 37°C internalization proceeded suggesting that occupied EGFR dimers redistribute normally. In the present work, EGFR was stimulated immediately after preservation and thus QDs binding was expected to occur shortly after EGF-QDs addition. We observed that following rewarming, QDs are internalized in cell cold stored 24 h but not 72 h in UW solution. In this group, QDs do not wash off after removing excess complexes indicating that indeed dimerization and activation occurred in order to anchor the QDs to the cell surface, but endocytic uptake was impaired.

Recent findings demonstrated that direct and indirect association of actin cytoskeleton with the plasma membrane profoundly affects the dynamics and functions of transmembrane receptors, as well as their interactions [3; 5; 13]. Up to date and to our knowledge, the effect of hypothermia in F-actin cytoskeleton of cultured hepatocytes has been addressed solely by Stefanovich et al. [42]. This group showed a correlation between irreversible cytoskeletal alterations and loss of function and membrane integrity after 24 h cold storage in UW solution and in Leibovitz 15 (L15) medium. Based on these findings and our observations, we hypothesized that long term cooling (72 h) prevent subsequent vesicular transport due to deterioration in actin cytoskeleton while membrane integrity is still maintained. To rule out whether impaired uptake was the result of loss of membrane integrity, LDH retention was evaluated in each experimental group. We demonstrated that cells preserved 24 h, which are endocytic competent; maintain membrane integrity/viability similar to non-preserved cells (~90%). In hepatocytes preserved 72 h in UW solution, however, none of the

observed cells internalized QDs although hepatocytes maintained 70% LDH retention after rewarming.

Cortical cytoskeleton is involved in earlier steps of clathrin-mediated endocytosis and facilitates the clustering of active EGFR receptors and downstream effectors to increase the efficiency of signaling upon ligand stimulation [16; 33]. Furthermore, F-actin itself is a target of downstream kinases following EGFR activation [43] and is physically linked through adaptors proteins to nascent endocytic vesicles [33]. Preservation injury is associated with loss of cellular adenosine triphosphate (ATP) which will rapidly disrupt the actin cytoskeleton [2; 7]. In our study, rat hepatocytes preserved 72 h in UW solution, supplemented with adenosine, intracellular ATP decreases to values comparable to those of cells preserved 24 h, which are endocytic competent. However, in hepatocytes cold stored 72 h a marked disappearance of subcortical actin occurs during rewarming. This data suggests a net depolymerization of actin, further supported by the altered cell morphology such as rounding up of hepatocytes, loss of cell-cell contact and thus biliary canaliculi. Under this scenario, a plausible explanation is that after 72 h preservation in UW solution at 4°C followed by oxygenated rewarming to 37°C, EGFR is still efficiently activated when stimulated with EGF but the altered subcortical actin network prevents subsequent interaction of the phosphorylated kinase receptor with actin binding proteins and adaptors proteins required for the functional assembling of the endocytic machinery. However, further correlation between ATP levels with EGFR autophosphorylation and ATP dependent actin polymerization should be addressed.

In conclusion, these findings suggest that 72 h cold storage in UW solution at 4°C leads to irreversible cytoskeleton disorganization during rewarming that inhibits earlier steps in the vesicular transport mediated by EGFR. In our hepatocyte culture model of hypothermic preservation, targeted Quantum dots proved suitable as sensors of cold impaired endocytic competence. Our approach can be applied on other receptor systems and on other hypothermic preservation solutions to further understand the effect of cooling in endocytic transport and to improve cold storage conditions.

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535 **Figure Captions**

536

537 **Figure 1. Hepatocyte morphology in culture and after 24 and 72 h cold storage followed by**
538 **120 min rewarming.** Phase contrast photographs of **A)** Hepatocytes cultured 24 h**,** non –preserved
539 controls HC; **(B-D)** preserved cells rewarmed 120 min. NR at 37°C was performed in WE medium
540 (serum free) pH 7.4 under 5 % CO_2. Scale bar: 20 μm.

541

542 **Figure 2. Internalization of EGF-QD complexes during rewarming in Tyrode's buffer**. Live
543 cell confocal images of **(A-B)** Cultured hepatocytes after 5 and 30 min rewarming; (**D-E)** HP24
544 after 5 and 30 min rewarming; **(G-H)** HP72 after 5 and 30 min rewarming; **(C, F, I)** corresponding
545 DIC images after 30 min rewarming. QDs bound to membranes (*filled triangles*), internalized QDs
546 (*open triangles*). NR was carried out in Tyrode´s buffer pH 7.2 under air in the microscope stage.
547 Images are z-projections of five confocal planes excluding top and bottom cell surface. Scale bar:
548 20 μm.

549

550 **Figure 3. Distribution of EGF-QD_{655} after rewarming in WE medium.** Confocal images of
551 fixed cells; **A)** HC-t_{30} , **C)** HP24-t_{30}, **E)** HP72- t_{30}, **G)** HP72-t_{30} +QD_{655} (-EGF); **(B, D, F and H)** the
552 corresponding DIC images. QDs bound to membranes (*filled triangles*), internalized QDs (*open*
553 *triangles*). NR at 37°C was performed in WE medium (serum free) pH 7.4 under 5 % CO_2. Images
554 are z-projections of five confocal planes excluding top and bottom cell surface. Scale bar: 20 μm.

555

556 **Figure 4. Effects of hypothermic preservation and rewarming in actin filaments distribution.**
557 Fluorescence images of Phalloidin-Alexa633 stained F-actin. **A)** Non-preserved hepatocytes HC;
558 **(B-C)** HP24 after 0 and 30 min rewarming respectively; **(D-E)** HP72 after 0 and 30 min rewarming
559 respectively. NR at 37°C was performed in WE medium (serum free) pH 7.4 under 5 % CO_2. Scale
560 bar: 20 μm.

561 **Figure 5. LDH intracellular activity before and after 30 and 120 min NR at 37°C in WE**
562 **medium.** ANOVA test, p value < 0.05 was considered significant; * Different from HC-t_{30} and HC-
563 t_{120} and from HP24-t_{30} and t_{120}, # Different from HP72-t_{30}.
564

Table 1: Normothermic rewarming conditions

Rewarming conditions	Rewarming period (37°C)	Analysis
In *Tyrode´s buffer* in thermostated chamber under air	30 min	Live cell confocal microscopy during NR
In *WE medium(serum free)*[a] in incubator under 5% CO_2	0 and 30 min	Confocal microscopy of fixed cells after NR, actin staining, LDH retention, ATP content
	120 min	LDH retention, phase contrast microscopy

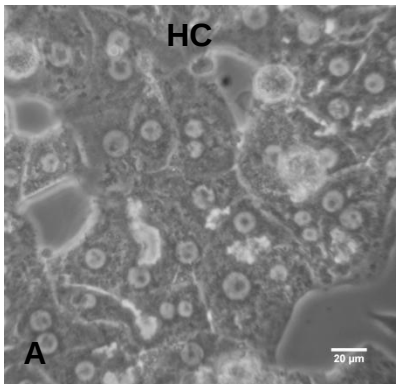
[a] *Attachment medium without serum*

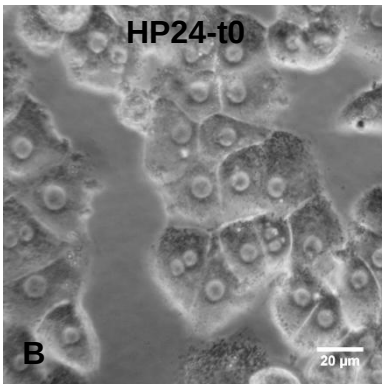
567
568 Table 2: Intracellular content of ATP during rewarming in Williams E medium
569

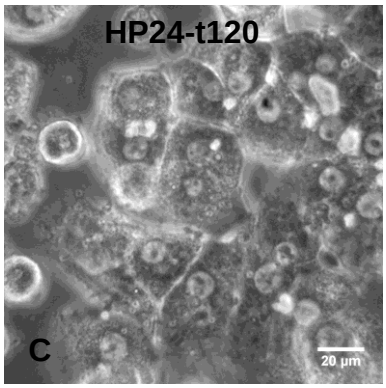
Experimental groups	ATP (nmoles/10^6 hepatocytes)[a]	
	$t_{0\ min}$	$t_{30\ min}$
HP24 (n=2)	4.4 ± 0.7	4.0 ± 0.3
HP72 (n=3)	7.7 ± 2.1	3.3 ± 1.3

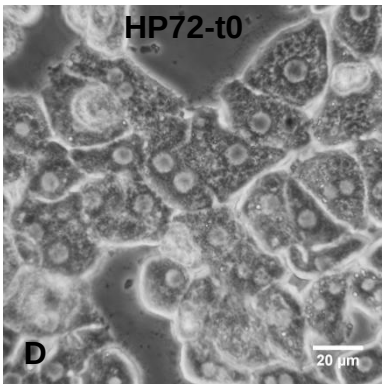
570 [a] expressed as mean ± SE
571

Figure 1


HC
A
20 µm


HP24-t0
B
20 µm


HP24-t120
C
20 µm


HP72-t0
D
20 µm

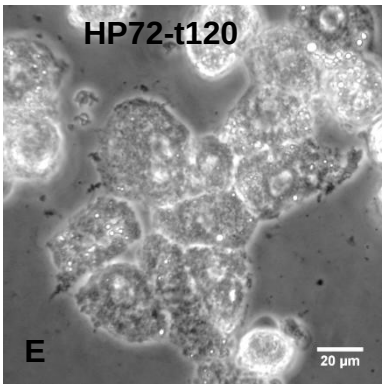

HP72-t120
E
20 µm

Figure 2

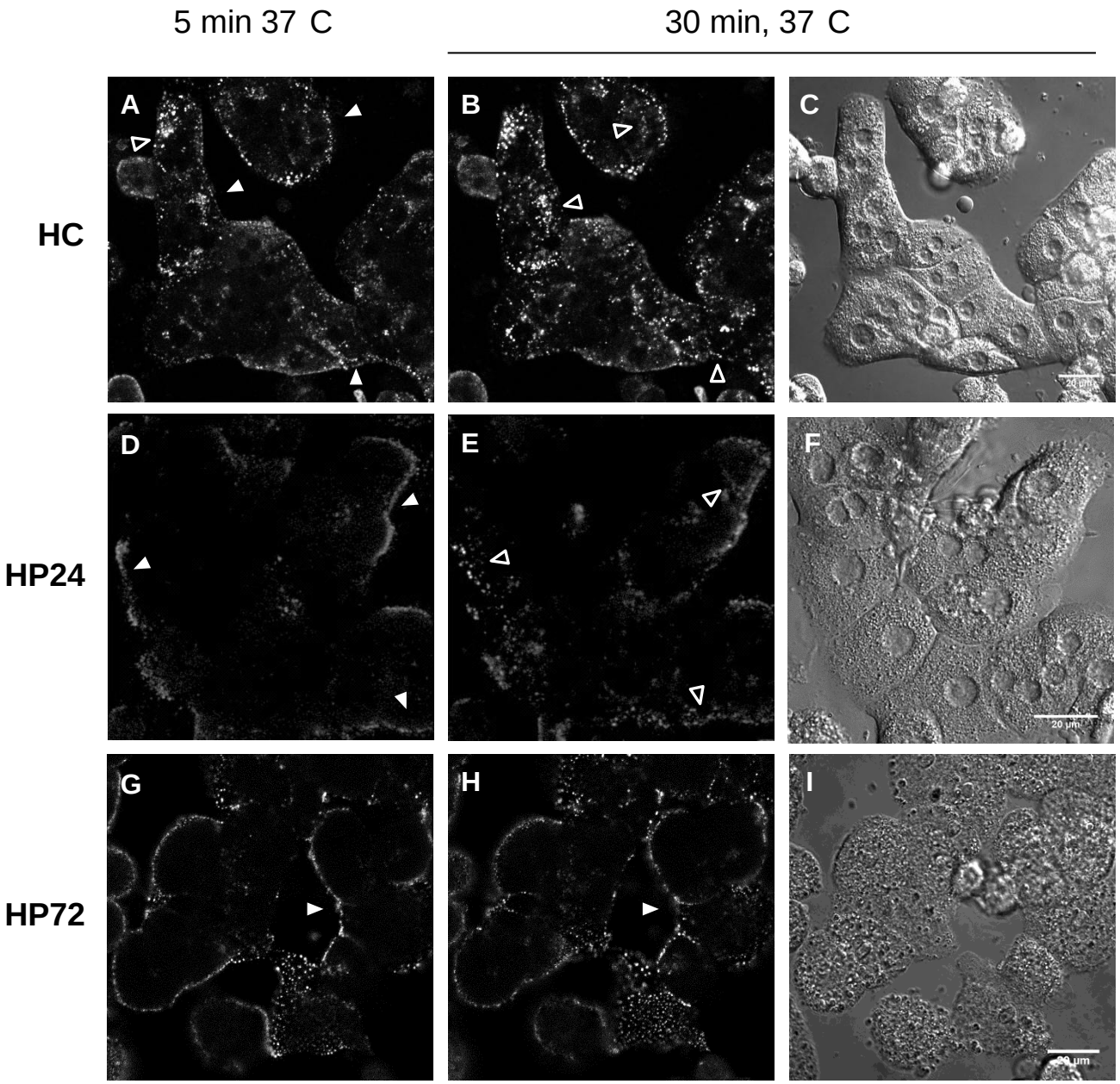


Figure 3

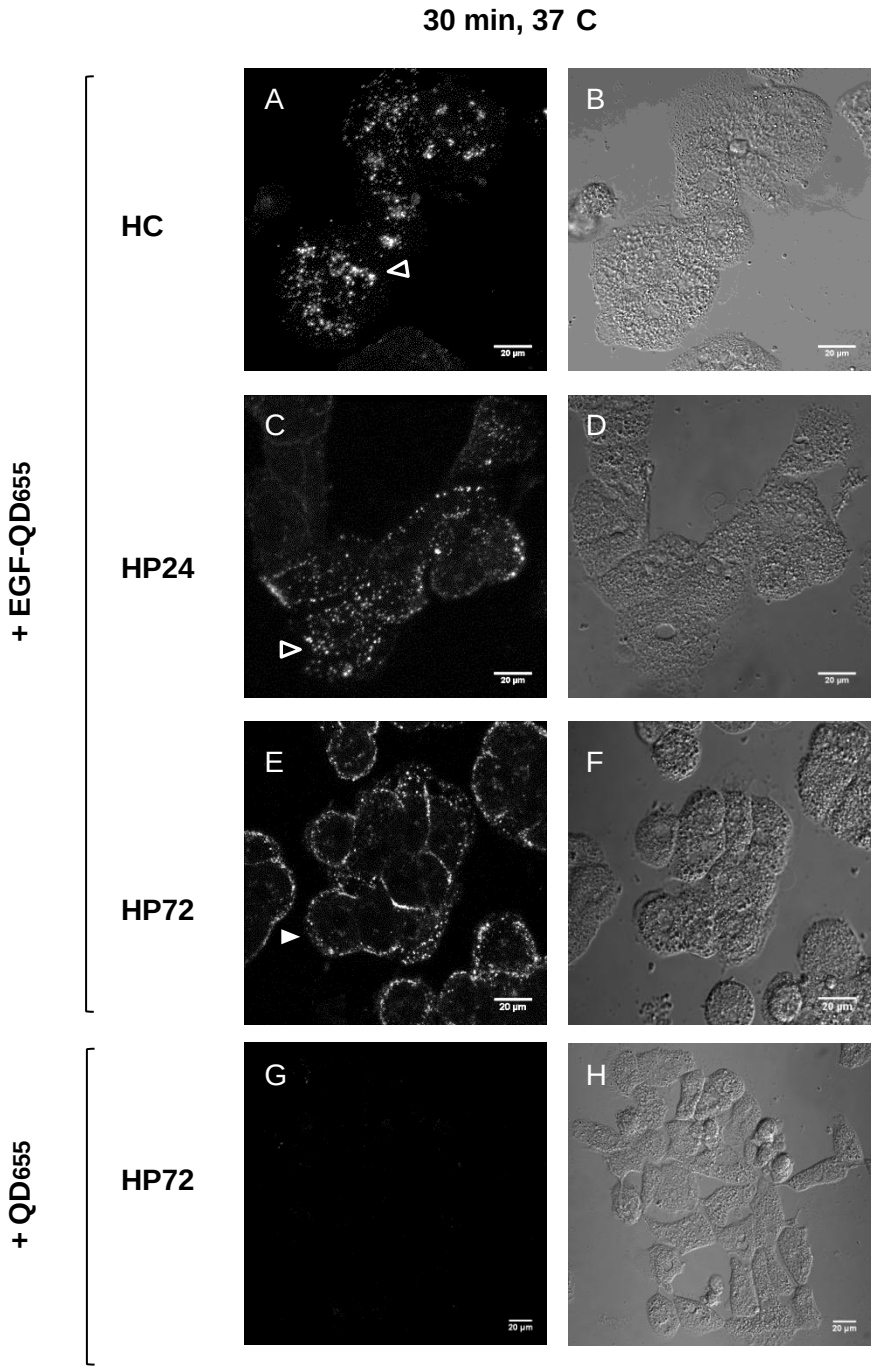


Figure 4

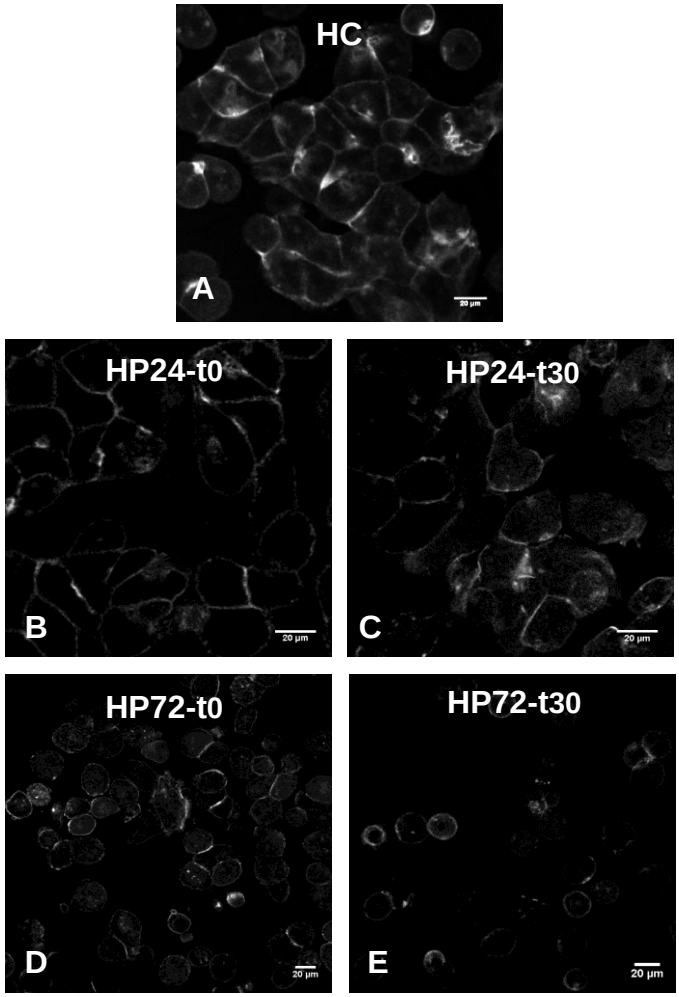


Figure 5