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PROLIFERATION, DIFFERENTIATION AND APOPTOSIS OF CHOLINE DEFICIENT ETHIONINE SUPPLEMENTED DIET-RAT OVAL CELLS UNDER THE INFLUENCE OF 2-METHOXYESTRADIOL

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Since numerous studies indicate that 2-methoxyestradiol (2-ME) as a metabolite of 17beta estradiol (17β-E_2) may exert antitumor activity by unclear mechanism, we undertake the study to elucidate the effect of 2-ME on oval cells (OC) activated by a carcinogenic choline deficient ethionine supplemented diet (CDE diet). Isolated OC were treated with different concentrations of 2-ME for 24, 48 and 72 hours. In these periods of time phenotypic studies, apoptosis detection and proliferative activity of cells were performed. A marked inhibition of OC proliferation was observed at the presence of 1.0 μM of 2-ME, with the lowest value obtained after 48 h. However, at the end of the cells' incubation, maximally reduced proliferative response of OC was attributed to 2.0 μM of 2-ME. Simultaneously with the time of incubation the amount of Thy-1-positive cells decreased slightly from 50.5±1.4% to 31.5±3.6%. Contrary to 1.0 and 2.0 μM of 2-ME, its lowest value (0.5 μM) reduced Thy-1 positive cells after 48 hours. The same 2-ME concentration resulted in the elevation of the cell number expressing CK-19. In turn, the marked increase of albumine-positive cells was observed under 1.0 μM of 2-ME and reaching 21.5±6.2 % and 23.9±5.7% after 48 and 72 hours, respectively. Although the presence of 1.0 μM of 2-ME dramatically intensified apoptosis within 24 h of cell culture, the percentage of apoptotic cells remained unchanged under 2.0 μM of 2-ME. When subjected to the carcinogenic effect of CDE, 2-ME exerts anti-proliferative, proapoptotic, and differentiation effects in OC.

Key words: *2-methoxyestradiol, oval cells, 17beta estradiol, hepatocellular carcinoma, proliferation, apoptosis, thymus cell antigen-1*

INTRODUCTION

2-ME, a natural endogenous metabolite of 17β-E_2, is a potent molecule that has shown promising anti-tumour activity in a number of cancers in both humans and animals (1, 2). Acting independently on oestrogens receptors, 2-ME has been reported to have unique properties including cytotoxic, anti-proliferative and apoptotic effects in the course of many kinds of tumours, including liver tumours (1, 3-5). The anti-proliferative activities of 2-ME have been attributed to several mechanisms, such as the influence on tubulin polymerization and depolymerisation, up-regulation of p53 and inhibition of superoxide dismutase enzymatic activity (5). Through activation of the death receptors family (DRs) (Fas, TNF receptor, DR4 and DR5), 2-ME induced and intensified the caspases cascade, resulting in G2-M cell cycle arrest in carcinogenic cells but not in normal cells (6). Additionally, the pro-apoptotic action of 2-ME has been linked to the stimulation of cellular reactive oxygen species (ROS) production, causing the release of cytochrome *c* from mitochondria and finally stimulation of caspases (7, 8).

Among the wide range of tumours, which are responsive to 2-ME action, the hepatocellular carcinoma (HCC) occupies the central position (1, 4). HCC is a major malignancy cancer which, according to many authors, may arise from transformed adult hepatic progenitor cells, called "oval cells" (9-11). These cells originated from the canals of Hering, are characterised by many cell-surface markers, including especially the hematopoietic markers Thy-1 (thymus cell antigen-1), which has become an accepted cell surface marker to sort hepatic oval cells (12-18). In addition, OC may also express both biliary epithelium (cytokeratine 19 (CK-19)) and hepatocyte lineages (albumine (ALB)). Thus, OC having hepatocytic and cholangiocytic markers, are believed to be the precursors of both of these hepatic epithelial lineages *in vivo* as well as *in vitro* appropriate condition (19-22). On the one hand the proliferation and differentiation of OC are important since they are responsible for liver regeneration, on the other hand, however, the excessive proliferative response and altered differentiation of OC can induce HCC development (9-11, 19, 23). Although recent observations have suggested the involvement of OC in rats' HCC, the molecular role of OC has not been fully understood yet.

Therefore feeding laboratory rats with a CDE diet as a stimulus of OC towards hepatocarcinogenesis, we sought to investigate the *in vitro* effects of 2-ME on proliferation,

differentiation and apoptosis of the primary culture of OC obtained from these animals.

MATERIALS AND METHODS

2-ME (Sigma Aldrich, Poland) was prepared as a stock solution of 10 mM in DMSO (Sigma Aldrich, Poland) and stored at –80°C (13). The compound was diluted in culture medium immediately before each experiment and obtained final concentration of DMSO (>0.5% (v/v), had no toxic effect on OC in experiments conducted.

Animals and experimental design

Ten-week old female Wistar rats (n=10) weighing 200–250 g were used in this study. Animals were kept in a room with controlled temperature and humidity and a 12 h light-dark cycle. To elucidate the response of OC we employed a CDE diet (0.1% w/w DL-ethionine) (MP Biomedicals, Inc), a common method for stimulating an OC response in rodent liver (9, 24, 25). Rats were fed a CDE diet for 6 weeks with free access to water throughout the experimental period. All experimental procedures were approved by the Local Ethics Committee on Animal Care at the University of Life Sciences.

After six weeks of taking CDE, OC were isolated as described previously with minor modifications (22, 26, 27). The liver was perfused *in situ* through the portal vein by Krebs-Ringer buffer (Sigma Aldrich, Poland) containing: a) EGTA, b) without Ca^{2+} and chelating agent and c) with type IV collagenase (Sigma Aldrich, Poland). After the perfusion, the liver was transferred to a mixture of DMEM/HAM'S F-12 (1:1 v/v) culture medium (Sigma Aldrich, Poland) and digested (1 h; 37°C) in PBS containing 0.1% collagenase, 0.1% protease E (Sigma Aldrich, Poland), 0.25% trypsin (Sigma Aldrich, Poland) and 0.004% DNase I (Sigma Aldrich, Poland). In the presence of protease E, after 1 hour incubation, all parenchymal cells are destroyed. After that, the mixture of non parenchymal cells (NPC) was decanted through a 70 µm nylon mesh, followed by a 40 µm-nylon mesh and centrifuged at 50×g for 3 minutes, to remove necrotic cells and debris. Finally, OC were purified by centrifugation through a discontinuous gradient of 20% and 50% Percoll™ (Amersham, Biosciences) in PBS at 1400×g for 20 min. This produced one thick band in the interface of Percoll fraction, which was collected separately and washed with an antibiotic-supplemented medium containing 10% foetal calf serum (PAA, Laboratories GmbH). Afterwards cells were plated on plastic dishes at 250,000 cells per well in 1000 µl of medium and incubated in a humidified atmosphere with 5% CO_2 at 37°C. The viability of the cell was estimated by the Trypan-blue exclusion method, ranged between 75–85%. The cultures were determined to be ~90% pure by immunocytochemical analysis for the oval cell antigens, hematopoietic Thy-1 and additionally CD34 markers (not published data). The contaminating cells were primarily endothelial cells, with occasional Kupffer cells (positive for ED1) and myofibroblast (positive for alpha smooth muscle actin (ASMA)) also apparent. No hepatocyte contamination was observed.

Finally, the adhered oval cells were cultured in DMEM/HAM'S F-12 (v/v) medium: without 2-ME (control), with 0.5; 1.0 and 2.0 µM of 2-ME respectively. After 24, 48, 72 h of incubation the OC and the media were collected for analysis.

Analytical procedures

1. Flow cytometry (*FCM*): cytometric analysis was performed in a Coulter Epics XL flow cytometer (Beckman Coulter). Cells used for cytometric examination were dissociated with trypsin and then washed twice with DMEM/HAM'S F-12 medium and adjusted to a concentration of 1×10^6 cells/ml. A 100 µl of cell suspension was used with each staining with monoclonal antibodies (28).

2. Cluster of differentiation 90 (*CD90*) (*anti Thy-1*) (*surface staining*): a 10 µl of CD90 FITC monoclonal antibody (Serotec) was added to each sample tube containing 10^5 cells in 100 µl. Then the sample tube was vortexed and incubated for 20 minutes at room temperature protected from light. Next a flow cytometric analysis was conducted (16).

3. CK 19 and ALB (*intracellular staining*): for cell membrane permeabilisation, a 100 µl of IntraPrep fixation reagent (Beckman Coulter) was added. Sample tubes were vortexed and then incubated in the dark for 15 minutes. Subsequently cells were washed with 4 ml of PBS; then the tubes were centrifuged at 300×g for 5 minutes. The supernatant was discarded, 100 µl of IntraPrep permeabilisation reagent was added, and probes were mixed gently without vortexing and incubated for 5 minutes. The direct staining procedure was applied in order to detect intracellular albumin. A 10 µl of monoclonal anti-albumin antibody (Serotec) was added to label 10^5 cells in 100 µl. After 20 minutes of incubation in the dark at room temperature the cells were washed in PBS, centrifuged, suspended in PBS+0.5% formaldehyde solution, and then analysed by flow cytometry. 10 µl of CK19 antibody (Serotec) was added and samples were incubated for 20 minutes in the dark at room temperature. After being washed with 4 ml of PBS the cells were labeled with a 10 µl aliquot of secondary FITC conjugated antibody and the samples were incubated for 20 minutes at room temperature and away from light. Next, the cells were washed with PBS and resuspended in PBS+0.5% formaldehyde solution, and finally analysed by flow cytometry.

4. Apoptosis detection: cells were washed in PBS, centrifuged, and the supernatant was discarded. Then the cell pellet was suspended in binding buffer and adjusted to a concentration of 5×10^5 cells/ml. Next 5 µl of annexin V-FITC solution was added (Kit Annex 100F, Serotec) and the cells were incubated for fifteen minutes at room temperature in the dark. 10 µl of propidium iodide was added and the probes were incubated for fifteen minutes in the dark at room temperature. Afterwards the suspension was centrifuged at 300×g for 5 minutes, resuspended in 200 µl of binding buffer, and analysed by flow cytometry.

5. Western blotting (*WB*): At various points in time OC were harvested in a lysis buffer (Complete, Roche) and cell lysates were separated by PAGE under a reducing condition. For immunodetection, proteins were transferred to polyvinylidene difluoride membranes (BioRad, Poland) and incubated for one hour at 20°C with PBS containing 5% low-fat milk. After washing, the membranes were incubated overnight with primary antibodies against Thy-1 (1/200) (Dako, Poland), CK-19 (1/100) (Dako, Poland), and albumin (1/1000) (Dako, Poland). The washed membranes were incubated with alkaline phosphatase-labeled goat, anti-mouse, or anti-rabbit immunoglobulins. (1/15,000) Protein bands were visualised by colorimetric detection. (GelDog XR System, BioRad, Poland) and analysed using Quantity One 1-D Analysis Software (22).

6. Cell proliferation assay: MTT analysis-assessment of cell proliferation was performed according to Wojcik *et al*. (22). Cultures were pulsed with 15 µl of the MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma Aldrich, Poland) for 3 hours at 37°C and solubilized to dissolve the dark blue crystals overnight. The optical density (OD) was measured at the wavelength of 600 nm and the results were expressed as a proliferation index (PI).

7. HPLC determination of 2-ME was performed according to Lakhani *et al.* with our modifications (29, 30). 2-ME were

extracted from the medium with dichloromethane and then analysed by high performance liquid chromatography, Beckman, Gold System USA with UV detection (220 nm). The medium concentration of 2-ME was calculated according to the following formula: $C_p=C_s/A_s \times A_p$, C_p – sample concentration of 2-ME; C_s – standard concentration; A_s – standard peak area; A_p – sample of 2-ME peak area.

8. *Statistical analysis*: Values examined by Student's t-test were compared using Microsoft Excel and STATISTICA.PL analysis software. All data represent the average and S.D. (means±S.D.) of twenty wells (two wells from each of ten independent isolations). A *p* value of ≤0.05 was considered significant.

RESULTS

As shown *Fig. 1B* we have found that isolated adhered OC grow best on plastic dishes in 2-ME free DMEM/HAM'S F-12 medium. After 72 h proliferating cells continue to expand and begin to form colonies (*Fig. 1B*). If OC were cocultured with 2.0 µM of 2-ME, they start to form clusters of cells already in first 24 h

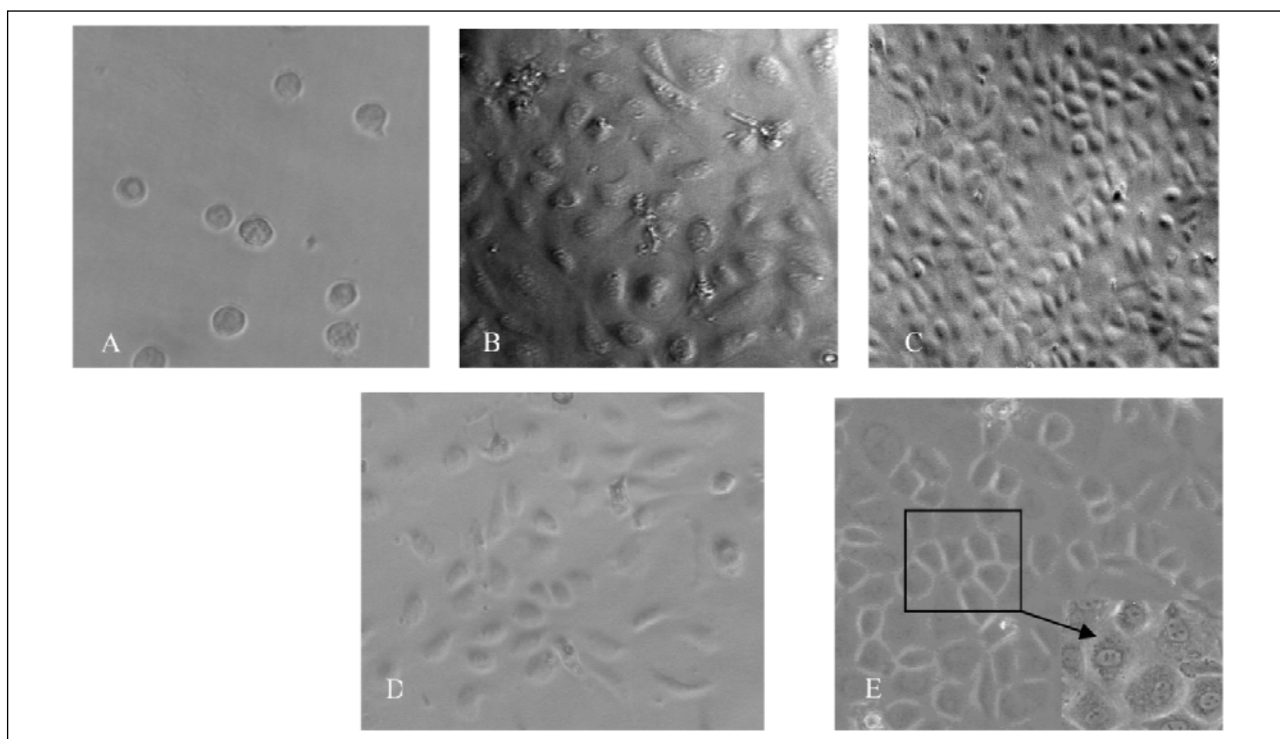

Fig. 1. Phase-contrast micrograph of oval cells: (A) oval cells immediately after isolation. The cells contain a small nucleus with scant cytoplasm; (B) 48 h and (C) 72 h of control OC culture; (D) 48 h and (E) 72 h of OC cultured with 2.0 µM of 2-ME. At this stage of experiment, cells with abundant cytoplasm and round nuclei are present (narrow). Under control as well as experimental condition, neither of cells had the hepatic myofibroblast-like appearance. Magnifications are original microscope magnification (200× and 400×).

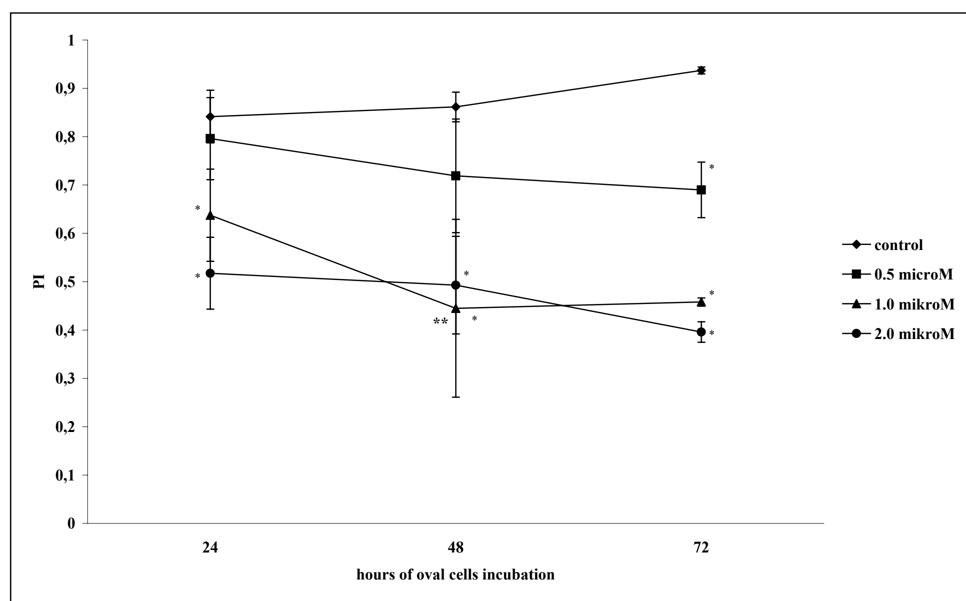

Fig. 2. Influence of 2-ME on proliferative activity of oval cells *in vitro*; (means±S.D.); *p≤0.05 when compared to control values in appropriate time of cell incubation. **significantly differences at p≤0.05 (*vs*. value obtained at 24 hours of cells incubation).

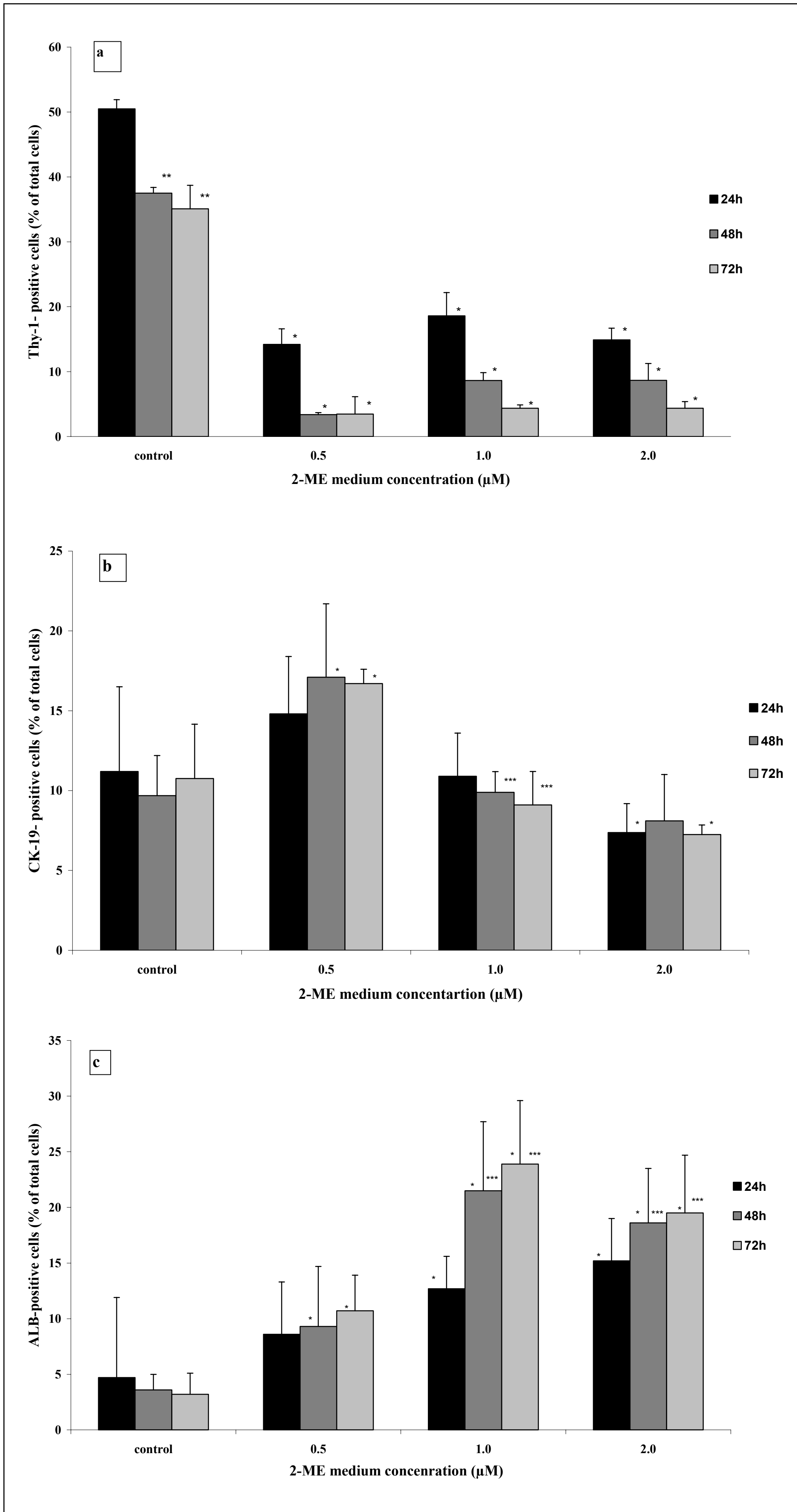

Fig. 3. Influence of 2-ME on number of Thy-1; (a), CK-19 (b) and ALB (c) positive cells analysed by FCM (I) and expression of these markers, detected by Western blot method.
*different from appropriate control values at p≤0.05; **significantly differences at p≤0.05 (*vs*. value obtained at 24 hours of control cells incubation); ***significantly differences at p≤0.05 (*vs*. value obtained at 48 and 72 hours of cells incubation).

of incubation. After 72 h, OC exposed to 2-ME, stopped to extend, build the tight colonies and appeared to become morphologically changed oval cells lines resembling small hepatocytes (*Fig. 1E*).

As shown in *Fig. 2* the control proliferation index of OC increased from 0.841±0.05 to 0.937±0.007 after 24 and 72 hours of incubation respectively. The lowest amount of 2-ME added to the culture medium resulted in a decrease of proliferative activity of OC, but without statistical significance. Markedly ($p \le 0.05$) inhibited cell proliferation was observed at the presence of 1.0 µM of 2-ME, with the lowest value obtained after 48 hours of incubation (PI=0.445±0.183). At this stage of our study, the difference between 1.0 and 2.0 µM of 2-ME in inhibitory effect, was lower than in 24 hours, but was maintained in the same level after 72 hours. However, at this point in the cell incubation period the most reduced proliferative response of OC was attributed to 2.0 µM of 2-ME (PI=0.396±0.02).

Concomitant with the control period of OC incubation the number of Thy-1 positive cells decreased slightly from 50.5±1.4% to 35.1±3.6%. The presence of 2-ME markedly diminished the percentage of Thy-1-expressed cells, especially after 48 and 72 hours of the experiment period (*Fig. 3a*). Contrary to 1.0 and 2.0 µM of 2-ME, the 2-ME's lowest value reduced significantly and permanently Thy-1 expressed cells. Conversely, the minimal 2-ME concentration resulted in the elevation of cell number expressed CK-19, which is the typical marker of cholangiocytes (*Fig. 3b*). The quantity of CK-19 positive cells averaged 17.1±4.6% and 16.7±0.9% after 48 and 72 hours, respectively. Exposure of cells to 1.0 µM of 2-ME caused far fewer ($p \le 0.05$) cells to be expressed CK-19, especially at the end of the experiment period. At the highest concentration of 2-ME, the amount of CK-19 positive cells did not exceed 8.1±2.9% of all cells. As shown in *Fig. 3c*, the incubation of OC without 2-ME resulted in low quantity of Alb expressed cells, which was had been maintained at a comparable level during the experiment. A marked increase of Alb-positive cells under 1.0 µM of 2-ME was observed and reached 21.5±6.2% and 23.9±5.7% after 48 and 72 hours, respectively. The highest value of 2-ME did not reduce the amount of Alb-positive cells significantly. However, the number of these cells was much greater than in the control group and 0.5 µM of 2-ME circumstances.

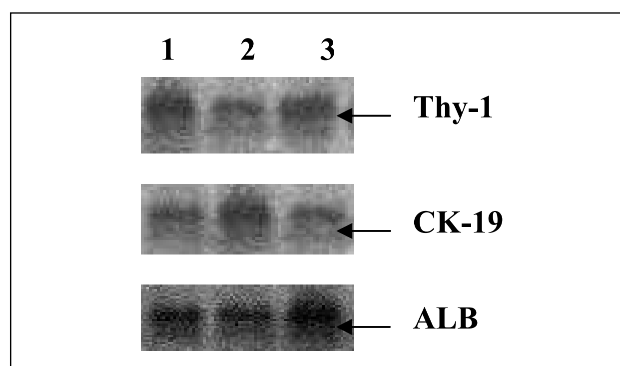

Fig. 4. Influence of 2-ME on number of Thy-1; (1) control condition after 48 h; (2) 0.5 µM of ME condition after 48 h and (3) 1.0 µM of ME condition after 48 hours of cells incubation.

Under control conditions the percentage of apoptotic cells did not exceed 5.7±1.2 (*Fig. 5*). After 24 hours of incubation with 1.0 and 2.0 µM of 2-ME, the number of apoptotic cells significantly increased to 42.1±4.3% and 48.9±4.8%, respectively. The presence of initial 1.0 µM of 2-ME in the culture medium dramatically intensified apoptosis within the next 24 hours of cell culture, and remained unchanged under 2.0 µM of 2-ME. After 72 hours the quantity of apoptotic cells was higher under 1.0 µM of 2-ME than 2.0 µM of 2-ME and reached 70±3.8% of the all cells.

We found that, in 1.0 µM of the 2-ME condition almost 50% of this metabolite was removed from the culture medium during the first 48 hours of cell incubation, while under other concentrations the metabolite did not exceed 19.0±2.6% and 24.7±2.4% (0.5 and 2.0 µM of 2-ME, respectively) (*Fig. 6B*). In turn, during the last 24 hours of the experiment we observed maximum disappearance (61.8±6.3%) of 2-ME under 2.0 µM of 2-ME.

DISSCUSION

In this study, we present evidence for the *in vitro* involvement of 2-ME, an endogenous 17β-E_2 metabolite, in suppression of proliferation, intensification of differentiation, and pro-apoptotic activity of hepatic OC, obtained from CDE-rats. The results of our study show that the response of

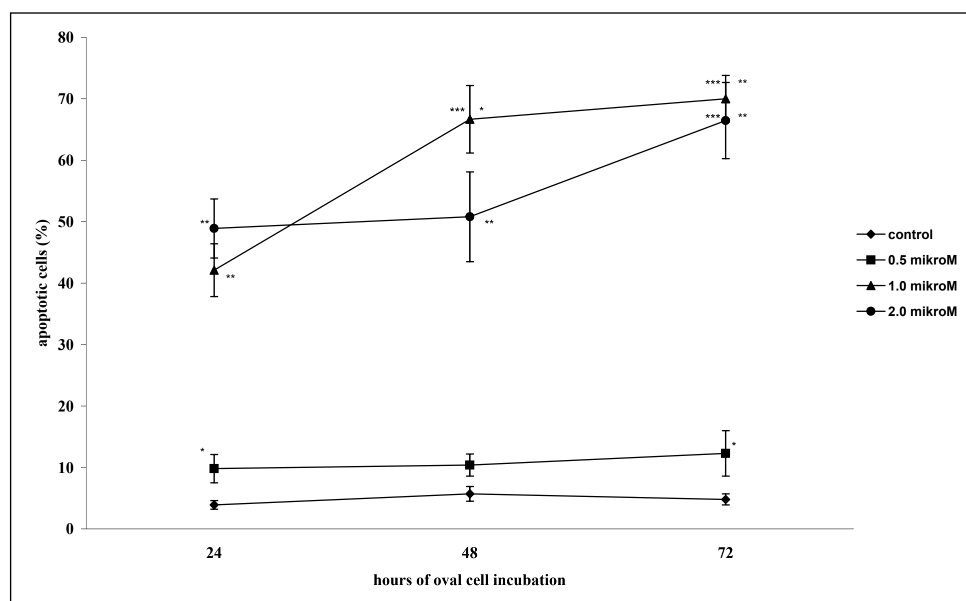

Fig. 5. Effect of 2-ME on percentage of apoptotic cells (means±S.D.); *$p \le 0.05$ when compared to control values in the same time of cell incubation; **$p \le 0.01$ when compared to control values in the same time of cell incubation; ***significantly differences at $p \le 0.05$ (*vs*. value obtained at 24 hours of cells incubation).

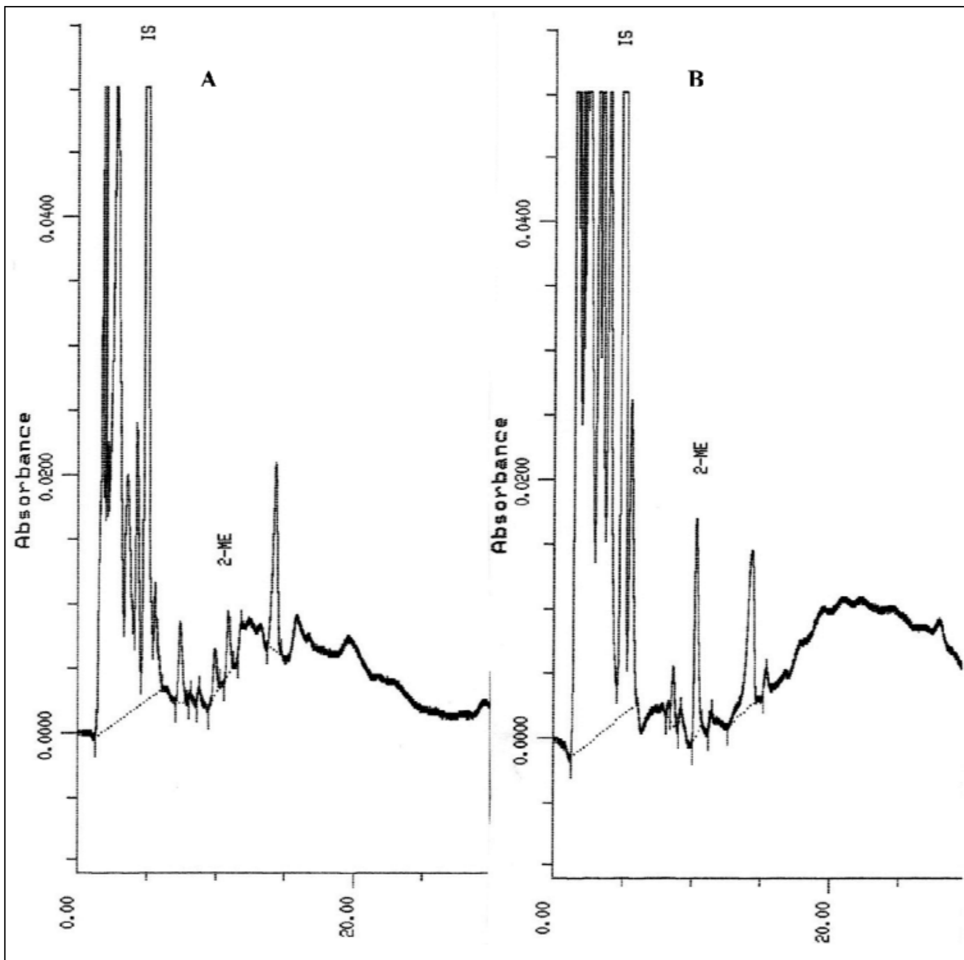

Fig. 6. HPLC analysis of 2-ME in culture medium obtained after 48 hours of cells incubation under 1.0 of µM of ME (A) and 2.0 µM of ME (B).

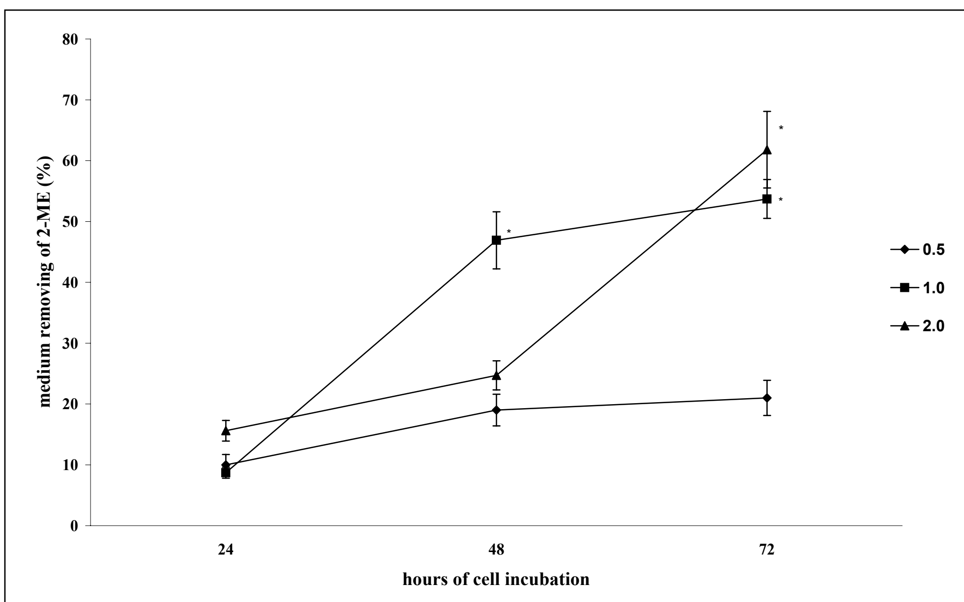

Fig. 7. HPLC analysis of 2-ME. Percentage of 2-ME removing from culture medium within experimental procedure. $*p \leq 0.05$ when compared to value obtained at 24 hours of cells incubation.

proliferative activity in rat OC differs depending on the dose of 2-ME. Although proliferation of OC under the initial lowest 2-ME culture medium concentration remained at a high level, the intermediate and highest concentration significantly reduced cells proliferation within the whole period of the experiment. The response of our OC clearly broadens the knowledge concerning numerous *in vitro* observations on other cells which prove that 2-ME exerts dose- and time-dependent effect on cell proliferation (1, 5, 8, 29). According to Thaver *et al.*, 2-ME might display a biphasic pattern on cell proliferation, namely that a low concentration has a stimulatory effects, whereas high concentration inhibitory ones (8). Correspondingly, *in vivo* studies have also revealed its influences on carcinogenic cells and proliferation in liver tumours (31). It is noteworthy that in

some cases 2-ME inhibited hepatic tumour progression predominantly by the production of a significantly high percentage of apoptotic cells (1, 4). With respect to OC, which are considered initial in HCC development, our results have shown that 2-ME in these cells also exerts pro-apoptotic effects. Each concentration of 2-ME used in our study intensified the process of apoptosis in these cells. However, only under 1.0 and 2.0 μM of 2-ME conditions did the number of apoptotic cells elevate dramatically. This pro-apoptotic action of 2-ME has been attributed to several mechanisms including up-regulation of p53 as well as the activation of intrinsic and extrinsic apoptotic pathways leading to induction of caspases cascade (5, 6). However, the results obtained by El Naga *et al.* on hepatocellular carcinoma cell lines showed that caspase-9 enzymatic activity was higher in 48-hour-treated cells than in cells treated with 2-ME for 72 hours (1). Our study produced similar results when the OC were exposed to 1.0 μM of 2-ME. Admittedly, under such conditions the percentage of anexine V-positive apoptotic cells was highest in 72 h (70±3.8%), but it was not much more than in 48 h (66.67±5.5%).

OC, as stem cells of the adult liver, can differentiate into hepatocytes and biliary epithelial cells, leading to liver regeneration when mature hepatocytes are damaged (12, 19, 32). On the other hand, OC can trigger hepatic cancer, especially when the process of normal differentiation is disturbed (10, 19, 32). There is strong interest by numerous investigators in identifying the hepatic OC with respect to the origin, mechanism of activation, and their final lineage destination (33, 34). It has been shown that, in addition to traditional OC markers (AFP, albumine, CK-18, CK-19, OV-6) these cells express the hematopoietic stem cell markers, among others Thy-1 (14, 34). Contrary, Dezso *et al.*, did not find Thy-1 expression in the hepatic oval/progenitor cells. Instead, authors recognised Thy-1 positive cells in subpopulation of stellate/myofibroblasts and suggest that use of Thy-1 as a cell surface marker for identification of OC shouldn't be recommended (35). However these observation was found in stem cell-mediated regeneration process but not hepatocarcinogenesis (4, 35). In respect to malignancies such as hepatocellular carcinoma, the Thy-1 expression appears to be restricted only to OC, which additionally have been characterised as a being functionally and phenotypically primitive cells (14, 17, 18, 34, 36).

Based on FLC and WB methods, we found that the amount of cells expressing the Thy-1 marker, which is characteristic for undifferentiated cells, was high only under the control conditions, *i.e*. OC cultured without 2-ME. The addition of 2-ME to the culture medium, independent of its dose, resulted in a small percentage of Thy-1 positive cells, which indicates that OC are differentiated. Similarly, Craig *et al*. presented data showing that Thy-1 expression decreased as the immature OC began to increase expression of markers of more committed/differentiated cell types (33). In our study, we found differences in albumin expression as well as the number of Alb-positive cells under the influence of 2-ME. Both augmented expression and its broader appearance in OC are a sign of their intensifying differentiation process. It must be emphasized that this effect was proportional to the concentration of 2-ME in culture medium. However, such an interpretation is not quite univocal, because another analysed factor, CK-19, predominantly rose at the lowest 2-ME medium concentration. Strong staining for Ck-19 may suggest that differentiation of cultured cells leads mainly to cholangiocytes.

2-ME exerts anti-proliferative, pro-apoptotic and differentiation effects on oval cells which have been subjected to the carcinogenic effect of CDE. The anti-proliferative and pro-apoptotic effects of 2-ME are highest at the intermediate doses. In addition, the pro-apoptotic action of 2-ME was progressively higher with the increased time of OC incubation. Based on the percentage of CK-19 positive cells, we have shown that at the lowest 2-ME medium concentrations, OC differentiates into cholangiocytes, but at high concentrations these cells create precursors for hepatocytes, confirmed by the elevation of cells expressed albumin.

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Conflict of interest: None declared.

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