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Modulation of cell proliferation and polyamine metabolism in rat liver cell cultures by the iron chelator O-trensox

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Abstract

The antiproliferative effects of the iron chelator O-trensox and the ornithine-decarboxylase (ODC) inhibitor alpha-difluoromethylornithine (DFMO) were characterized in the rat hepatoma cell line FAO, the rat liver epithelial cell line (RLEC) and the primary rat hepatocyte cultures stimulated by EGF. We observed that O-trensox and DFMO decreased cell viabilty and DNA replication in the three culture models. The cytostatic effect of O-trensox was correlated to a cytotoxicity, higher than for DFMO, and to a cell cycle arrest in G0/G1 or S phases. Moreover, O-trensox and DFMO decreased the intracellular concentration of spermidine in the three models without changing significantly the spermine level. We concluded that iron, but also polyamine depletion, decrease cell growth. However, the drop in cell proliferation obtained with O-trensox was stronger compared to DFMO effect. Altogether, our data provide insights that, in the three rat liver cell culture models, the cytostatic effect of the iron chelator O-trensox may be the addition of two mechanisms: iron and polyamine depletion.

Introduction

In genetic and secondary hemochromatosis, a progressive accumulation of iron in the body, particularly in the liver which is the main iron storage site, leads to iron overload which is toxic and can induce hepatocellular carcinoma development (Deugnier et al. 1993a; Hsing et al. 1995). In addition, iron is known to be necessary for cellular proliferation and we have previously demonstrated that iron overload increased DNA synthesis and mitotic index in rat hepatocyte cultures stimulated by Epidermal Growth Factor (Chenoufi et al. 1995). Moreover, several studies, using different models, show that iron is implicated in tumoral cell proliferation (Hann et al. 1988; Thompson et al. 1991) and the risk of developing an hepatocellular carcinoma appears to be related to the level and duration of iron overload (Tarao et al. 1992; Ballardini et al. 1994). This is probably why hemochromatosis is frequently complicated by an hepatocellular carcinoma. Moreover, iron depletion, obtained by different iron chelators, has been shown to inhibit proliferation of various cell lines. Indeed, we demonstrated previously that iron depletion, induced by desferrioxamine (DFO), the hydroxypyridinones (CP20, CP411) or O-trensox decreased DNA synthesis in both normal and transformed hepatocytes (Chenoufi et al. 1997, 1998; Rakba et al. 2000; Gaboriau et al. 2004).

Polyamines are ubiquitous chemical entities which play an important role in cell prolifera-

tion (Bettuzi et al. 1999) and in the synthesis of proteins and nucleic acids (for a review Moinard et al. 2005). Their effect on cell proliferation may involve their interaction with the activity of the nuclear phosphoprotein p53, which plays an essential role in the control of genes involved in cell proliferation and death (Moinard et al. 2005). The polyamine putrescine is synthesised from ornithine by a reaction catalysed by ornithine-decarboxylase (ODC), the limiting enzyme in polyamine synthesis (Coffino 2000). The two other polyamines derive from putrescine by successive attachment of two propylamine groups by the action of aminopropyl-transferases, namely spermidine and spermine synthetase. The propylamine group donor is *S*-adenosyl-*S*-methyl homocysteamine, derived from *S*-adenosyl-methionine (SAM) by the action of SAM-decarboxylase (SAMdc) (Jeevanandam & Petersen 2001). Spermine and spermidine can also be recycled respectively into spermidine and putrescine. These reactions require the formation of N_1-acetylspermine and N_1-acetylspermidine by the action of a cytosolic acetyl-CoA: spermidine/spermine N_1-acetyltransferase (SSAT) and polyamine oxydase (PAO) (Jeevanandam & Petersen 2001). Polyamines are metabolised into a very large number of derivatives by the action of various oxidases and acetyl-tranferases (Moinard et al. 2005). Moreover, the intracellular polyamine content is regulated by cellular uptake and efflux. The polyamine transport system (PTS) has been well characterized in *Escherichia coli* (Seidel & Scemama, 1997), but the gene for the transporter has not yet been identified in mammals. Finally, the intracellular polyamine content is the resultant of the simultaneous regulation of their synthesis, catabolism, uptake and elimination (Seiler et al. 1996; Persson et al. 1998).

Using three rat culture models, the aim of the present study was: (1) to analyse the effect of iron chelation and polyamine depletion on cell proliferation; (2) to check if iron chelation may also modify polyamine metabolism; (3) to study the influence of exogenous polyamines on cell proliferation. Iron and polyamine depletion were obtained respectively by using the hexadentate hydroxyquinoline iron chelator O-trensox and the ODC inhibitor alpha-difluoromethylornithine (DFMO).

Materials and methods

Cell cultures

Adult hepatocytes were isolated from 2 month old male Sprague–Dawley rats (250–300 g) by perfusing the liver with a liberase solution (0.007% liberase^{TM} RH purified enzyme blend from Boehringer Mannheim, Meylan, France; 0.075% $CaCl_2$ buffered with 0.1 M HEPES, pH 7.6 from Calbiochem, Meudon, France) according to Seglen's method with some modification (Seglen et al. 1972; Guguen et al. 1975). Rats were maintained on a 12 h light/dark cycle and were given diet and water *ad libitum*. All procedures involving animals were done in compliance with French rules and regulations. The hepatocytes were collected in Leibovitz medium containing per ml: glutamine (2 μmol), bovine serum albumin (2 mg). The cell suspension was filtered through gauze and allowed to sediment for 20 min to eliminate cell debris, blood and sinusoidal cells. The cells were then washed three times by centrifugation (700 rpm/min during 2 min), tested by Trypan blue dye exclusion for viability (always in the range of 85–95%). The hepatocytes were then suspended in a mixture of 75% Eagle's minimun essential medium and 25% medium 199 containing per ml: glutamine (2 μmol), $NaHCO_3$ (2.2 mg), penicillin (50 IU), streptomycin (50 μg), bovine insulin (5 μg), bovine serum albumin (1 mg) and fetal calf serum (0.05 ml). The medium, supplemented with hydrocortisone hemisuccinate (7×10^{-7} M), was changed 3–4 h after plating the hepatocytes and proliferation was obtained by adding 0.5 ng of human recombinant EGF per ml of medium at 24 h of culture.

The rat hepatoma cell line FAO used in this study was obtained by Deschatrette & Weiss (1974) and maintained by subculture in the following medium – 50% HAM F12 medium and 50% NCTC 135 medium (Eurobio, Les Ulis, France) – containing per ml: glutamine (2 μmol), $NaHCO_3$ (2.2 mg), penicillin (50 IU), streptomycin (50 μg) and fetal calf serum (0.05 ml).

The rat liver epithelial cell line (RLEC) used in this study was obtained as described in Morel-Chany et al. (1978). The RLEC cell line was maintained in our laboratory by subculture using the Williams medium containing per ml: $NaHCO_3$ (2.2 mg), glutamine (2 μmol), penicillin

(50 IU), streptomycin (50 μg) and fetal calf serum (0.07 ml).

For the experiments, the hepatocytes, the FAO and the RLEC cells were maintained in the same medium as above but deprived of fetal calf serum.

Cell cultures were maintained during 48 h in the control condition or in the presence of the iron chelator O-trensox or DFMO, an enzyme ODC inhibitor.

Iron and polyamine depletion

Iron and polyamine depletion were obtained respectively using the hexadentate hydroxyquinoline iron chelator O-trensox (Rakba et al. 2000) and DFMO purchased from Merrel Down Research Institute (Strasbourg, France).

Antiproliferative effect of O-trensox and DFMO

In order to evaluate DNA synthesis, [^{3}H] methyl-thymidine (Amersham, Les Ulis, France) was added to the culture medium at a final concentration of 1 μCi/ml during 24 h, before cell harvesting. DNA synthesis was evaluated by measuring [^{3}H] methyl-thymidine incorporation in the absence or in presence of O-trensox or DFMO. The total protein content of the cultures was evaluated by the method of Bradford (1976). Results were expressed as percent of control values.

Analysis of DNA content was performed by flow cytometry. After cell trypsinisation, DNA content was measured in cells using cycle test Plus kit (Becton Dickinson, San Jose, CA, USA). Cell cycle analysis was performed using FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) equipped with an argon laser (488 nm). Data analysis was done using the Modfit Software.

In vitro evaluation of cell viability (MTT assay)

The effect of O-trensox or DFMO on cell viability was determined using the formazan formation from 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Mosmann 1983). MTT measurements were done from absorbance readings at 535 nm using a 'Fusion^{TM}Packard' microreader.

Enzyme assay

Lactate dehydrogenase (LDH) activity was measured in both the culture medium and intracellularly as an index of cytotoxicity, employing an LDH kit (Roche Diagnostics, Mannheim, Germany). Extracellular LDH activity was measured on an aliquot of cell free medium obtained by centrifugation of the medium (2500 rpm/min during 5 min). Intracellular LDH activity was evaluated on hepatocytes previously lysed in phosphate saline buffer by sonication for 15 s and centrifuged as above. Experimental results were expressed in terms of LDH release into the medium given as a percentage of the total activity of the culture.

Statistical analysis

Results from at least four replicates were expressed as means $\pm$SD. Statistical analysis was performed using the Mann–Whitney test. The significant level was set at 0.05.

Results

Effect of O-trensox on cell viability, DNA replication and cell cycle distribution

The cytotoxicity of O-trensox was evaluated by measuring the extracellular LDH activity. After 48 h of incubation, 50 μM of O-trensox exhibited a significant toxic effect in the FAO cell line ($+150\%$; $P<0.001$), the RLEC cell line ($+275\%$; $P<0.001$) and the rat hepatocyte cultures ($+175\%$; $P<0.001$); Figure 1a–c.

SDH activity and thymidine incorporation were measured respectively as indexes of cell viability and DNA replication. After 48 h of incubation, 50 μM of O-trensox decreased cell viability in the FAO cell line (-30%; $P<0.001$), the RLEC cell line (-40%; $P<0.001$) and the rat hepatocyte cultures (-60%; $P<0.001$); Figure 1a–c. In the same experimental conditions, a clear drop in DNA replication was observed in the FAO cell line (-65%; $P<0.001$), the RLEC cell line (-80%; $P<0.001$) and the rat hepatocyte cultures (-90%; $P<0.001$); Figure 1a–c.

In the RLEC cell line, after 48 h of treatment with 50 μM of O-trensox, we observed an accumulation of the cells in G0/G1 phase (90% vs. 55% in the control; $P<0.001$; Figure 2a). In the primary rat hepatocyte cultures, the cell cycle arrest was observed in S phase (35% vs. 20% in the control; $P<0.001$; Figure 2b).

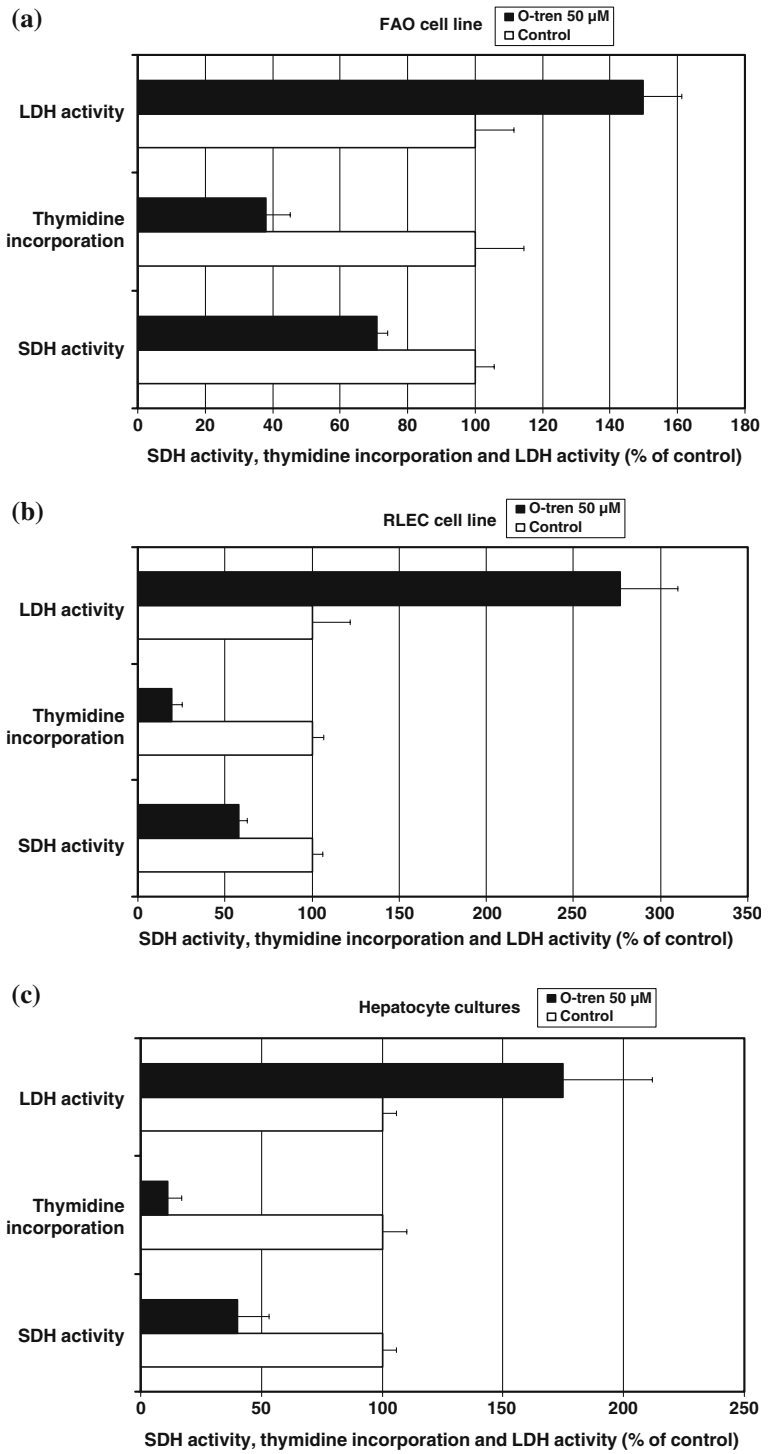

Figure 1. Cell viability, DNA replication and cytotoxicity in rat hepatoma FAO cells (a), liver epithelial cells RLEC (b) and hepatocytes (c) maintained in culture for 48 h in the absence (control) or presence of 50 μM O-trensox (O-tren 50 μM).

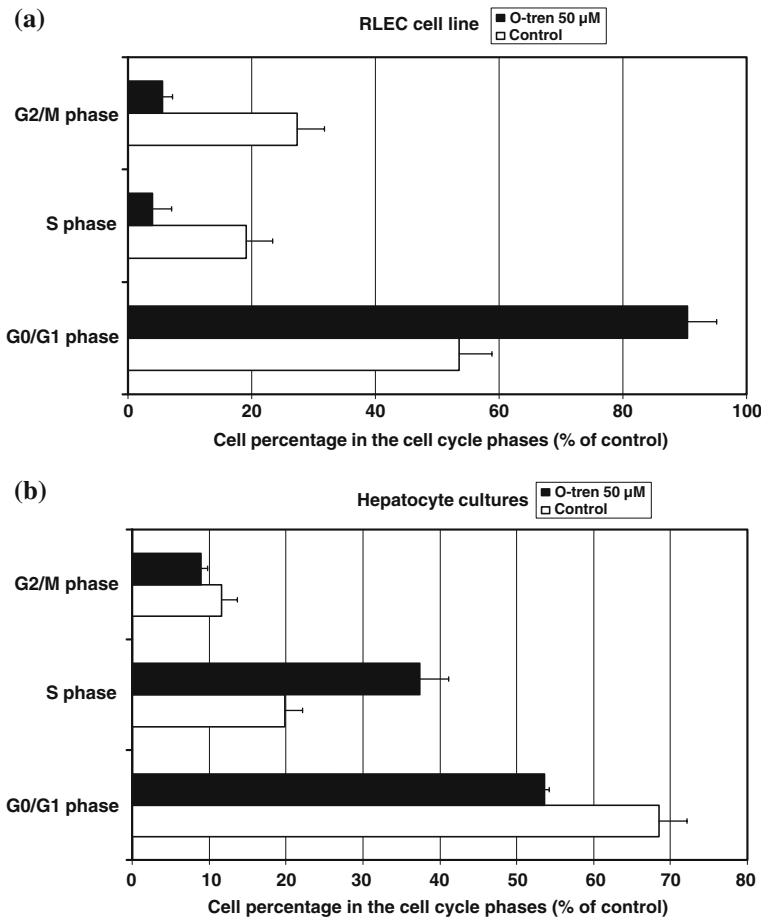

Figure 2. DNA content analysis measured by flow cytometry in rat liver epithelial cells RLEC (a) and hepatocytes (b) maintained in culture for 48 h in the absence (control) or presence of 50 μM O-trensox (O-tren 50 μM).

Effect of DFMO on cell viability and DNA replication

In the same cell cultures, 20 mM DFMO, after 48 h of exposure, did not exert any toxic effect except a slight increase in extracellular LDH activity in the FAO cell line (+135%; $P<0.05$; Figure 3a–c).

After 48 h of treatment, 20 mM of DFMO decreased also cell viability in the FAO cell line (−30%; $P<0.001$), the RLEC cell line (−30%; $P<0.001$) and the rat hepatocyte cultures (−20%, $P<0.01$); Figure 3a–c. In the same conditions, we observed also a DNA replication decrease in the FAO cell line (−45%; $P<0.001$), the RLEC cell line (−30%; $P<0.001$) and the rat hepatocyte cultures (−35%; $P<0.001$); Figure 3a–c.

Effect of O-trensox and DFMO on spermidine and spermine intracellular levels

Spermidine and spermine are the two polyamines easily measurable in our cell culture models since they are expressed at a sufficient high level. After 48 h of incubation with 50 μM of O-trensox a significant decrease in the spermidine intracellular concentration was observed in the FAO cell line (−40%; $P<0.001$), the RLEC cell line (−30%; $P<0.001$) and in the rat hepatocyte cultures (−15%; $P<0.05$); Figure 4a. In the same cultures treated with 50 μM of O-trensox, any significant effect on intracellular spermine level was observed (Figure 4b).

After 48 h of culture in the presence of 20 mM of DFMO, a 30% decrease in the intracellular

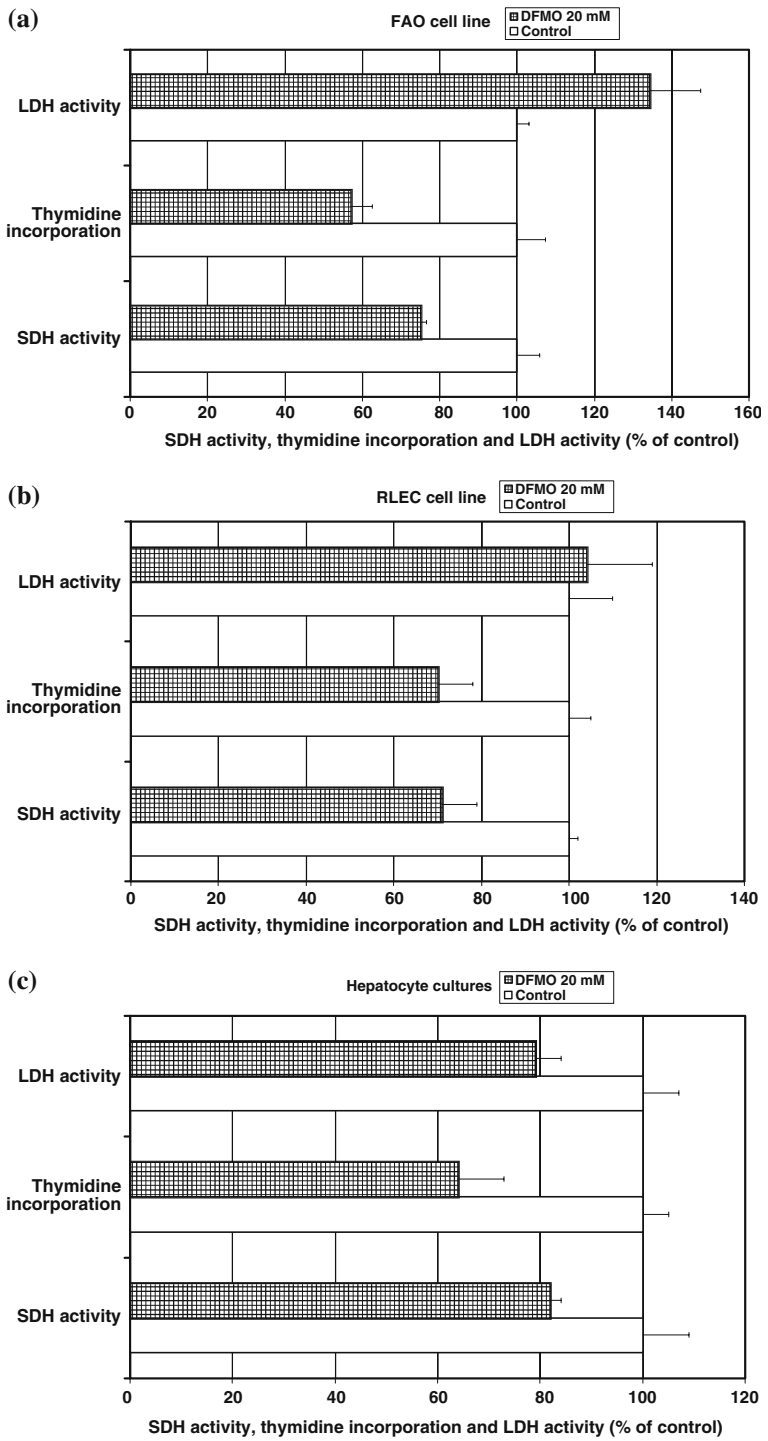

Figure 3. Cell viability, DNA replication and cytotoxicity in rat hepatoma FAO cells (a), liver epithelial cells RLEC (b) and hepatocytes (c) maintained in culture for 48 h in the absence (control) or presence of 20 mM DFMO (DFMO 20 mM).

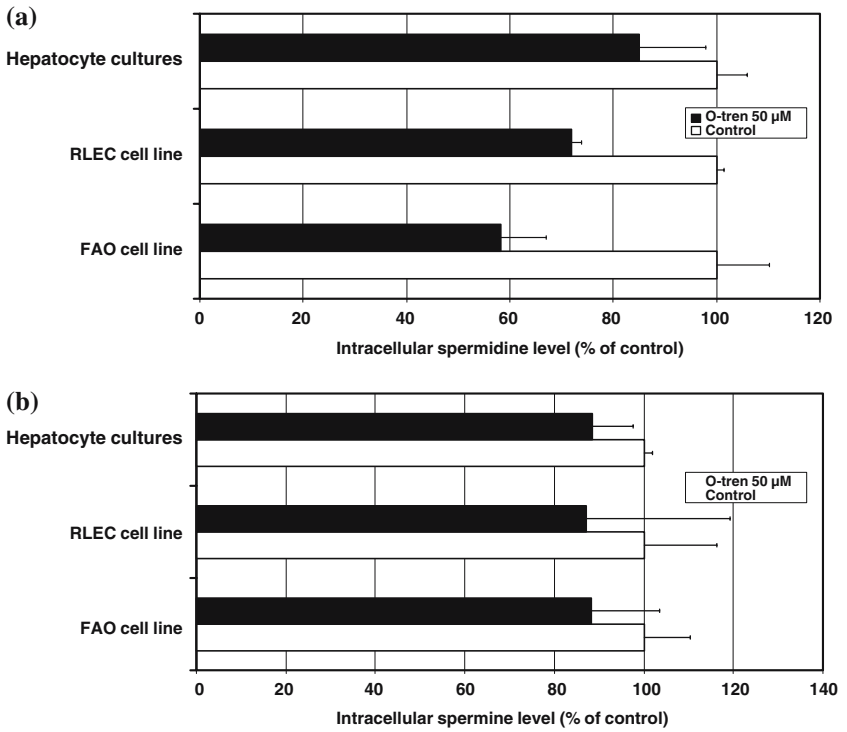

Figure 4. Intracellular levels of spermidine (a) and spermine (b) in rat hepatoma FAO cells, liver epithelial cells RLEC and hepatocytes maintained in culture for 48 h in the absence (control) or presence of 50 μM O-trensox (O-tren 50 μM).

spermidine level was measured in the FAO cell line, the RLEC cell line and the rat hepatocyte cultures ($P < 0.001$; Figure 5a). In the same experimental conditions, the spermine intracellular level did not change significantly (Figure 5b).

Effect of spermidine on DNA replication

After 48 h of incubation in the presence of 1 or 3 mM of spermidine, a dose dependent increase in DNA replication, ranging from +130% to +150%, was observed in the FAO cell line ($P < 0.001$; Figure 6).

Discussion

In some pathological conditions such as iron-overload diseases, iron which is usually bound to transferrin under physiological conditions, is found in plasma as non-transferrin bound iron (NTBI). This NTBI form has been demonstrated to exist mainly as complexed to citrate (Grootveld et al. 1989). NTBI, is assumed to play an important role in the formation of reactive oxygen species, such as the highly reactive hydroxyl radical which induces oxidative cell damages (Halliwell & Gutteridge 1986). Hepatocytes are crucially important not only in general intermediary metabolism, but also in iron metabolism. Hepatocyte iron uptake from non-transferrin sources may be extremely important in parenchymal iron loading which leads to oxidative damages (Brissot et al. 1985; Wright et al. 1986; De Silva et al. 1996). In hemochromatosis or secondary iron overload situations, this iron excess deposition is associated with fibrosis, cirrhosis, hepatocarcinoma, arthropathy and a dysmetabolic syndrom (Deugnier et al. 1993b; Turlin & Deugnier 2002). So, hepatocyte cell cultures appeared to be a useful *in vitro* model to investigate the protective efficiency of new iron chelators prior to their evaluation in *in vivo* models. Thus, our experimental models, the rat hepatoma cell line FAO, the RLEC and the primary rat hepatocyte cultures stimulated by EGF appeared to be suitable models to investigate the efficiency of the iron chelator O-trensox and the ODC inhibitor DFMO on cell proliferation and polyamine metabolism.

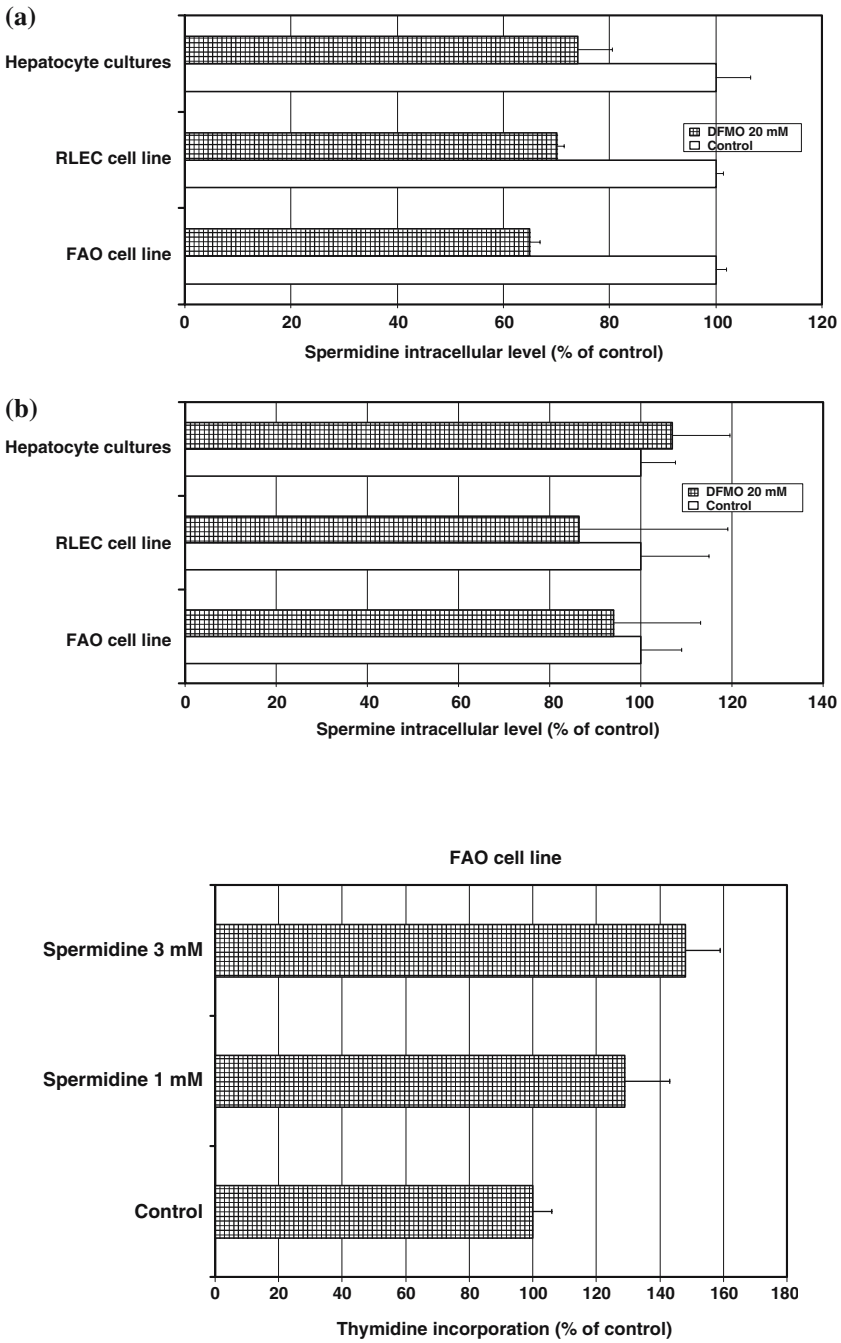

Figure 6. DNA replication in rat hepatoma cell line cultures maintained 48 h in the presence of increasing concentrations of spermidine (1–3 mM).

In our three experimental models, we observed that O-trensox decreased significantly cell viability, from 30% in the FAO cell line to 60% in the primary hepatocyte cultures stimulated by EGF. In a parallel manner, a drop in DNA replication ranging from 65% in the FAO cell line to 90% in the primary hepatocyte cultures was induced by O-trensox. However, this cell proliferation inhibition was correlated to a cytotoxic effect of the chelator in the three culture models. So, the present results,

confirmed the cytostatic effect of O-trensox previously observed in the human hepatoma cell line HepG2 (Rakba et al. 2000). Moreover, the cytostatic effect of O-trensox was associated to a block in the G0/G1 phase of the cell cycle for the FAO cell line and in the S phase for the hepatocyte cultures. These results confirmed our previous observations indicating that the cell cycle arrest depends of the chelator but also of the cell model for a same chelator (Chenoufi et al. 1997, 1998; Rakba et al. 2000; Gaboriau et al. 2004).

In the same three culture models, the ODC inhibitor DFMO decreased also cell viability, 30% in the FAO and the RLEC cell lines and 20% in the hepatocyte cultures. This decrease in cell viability, observed without significant cytotoxic effect in the RLEC cell line and the hepatocyte cultures, was also correlated to an inhibition of DNA replication ranging from 30% to 45% in the three cell models. In the three experimental culture models, DFMO decreased also the intracellular level of spermidine without affecting spermine. These results demonstrate a cytostatic rather than a cytotoxic effect of DFMO in the liver cell cultures and confirm the antimitotic property of this drug observed in previous work (Mamont et al. 1976; Poso & Pegg 1982). Many studies using DFMO have also shown an effective depletion of spermidine while spermine content was often unaffected (Gerner & Mamont 1986). The present data confirm the inhibiting action of DFMO on polyamine metabolism which was probably mediated in our hepatocyte culture models by an inactivation of the enzyme ODC. This polyamine depletion leads to cell growth arrest, an effect that can be reversed by exogenous spermidine in our experimental conditions. Moreover, it is already reported that growth arrest of cells in response to DFMO occurs predominantly at the G1 phase of the cell cycle (Li et al. 2002) but an increase of S phase length has also been observed in CHO cells (Fredlund & Oredsson 1996). Thus, it appeared that DFMO blocks likely the cell cycle at the same phases as O-trensox in the present models.

In the present data, we observed also that O-trensox inhibits DNA replication, blocks the cell cycle at the G0/G1 or S phase and decrases the intracellular concentration of spermidine without modifying spermine expression. This effect of O-trensox on polyamine metabolism was quite similar to the result obtained by DFMO and let us think to a possible inhibition of ODC by the chelator. However, O-trensox exerts a cytostatic effect which was about two times higher than for DFMO. Since iron and polyamines play a critical role in cell proliferation, the antiproliferative effect of O-trensox may be the consequence of two mechanisms: iron and polyamine depletion.

In conclusion, we have demonstrated in the rat hepatoma cell line FAO, the RLEC and the primary rat hepatocyte cultures stimulated by EGF, that iron and/or polyamine depletion decrease cell proliferation. Moreover, in these rat cell culture models, the iron chelator O-trensox antiproliferative effect may be the additive consequence of iron and polyamine depletion.

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