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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induced autophagy in a bovine kidney cell line

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ABSTRACT

The administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to a variety of cultured cells may alter their ability to proliferate and die. In a previous study we demonstrated that TCDD induced proliferation in Madin-Darby Bovine Kidney (MDBK) cells where no signs of apoptosis were observed, but herein, analysis of MDBK cell morphology, in a large number of exposed cells, revealed some alterations, as expanded cytoplasm, an increase of intercellular spaces and many pyknotic nuclei. Hence, the aim of the current study was to elucidate the influences of dioxin on cell proliferation and cell death. We found that dioxin increased proliferation, as well as, activated cell death with autophagy, as we detected by increased amount of LC3-II, an autophagosome marker. Furthermore, formation of acidic vesicular organelles was observed by fluorescence microscopy following staining with the lysosomotropic agent acridine orange. These results were accompanied by down-regulation of telomerase activity, bTERT and c-Myc. Key tumor-suppressor protein p53 and expression of cell cycle inhibitor p21Waf1/Cip1 were activated after TCDD exposure. These changes occurred with activation of ATM phosphorylation in the presence of a decrease in Mdm2 protein levels.

Taken together, these results support the idea that TCDD in MDBK cells, may exert its action, in part, by enhancing cell proliferation, but also by modulating the incidence of induced cell death with autophagy.

1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), commonly known as dioxin, is a highly toxic and widely distributed environmental contaminant. Many studies have established that TCDD is persistent and bioaccumulative in the body and cause a wide range of tissue- and species-specific toxic effects such as carcinogenicity, teratogenicity, immune suppression and endocrine disruption (DeVito et al., 1995; Mandal, 2005). Humans are generally exposed to TCDD, which is incorporated into food, drinking water, soil, dust, smoke and air (Mandal, 2005). In the last years, levels of TCDD, exceeding the European Union tolerance, were detected in dairy products and milk from cow and water buffalo, raised on some areas of Campania Region (South Italy) (Diletti et al., 2003; Santelli et al., 2006). The mechanism of action by which TCDD exerts the biochemical effects on vertebrate species is through activation of the Aryl hydrocarbon Receptor (AhR), a ligand-activated basic helix–loop–helix transcription factor, and a member of the PER-ARNT-SIM (PAS) superfamily of transcription factors. Binding of AhR/ARNT leads to changes including induction of the cytochrome P-450 1A1 (Mandal, 2005). Although the molecular mechanisms for carcinogenicity by TCDD have not been elucidated so far (Mandal, 2005; Knerr and Schrenk, 2006), in 1997, TCDD was classified as a cancer promoter (IARC, 1997). This classification is based on studies performed in laboratory animals which have shown that TCDD promotes the formation of neoplastic lesions in the liver, lung, oral mucosa and skin. For instance, in mouse, TCDD exposure induces continued proliferation of epithelial cells within the skin tissue in a tissue-dependent manner (Ray and Swanson, 2009).

In a previous study, we showed that TCDD induced cell proliferation in Madin-Darby Bovine Kidney (MDBK) cells (Fiorito et al., 2008a). Furthermore, in a recent study, we demonstrated that MDBK cells exposed to TCDD induced a concomitant Iron Regulatory Protein (IRP) 1 down-regulation and IRP2 up-regulation

Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; MDBK cells, Madin-Darby bovine kidney cells; LC3, microtubule associated light chain protein 3; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; AVOs, acidic vesicular organelles; bTERT, bovine telomerase reverse transcriptase.

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thus determining a marked enhancement of transferrin receptor 1 expression and a response in ferritin content which impairs cellular iron homeostasis, leading to changes in the labile iron pool extent (Santamaria et al., 2011). Deregulation of cell proliferation/differentiation processes induced by TCDD in MDBK cells coupled to an iron excess, as previously described (Fiorito et al., 2008a; Santamaria et al., 2011), could be associated to the neoplastic transformation of the bovine cell and to cancer development (Toyokuni, 2009), as supported by a recent work which demonstrates a pro-oncogenic activity for IRP2 to induce the growth of tumor xenografts (Maffettone et al., 2010).

In the present study, by analyzing MDBK cell morphology by Giemsa staining, in a large number of cells exposed to TCDD, some death signs were detected. Cell death is associated with at least three morphologically distinct processes that have been named apoptosis, necrosis and autophagic cell death (Kroemer and Levine, 2008). Our previous study demonstrated that different concentration of TCDD (0.01–100 pg/ml) induced no signs of apoptosis in bovine cells, in fact neither chromatin condensation by acridine orange staining nor DNA laddering were observed (Fiorito et al., 2008a), furthermore Western blot analysis showed no activation of caspases 8, 9 or 3 (data not shown). During tumor development, autophagy has paradoxically been reported having roles in promoting both cell survival and cell death (Rosenfeldt and Ryan, 2011). Autophagy is a homeostatic "self-eating" process that has been conserved among eukaryotic cells, and which involves the digestion of cytoplasmic components via the lysosomal pathway (Klionsky, 2007). The autophagic pathway begins with the formation of the autophagosome, which consists of cytoplasmic material sequestered inside a double membrane vescicle. Then, the autophagosome fuses with the lysosome and the cytoplasmic material is digested by hydrolases (Demarchi et al., 2006). Several studies have established a link between autophagy and tumor development. The last one is caused by the successive acquisition of mutations and epigenetic changes that prevail reliable mechanisms such as cell death and cell cycle arrest which normally prevent tumor development. Indeed, many autophagy genes have been found to be inactivated in human cancers (Rosenfeldt and Ryan, 2011). Based on these observations, in the current study we examined if TCDD induces autophagy in MDBK cells. So, herein, in MDBK cells exposed to different concentrations of TCDD, at various times after exposure, we analyzed the levels of LC3-II, as autophagosome marker, by Western blot and immunofluorescence. Furthermore, detection of acidic vesicular organelles (AVOs), as autophagic signs, was carried out by using lysosomotropic agent acridine orange.

A variety of tumor suppressor mechanisms exist to protect cells from cancer (Campisi, 2005), as the inhibition of telomerase activity and up-regulation both of key tumor-suppressor p53 and of cell cycle inhibitor p21 (Caino et al., 2009). For example, it has been shown, in a hepatocellular carcinoma cell line, that an anti-cancer compound induced its activity by involving both inhibition of telomerase and autophagy (Ko et al., 2009). The enzyme responsible for telomere elongation, called telomerase, is a cellular reverse transcriptase that catalyzes the synthesis and extension of telomeric DNA repeats (Greider and Blackburn, 1989). Structurally, telomerase is a ribonucleoprotein enzyme complex that contains a catalytic protein subunit and an essential RNA component. The catalytic protein subunit contains the telomerase reverse transcriptase activity. In humans, the telomerase reverse transcriptase (hTERT) catalytic subunit is a protein which is actively up-regulated by c-Myc (Wu et al., 1999). Telomerase activity is specifically expressed in immortal cells, cancer and germ cells where it compensates for telomere shortening during DNA replication and thus stabilizes telomere length (Autexier and Greider, 1996). A variety of cell lines and malignant tumors have been found to express high levels of telomerase activity (Greider, 1996), suggesting that telomerase activation may be a critical step in cell immortalization and oncogenesis. Thus, herein, we evaluated the effects of dioxin on telomerase activity by TRAP assay, and we analyzed bovine TERT (bTERT) and c-Myc by Western blot.

In response to oncogenic activation or other forms of stress, the "cellular gatekeeper" p53 acts, through transcription-dependent and -independent mechanisms, in order to transmit a variety of stress-inducing signals to different antiproliferative cellular responses (Zilfou and Lowe, 2009). p53 controls autophagy in an ambiguous fashion, in particular, autophagy induction by p53 may either contribute to cell death (Crighton et al., 2007) or constitute a physiological cellular defense response (Amaravadi et al., 2007). A p53-dependent induction of autophagy has been documented by several groups, p53 can transactivate an autophagy inducing gene, DRAM, which codes for a lysosomal protein (Maiuri et al., 2009). Recently, in several non-transformed or malignant human cell lines, it was observed that inactivation of p53 by deletion, depletion or inhibition also can trigger autophagy, both in vitro and in vivo (Maiuri et al., 2009). Herein, we examined p53, phospho-p53 (p-p53) protein levels and the expression of proteins that are stimulated by p53, such as p21. Mdm2 is the main regulator of p53 and, DNA damage signaling kinases, such as ataxia telangiectasia mutated (ATM), may target both p53 and Mdm2 (Maya et al., 2001). Therefore, in bovine cells exposed to TCDD, we evaluated also ATM phosphorylation and Mdm2 protein levels. In this study, within a research project aimed to investigate the dioxin effects on cattle, we evaluated the molecular effects of TCDD on cell proliferation and cell death using MDBK, an useful and standardized in vitro model for studying TCDD exposure in mammalian cells, at doses and times herein reported (Fiorito et al., 2008a,b, 2010; Santamaria et al., 2011).

2. Materials and methods

2.1. Materials

MDBK cells (American Type Culture Collection, CCL22) were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 2% foetal calf serum (FCS), 1% L-glutamine, 1% penicillin/streptomycin, 0.2% sodium pyruvate and 0.1% tylosin, a macrolide-class antibiotic. Cells were maintained in an incubator at 37 °C (in 5% CO_2/95% air). This cell line was maintained free of mycoplasma and of bovine viral diarrhoea virus. We used TCDD, 10 μg/ml in toluene (Supelco, 48599). TCDD was initially diluted to give a 10,000 pg/ml stock solution by mixing with DMEM. This stock solution was then diluted to give working solutions of 0.01, 1 and 100 pg/ml (from 3.1×10^{-14} to 3.1×10^{-10} M) in DMEM, which were added to cultures, according to Fiorito et al. (2008a,b, 2010) and Santamaria et al. (2011). Proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) (Calbiochem, 474790) was dissolved in Me_2SO and added to the media to a final concentration of 20 μM, according to Li and Elsasser (2005).

All other chemicals were of the highest commercially available purity.

2.2. Cells and TCDD exposure

MDBK cells, at confluency, were washed with DMEM and then exposed or not to different concentrations of TCDD (0.01, 1 and 100 pg/ml) for 4, 8, 12, 24, 36 and 48 h and then processed.

2.3. Cell viability and proliferation

Cell viability was evaluated by MTT assay, as we previously described (Fiorito et al., 2008a; Santamaria et al., 2011). The principle of this method is that 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma, M2003), a soluble tetrazolium salt, is converted to insoluble formazan by active mitochondrial dehydrogenases of living cells. Such conversion from yellowish soluble tetrazolium to purple formazan can be assayed spectrofluorimetrically. MDBK cells (2×10^4 cells/well), in 96-well plates, at confluency, were exposed or not at TCDD (0.01, 1, or 100 pg/ml) and incubated for 4, 8, 12, 24, 36 and 48 h. MTT (5 mg/ml) was added to cells and after further 4 h of incubation, the medium was removed and replaced with DMSO to solubilize the MTT formazan crystals. The spectrophotometer absorbance at 570 nm was determined. Data are calculated as a percentage

of the control, and results are the mean ± S.E.M. of four independent experiments performed in duplicate.

To evaluate cell proliferation, MDBK cells (30×10^4 cells/flask) were plated in 25 cm^2 flask and, after reaching the confluence, were exposed or not at TCDD (0.01, 1, or 100 pg/ml) and incubated for 4, 8, 12, 24, 36 and 48 h. At different times of incubation, adherent cells, removed from the culture substrate by treatment with trypsin–EDTA solution, were mixed with cells previously collected by centrifugation in supernatant from the same flask and resuspended at an adequate concentration in PBS. Thus, the entire cell population of the culture was reconstituted for determination of the cell number by counting them in a Burker chamber. Data are expressed as a percentage of the control, and results are the mean ± S.E.M. of four independent experiments performed in duplicate.

Moreover, cell proliferation/viability was assessed by Trypan blue (TB) exclusion test. Cells were collected by trypsinization, at the indicated times after TCDD exposure, and an aliquot of the cell suspension was mixed with an equal volume of 0.2% Trypan-blue (Sigma) in 1 × phosphate-buffered saline (PBS). After 10 min, cells were counted using a Burker chamber under a phase contrast microscope. Cell proliferation/viability was calculated as percentage of live cells over the total cells number, and results are the mean ± S.E.M. of four independent experiments performed in duplicate. Stained blue cells with damaged cell membrane represented non-viable cells.

2.4. Nuclear staining with propidium iodide

MDBK cells, grown on coverslips, at the indicated times after TCDD exposure, were fixed in ice-cold methanol/water (8/2, v/v), at −20 °C for 30 min, washed with 1 × PBS and stained with propidium iodide (2.5 g/ml in PBS) for 4 min. The coverslips were mounted onto glass slides with PBS/glycerol (1/9, v/v) containing 0.1% (w/v) phenylenediamine. The stained cells were then examined at a fluorescence microscope. Nuclei were stained with propidium iodide to visualize chromatin condensation or necrosis.

2.5. Examination of cell morphology

Cell morphology was examined by light microscopy following Giemsa staining. Briefly, monolayers of MDBK cells (10^5 cells per well), in 16-well culture chambers, were exposed or not at TCDD (0.01, 1, or 100 pg/ml) and incubated at 37 °C. After 4, 8, 12, 24, 36 and 48 h of exposure, cells were washed twice with PBS. Cells were fixed with 95% ethanol, drained and dried. Afterward, cells were stained with a 5% Giemsa solution (Merck, 109203). After 30 min cells were rinsed with tap water and H_2O. The slides were mounted in Entellan (Merck, 100869) and coverslipped. Light microscopic studies and photomicrographs observations were carried out. Non-apoptotic cell death was identified by the criteria described previously (Kroemer and Levine, 2008; Zakeri et al., 2008; Lamparska-Przybysz et al., 2005). Percentage of cell death was determined by counting a total of >500 cells a sample.

2.6. Protein extraction and Western blot analysis

MDBK cells in 75 cm^2 flask, at confluency, were exposed at different concentrations of TCDD (0.01, 1 or 100 pg/ml). After 4, 8, 12, 24, 36 and 48 h of exposure, adherent cells were washed twice with PBS and removed from the flask by treatment with trypsin–EDTA solution. Then cells were mixed with cells previously collected by centrifugation in supernatant from the same flask and resuspended at an adequate concentration in PBS. The pellets, obtained by centrifugation, were stored at −20 °C. Cells were homogenized directly into lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 0.5 mM sodium orthovanadate, and 20 mM sodium pyrophosphate). The lysates were clarified by centrifugation at 14,000 rpm × 10 min. Protein concentrations were estimated by an assay (Bio-Rad) and boiled in Laemmli buffer [0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue] for 5 min before electrophoresis. Proteins were subjected to SDS-PAGE (12.5% polyacrylamide). After electrophoresis, proteins were transferred to nitrocellulose membranes (Millipore, Immobilon); complete transfer was assessed using pre-stained protein standards (Bio-Rad). After blocking with Tris–buffered saline–BSA [25 mM Tris (pH 7.4), 200 mM NaCl, and 5% BSA], the membrane was incubated with the primary antibodies. The following antibodies, dissolved in 5% bovine serum albumin-TBST, were used: anti-p53 MAb (Santa Cruz Biotechnology, sc-99) (1:1000), anti-$p21^{Waf1/Cip1}$ PAb (Santa Cruz Biotechnology, sc-397) (1:1500), anti-c-Myc MAb (Santa Cruz Biotechnology, sc-70463) (1:2000), anti-Mdm2 PAb (Santa Cruz Biotechnology, sc-812) (1:1000), anti-ATM PAb (Santa Cruz Biotechnology, sc-1214) (1:1000) and anti-phospho-ATM MAb (Santa Cruz Biotechnology, sc-47739) (1:1000). To detect bTERT, we selected hTERT as the target molecule due to its 80% nucleotides sequence homology with bovine TERT (Iqbal et al., 2008). Antibodies were used for hTERT PAb (Rockland, 600-401-252) (1:1000); anti-phospho-p53 PAb (Ser315) (Cell Signaling, 2528) (1:1000); anti-β-actin MAb (Sigma, A5316) (1:7500). Rabbit polyclonal microtubule-associated protein light chain 3 (LC3) antibody PAb was used (Novus Biologicals, NB100-2220) (1:1500). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:1000) (at room temperature), and the reaction was detected with an enhanced chemiluminescence system (Amersham Life Science). The images of Western immunoblot specific bands on X-ray films were imported into a computer by a scanner and captured as digital TIFF images. The results were plotted in a graph after densitometry analysis of the blots obtained. Moreover, both p-p53/p53 and p-ATM/ATM ratios in control groups or in TCDD exposed groups were obtained by densitometric evaluation.

2.7. Immunofluorescence and confocal analysis

Monolayers of MDBK cells grown on glass coverslip, at confluency, were washed with DMEM and exposed to different concentrations of TCDD (0.01, 1 and 100 pg/ml) for 4, 8, 12, 24, 36 and 48 *h*. After being incubated with TCDD, cells were washed with PBS and then fixed with paraformaldehyde (4%, w:v). After rinsing in PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 1 min and blocked in 4% BSA in PBS for 30 min. This was followed by incubation in rabbit polyclonal microtubule-associated protein light chain 3 (LC3) antibody (1:200) (Novus Biologicals, NB100-2220), for 24 h at 4 °C in a humidified chamber. After 3 washes in PBS, the cells were incubated in anti-rabbit IgG conjugated to Cy3 (1:150) (Sigma, C2306) for 1 h at room temperature. Finally, cells were rinsed in PBS, coverslipped and examined with a confocal microscope (Zeiss).

2.8. Detection of acidic vesicular organelles with acridine orange

Autophagy is the process of sequestrating cytoplasmic proteins into the lytic component and characterized by the formation and promotion of AVOs. To detect the AVO in TCDD-treated cells, we performed the vital staining with acridine orange. In acridine orange-stained cells, the cytoplasm and nucleolus fluoresce bright green and dim red, whereas acidic compartments fluoresce bright red as described previously (Traganos and Darzynkiewicz, 1994; Chiu et al., 2009). The intensity of the red fluorescence is proportional to the degree of acidity of the cellular acidic compartment. Therefore, we could measure a change in the degree of acidity of their cellular acidic compartment. Monolayers of MDBK cells grown on glass coverslip, at confluency, were washed with DMEM and exposed to different concentrations of TCDD (0.01, 1 and 100 pg/ml) for 4, 8, 12, 24, 36 and 48 *h*. After exposure to TCDD, cells were washed with PBS and stained with acridine orange (Sigma, A6014) at a final concentration of 1 μg/ml for 15 min.

Stained cells were placed on a microscope slide and observed under UV with a fluorescence microscope (Nikon).

2.9. Detection of telomerase activity

Telomerase activity was evaluated by Telomeric Repeat Amplification Protocol (TRAP) assay using the TeloTAGG Telomerase PCR ELISA^{PLUS} (Roche Applied Science, 12013789001) which is an extension of the original method described by Kim et al. (1994), as our group previously described (Pagnini et al., 2006). Briefly, cell extract ($1–3 \times 10^5$ cell equivalents) were employed in the first step, in which telomerase adds telomeric repeats (TTAGGG) to the 30-end of the biotin-labeled synthetic P1-TS primer. These elongation products, as well as, the internal standard (IS) were amplified by PCR. In the second step the PCR products were split into two aliquots, denatured and hybridized separately to digoxigenin-(DIG)-labeled detection probes, specific for the telomeric repeats and for the IS, respectively. Results obtained by densitometric analysis were normalized upon the data of IS and expressed as relative telomerase activities (RTA).

2.10. Statistical analysis

Data are presented as mean ± S.E.M. One-way ANOVA with Tukey's post-test was performed using GraphPad InStat Version 3.00 for Windows 95 (GraphPad Software, San Diego, CA). p value <0.05 was considered statistically significant.

3. Results

3.1. TCDD enhances MDBK cells viability and proliferation

We analyzed the effect of TCDD exposure on MDBK cells viability and proliferation by measuring the mitochondrial redox capacity with the MTT assay and by evaluation of cell number. Confluent monolayers of MDBK cells were incubated in the presence of 0.01, 1 and 100 pg/ml of TCDD and then the MTT assay was performed at different times of exposure (4, 8, 12, 24, 36 and 48 h). As shown in Fig. 1A and, as previously reported (Fiorito et al., 2008a; Santamaria et al., 2011), the exposure of MDBK cells to various amount of TCDD caused a significant ($p < 0.001$, $p < 0.01$ and $p < 0.05$) increase in the mitochondrial dehydrogenases with respect to control cells from 24 h to the end of incubation. Concomitantly, the number of cells significantly ($p < 0.001$, $p < 0.01$ and $p < 0.05$) increased at

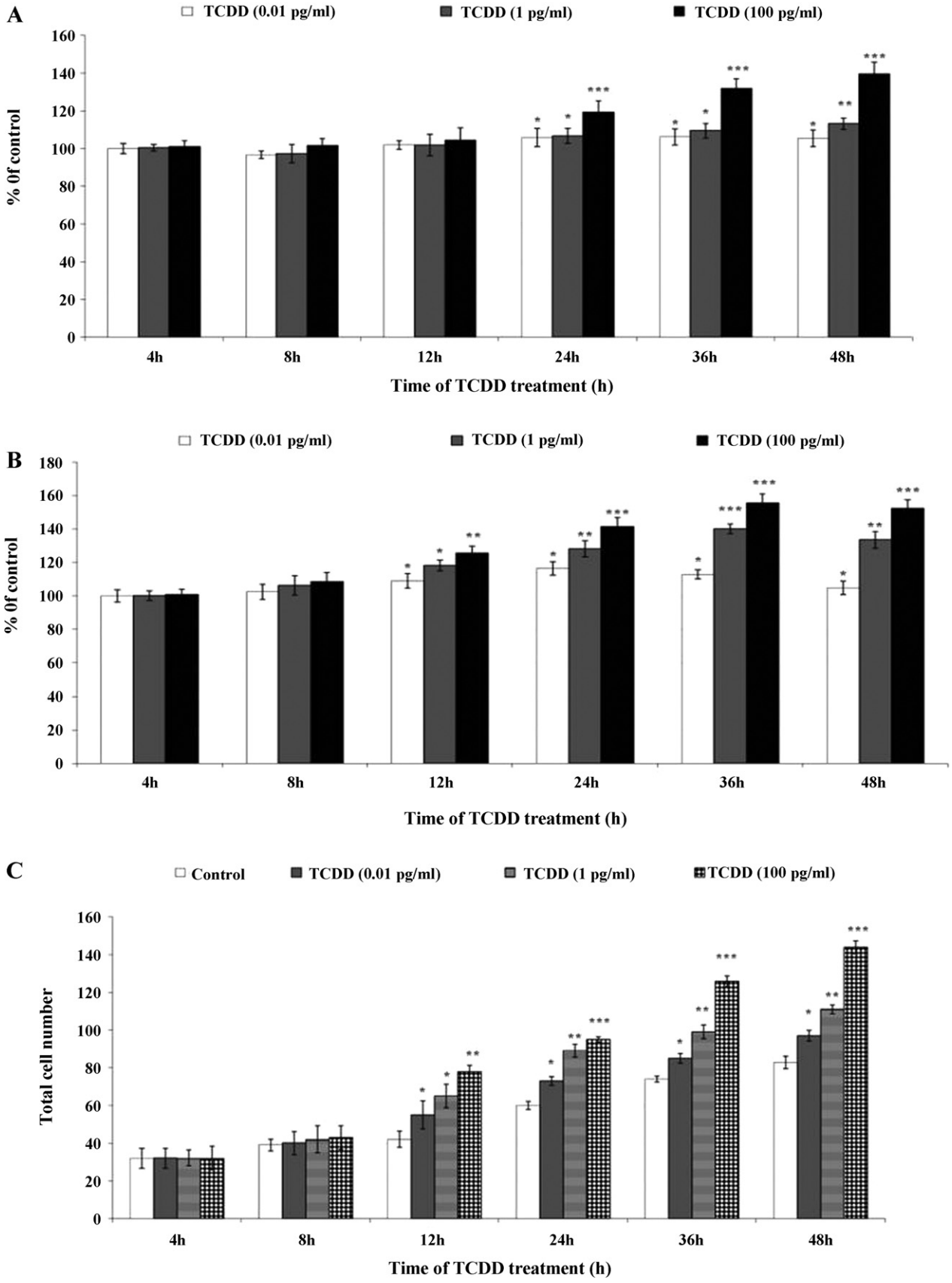

Fig. 1. (A) Dose response curve of MDBK cells treated with different concentrations of TCDD and observed at different times on cell viability. Viable, adherent cells were stained with MTT at different times of incubation and the absorbance assayed as described in Section 2. Data are presented as a percentage of the control, and results are expressed as the mean ± SE of four independent experiments performed in duplicate. (B and C) Effects of different concentrations of TCDD on MDBK cell proliferation. MDBK cells were incubated with TCDD (0.01, 1 and 100 pg/ml) for 4, 8, 12, 24, 36 or 48 *h*. At different times of exposure, the number of cells was evaluated by counting in a Burker chamber (B) or cells were stained with Trypan-blue and scored with a Burker chamber at a light microscope (C). Data are presented as mean ± S.D. of four independent experiments performed in duplicate. Significant differences between unexposed groups and TCDD-exposed groups are indicated by probability p. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

all concentrations of TCDD studied from 12 to 48 h of exposure (Fig. 1B). Similar results were obtained by performing Trypan Blue exclusion test. As we showed in Fig. 1C, from 12 h to the end of treatment, TCDD increased total cell number in significant manner ($p < 0.001$, $p < 0.01$ and $p < 0.05$).

Thus, TCDD exposure determined an increase in cells viability and proliferation, in dose-dependent manner.

3.2. TCDD does not induce cell death in MDBK

In order to evaluate the effects of TCDD on cell death we performed nuclear staining with propidium iodide. As we displayed in Fig. 2A, neither nuclear (apoptosis) nor membrane (necrosis) alterations in MDBK cells, exposed or not to TCDD, at all times studied, were observed after 48 h of treatment (at the end of TCDD exposure time), where TCDD exposed groups (1 pg/ml) were compared to control cells. Moreover, Trypan-blue exclusion test also indicated that no significant differences were observed between control and TCDD-treated groups in time dependent alteration of cell membrane permeability (TB permeable cells), at all doses tested (Fig. 2B).

These data, taken together to the results of our previous study in which we demonstrated that different concentration of TCDD (0.01–100 pg/ml) induced neither signs of apoptosis in bovine cells (Fiorito et al., 2008a), nor activation of caspases 8, 9 or 3 (data not shown), showed that TCDD did not induce cell death in MDBK cells.

3.3. TCDD induces morphological alterations in MDBK cells

To examine cell morphology, all TCDD-exposed groups were examined using light microscopy of Giemsa-stained cells. Herein, we showed the results only at 24 h which represented the exposure time where all TCDD concentrations displayed significant signs of morphological death. As shown in Fig. 3A, already at 24 h after exposure, in TCDD exposed group (1 pg/ml), compared to controls, a large number of cells exhibited some alterations, such as expanded cytoplasm (magnified cells), an increase of intercellular spaces, an elevated degree of vacuolization, and some pyknotic nuclei with central condensed chromatin. These features represent characteristic signs of autophagy, as previously described (Kroemer and Levine, 2008; Zakeri et al., 2008; Lamparska-Przybysz et al., 2005). Morphological cell death in the Giemsa-stained samples was reported in Fig. 3B. From 8 h to the end of exposure, TCDD significantly ($p < 0.001$, $p < 0.01$ and $p < 0.05$) enhanced the percentage of morphological cell death, in a dose-dependent manner (Fig. 3B).

Our results displayed that TCDD both increased the percentage of morphological cell death and induced some MDBK cells to undergo autophagy.

3.4. TCDD induces autophagy in MDBK cells

The effects of different concentrations of TCDD (0.01, 1 and 100 pg/ml) on autophagic markers were evaluated in MDBK cells at various times post exposure. LC3 is a recognized marker for

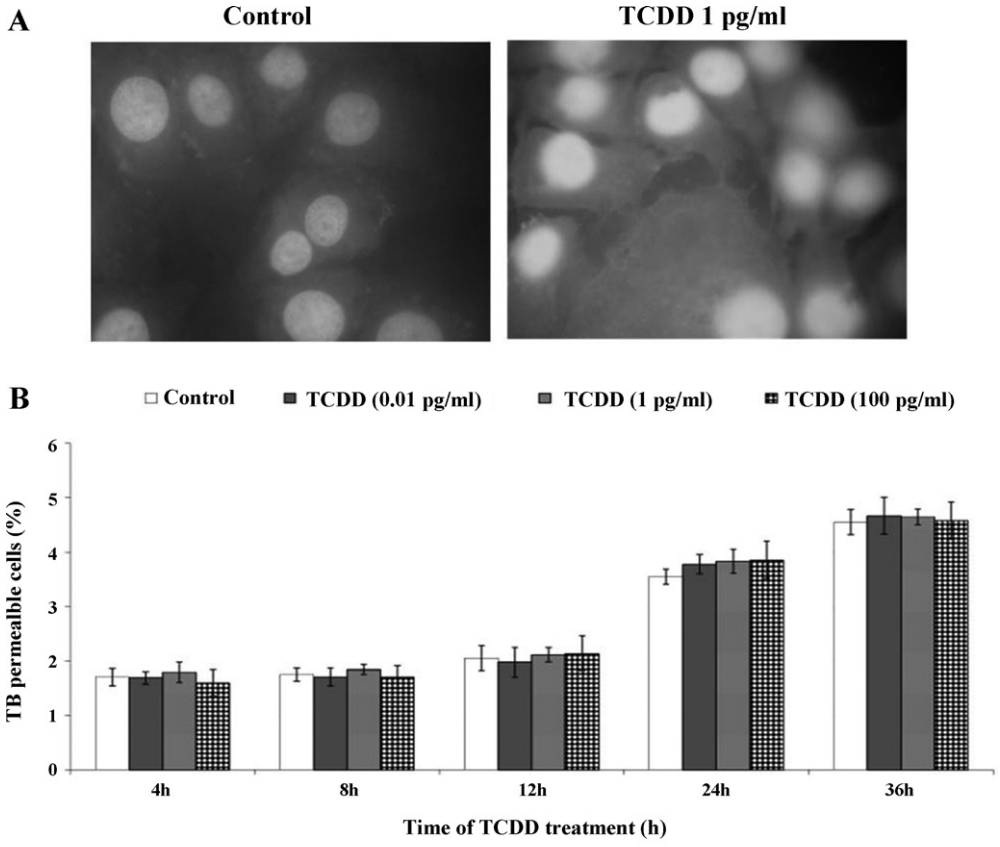

Fig. 2. Effects of different concentrations of TCDD on MDBK cell death. (A) MDBK cells were treated with different concentrations of TCDD and incubated. After different times of exposure, cells were fixed, and nuclei are stained with propidium iodide to visualize chromatin as described in Section 2. Neither nuclear (apoptosis) nor membrane (necrosis) alterations in MDBK cells, exposed or not to TCDD (1 pg/ml), were observed after 48 h of treatment condensation. (B) Effects of TCDD on alteration of cell membrane permeability. Values were percentage of non-viable cells, as determined by assessment of cells permeable to Trypan-blue. Data are presented as a percentage of the control, and results are expressed as the mean ± S.D. of four independent experiments performed in duplicate.

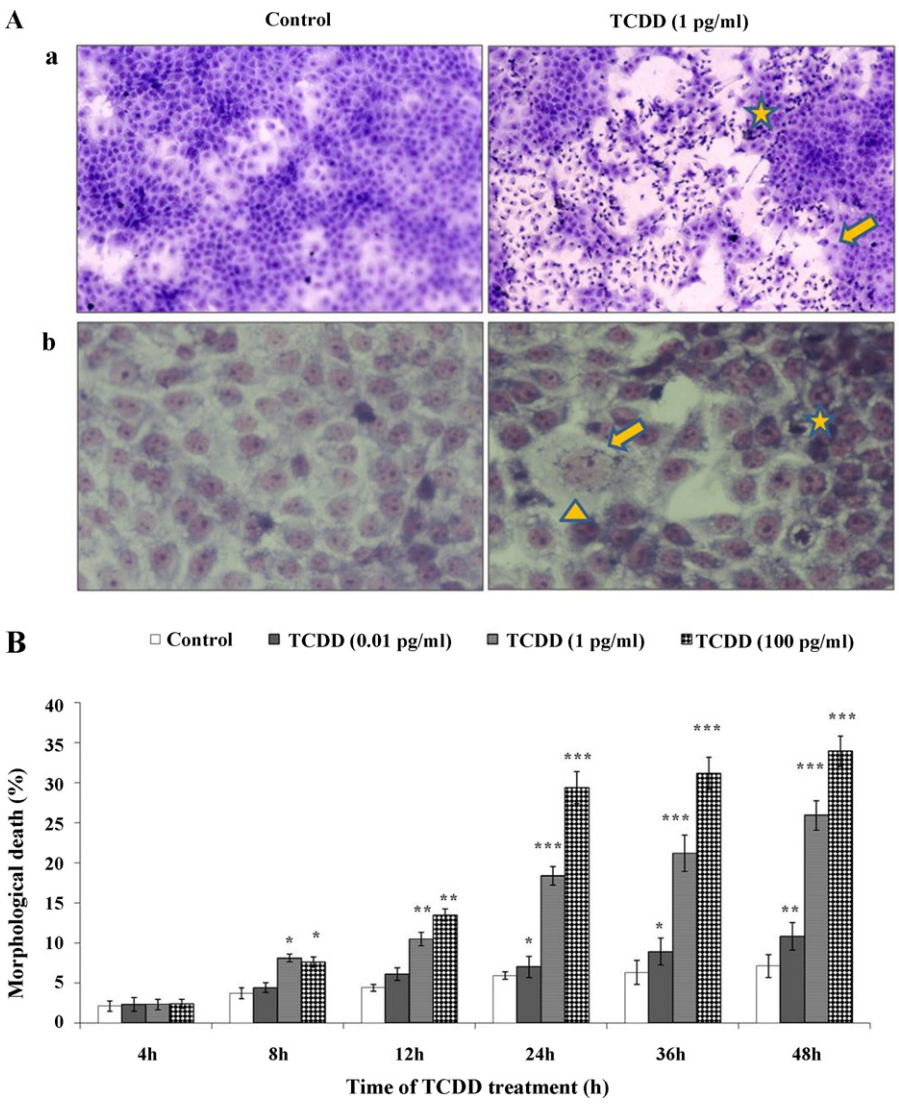

Fig. 3. Effects of different concentrations of TCDD on MDBK cell morphology. MDBK cells were treated with different concentrations of TCDD and incubated. After different times of exposure, cells were fixed, stained with Giemsa solution and then observed under light microscope, as described in Section 2. (A) Photomicrographs showing morphology of MDBK cells stained with Giemsa. After 24 of exposure, TCDD exposed groups (1 pg/ml), compared to the control groups, displayed magnified cells (arrows), an increase of intercellular spaces, an elevated degree of vacuolization (arrowheads), and many pyknotic nuclei with central condensed chromatin (star) (a, magnification ×150); (b, magnification ×400). (B) Percentages of morphological cell death at all times studied. Data are presented as mean ± S.D. of four independent experiments performed in duplicate. Significant differences between unexposed groups and TCDD-exposed groups are indicated by probability p. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

mammalian autophagocytosis (Kabeya et al., 2000; Wu et al., 2006). Protein levels of LC3 were studied by Western blot. LC3 protein is processed during autophagocytosis from an 18 kDa protein (LC3-I) to a membrane bound 16 kDa protein (LC3-II) (Wu et al., 2006). As we reported in Fig. 4, TCDD induced autophagic features in MDBK cells. From 8 h to the end of treatment, TCDD significantly ($p < 0.001$) increased the amount of LC3-II (Fig. 4A and B), indicating that these cells evidenced a high rate of autophagy. The rate of LC3-II increased in dose-dependent manner, when bovine cells were exposed to TCDD from 4 to 36 h. Whereas, examining treated cells at 0.01 and 100 pg/ml of TCDD compared to 1 pg/ml of TCDD, at 48 h, we observed an inverted U-shaped dose responsiveness in LC3-II protein levels (Fig. 4A and B). These modulations with an inverted U-shaped dose responsiveness induced by dioxin were consistent with the results of previous studies (Ahn et al., 2005; Fiorito et al., 2008a,b, 2010).

Furthermore, after 24 h of treatment, TCDD increased punctate staining for LC3 compared to controls, as detected by immunofluorescence for LC3, using epitope-specific antibody on methanol-fixed cells (Fig. 4C). In particular, when MDBK cells were treated with TCDD, there was a strong accumulation of LC3-II (Fig. 4C). Such LC3-II accumulation was more abundant when the cells were treated with the highest dose of dioxin (data not shown). As showed in Fig. 4C, LC3 was sometimes strongly detected in the nucleus and sometimes not. It is well known that the amount of LC3 is correlated with the extent of autophagosome formation. Indeed, when autophagosome–lysosome fusion is blocked, larger autophagosomes are detected, possibly due to autophagosome-autophagosome fusion (Klionsky et al., 2008). Moreover, we hypothesized that TCDD, as other toxins (Cummings et al., 1998), could induce toxic proteins formation and their import to the nucleus, which represents the primary site of action of proteotoxins. The data presented herein suggested that some nuclei were protected environments that provided a place for toxic protein aggregates to escape degradation by autophagy. Autophagy thus appeared to be relatively ineffective to clear toxic protein aggregates which accumulated within some nuclei. Then, we detected also the formation of AVOs. Autophagy is a process of sequestrating cytoplasmic proteins into the lytic component and to evaluate the AVOs in TCDD-treated cells, we performed the vital

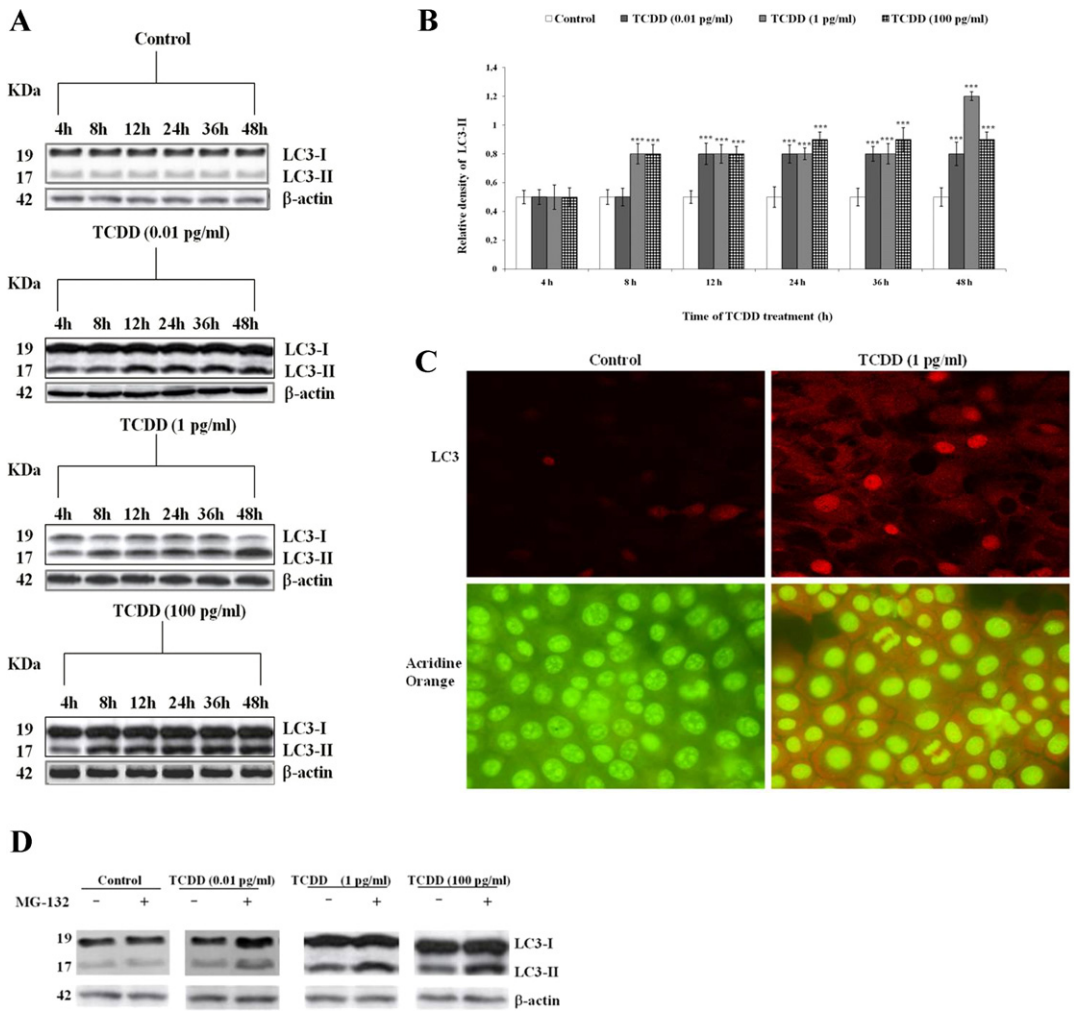

Fig. 4. TCDD induces autophagy in MDBK cells. (A) Whole-cell lysates were prepared from untreated cells (lane Control) or exposed to different concentrations of TCDD (lane TCDD 0.01 pg/ml), (lane TCDD 1 pg/ml) or (lane TCDD 100 pg/ml) cells and, after 4, 8, 12, 24, 36, or 48 h, Western blot analysis was performed with an antibody which specifically recognized LC3 or β-actin, as described in Section 2. β-Actin was used as an internal loading control. The molecular weight (in kDa) of protein size standards is shown on the left hand side. Blots are representative of at least three separate experiments. (B) Densitometry analysis of the blots shown in (A). Results are the mean ± S.D. of three separate experiments. Significant differences between unexposed groups and TCDD-exposed groups are indicated by probability *p*. ***$p < 0.001$. (C) Immunofluorescence for LC3 in MDBK cells treated or not with TCDD (1 pg/ml) for 24 *h*, studied as described in Section 2; microphotographs of cells staining with acridine orange using a fluorescence microscope that revealed the induction of acidic vesicular organelles. Detection of green and red fluorescence in acridine orange-stained cells was performed using a fluorescence microscope in MDBK cells treated or not with TCDD (1 pg/ml) for 24 *h*. (D) Whole-cell lysates were prepared from untreated cells exposed or not to MG-132 (lane Control), or exposed to different concentrations of TCDD (lane TCDD 0.01 pg/ml), (lane TCDD 1 pg/ml) or (lane TCDD 100 pg/ml) in the presence or not of MG-132 cells and, after 12 h, Western blot analysis was performed with an antibody which specifically recognized LC3 or β-actin, as described in Section 2. β-Actin was used as an internal loading control. The molecular weight (in kDa) of protein size standards is shown on the left hand side. Blots are representative of at least three separate experiments.

staining with acridine orange. At 24 h of treatment, in acridine orange-stained cells, the cytoplasm and nucleolus fluoresced bright green and dim red, as resulted in control group (Fig. 4C), whereas, TCDD treatment (1 pg/ml) enhanced acidic compartments which fluoresced in bright red (Fig. 4C). The intensity of the red fluorescence, proportional to the degree of acidity of the cellular acidic compartment, was increased by dioxin in dose-dependent manner (data not shown).

Treatment of MDBK cells with proteasome inhibitor MG-132 in the presence or absence of TCDD was used to assess the autophagic flux by TCDD. Fig. 4D shows that MG-132 increased LC3-II accumulation in the presence of all doses of TCDD at 12 h post exposure, when significant TCDD-induced autophagy started (Fig. 4A and B). These results confirmed that TCDD activated autophagic flux, indeed autophagy can increase as a compensatory means of protein degradation when the proteasomal pathway is blocked (Pandey et al., 2007).

By summarizing, these experiments indicated that TCDD exposure increased autophagy in MDBK cells, as compared to untreated controls, in time and dose-dependent manner.

3.5. *TCDD down-regulates telomerase activity, bTERT and c-Myc protein levels in MDBK cells*

In order to evaluate the effects of TCDD on telomerase activity, MDBK cells were exposed or not to different concentrations of TCDD (0.01, 1 or 100 pg/ml) and analyzed, at different hours post exposure, by TRAP assay, as described in methods section. Following the kinetic of telomerase activity in MDBK cells exposed to TCDD, high levels of telomerase activity were detected at 4 h, which significantly ($p < 0.001$) decreased from 8 h to the end of exposure, in the presence of 1 and 100 pg of TCDD (Fig. 5A). Differently, 0.01 pg of TCDD induced a significant ($p < 0.05$) decrease of telomerase activity only from 36 to 48 h of exposure (Fig. 5A). Interestingly,

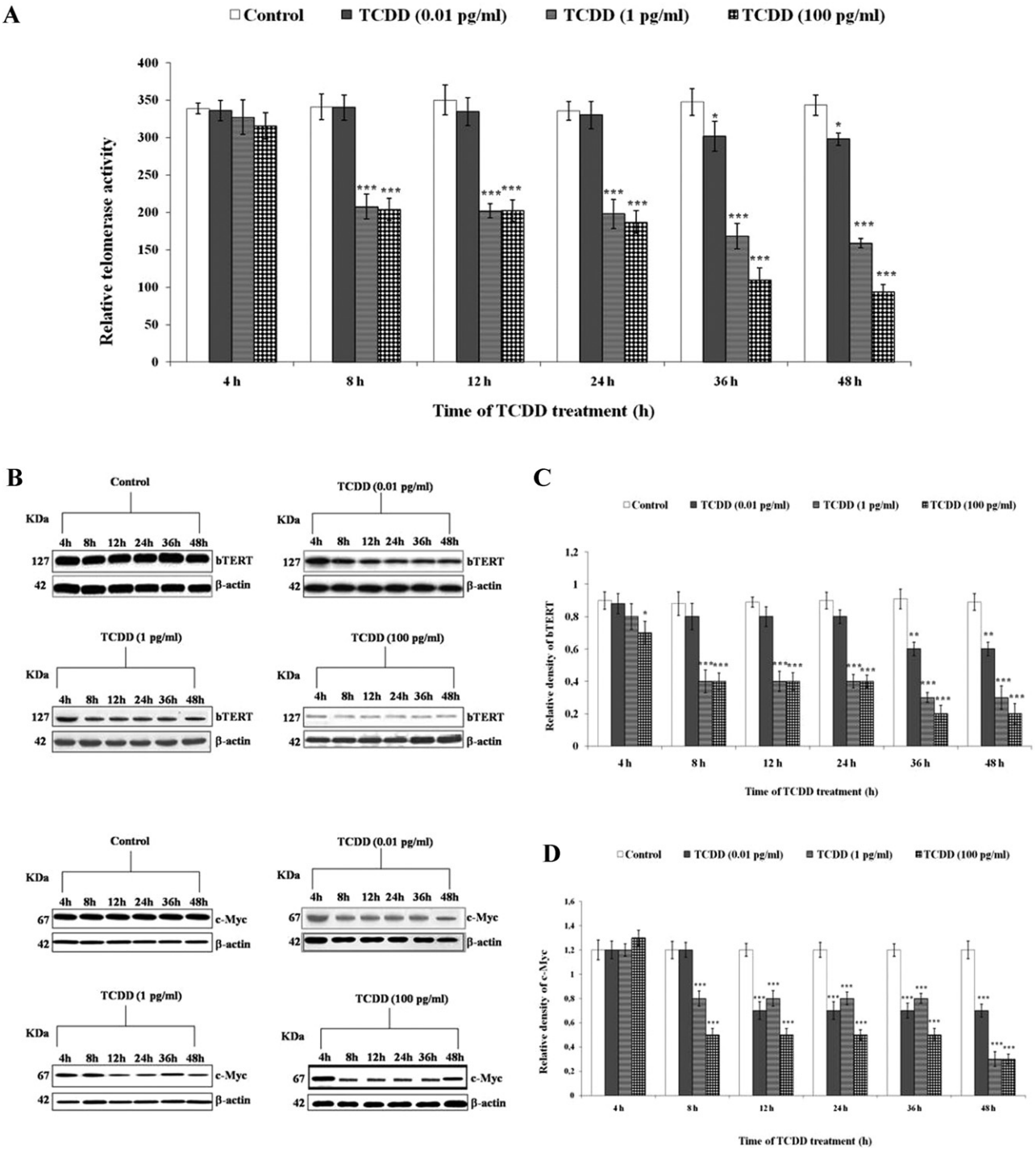

Fig. 5. TCDD down-regulates telomerase activity, bTERT and c-Myc protein levels in MDBK cells. (A) Telomerase activity in MDBK cells, exposed or not to TCDD, after 4, 8, 12, 24, 36, or 48 h of exposure. Telomerase activity was measured by the TRAP assay and results are expressed as relative telomerase activity (RTA). Results are the mean ± S.D. of three separate experiments performed in duplicate (B) Whole-cell lysates were prepared from untreated cells (lane Control) or exposed to different concentrations of TCDD (lane TCDD 0.01 pg/ml), (lane TCDD 1 pg/ml) or (lane TCDD 100 pg/ml) cells and, after 4, 8, 12, 24, 36, or 48 h, Western blot analysis was performed with antibodies which specifically recognized bTERT, c-Myc or β-actin, as described in Section 2. β-Actin was used as an internal loading control. The molecular weight (in kDa) of protein size standards is shown on the left hand side. Blots are representative of at least three separate experiments. (C) Densitometry analysis of bTERT blots shown in (B). (D) Densitometry analysis of c-Myc blots shown in (B). Results are the mean ± S.D. of three separate experiments. Significant differences between unexposed groups and TCDD-exposed groups are indicated by probability p. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

we observed that TCDD exposure decreased telomerase activity in MDBK cells in dose-dependent manner (Fig. 5A).

Among the various components associated with telomerase complex, the telomerase catalytic subunit hTERT has been demonstrated to be the rate-limiting determinant of telomerase activity (Nakamura et al., 1997). Therefore bTERT protein levels were examined to establish if telomerase inhibition by TCDD was indeed mediated through modulation of the catalytic subunit bTERT. As shown in Fig. 5B and C, the levels of bTERT protein significant decreased following TCDD treatment when compared to untreated

cells. In the presence of higher doses of TCDD, we detected a significant ($p < 0.001$; $p < 0.01$) down-regulation of bTERT from 8 h to the end of exposure. Differently, 0.01 pg of TCDD impaired bTERT levels in significant manner ($p < 0.01$) from 36 to 48 h of exposure (Fig. 5B and C).

In addition, by comparing telomerase activity and bTERT protein level measurements, we observed a good correlation, showing a similar trend. These data were in agreement with a previous study carried out on esophageal epithelial cells, describing an evident correlation between telomerase activity and the amount of cellular hTERT mRNA (McGruder et al., 2006). To determine if the regulation of c-Myc was also involved in telomerase down-regulation by TCDD, MDBK cells were treated with TCDD and total cell lysates were subjected to Western blot analysis. As we showed in Fig. 5B–D, from 8 to 48 h, dioxin significantly ($p < 0.001$) down-regulated c-Myc protein levels, at all doses tested. Analyzing treated cells at 0.01 and 100 pg/ml of TCDD compared to 1 pg/ml of TCDD, at 12, 24 and 36 h of exposure we distinguished an inverted U-shaped dose responsiveness (Fig. 5B–D), as reported above.

Our results indicated that TCDD exposure down-regulated telomerase activity, bTERT and c-Myc protein levels in MDBK.

3.6. TCDD exposure up-regulates key tumor suppressor protein p53 and modulates the levels of $p21^{Waf1/Cip1}$, ATM and Mdm2

Since the tumor suppressor p53 typically contributes to cell death, we wondered if in bovine cells TCDD treatment induced changes both in p53 phosphorylation and protein levels. Ser315 locates within the nuclear localization signal (amino acids 305–322) of the C-terminal region of p53. Ser315 is phosphorylated by kinases involved in cell cycle progression, such as Aurora kinase A and the cyclin-dependent kinase cdk9 (Katayama et al., 2004; Radhakrishnan and Gartel, 2006). It has been shown that Ser315 phosphorylation is required for the E2F family to enhance p53-mediated transcription and apoptosis (Fogal et al., 2005). Thus, we selected to study Ser315 phosphorylation because, although Aurora kinase A (involved in chromosome segregation and function) phosphorylates p53 at Ser315 leading to results in its destabilization (Katayama et al., 2004), phosphorylation at Ser315 in the other instances seems to elevate p53 stability and positively contribute to tumor suppression. We found that TCDD induced p53 phosphorylation at serine 315 as early as 8 h to the end of treatment (Fig. 6A and B), suggesting both activation by dioxin and phosphorylation levels increased with prolonged incubation. As shown

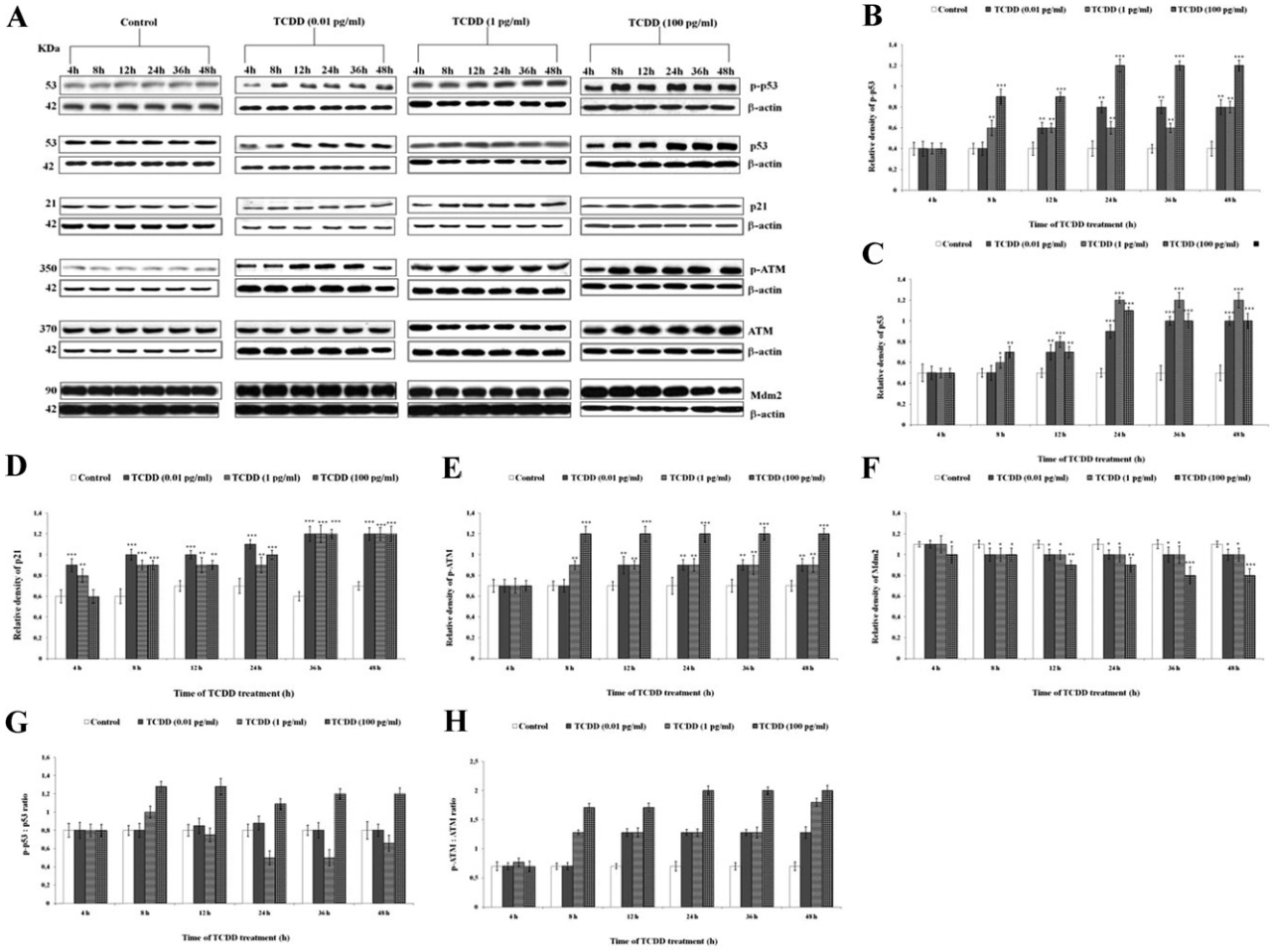

Fig. 6. TCDD exposure up-regulates key tumor suppressor protein p53 and modulates the levels of $p21^{Waf1/Cip1}$, ATM and Mdm2 in MDBK cells. (A) Whole-cell lysates were prepared from untreated cells (lane Control) or exposed to different concentrations of TCDD (lane TCDD 0.01 pg/ml), (lane TCDD 1 pg/ml) or (lane TCDD 100 pg/ml) cells and, after 4, 8, 12, 24, 36, or 48 h, Western blot analysis was performed with antibodies which specifically recognized p-p53, p53, p-21, p-ATM, ATM, Mdm2 or β-actin, as described in Section 2. β-Actin was used as an internal loading control. The molecular weight (in kDa) of protein size standards is shown on the left hand side. Blots are representative of at least three separate experiments. (B) Densitometry analysis of p-p53 blots shown in (A). (C) Densitometry analysis of p53 blots shown in (A). (D) Densitometry analysis of $p21^{Waf1/Cip1}$ blots shown in (A). (E) Densitometry analysis of p-ATM blots shown in (A). (F) Densitometry analysis of Mdm2 blots shown in A. (G) p-p53/p53 ratio were obtained by densitometry analysis of the relative blots shown in (A). (H) p-ATM/ATM ratio were obtained by densitometry analysis of the relative blots shown in A. Results are the mean ± S.D. of three separate experiments. Significant differences between unexposed groups and TCDD-exposed groups are indicated by probability *p*. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

in Fig. 6A–C, TCDD treatment led to significant increase of p53 protein levels from 8 h to the end of exposure. Our data demonstrated that the levels of both p-p53 and p53 protein levels were significantly ($p < 0.001$, $p < 0.01$ and $p < 0.05$) increased by TCDD, at all times studied (Fig. 6A–C). In particular, analyzing treated cells at 0.01 and 100 pg/ml of TCDD compared to 1 pg/ml of TCDD, from 24 h to 48 h, we observed an U-shaped dose responsiveness in p-p53 activation (Fig. 6A–C), and from 12 to the end of treatment, we detected an inverted U-shaped dose responsiveness in p53 protein levels (Fig. 6A and B), as reported above. Moreover, by measuring the p-p53/p53 ratio, we detected an increase in exposed groups only in the presence of 0.01 or 100 pg/ml of TCDD (Fig. 6G), at all times studied, while 1 pg/ml of TCDD induced, after an increase at 8 h, a decrease from 12 h to the end of exposure (Fig. 6G). It is well known that p53 is phosphorylated at 23 sites, mostly found within the N- and C-terminal domains that form the molecule's regulatory regions, but generally, even if the total quantity of p-p53 might be important to consider for its transcription function, a decrease of the ratio might also lead to a dilution effect. In the presence of 1 pg/ml of TCDD, we detected an increase of p53 protein levels even if the ratio decreased, suggesting that this dose of TCDD was sufficient to induce maximal effects on parameters studied here. A strange modulation of p53 phosphorylation induced by TCDD has already been found by Pääjärvi et al. (2005).

A p53-inducible gene product, $p21^{Waf1/Cip1}$, which is a component of p53-dependent G1 arrest, is required for cell cycle arrest (Cox, 1997). Herein, analysis of cell cycle inhibitor p21 indicated a significant ($p < 0.001$, $p < 0.01$ and $p < 0.05$) time-dependent up-regulation of protein levels, in the presence of all doses tested (Fig. 6A–D).

In general, ATM is considered a protein kinase which serves as a critical mediator of signaling pathways facilitating response of mammalian cells to agents that induce DNA damage (Kastan and Bartek, 2004). Mdm2 is the main regulator of p53, and ATM may target both p53 and Mdm2 (Maya et al., 2001). Herein, analysis of MDBK cells exposed to TCDD by Western blot, displayed a significant ($p < 0.001$ and $p < 0.01$) time-dependent augment of both ATM phosphorylation (Fig. 6A–E) and p-ATM/ATM ratio (Fig. 6H), in the presence of a slow, but significant ($p < 0.001$, $p < 0.01$ and $p < 0.05$) time-dependent decline in Mdm2 protein levels (Fig. 6A–F).

These results indicated that both p53 and $p21^{Waf1/Cip1}$ activations may be involved in stimulation of cell death in TCDD-treated MDBK cells. Furthermore, exposure of cells to dioxin activated the ATM checkpoint pathway, inducing both ATM and p53 phosphorylation, and inhibiting Mdm2 protein levels.

4. Discussion

Autophagy, in mammalian cells, is a largely unknown process and only recently mammalian homologues of yeast genes have been discovered and used as molecular markers for autophagy (Demarchi et al., 2006). The role of autophagy extends beyond the general homeostatic removal, degradation and recycling of damaged proteins and organelles to many specific physiological and pathological processes such as development, immunity, energy homeostasis, cell death or tumourigenesis (Rosenfeldt and Ryan, 2011). Dioxin is recognized to act as a potent tumor promoter in various model of carcinogenesis (White and Birnbaum, 2009), but the mechanisms by which it induces this effect is poorly understood. However, it is well established that dioxin can induce tissue- and species-specific carcinogenicity (DeVito et al., 1995; Mandal, 2005). Several studies, performed using laboratory animals, have shown that TCDD promotes the formation of neoplastic lesions in the liver, lung, oral mucosa and the skin (Whysner and Williams, 1996; Knerr and Schrenk, 2006; Walker et al., 2006), as well as, increases proliferation of cultured cells, such as human keratinocytes (Milstone and LaVigne, 1984; Ray and Swanson, 2003), late gestational ureteric cells (Bryant et al., 2001), human breast cells (Ahn et al., 2005). As we described in previous studies (Fiorito et al., 2008a; Santamaria et al., 2011), and as well as in this one, TCDD induced cell proliferation and increased cell viability in MDBK, which is a kidney epithelial cell line (Fig. 1). These findings are supported by a previous study that, by examining the tumor-promoting activities of dioxin, indicated that epithelial cells are the most responsive to the effects of dioxin (Poland et al., 1982).

Although TCDD induced cell proliferation and increased cell viability in bovine cells, the morphological investigations displayed that, already at the lowest dose level, some exposed cells exhibited pyknotic nuclei. Increasing cell size was observed, as well as presence of vacuoles (Fig. 3A and B). Similar features were reported in some seminiferous tubuli of male offspring rats exposed to TCDD throughout pregnancy and lactation (Faqi et al., 1998), as well as, in mice thymocyte, where pyknotic cells were increased by TCDD treatment in the cortical zones (Besteman et al., 2005). On the basis of morphological features of cells undergoing death, it is possible to distinguish apoptosis, necrosis and autophagic cell death (Kroemer and Levine, 2008). As above reported, exposition to different concentrations of TCDD, for 48 h, induced neither signs of apoptosis in MDBK cells (Fiorito et al., 2008a), nor signs of necrosis (Fig. 3A). Moreover, by performing nuclear staining with propidium iodide, neither nuclear (apoptosis) nor membrane (necrosis) alterations in MDBK cells, exposed or not to TCDD, were observed after 48 h of treatment (Fig. 2A). Necrosis is characterized by swelling of organelles and plasma membrane rupture and subsequent loss of intracellular contents (Kroemer and Levine, 2008). Herein, the treatment of bovine cells with TCDD determined detectable features of autophagy (Fig. 3A), such as magnified cells, an increase of intercellular spaces, vacuolization of the cytoplasm, and many pyknotic nuclei with central condensed chromatin (Kroemer and Levine, 2008; Zakeri et al., 2008; Lamparska-Przybysz et al., 2005). There are many methods to evaluate autophagy, a facet of normal homeostasis, but none of these techniques specifically distinguish autophagy from autophagic cell death (Kroemer and Levine, 2008; Zakeri et al., 2008). Autophagy is a process by which the cell, using membranes to isolate organelles or regions of cytoplasm, eliminates damaged organelles or consumes intracellular components as resources during starvation or other limiting conditions (Zakeri et al., 2008). Autophagic cell death (or cell death by autophagy) is not only accompanied by the accumulation of autophagic vacuoles, but also involves an increase in autophagy that contributes to cell death (Kroemer and Levine, 2008; Zakeri et al., 2008). Moreover, if cell death occurs by autophagy, the specific inhibition of autophagy could alter the fate of the cell and result in long-term cellular survival (Kroemer and Levine, 2008). Hence, based mostly on the growing evidence that autophagy exerts cytoprotective effects, it is shared the opinion that autophagic cell death is rarely, if ever, the mechanism by which cells actually die (Kroemer and Levine, 2008). Thus, since TCDD increased the percentage of morphological cell death (Fig. 3B), we simply presumed that some treated cells partially lost the capacity to proliferate and TCDD induced some MDBK cells to undergo cell death with autophagy. Then, by using LC3-II, as autophagy marker, we distinguished an increase in both LC3-I and II (Fig. 4A and B). The accumulation of LC3-II and the detection of LC3 by immunofluorescence, as we reported in Fig. 4C, are considered reliable methods for monitoring autophagy (Tanida et al., 2008). To date, LC3-II is the only well-characterized protein that is specifically localized to autophagic structures throughout the process from phagophore to lysosomal degradation (Tanida et al., 2008). Moreover, by performing the vital staining with acridine orange, we detected in

TCDD-treated cells the formation and promotion of acidic vesicular organelles (Fig. 4C), that further characterize autophagy (Chiu et al., 2009). For the first time, we described signs of autophagy induced by TCDD, a cancer promoter. Tumor promotion is often thought to be a state that involves the disruption of cellular homeostasis, which is dependent on the precise regulation of processes such as proliferation, differentiation, replicative senescence, apoptosis and autophagy. In general, the regulated lysosomal degradation pathway of autophagy prevents cellular damage and thus protects from malignant transformation (Rosenfeldt and Ryan, 2011). The involvement of autophagy in tumor development is unquestioned, but it is until now incompletely understood. Deregulation of autophagy is known to affect many processes that can control the formation and existence of a cancer cell, but contradictions still exist about the relationship between autophagy and cancer (Rosenfeldt and Ryan, 2011). It has been demonstrated that anti-cancer compounds could induce anti-tumor activity through both inhibition of telomerase activity and autophagy modulation (Ko et al., 2009). Until now there are very few reports describing how TCDD impacts on telomerase activity. In particular, TCDD did not affect telomerase activity either in normal human epidermal cells or immortalized cells or malignant keratinocytes (Rea et al., 1998). Nevertheless, a report has shown that human choriocarcinoma cells (BeWo), treated with two concentrations of TCDD, showed a dose-dependent increase in telomerase activity, with an increase in hTERT copy number (Sarkar et al., 2006). Conversely, herein, analysis of telomerase activity showed that TCDD down-regulated telomerase activity in MDBK cells (Fig. 5A), with a significant decrease in bTERT protein levels (Fig. 5A and B), in the presence of cell proliferation. Although this may seem a paradox, it has been shown that telomerase activity was reported in liver samples from patients with hepatitis and liver cirrhosis but no tumor pathology (Greider, 1996). Moreover, as reported in a recent study, approximately 10–15% of human cancers lack detectable telomerase activity (Heaphy et al., 2011). As reported above, hTERT is actively regulated by c-Myc (Wu et al., 1999). hTERT proximal core promoter contains two binding sites for c-Myc, which acts as a regulatory factor for hTERT transcription (Wu et al., 1999; Kyo et al., 2000). Results from our analysis showed that in MDBK cells there was significant repression of c-Myc protein levels by TCDD, in a dose-dependent manner (Fig. 5A–C). Expression of c-Myc has been closely associated with cellular proliferation/cell death and hTERT activity, which were consistent with the inhibition of c-Myc by TCDD and the resulting decrease in telomerase activity. Western blot analysis of key tumor-suppressor proteins as p53 and of inhibitor of the cell cycle $p21^{Waf1/Cip1}$ showed that in bovine cells TCDD up-regulated their expression levels, concomitant to activation of p53 phosphorylation (Fig. 6). Phosphorylation of Ser315 can have an inhibitory or a stimulatory role in modulating p53-dependent transcription, depending on the context (Liu et al., 2004) but, as above reported, phosphorylation at Ser315 often seems to elevate p53 stability and positively contribute to tumor suppression (Fogal et al., 2005; Radhakrishnan and Gartel, 2006). These results, together with inhibition of telomerase activity, could represent some of the tumor suppressor mechanisms to protect bovine cells from proliferation induced by dioxin, as previously reported by others (Campisi, 2005; Caino et al., 2009). Indeed, dysfunctional telomeres activate p53 to initiate cellular cell death in order to suppress tumorigenesis (Deng and Chang, 2007). Furthermore, in response to DNA damage or oncogene activation, p53 can be activated and subsequently it can orchestrate biological outputs such as modulation of autophagy (Yee and Vousden, 2005). Genotoxic stresses activate p53 which initiates tumor suppressor processes such as growth arrest. Although p53 can affect cell growth and proliferation by activating cyclin-dependent kinase p21, recent observations indicate that once activated, p53 also inhibits mTOR activity and up-regulates autophagy (Botti et al., 2006). We also observed that TCDD in MDBK cells induced a slight decrease of Mdm2 protein levels, in the presence of an activation in ATM phosphorylation (Fig. 6). These results correlate with the finding that TCDD up-regulated key tumor-suppressor proteins as p53. p53 and Mdm2 interact in an autoregulatory loop. Indeed, p53 protein generally accumulates and trans-activates target genes, including Mdm2 and genes inducing growth arrest (Oren, 2003). However, Mdm2 is not only targeted by p53 but may partially be regulated independently. For example, Mdm2 is phosphorylated by DNA-damage signaling kinases such as ATM (Appella and Anderson, 2001). An up-regulation of Mdm2 may attenuate a p53 response and protect cells from inhibitory effects of p53 (Leri et al., 1999). A previous study reported that TCDD increased basal levels of Mdm2 protein and lead to the rapid onset of p53 degradation in rat hepatocytes, where dioxin decreased apoptosis rates (Pääjärvi et al., 2005). Conversely, our data indicated that TCDD induced cell death with autophagy in bovine cells where decreased Mdm2 and intensified p53 protein levels.

In conclusion, our results showed that TCDD in MDBK cells induced alterations in the balance between cell replication and cell death. Herein, TCDD exerted their proliferative effects not only via stimulation of cell replication, but also by modulating the

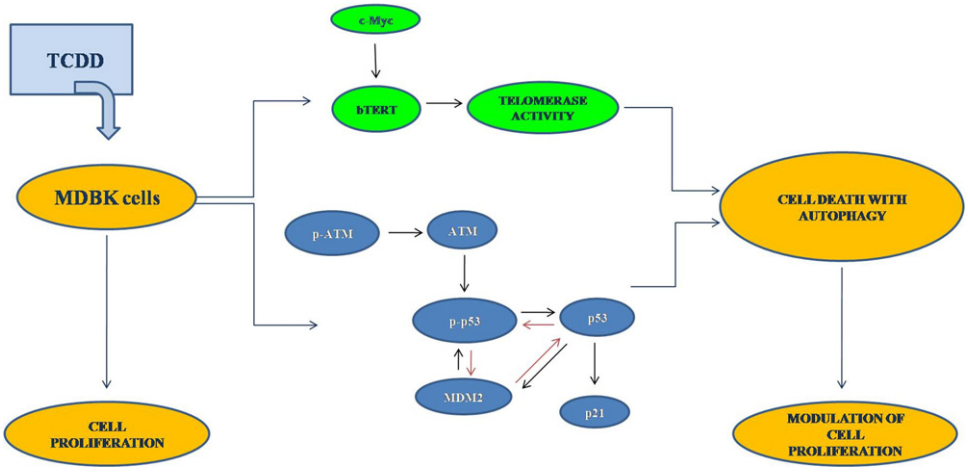

Fig. 7. Schematic diagram illustrating the hypothesized mechanisms of action as to how TCDD exerted its effects on the multiple targets, herein evaluated, resulting in increased proliferation and cell death.

incidence of induced cell death with autophagy, both by down-regulated telomerase activity and by up-regulating the expression levels of key tumor-suppressor proteins (see Diagram in Fig. 7). Although this may seem a paradox, it should be noted that non-genotoxic (epigenetic) carcinogens, such as dioxin, are a diverse group of chemicals that induce tumor formation by mechanisms different from direct DNA damage (Mally and Chipman, 2002). When a somatic cell proliferates, a network of positive and negative signals tightly regulates progression of the mitotic cycle (Sherr and Roberts, 1999). Indeed, the increased cell proliferation may increase probability of initiated clonal cell expansion, while simultaneously it may increase the risk of DNA modification during its replication (Klaunig et al., 2000). Moreover, a finest cellular damage response requires repair of damage, coordination of critical cellular processes and cell cycle progression (Kastan and Bartek, 2004). Several studies have implicated AhR in the regulation of cell cycle, cell proliferation, and cell death as a potential mechanism underlying the promotional role of AhR in carcinogenesis (Barouki et al., 2007). However, others groups have suggested that AhR ligands might sometimes act independently of the receptor, as recently reported, environmental Polycyclic aromatic hydrocarbons trigger intracellular calcium concentration induction in an AhR-independent manner (Mayati et al., 2011). Furthermore, AhR ligands might act through a membrane-dependant pathway, such as the protein kinase c-Src, providing a potentially important mechanism by which TCDD can alter cell growth and differentiation (Blankenship and Matsumura, 1997). Future studies will be crucial to elucidate the role of AhR in the effects herein described in MDBK cells.

Finally, the role of autophagy in cancer, is more complex. There is evidence that autophagy may be oncogenic in some contexts, whereas in others it clearly contributes to tumor suppression (Rosenfeldt and Ryan, 2011). Indeed, a recent study established the importance of autophagy for the maintenance of adult hematopoietic stem cells and provided evidences suggesting that autophagy protects against leukemia development (Mortensen et al., 2011). Our study is the first establishing the requirement for autophagy in the maintenance of MDBK cells exposed to dioxin, as well as, the first to provide evidences suggesting that autophagy protects against proliferative effects induced by non-genotoxic compounds such as TCDD.

Until now, there are no reports in the literature describing autophagy induced by TCDD. This is the first evidence that administration of TCDD to an epithelial line of mammalian cells may induce cell death with autophagy.

Recently, as above reported, levels of TCDD, exceeding the European Union tolerance, were detected in dairy products and milk from cow and water buffalo, raised on some areas of Campania Region (South Italy) (Diletti et al., 2003; Santelli et al., 2006). Since very low (0.01 pg/ml) of TCDD doses were sufficient to induce the results herein showed in vitro, these effects might be taken into account in the care of farm animal health.

Conflict of interest

None

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