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# Accelerated internalization of junctional membrane proteins (connexin 43, N-cadherin and ZO-1) within endocytic vacuoles: An early event of DDT carcinogenicity

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**Abstract**

Stability of cell-to-cell interactions and integrity of junctional membrane proteins are essential for biological processes including cancer prevention. The present study shows that DDT, a non-genomic carcinogen used at a non-cytotoxic dose (1 μM), rapidly disrupted the cell–cell contacts and concomitantly induced the formation of cytoplasmic vacuoles close to the plasma membrane in the SerW3 Sertoli cell line. High-resolution deconvolution microscopy reveals that this vacuolization process was clathrin-dependent since a hyperosmotic media (0.2 M sucrose) blocked rhodamine–dextran endocytosis. In response to DDT, junctional proteins such as Cx43, N-Cadherin and ZO-1 were internalized and present in vacuoles. In Cx43-GFP transfected cells, time lapse videomicroscopy demonstrates that DDT rapidly enhanced fragmentation of the gap junction plaques and abolished the gap junction coupling without major modification of Cx43 phosphorylation status. Repeated exposure to DDT resulted in chronic gap junction coupling injury. The present results demonstrate that one of the early effect of DDT is to interfere with the plasma membrane and to perturb its function, specifically its ability to establish cell–cell junctions that are essential for tissue homeostasis and control of cell proliferation and differentiation. Such an alteration may play a specific role during carcinogenesis.


*Keywords:* DDT; Endocytosis; Junctional proteins; Connexin 43; N-cadherin; ZO-1; Carcinogen; Sertoli cell

## 1. Introduction

DDT (1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl)ethane) is an environmental pollutant that has been largely used for the eradication of malaria. In spite of its obvious value in combatting this disease, DDT exerts adverse effects on human health and has been classified as a non-genotoxic mitogenic hepatocarcinogen in rodent and a potential human carcinogen (group 2B) [1]. As a result of these potential adverse effects, the use of DDT was restricted or banned in most developed countries. However, the risk of DDT on human health versus its benefit has been reviewed (for a review, see Ref. [2]), and the World Health Organization (WHO) has recently endorsed the use of DDT for malaria control in several sub-Saharan Africa countries [3].

There is compelling evidence that induced hepatocarcinogenesis by DDT in rodent may be associated with different cell dysfunctions: altered metabolism (microsomal enzymes, oxidative stress and on lipid peroxidation) [4,5] and inhibition of the gap junctional intercellular communication [6]. In human, epidemiological studies strongly supported its involvement in the aetiology of other human diseases such as pancreatic cancer, neuropsychological and reproductive dysfunctions [7]. However, the fine early molecular mechanisms by which DDT exerts its toxic effect(s) on cell function are still unknown.

In the testis, Sertoli cells, the somatic cells that support germ cell proliferation and differentiation into spermatozoa, are the first and major target of testicular toxicants [8]. Morphological alterations (vacuolization) and metabolism dysfunctions

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(altered lactate and inhibin secretions) of Sertoli cells are the two main adverse effects reported after exposure to a large variety of toxicants in laboratory animals [8,9]. Vacuolization is a common feature of Sertoli cell dysfunction and injury also described in pathological human testis with major spermatogenic anomalies [9]. This process can occur from two different pathways: by swelling of membrane-bound organelles such as the endoplasmic reticulum and identified as autophagic vacuoles [10] or by endocytosis resulting from invagination of the plasma membrane and formation of membrane vesicles (for a review, see Ref. [11]).

Within the seminiferous epithelium, there is evidence that dynamic remodeling of tight, adherens and gap junctions, which form the blood–testis barrier are mediated by endocytosis of these membranous structures during normal spermatogenesis. It has been previously reported that toxicants disturb this process by modifying the internalization of these junctional proteins [12,13]. Indeed, testicular toxicants such as DDT, lindane and phthalates, are not only able to induce vacuoles [14] but also to delocalize the gap junction protein connexin 43 (Cx43), zonula occludens 1 (ZO-1), a tight junction associated protein [15–18] and flamingo1, a protein localized to sites of cell adhesion in Sertoli cells [19]. Adherens junctions and one of its constitutive proteins, N-cadherin, were detected in the membranes of vacuoles in Sertoli cells from a pathological mutant mice displaying a defect in cell adhesion in the testis confirming high endocytic activity in some testicular pathological situations [20].

In this study, we have analyzed the vacuolization capacity of a Sertoli cell line (SerW3) in relation with the internalization of the junctional proteins, Cx43, ZO-1 and N-Cadherin in response to DDT. The present data demonstrate that rapid Sertoli cell vacuolization mainly results from plasma membrane fragment endocytosis. The accelerated internalization of junctional proteins Cx43, ZO-1 and N-cadherin may reflect an early event of Sertoli cell response to non-cytoxic doses of DDT.

## 2. Materials and methods

### 2.1. Chemicals and antibodies

Tissue culture supplies, Dulbecco's modified Eagle's medium and fetal calf serum (FCS), were obtained from Gibco BRL (Cergy Pontoise, France). EGF, insulin and transferrin, selenium, testosterone, ATP 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), DDT (99.7% purity) and rhodamine–dextran were from Sigma (St. Louis, MO), calcein-AM (acetoxymethylester) was provided by Invitrogen SARL (Cergy Pontoise, France) and Mowiol medium by Calbiochem (USA). Polyclonal antibody to ZO-1 and monoclonal antibody to N-cadherin were obtained from Zymed Laboratories (CA) and anti-rabbit IgG from Dako (Trappes, France). Cx43-GFP (Green Fluorescent Protein) vector was a generous gift from Dr. Mathias Falk (Department of Biological Sciences, Bethlehem, PA) and the transfection reagent, Lipofectamin, was purchased from Invitrogen SARL (Cergy-Pontoise, France).

### 2.2. Cell culture and toxicant treatment

The SerW3 Sertoli cell line was maintained in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and cultured at 32 °C as previously described [21]. Cells were seeded at $0.2\times10^6$ cell/well in 12-well plates. After 2 days in DMEM containing 5% SVF, cells were washed twice with DMEM without serum and incubated for 12 h in serum-free medium composed of DMEM supplemented with EGF (10 mg/l), insulin and transferrin (10 μg/ml), selenium (10 ng/ml) and testosterone (0.1 mM). The culture medium was then replaced by fresh serum-free medium completed with $NH_4Cl$ (5 mM) and cells were cultured in the presence or absence (control) of DDT. DDT was diluted in dimethyl sulphoxide at a final concentration of 0.1% DMSO in culture medium (v/v). In the majority of experiments, DDT was used at concentrations that did not alter cell viability, at least 17 times lower than the $EC_{50}$ as determined in Fig. 1. Control SerW3 Sertoli cells cultured in the usual serum free medium in the presence of 0.1% DMSO did not exhibit vacuoles. For the analysis of vacuolization, approximately 120 cells were observed by contrast phase light microscopy in three different experiments and the percentage of vacuolized cells was quantified in 2 different fields.

### 2.3. Cell viability assay

Cell viability was evaluated as previously described [16]. SerW3 cells were plated at a density of 48,000 cells/well in the absence or presence of increasing concentrations of DDT for 24 h. Tetrazolium salt MTT (0.5 mg/ml) was added for 2 h. The formazan crystals formed were solubilized 1 h with a solution of isopropanol containing HCl 0.1 N and 10% Triton X100 and quantified at 570 nm with a microplate reader Softmax Pro spectrophotometer (Molecular Devices).

### 2.4. Quantification of Cx43 gene expression by real-time quantitative RT-PCR

Total RNA was isolated using RNeasy (Qiagen, 91974 Courtaboeuf, France) as previously described [22]. RNA quality and quantity were determined by measuring absorbance at 260 and 280 nm. cDNA synthesis was performed using high-capacity cDNA archive kit (Applied Biosystems, ZA, Courtaboeuf, France). Probe and primer sequences for Cx43 were chosen according to the Applied biosystems design service (forward: caattcctcctgccgcaat; probe: acaacaagcaagccag; reverse: tcgttttgacccgcttaatgtc). For the endogenous control *18S* gene, primers and probe were obtained from Applied Biosystems (4333760t). Real-time PCR was performed with the Singleplex PCR reaction Mix from Applied Biosystems. Briefly, cDNA in a 25 μl total volume solution containing 12.5 μl TaqMan Universal PCR Master Mix, 0.415 μl assay mix 60× was denatured for 2 min at 50 °C and 10 min at 95 °C. Forty cycles, each consisting of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min, were performed. Amplification was visualized by the emission of the probe fluorescence. Quantitative normalization of cDNA in each sample was evaluated to the endogenous control in order to normalize quantity of cDNA samples. The relative quantification was performed with the ABI Prism 7700 Sequence detection system using the standard threshold cycle (Ct) method. Three independent total RNA extractions were carried out and DNA follow-up samples were measured in duplicate.

### 2.5. Cx43-GFP transfection

For Cx43-GFP transient expression, cells (70% confluency) were transfected using Lipofectamin (Invitrogen) with a Cx43-GFP probe as recently reported [23] and cultured in the presence or absence of DDT. Positive transfected clones were identified by intense fluorescence observed between adjacent cells.

### 2.6. Immunofluorescence analysis

Cells were fixed in cold methanol at −20 °C for 5 min. Cells were then incubated with anti-ZO-1 or anti N-Cadherin (1:100) in PBS containing 0.1% BSA, 0.2% gelatine and 0.5% saponin, overnight at 4 °C as previously reported [16]. Cells were rinsed with PBS 0.5% saponin and then incubated for 1 h with a FITC-conjugated anti-rabbit IgG or anti-mouse IgG (1:100) in PBS containing 0.1% BSA, 0.2% gelatine, and 0.5% saponin. After washing, the slides were then mounted in Mowiol medium for immunofluorescence and were examined with a laser scanning confocal microscope (Leica TCS SP). Cells incubated without the primary antibodies showed no staining (data not shown). Nuclear staining of cells was performed by incubating slides in a solution of 2 mg/ml DAPI (Sigma).

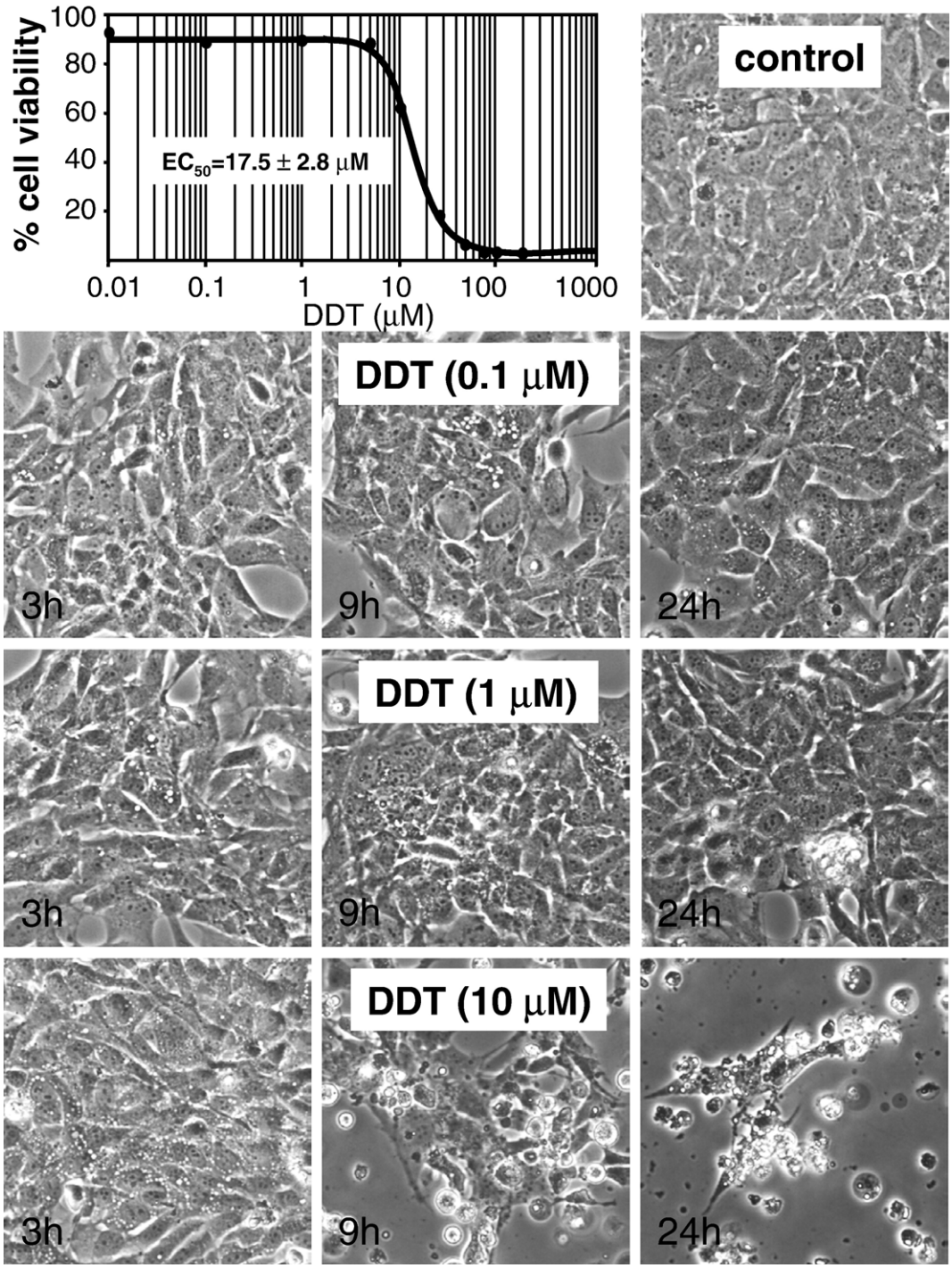


Fig. 1. Effect of DDT on cell viability and cell vacuolization in SerW3 Sertoli cells. For the analysis of cytotoxicity potency, cells were treated with the indicated concentrations of DDT for 24 h and the cell viability was assessed with the MTT assay as described in Materials and methods. Cell vacuolization was analyzed in cells cultured for different times (only 3-, 9- and 24-h exposures are presented) in the presence or absence of DDT at doses near the $EC_{50}$, and 10 or 100 times lower. Representative of 3 different experiments.

### 2.7. SDS-PAGE and immunoblotting

The whole cell lysates (75 μg) of cultured cells were subjected to one-dimensional SDS-PAGE (9%). For immunoblotting, proteins were transferred onto a polyvinylidene fluoride membrane (PVDF Immobilon-P, Millipore) which was then incubated with the anti-Cx43 (1:2000) from Transduction Laboratories (Lexington, KY) as previously reported [16]. The presence of the primary antibody was revealed with horseradish peroxidase-conjugated secondary antibody:anti-mouse (1:5000) IgG (Dako, Trappes, France), and visualized with an enhanced chemiluminescence detection system (ECL, Amersham, UK).

### 2.8. Dye coupling analysis

The cell to cell coupling was measured by the gap FRAP analysis as previously described [23]. Briefly, cells were loaded for 30 min at 32 °C with

calcein-AM diluted in PBS (5 μM). After three PBS washes, cells were exposed to DDT or to vehicle alone. Dye transfer was then monitored by using an interactive laser cytometer (Zeiss LSM 510, Service Commun de Microscopie, IFR 95, University Paris 5, France). Cells were bleached with a 488-nm laser and recovery of fluorescence intensity was monitored at 2-min intervals. Analysis of the fluorescence recovery was quantified using a Zeiss LSM software.

### 2.9. Measure of endocytic capacity

To determine the effect of DDT on endocytosis, rhodamine–dextran (RD, 0.5%) was applied to cell culture with or without DDT for 10 min to fill vesicules. In order to determine if the clathrin–endocytic pathway was involved, cells were pretreated with 0.2 M sucrose (known to inhibit clathrin-dependant internalization) for 3 h as previously described [24].

### 2.10. Fluorescence microscopy and image analysis

For live observations, cells were cultured on labtek coverslides for 24 h. Images of cells were collected at 32 °C with a Nikon TE2000E deconvolution microscope equipped with a 100× oil immersion objective (PlanApo VC/1.40 N.A.), through a cooled charge-coupled device camera (Roper CoolSnap HQ2). Time lapse images collected every 30 s for 180 min were accumulated and deconvolved in AutoQuant software.

### 2.11. Electron microscopy

Briefly Sertoli cell pellets were fixed with 3.5% glutaraldehyde in phosphate-buffered saline (0.1 M, pH: 7.4) for 1 h at room temperature. Cell pellets were postfixed in reduced osmium, dehydrated and embedded in epon. Sections were counterstained with lead citrate and analyzed with a Philips CM 10 electron microscope (Institut A. Feyssard, CNRS, Gif sur Yvette, France).

### 2.12. Statistical analysis

Data are expressed as mean±S.E. Comparisons between treated cells and corresponding controls without treatment were analyzed by one-way analysis of variance (ANOVA) and Student's *t*-test. Statistical significance was evaluated at $P<0.05$ and 0.01.

## 3. Results

Exposure of SerW3 Sertoli cells to increasing concentrations of DDT ranging from 0.01 to 200 μM shows that DDT was cytotoxic for doses higher than 5 μM (Fig. 1). The $EC_{50}$ calculated at 24 h from the different dose–response curves was 17.5±2.8 μM (M±S.E., $n=6$). In the absence of DDT in the culture medium, no vacuoles were observed by phase contrast microscopy in the cytoplasm of SerW3 cells (Fig. 1). In the presence of 0.1 and 1 μM DDT, doses 10 or 100 times lower that the $EC_{50}$, around 20% of the cells exhibited intracytoplasmic vacuoles. The vacuoles were observed at 3-, 9- and 24-h treatment with the two doses of DDT (0.1 and 1 μM) but were undetectable when cells were cultured for more than 48 h in the presence of DDT (data not shown). Approximately 80% of the cells exhibited vacuoles when cultured for 3 h in the presence of 10 μM DDT, a dose near the $EC_{50}$. For longer times of culture (9 and 24 h) most of cells died.

In order to discriminate between autophagic and endocytic vacuoles, SerW3 Sertoli cells were exposed to DDT, at a dose (1 μM) and at a time period that did not alter cell viability, and vacuole formation was analyzed by time lapse video microscopy. As shown in images captured at time 5, 7, 8 and 12 min (Fig. 2, left panels), no major modifications of the contact zone between two adjacent cells, identified by calcein-AM staining, were observed in control cells. In contrast, in the presence of DDT, the spaces between adjacent viable cells rapidly enlarged, within 5–7 min, leading to clear disruption of the close cell–cell contacts (Fig. 2, right panels). Fine microscopy analysis demonstrated that many vacuoles were concomitantly distributed close to the plasma membranes. Due to this location, the possibility that these vacuoles exhibited an endocytic origin was put forward.

To verify this hypothesis, cells were cultured in the presence of rhodamine–dextran with or without 1 μM DDT. As shown in Fig. 3B, numerous red vacuoles loaded with rhodamine–dextran were detected in cells exposed to the pesticide, whereas only few spots were present in control cells (Fig. 3A). To test the potential role of clathrin-mediated endocytosis in the vacuole formation, cells were incubated in the presence of DDT in an hypertonic media known to prevent the formation of clathrin-coated pits. The presence of 0.2 M sucrose in the culture medium totally blocked the formation of endocytic vacuoles (Fig. 3C). Electron microscopy analysis revealed that the vacuole membrane contained cluster of electron-dense membranous elements suggesting the internalization of membranous components (Fig. 3D–F). It is noteworthy that the dose of DDT, which induced both disruption of cell–cell contacts and endocytic vacuolization, was also capable of altering the localization of junctional proteins such as N-cadherin and ZO-1. Immunofluorescent staining demonstrated that N-cadherin and ZO-1 were present at the zone of contact between adjacent cells in control cells (Fig. 3G, J) and detected in the cytoplasmic compartment after treatment of the cells with DDT (Fig. 3H, K). Thus, we hypothesized that these proteins could be components of the endocytic vacuole membranes of cells exposed to DDT. High magnifications revealed that specific immunoreactive spots were present in small vesicles near the plasma membrane (Fig. 3I, L) and sometimes in plasma membrane invagination (Fig. 3I).

Cx43-GFP transfected cells form large gap junction plaques whose internalization can be easily analyzed in real-time by deconvolution microscopy. In control cells, large gap junction plaques were present between adjacent cells (Fig. 4A, left panel), whereas in DDT-treated cells, only small fluorescent spots containing Cx43-GFP were detected in the cytoplasm (Fig. 4A, middle panel). When cells were loaded with rhodamine–dextran and exposed to DDT, the red dye colocalized with the green intracytoplasmic fluorescence, indicating the presence of Cx43 within the endocytic vacuoles (Fig. 4A, right panel). In order to study the effect of DDT on *Cx43* gene expression and protein level, real-time quantitative PCR and Western blot analyses were performed on cells exposed to 1 μM DDT. Fig. 4B showed that DDT treatment was unable to affect Cx43 expression standardized to 18S. In addition, Western blot analysis revealed that Cx43 was present, as expected, as three distinct bands corresponding respectively to the non-phosphorylated ($P_0$) and the two phosphorylated ($P_1$ and $P_2$) isoforms (Fig. 4C). This figure also showed that the dose of DDT that was able to induce

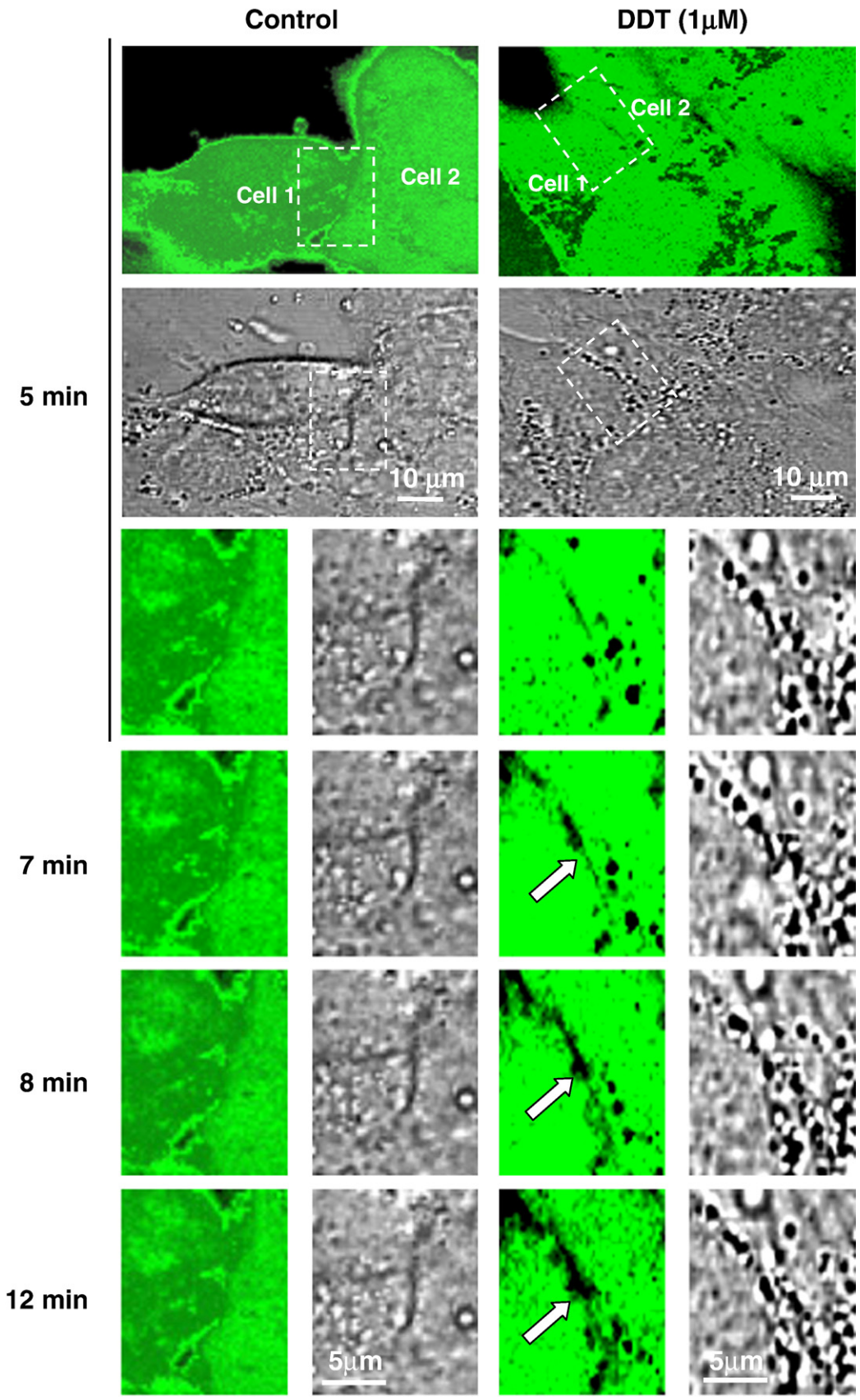


Fig. 2. Time lapse video microscopy analysis of the short-term effect of DDT on cell membrane stability and vacuole formation. SerW3 Sertoli cells, loaded with calcein-AM, were cultured in the presence or absence of 1 μM DDT. Note that rapidly after DDT exposure, within 7 min, the space between adjacent cells enlarged (open arrows) leading to the disruption of the close cell–cell contact and small vacuoles were apparent near the plasma membrane. Representative of 4 different experiments.

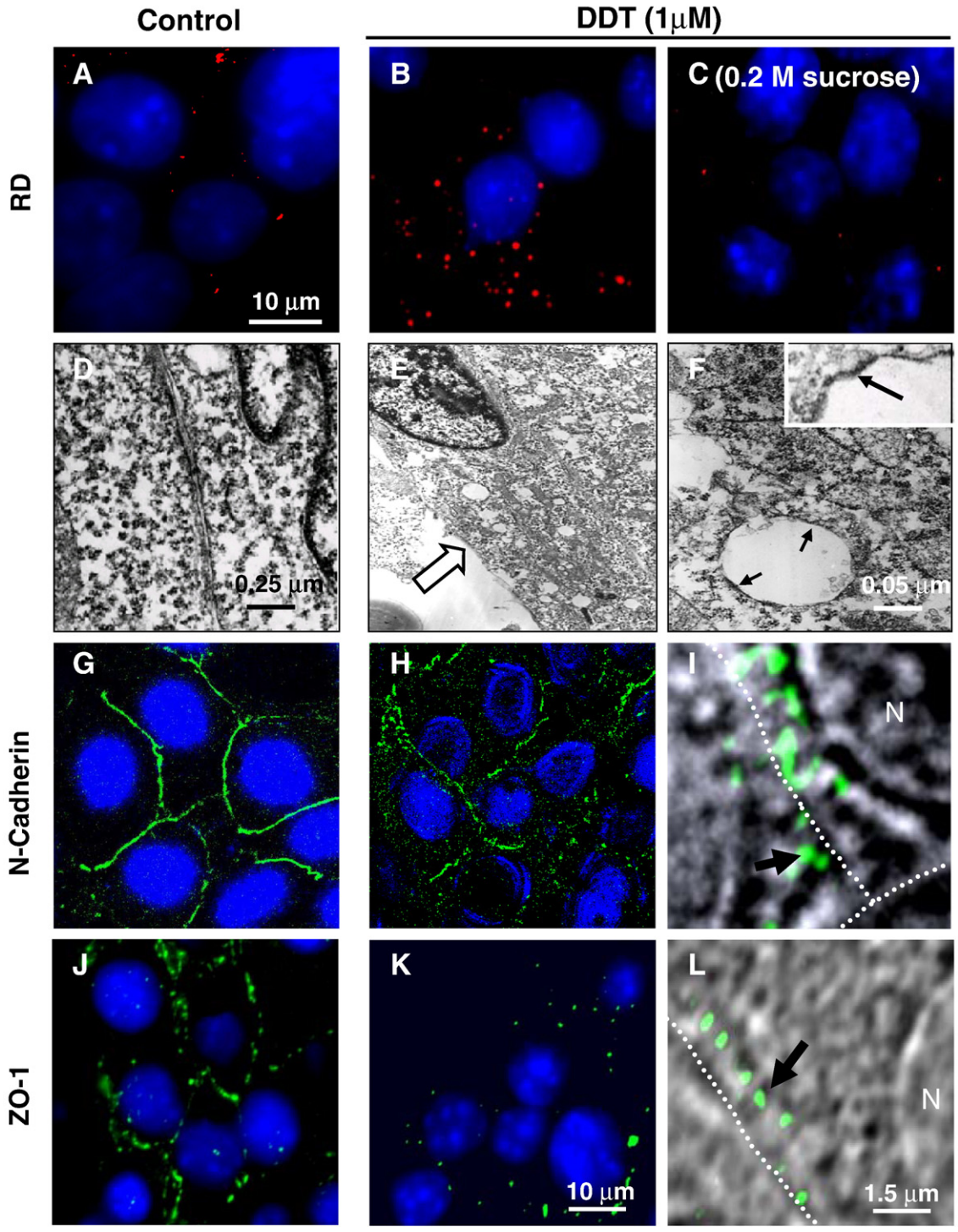


Fig. 3. Stimulatory effect of DDT on endocytosis and internalization of junctional proteins, ZO-1 and N-cadherin. SerW3 Sertoli cells were cultured in the presence of rhodamine–dextran, to analyze the endocytic capacity, with or without 1 μM DDT (A, B). A hyperosmotic media (0.2 M sucrose) totally reversed the formation of rhodamine–dextran positive endocytic vacuoles (red spots) induced by DDT (C). Electron microscopy analysis confirmed disruption of contact between cells exposed to DDT (large arrow) and revealed that the vacuole membrane contained cluster of electron-dense membranous elements (F, arrows). In the presence of DDT the distribution of the immunosignals for N-cadherin and ZO-1 switched from the plasma membrane (G, J) to a more cytoplasmic localization (H, K). High magnification revealed that the immunoreactive signals for N-cadherin (I) and ZO-1 (L) were present in the invagination of the plasma membrane and in small vesicles close to the plasma membranes indicated by dotted lines. N=nucleus. Representative of 3 different experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

endocytic vacuoles and internalization of Cx43 was unable to alter the levels of Cx43 or its SDS gel mobility during the same time period (Fig. 4C).

Time lapse video microscopy showed that internalization of gap junction plaques was characterized in control cells by the formation of large annular gap junctions, the constitutive

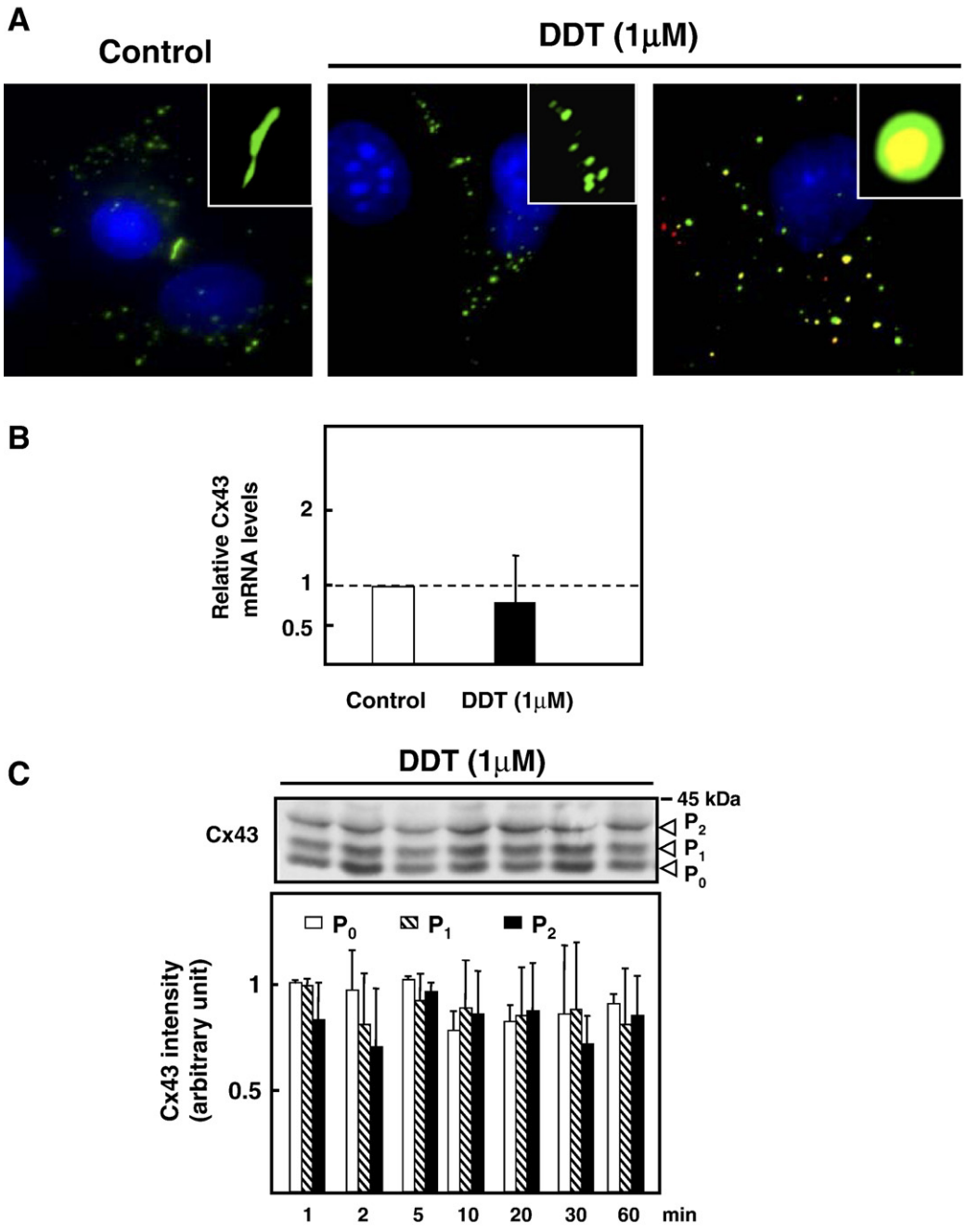


Fig. 4. Effect of DDT on gap junction plaque endocytosis and Cx43 expression. (A) SerW3 Sertoli cells transfected with Cx43-GFP exhibited large gap junction plaques (left panel and insert) that were fragmented in the presence of DDT (middle panel and insert). Green fluorescence corresponding to internalized Cx43 colocalized with rhodamine–dextran (yellow fluorescence) in endocytic vacuoles (right panel). (B) Cx43 mRNA levels analyzed by quantitative RT-PCR in cells cultured for 24 h in the presence or absence of 1 μM DDT. Values represent the means ± S.E. of 3 different experiments analyzed in duplicate and normalized to 18S. Cx43 expression in DDT-treated cells was normalized to control cells corresponding to the arbitrary value of 1. (C) Western blot analysis of Cx43 in SerW3 exposed for increasing time periods to 1 μM DDT. The anti-Cx43 antibody detected the presence of unphosphorylated ($P_0$) and phosphorylated ($P_1$ and $P_2$) Cx43 isoforms. The positions of Cx43 isoforms are shown by arrowheads. Densitometric scannings of $P_0$, $P_1$ and $P_2$ Cx43 isoforms are shown in the lower panel. Values are means ± S.E. of 3 different experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mechanism by which gap junction plaques are degraded within cells (Fig. 5A). In contrast, in the presence of 1 μM DDT, the Cx43-GFP gap junction plaques were disintegrated to form cytoplasmic vacuoles containing Cx43-GFP membranous fragments (Fig. 5A). DDT affected not only the endocytic mechanism of gap junction internalization but also the kinetic of endocytosis. As shown in Fig. 5B, the mean time before complete internalization of a gap junction plaque was of 180.2 ± 14.8 min in control cells whereas this time period was significantly reduced to 35.8 ± 12.5 min in the presence of 1 μM DDT ($P < 0.01$).

Results presented in Fig. 6 showed that the deleterious effect of low doses of DDT on Cx43 was a transient process characterized on both the number of Cx43-GFP gap junction plaques and the gap junctional coupling. Indeed, DDT reduced by 50%

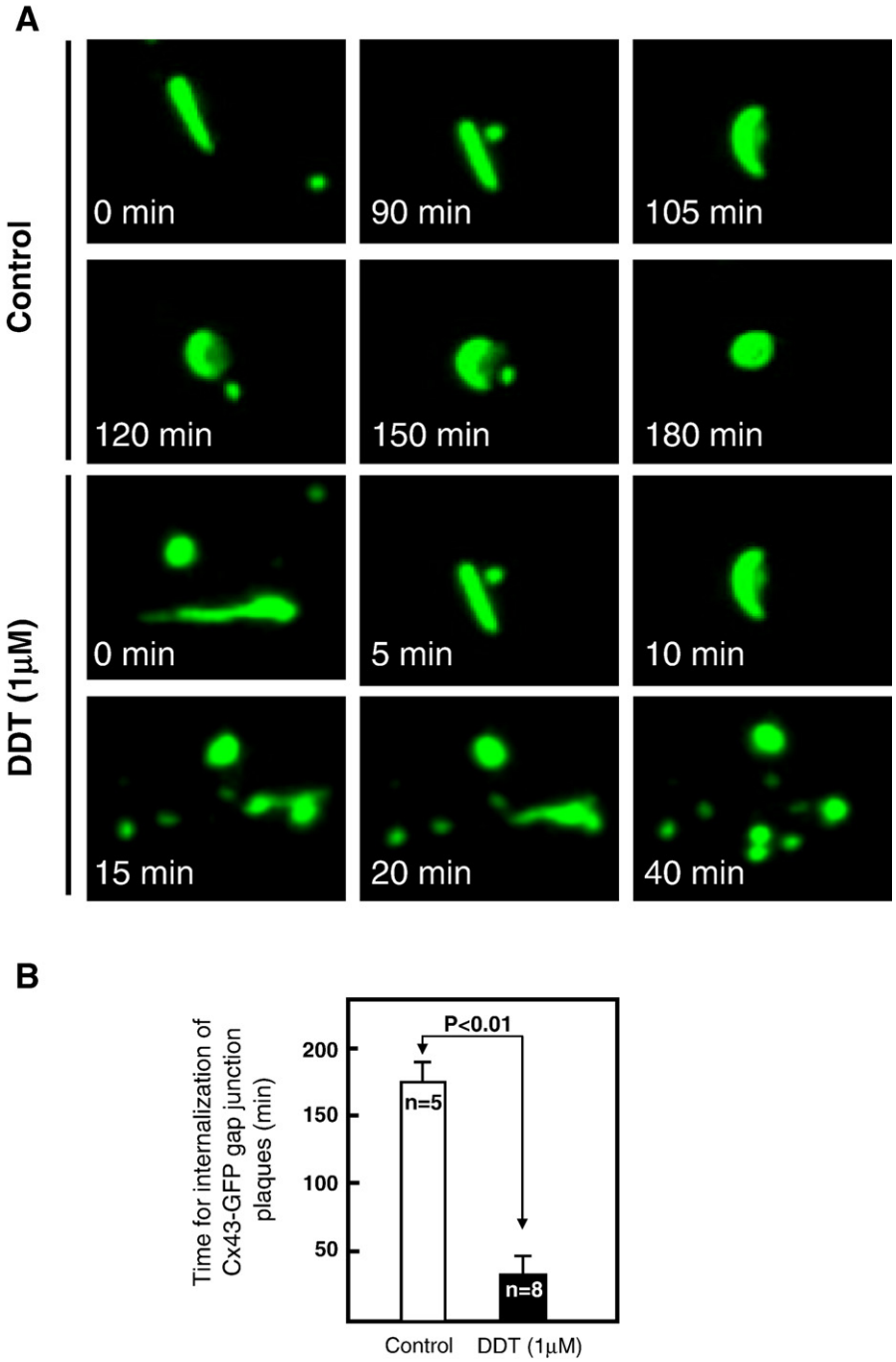


Fig. 5. Time lapse video microscopy analysis of Cx43 gap junction plaque endocytosis in control cells or in presence of DDT. (A) In control cells, endocytosis of the complete gap junction plaque occurred slowly with the formation of large vacuoles identified as annular gap junctions. In contrast, the plaque was rapidly disintegrated in DDT-treated cells and small plaques fragments were internalized and formed cytoplasmic Cx43-GFP vesicles. (B) Quantitative analysis of the mean time (±S.E.) before total Cx43-GFP gap junction plaque internalization in cells cultured in the presence or absence of 1 μM DDT. The number of independent experiments is indicated in the columns.

the number of Cx43-GFP plaques after 1-h exposure ($P<0.01$), by 20% after a 3-h treatment ($P<0.05$), whereas no significant effect of DDT on the number of plaques was evidenced after a 6-h exposure (Fig. 6A). The decreased number of gap junction plaques and its turn to control values in function of time was confirmed by the analysis of the intercellular coupling measured by gap FRAP (Fig. 6B). In this case, as reported for gap junction plaque number, the kinetic of the junctional coupling appeared down-regulated during the first hour and up-regulated afterwards. Gap junctional coupling in treated cells was significantly decreased at 20 ($P<0.05$) and 30 min ($P<0.01$) with a maximal reduction observed at 1 h ($P<0.01$). Thereafter, the gap junctional coupling was recovered and values near the control levels were obtained for a time course exposure of 24 h. After washing the cells, exposure to the same dose of DDT for a second period of 24 h, after the first treatment, resulted in another

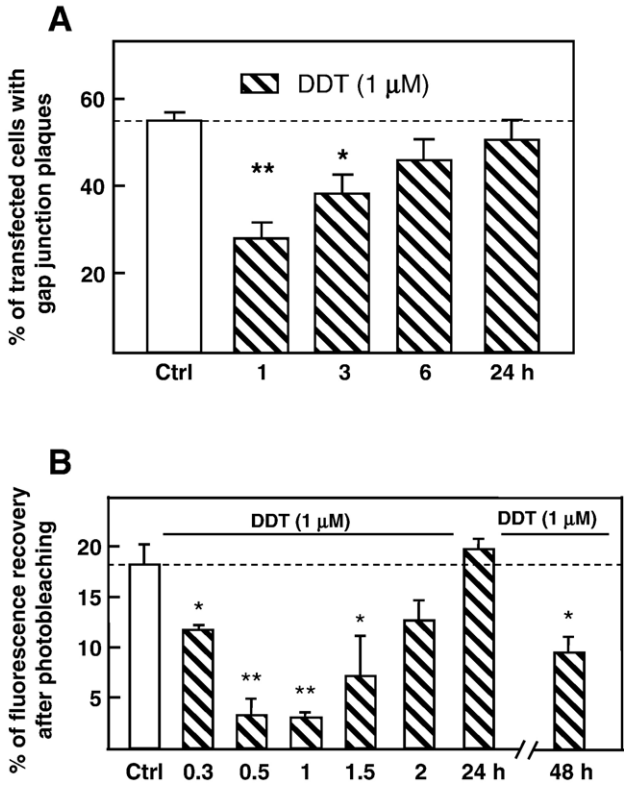


Fig. 6. Reversibility of the DDT effect on both gap junction plaque and cell coupling. (A) Cx43-GFP transfected SerW3 Sertoli cells were cultured in the presence or absence of 1 μM DDT and the number of gap junction plaques was quantified at different time culture periods. (B) As for gap junction plaque number, the level of cell coupling was restored after 24-h culture. Note that a second exposure to 1 μM DDT did not allow to return to control value. *$P<0.05$; **$P<0.01$ significantly different from control values.

disruption of gap junction functionality (Fig. 6B). However, in this case, the intercellular coupling values did not return to control values and the recovery was about 50% ($P<0.01$).

## 4. Discussion

Many studies have shown that DDT may exert its carcinogenesis effects through different mechanisms not entirely characterized [5]. Among them, the interaction of DDT with the plasma membrane and subsequently alteration of membrane structure and function has been suggested. However, the fine molecular and cellular mechanisms have not been clearly reported. By using the Sertoli cell line SerW3, we demonstrated here that DDT may exert a dual deleterious effect on cell function. Firstly, doses of the chemical, near the $EC_{50}$, induced the formation of vacuoles, probably of autophagic origin, and lead to cell death. Secondly, to lower non-cytotoxic doses of DDT, a time-dependent and reversible effect has been observed. The present data revealed that this early response was characterized by rapid changes in the membrane stability (disruption of cell–cell contacts) and function (loss of junctional proteins from plasma membrane).

We previously reported that Cx43, the predominant gap junction protein in the testis, ZO-1 (a tight junction associated protein) and N-cadherin (adherens junction) are delocalized in the cytoplasm of SerW3 cells after treatment with various toxicants including DDT [16]. The present results showed that DDT modified the endocytic process of junctional proteins from the plasma membrane. However, we cannot eliminate the possibility that this compound also altered trafficking of these junctional proteins toward the plasma membrane. The dose of the toxicant able to alter the expression and/or the localization of junctional protein is close to that reported to induce SerW3 vacuolation. The current immunofluorescence studies performed with ZO-1 and N-cadherin antibodies or in Cx43-GFP transfected cells showed that these junctional proteins were closely associated with vacuoles in SerW3 cells exposed to DDT. These observations support the idea that vacuoles induced by the chemical can reflect an exaggeration of the physiological internalization of junctional proteins. This hypothesis is reinforced by images of time lapse video microscopy analysis of Cx43 gap junction plaques that showed a rapid internalization, within about 30 min, whereas these events occurred only after 3 h in control cells.

Constitutive endocytosis of adherens, tight and gap junctions has been shown in numerous epithelial remodeling processes [25]. In the testis, junctional complexes located at the level of the blood–testis barrier between Sertoli cells are highly dynamic structures undergoing continuous remodeling. These junctions and their associated proteins are internalized by endocytosis and recycled to the new site of adhesion during translocation of germ cells from the basal to the adluminal compartment [26]. Exaggerated internalization of junctional complex proteins has been shown in response to various pathologic stimuli (for a review, see Ref. [27]). The present data show that environmental pollutants such as DDT can also accelerate endocytosis of junctional proteins and that the increase in vesicle number identified by rhodamine–dextran is the result of this endocytosis intensification.

The molecular mechanisms that drive this process are still unknown. Endocytosis of cell membrane proteins can occur by three major mechanisms: caveolin-coated vesicle, clathrin-coated vesicle or uncoated vesicle (for a review, see Ref. [27]). From the present data, it is obvious that DDT promotes endocytosis of cell surface junction proteins via a clathrin-mediated process. First, hyperosmotic media, known to inhibit clathrin-coated pit formation [23], totally blocked the internalization of extracellular rhodamine–dextran. Second, Cx43 was detected within the endocytic vacuoles in cells exposed to DDT. In addition, the precise mechanisms by which DDT stimulates endocytosis of plasma membrane fragment containing proteins implicated in the cell–cell communication are unknown. There is evidence that endocrine disrupters such as DDT, that has potent estrogenic activity, exert their deleterious effects through different mechanisms. They can trigger the specific receptor of natural hormones, block their action and/or alter their production, transport and metabolism. Recently, an inhibitory effect of DDT on the SerW3 FSH response was reported [28]. Another possibility is that DDT, which is highly lipophilic, accumulates in cell membranes and promotes lipid alterations [29]. Thus, the rapid effect of the chemical, observed in the present study, could

be caused by a non-specific effect on membrane stability rather than to a specific enhancement of junctional protein internalization. This possibility is supported by time lapse video microscopy experiments showing a disintegration of the gap junction plaque into small membrane fragments containing Cx43-GFP in cells exposed to DDT, whereas this process was never observed during constitutive endocytosis of gap junction plaque [30,31]. It is also reinforced by the lack of major Cx43 phosphorylation modification analyzed by Western blotting. It is interesting to note that a concentration of DDT (0.1 μM), 100 times lower than the $EC_{50}$, also induced the same plaque fragmentation and internalization (data not shown). Taken together these data suggest that high lipophilic non-genotoxic chemicals as DDT may alter junctional membrane proteins dependent on the lipid phase probably by inducing membrane-perturbing effects, whereas no-highly lipophilic agents affect junctional complexes by different other pathways such as activation of PKC for TPA [32,33] or ERK/mitogen-activated protein kinase pathway as demonstrated with another pesticide, lindane [17]. However, the current results do not totally exclude the possibility that DDT affect the clathrin-mediated endocytosis per se.

A markedly effect of DDT, independent of decreased *Cx43* gene expression, is the regulation of gap junction channel permeability. Such effect was described for short-term exposure to DDT and appeared not associated with the phosphorylation status of Cx43 in rat liver epithelial cells [14,34]. Exposure of granulosa cells, the homologous of Sertoli cells in the female, for 1 h also resulted in the down-regulation of the dye coupling without alteration of Cx43 expression [35]. Our data demonstrate that Cx43 internalization occurred without modification of SDS gel mobility suggesting no major alteration of the phosphorylation status. On the basis of these observations, it is tempting to suggest that the inhibition of gap junction intercellular communication, observed here in response to DDT, probably resulted from the removal of gap junctions from the plasma membrane through internalization. We previously demonstrated that Sertoli cell exposure to lindane enhanced the internalization of Cx43 in early endosomes [15,17]. Since in our experimental conditions, the effect of DDT was rapidly reversed, it is possible that internalized membranous proteins were sequestered in the early endosome compartment before being targeted to the plasma membrane via the recycling compartment instead of being transported to late endosomes for degradation in lysosomes and/or proteasomes, the two degradative pathways described for this protein [36]. Another possibility is that classical gap junction plaque formation from newly synthesized Cx could occur. The rapid recovery of the gap junction functionality in non-transfected cells (Fig. 6) plays in favour of the first hypothesis, but repeated DDT exposure reveals that the last possibility cannot be excluded because the toxicant decreased Cx expression after a 24-h exposure [16].

Our study also demonstrates that the effect of a low dose of DDT on vacuole formation and membranous junctional protein endocytosis is a non-persistent and transient process as evidenced by the time-dependent redistribution of Cx43-GFP at the plasma membrane level after the beginning of DDT exposure. This observation does not exclude that more long-term exposure to DTT might exert other more drastic effects at the transcriptional and post-translational levels. Indeed, we recently reported that this chemical is able to affect significantly the levels of membranous proteins implicated in Sertoli cell–cell communication [16].

Recent studies have revealed that Cx needs other junctional proteins such as N-cadherin and ZO-1 to form gap junction plaques. Indeed, E-cadherin is necessary to restore Cx43 at the cytoplasmic membrane and gap junction coupling gene into uncoupled skin papilloma cells [37]. In addition reduced expressions of E-cadherin, Cx32 and Cx43 resulted in impaired cell–cell adhesion and communication and were associated with dedifferentiation, invasion and metastatic potential of human prostate tumor cells [38]. The association of Cx43 and ZO-1 is required for stability [39] and internalization of the gap junction plaques [18,40]. The present data demonstrate that the adverse effect of DDT also concerns these two Cx43 partners. They support the idea that DDT globally modifies cell–cell interactions and junctions rather than Cx43 expression since neither the levels of Cx43 nor its phosphorylation status were altered after short-term treatments with the pesticide.

Cx is present in representatives of the major vertebrate groups [41] and is a key element in the maintenance of cell proliferation and tissue homeostasis [42]. There is evidence that reduced Cx expression is associated with carcinogenesis while forced expression of Cx in malignant cells suppresses tumorigenicity [43]. Most carcinogens have been reported to inhibit cell–cell communication through increased internalization and degradation of Cx [44]. However, toxicological studies have reported that the carcinogenic effect of DDT was only induced for high doses of this chemical, whereas low doses were inactive or even inhibitory in carcinogenesis [45]. The present data clearly show that, although functionality can be rapidly recovered after the first exposure to a low dose of DDT, the presence of a constant low dose of DDT added every 24 h maintained a reduced gap junction coupling suggesting that exposure during long-time period, even to low dose of DDT, may be responsible in non-genomic carcinogenesis. It remains to be determined whether the effect of DDT, demonstrated here, could be additive or synergistic with other agents as previously reported [46,47].

In conclusion, the present study indicates that DDT, at a low non-cytotoxic dose, induce abnormal disintegration of the gap junction plaque, and internalization of both Cx43 and its partners (ZO-1 and N-cadherin), through the formation of clathrin-dependent endocytic vacuoles. These effects are probably not specific to junctional complexes but rather result from a membrane disorganization as clearly evidenced by the loss of cell–cell membrane interactions. These events might represent an early response to deleterious DDT effects occurring prior to any appreciable biochemical changes.

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## References

[1] IARC, Working Group on the Evaluation of Carcinogenic Risks to Humans. Occupational exposure in insecticide application, and some pesticides in IARC, Monogr Eval Carcinog Risk Hum (1991) 179–249.
[2] W.J. Rogan, A. Chen, Health risks and benefits of *bis*(4-chlorophenyl)-1,1,1-trichloroethane (DDT), Lancet 366 (2005) 763–773.
[3] A. Mandavilli, Health agency backs use of DDT against malaria, Nature 443 (2006) 250–251.
[4] T. Harada, S. Yamaguchi, R. Ohtsuka, M. Takeda, H. Fujisawa, T. Yoshida, A. Enomoto, Y. Chiba, J. Fukumori, S. Kojima, N. Tomiyama, M. Saka, M. Ozaki, K. Maita, Mechanisms of promotion and progression of pre-neoplastic lesions in hepatocarcinogenesis by DDT in F344 rats, Toxicol. Pathol. 31 (2003) 87–98.
[5] M. Kushida, T. Sukata, S. Uwagawa, K. Ozaki, A. Kinoshita, H. Wanibuchi, K. Morimura, Y. Okuno, S. Fukushima, Low dose DDT inhibition of hepatocarcinogenesis initiated by diethylnitrosamine in male rats: possible mechanisms, Toxicol. Appl. Pharmacol. 208 (2005) 285–294.
[6] G. Tsushimoto, C.C. Chang, J.E. Trosko, F. Matsumura, Cytotoxic, mutagenic, and cell–cell communication inhibitory properties of DDT, lindane, and chlordane on Chinese hamster cells in vitro, Arch. Environ. Contam. Toxicol. 6 (1983) 721–729.
[7] J. Beard, DDT and human health, Sci. Total Environ. 355 (2006) 78–89.
[8] T.K. Monsees, M. Franz, S. Gebhardt, U. Winterstein, W.B. Schill, J. Hayatpour, Sertoli cells as a target for reproductive hazards, Andrologia 32 (2000) 239–246.
[9] K. Boekelheide, Sertoli cell toxicants, in: L.D. Russell, M.D. Griswold (Eds.), The Sertoli cell, Cache River Press, Clearwater, Florida, 1993, pp. 552–575.
[10] A.J. Meijer, P. Codogno, Regulation and role of autophagy in mammalian cells, Int. J. Biochem. Cell Biol. 36 (2004) 2445–2462.
[11] J.S. Bonifacino, B.S. Glick, The mechanisms of vesicle budding and fusion, Cell 116 (2004) 153–166.
[12] J.A. Guttman, Y. Takai, A.W. Vogl, Evidence that tubulobulbar complexes in the seminiferous epithelium are involved with internalization of adhesion junctions, Biol. Reprod. 71 (2004) 548–559.
[13] D.D. Mruk, C.Y. Cheng, Sertoli–Sertoli and Sertoli–germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis, Endocr. Rev. 25 (2004) 747–806.
[14] X. Guan, R.J. Ruch, Gap junction endocytosis and lysosomal degradation of connexin43-P2 in WB-F344 rat liver epithelial cells treated with DDT and lindane, Carcinogenesis 17 (1996) 1791–1798.
[15] N. Defamie, B. Mograbi, C. Roger, L. Cronier, A. Malassine, F. Brucker-Davis, P. Fenichel, D. Segretain, G. Pointis, Disruption of gap junctional intercellular communication by lindane is associated with aberrant localization of connexin43 and zonula occludens-1 in 42GPA9 Sertoli cells, Carcinogenesis 22 (2001) 1537–1542.
[16] C. Fiorini, A. Tilloy-Ellul, S. Chevalier, C. Charuel, G. Pointis, Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants, Reprod. Toxicol. 18 (2004) 413–421.
[17] B. Mograbi, E. Corcelle, N. Defamie, M. Samson, M. Nebout, D. Segretain, P. Fenichel, G. Pointis, Aberrant connexin 43 endocytosis by the carcinogen lindane involves activation of the ERK/mitogen-activated protein kinase pathway, Carcinogenesis 24 (2003) 1415–1423.
[18] D. Segretain, C. Fiorini, X. Decrouy, N. Defamie, J.R. Prat, G. Pointis, A proposed role for ZO-1 in targeting connexin 43 gap junctions to the endocytic pathway, Biochimie 86 (2004) 241–244.
[19] J.H. Richburg, K.J. Johnson, H.A. Schoenfeld, M.L. Meistrich, D.J. Dix, Defining the cellular and molecular mechanisms of toxicant action in the testis, Toxicol. Lett. 135 (2002) 167–183.
[20] E. Hellsten, D.J. Bernard, J.W. Owens, M. Eckhaus, S.F. Suchy, R.L. Nussbaum, Sertoli cell vacuolization and abnormal germ cell adhesion in mice deficient in an inositol polyphosphate 5-phosphatase, Biol. Reprod. 66 (2002) 1522–1530.
[21] F. Pognan, M.T. Masson, F. Lagelle, C. Charuel, Establishment of a rat Sertoli cell line that displays the morphological and some of the functional characteristics of the native cell, Cell Biol. Toxicol. 13 (1997) 453–463.
[22] N. Defamie, I. Berthaut, B. Mograbi, D. Chevallier, J.P. Dadoune, P. Fenichel, D. Segretain, G. Pointis, Impaired gap junction connexin43 in Sertoli cells of patients with secretory azoospermia: a marker of undifferentiated Sertoli cells, Lab. Invest. 83 (2003) 449–456.
[23] J. Gilleron, M. Nebout, L. Scarabelli, F. Senegas-Balas, S. Palmero, D. Segretain, G. Pointis, A potential novel mechanism involving connexin 43 gap junction for control of sertoli cell proliferation by thyroid hormones, J. Cell. Physiol. 209 (2006) 153–161.
[24] J.E. Heuser, R.G. Anderson, Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation, J. Cell Biol. 108 (1989) 389–400.
[25] M. Matsuda, A. Kubo, M. Furuse, S. Tsukita, A peculiar internalization of claudins, tight junction-specific adhesion molecules, during the intercellular movement of epithelial cells, J. Cell Sci. 117 (2004) 1247–1257.
[26] W.Y. Lui, W.M. Lee, Regulation of junction dynamics in the testis-transcriptional and post-translational regulations of cell junction proteins, Mol. Cell. Endocrinol. 250 (2006) 25–35.
[27] A.I. Ivanov, A. Nusrat, C.A. Parkos, Endocytosis of the apical junctional complex: mechanisms and possible roles in regulation of epithelial barriers, Bioessays 27 (2005) 356–365.
[28] L. Bernard, N. Martinat, C. Lecureuil, P. Crepieux, E. Reiter, A. Tilloy-Ellul, S. Chevalier, F. Guillou, Dichlorodiphenyltrichloroethane impairs follicle-stimulating hormone receptor-mediated signaling in rat Sertoli cells, Reprod. Toxicol. 23 (2007) 158–164.
[29] M.M. Donato, A.S. Jurado, M.C. Antunes-Madeira, V.M. Madeira, Membrane lipid composition of Bacillus stearothermophilus as affected by lipophilic environmental pollutants: an approach to membrane toxicity assessment, Arch. Environ. Contam. Toxicol. 39 (2000) 145–153.
[30] G. Gaietta, T.J. Deerinck, S.R. Adams, J. Bouwer, O. Tour, D.W. Laird, G.E. Sosinsky, R.Y. Tsien, M.H. Ellisman, Multicolor and electron microscopic imaging of connexin trafficking, Science 296 (2002) 503–507.
[31] U. Lauf, B.N. Giepmans, P. Lopez, S. Braconnot, S.C. Chen, M.M. Falk, Dynamic trafficking and delivery of connexons to the plasma membrane and accretion to gap junctions in living cells, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 10446–10451.
[32] P. Ren, P.P. Mehta, R.J. Ruch, Inhibition of gap junctional intercellular communication by tumor promoters in connexin43 and connexin32-expressing liver cells: cell specificity and role of protein kinase C, Carcinogenesis 19 (1998) 169–175.
[33] L. Warngard, H. Hemming, S. Flodstrom, S.K. Duddy, G.E. Kass, Mechanistic studies on the DDT-induced inhibition of intercellular communication, Carcinogenesis 10 (1989) 471–476.
[34] R.J. Ruch, W.J. Bonney, K. Sigler, X. Guan, D. Matesic, L.D. Schafer, E. Dupont, J.E. Trosko, Loss of gap junctions from DDT-treated rat liver epithelial cells, Carcinogenesis 15 (1994) 301–306.
[35] U. Tiemann, R. Pohland, W. Richter, K. Wollenhaupt, Influence of *tris* (4-chlorophenyl)methanol (TCPM) on gap junction-mediated intercellular communication of cultured bovine granulosa cells, Toxicol. In Vitro 16 (2002) 129–139.
[36] J.G. Laing, P.N. Tadros, E.M. Westphale, E.C. Beyer, Degradation of connexin43 gap junctions involves both the proteasome and the lysosome, Exp. Cell Res. 236 (1997) 482–492.
[37] W.M. Jongen, D.J. Fitzgerald, M. Asamoto, C. Piccoli, T.J. Slaga, D. Gros, M. Takeichi, H. Yamasaki, Regulation of connexin 43-mediated gap junctional intercellular communication by $Ca^{2+}$ in mouse epidermal cells is controlled by E-cadherin, J. Cell Biol. 114 (1991) 545–555.
[38] H. Habermann, W.Y. Chang, L. Birch, P. Mehta, G.S. Prins, Developmental exposure to estrogens alters epithelial cell adhesion and gap junction proteins in the adult rat prostate, Endocrinology 142 (2001) 359–369.
[39] T. Toyofuku, M. Yabuki, K. Otsu, T. Kuzuya, M. Hori, M. Tada, Direct association of the gap junction protein connexin-43 with ZO-1 in cardiac myocytes, J. Biol. Chem. 273 (1998) 12725–12731.
[40] R.J. Barker, R.L. Price, R.G. Gourdie, Increased association of ZO-1 with connexin43 during remodeling of cardiac gap junctions, Circ. Res. 90 (2002) 317–324.

[41] V. Cruciani, S.O. Mikalsen, The vertebrate connexin family, Cell. Mol. Life Sci. 63 (2006) 1125–1140.
[42] W.R. Loewenstein, B. Rose, The cell–cell channel in the control of growth, Semin. Cell Biol. 3 (1992) 59–79.
[43] J.E. Trosko, R.J. Ruch, Gap junctions as targets for cancer chemoprevention and chemotherapy, Curr. Drug Targets 3 (2002) 465–482.
[44] H. Yamasaki, C.C. Naus, Role of connexin genes in growth control, Carcinogenesis 17 (1996) 1199–1213.
[45] T. Sukata, S. Uwagawa, K. Ozaki, M. Ogawa, T. Nishikawa, S. Iwai, A. Kinoshita, H. Wanibuchi, S. Imaoka, Y. Funae, Y. Okuno, S. Fukushima, Detailed low-dose study of 1,1-*bis*(*p*-chlorophenyl)-2,2,2- trichloroethane carcinogenesis suggests the possibility of a hormetic effect, Int. J. Cancer 99 (2002) 112–118.
[46] B.V. Madhukar, M. Yoneyama, F. Matsumura, J.E. Trosko, G. Tsushimoto, Alteration of calcium transport by tumor promoters, 12-O-tetradecanoyl phorbol-13-acetate and *p*,*p*′-dichlorodiphenyltrichloroethane, in the Chinese hamster V79 fibroblast cell line, Cancer Lett. 3 (1983) 251–259.
[47] L. Warngard, S. Flodstrom, S. Ljungquist, U.G. Ahlborg, Interaction between quercetin, TPA and DDT in the V79 metabolic cooperation assay, Carcinogenesis 8 (1987) 1201–1205.