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Mini-Review

Dichlorodiphenyltrichloroethane, an old pesticide with a new mechanism of toxicity

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ABSTRACT

Dichlorodiphenyltrichloroethane (DDT) is an organochlorine derivative known for its detrimental effect on human health. It was abundantly used as a pesticide and finally banned in many countries for its toxicity. Because of its extremely long half-life (up to 10 years), DDT is still blamed to cause health problems, due to the accumulation in the environment. We have previously shown that *in vitro* exposure to DDT causes severe membrane shedding with the release of vesicular organelles such as exosomes and/or ectosomes. A large body of evidence has shown that these vesicles, other than being directly involved in physiological exchanges of cellular materials, are implicated in the pathogenesis of several diseases such as viral and neurodegenerative diseases as well as tumorigenesis. In this short review, we discuss how the increased release of extracellular vesicles could explain the enhanced risk of diseases in patients exposed to organochlorine derivatives such as DDT.

KEYWORDS: dichlorodiphenyltrichloroethane, extracellular vesicles, lipid rafts, exosomes, ectosomes, miRNA.

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1. Introduction

Dichlorodiphenyltrichloroethane (DDT) is an organochlorine compound known for its pesticide properties and negative effects on human health. The mechanism of action of this compound as pesticide is to block voltage-gated sodium channels, which results in a spontaneous neuronal firing that leads to muscle spasms and eventually to the insect death [1]. In the past, DDT has been widely and abundantly used, until eventually banned in many western countries for its toxicity on the endocrine system [2]. In some developing countries, however, most of them in Africa, it is still being used for controlling the spread of malaria through the eradication of mosquito species [3].

During the screening of several goitrogenic environmental factors, Santini *et al*. [4] found that many substances, including DDT, act as thyroid disruptors by inhibiting, *in vitro*, the thyroid stimulating hormone (TSH) receptor-stimulated cAMP production. The TSH receptor is a member of the large family of G protein-coupled receptors and is composed of a large extracellular domain anchored to a seven trans-membrane α helix structure that signals through G-protein [5] and β-arrestin [6] proteins.

We have extensively studied the effect of DDT on TSH receptor demonstrating that DDT inhibited both the basal and the TSH-stimulated

accumulation of cAMP in Chinese hamster ovary (CHO) cells stably transfected with the TSH receptor [7, 8]. Furthermore, we demonstrated that DDT was altering the TSH receptor activity, even when the receptor was deprived of its extracellular domain, suggesting that DDT was targeting the transmembrane part of the TSH receptor [7]. Strikingly, DDT was also causing TSH receptor retention on the cell surface of CHO cells preventing TSH-mediated internalization [9]. These effects of DDT appear quite specific as the function of other receptors positively coupled to the adenylyl cyclase, like the β_2-adrenergic, the dopamine D_1, and the adenosine A_2a, was not affected by DDT presence. Our observations were thus consistent with a model whereby DDT acts on the transmembrane part of the TSH receptor to inhibit its signalling and internalization.

DDT is also a high lipophilic compound that alters membrane fluidity by interacting with phospholipids and cholesterol [10, 11]. In particular, it has been shown that these interactions also lead to depletion of the membrane cholesterol content, and consequently alter the organization of the raft microdomains that segregate the TSH receptor [12]. In the presence of DDT, receptors and lipid rafts would become highly separated and dislodged along opposite cell poles, thus altering the raft-dependent internalization of the TSH receptor and its signalling.

2. DDT induced formation of extracellular vesicles

Unexpectedly, we observed that exposure to DDT led to a profound membrane remodelling characterized by the formation of vesicular buds that were shed from the membrane forming extracellular vesicles. We could speculate that this membrane shedding, as caused by DDT, could be a cell attempt to get rid of the contaminated lipid rafts (Figure 1). Interestingly, this DDT-mediated formation of extracellular vesicles could not simply be the results of its extreme lipophilicity, inasmuch as, another high lipophilic agent structurally related to DDT but devoid of chlorine atoms, the diphenylethylene (DPE), did not alter the organization of the raft microdomains and did not induce the formation of extracellular vesicles [12]. The results observed with DDT, then seem to be ascribed to the polyhalogenation of the aromatic hydrocarbons (chlorination) of DDT other than to its high lipophilicity. This also suggested that other organochlorine pesticides, such as the derivative structurally related to DDT Aroclor 1254 [7] that clearly alters the function of TSH receptors, might have the same effects.

Notably, two types of extracellular vesicles with different sizes and origins have been found: exosomes and ectosomes (also known as microvesciles). Exosomes (40-100 nm size) are formed in an intermediate edocytic compartment, the multivescicular body, and are released upon fusion of this compartment with the plasmamembrane. Ectosomes (100-1000 nm size) are formed upon membrane budding and shedding from the plasma membrane [13]. However, despite their considerable differences, the functions of ectosomes may be largely analogous to those of exosomes [14].

In our previous work DDT was implicated in the formation of ectosomes [12]; nevertheless, Osborne reported that DDT induces a pronounced increase in the release of exocytotic vesicles in nerve terminals [15].

3. Exstracellular vescicle-mediated Trojan horse transference or molecules promoting diseases

Extracellular vesicles are emerging as fundamental elements for transferring proteins, mRNA, microRNA (miRNA), DNA, lipids and transcriptional factors among cells. These organelles play an important role in protecting these factors from degradation [16].

Extracellular vesicle communication represents a substantially different type of cell to cell interaction. In fact, communication *via* neurotransmitters or hormones requires that the cells releasing the signal and the cells receiving it must have intrinsic receptor-mediated connections. On the contrary, communication *via* intracellular vesicles does not require any connection between the cells that release and respond to the signal [17]. Strikingly, once vesicles are released, the target cells on which extracellular vesicles will discharge their cargo cannot be known a priori but will be selected

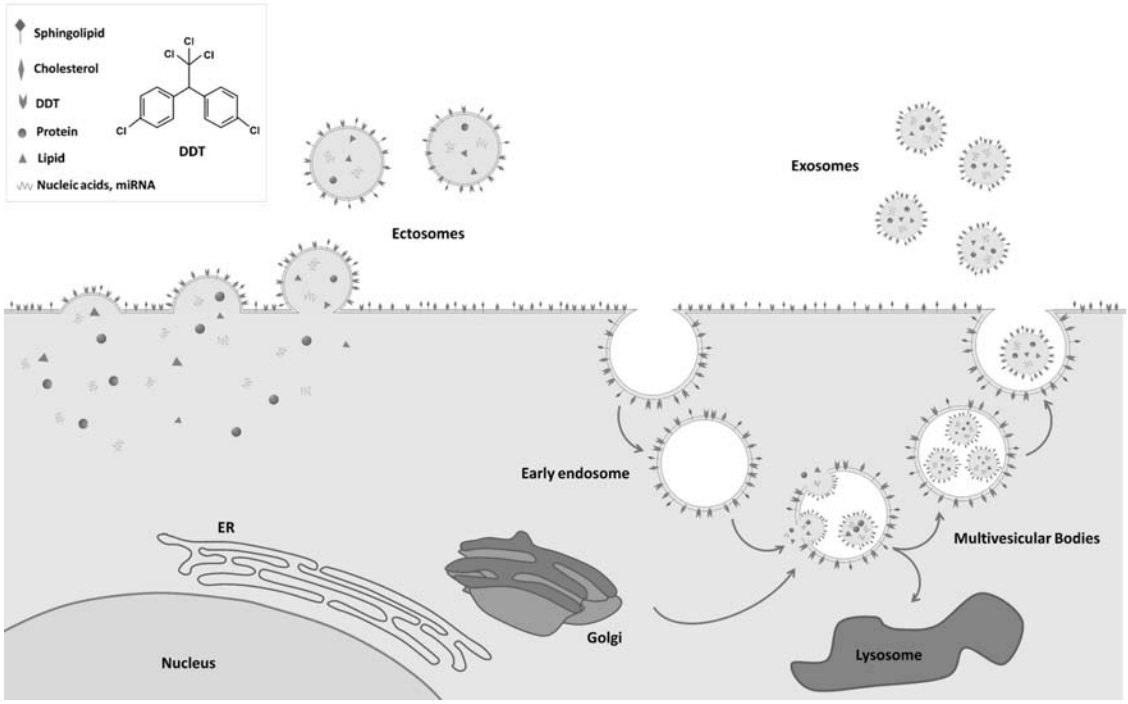

Figure 1. Formation of extracellular vesicles in the presence of DDT. Due to its high lipophilicity, and probably to its diffuse chlorination, DDT accumulates in cholesterol-rich regions like lipid rafts. The membrane tries to get rid of contaminated lipid rafts with the formation of extracellular vesicles. Two types of extracellular vesicles with different sizes and origins have been found: exosomes and ectosomes. Exosomes are extracellular vesicles that are released from cells upon fusion of an intermediate endocytic compartment, the multivesicular body, with the plasma membrane and are smaller in size (shown on the right). Ectosomes are shed from the plasma membrane and are larger in size (shown on the left). DDT increases the formation of both exo- and ectosomes, and by this way it could increase the delivery of extracellular vesicles and their harmful cargo to "healthy" cells.

on the basis of the surface properties of their enclosing membrane. Extracellular vesicles may enter the target cells by three different routes; they can be (1) fused directly with the plasma membrane, (2) taken up by phagocytosis or (3) internalized by receptor-mediated endocytosis [18]. Thus, these vesicles can play a variety of physiological roles depending on the targeted/ hosting cells and the nature of their limiting membrane and their cargo, which strictly depends on the type of cells from which they originated.

Besides their physiological role, extracellular vesicles are involved in the pathogenesis of several diseases such as neurodegenerative diseases [19] and tumorigenesis [20]; then, agents that increase their formation could play a role in these diseases.

At the time of the observation that DDT led to the formation of extracellular vesicles, the ecto/ exosomic field was just at the beginning [21]. Therefore, the possibility of extracellular vesicle formation as a possible delivery system was in its childhood, and the findings we previously described were not investigated for having potential toxicological effects [12]. In the light of the evidence presented above, on the role of extracellular vesicles in cell to cell communication, below we summarize some of the data that strongly suggest that DDT by inducing the formation of extracellular vesicles could increase the risk of neurodegenerative diseases and tumorigenesis.

4. Extracellular vesicle formation as a potential mechanism by which DDT could increase neurodegenerative diseases

Recent evidence strongly suggests that neuronal extracellular vesicles, mostly exosomes in the

brain, promote the spreading of neurodegenerative diseases [22] by carrying misfolded proteins of compromised cells to nearby healthy neurones [23].

The first pathogenetic protein aggregates that were identified in extracellular vesicles were prions [24]. Prions are self-replicating infectious agents entirely constituted by proteins that are the cause of transmissible diseases like spongiform encephalopathies, among others [25].

Moreover, there is strong evidence that misfolded α-synuclein and β-amyloid proteins, which are associated with Parkinson's disease and Alzheimer's disease, respectively, are delivered to healthy neurons through extracellular vesicles [24].

For instance, the soluble protein α-synuclein in pathological conditions aggregates to form insoluble fibrils that are released extracellularly through exosomes when the intracellular autophagic mechanism fails to degrade them [26]. Therefore, in this attempt of getting rid of these unphysiological protein aggregations, the sick neurones become the source of extracellular vesicles saturated with toxic materials that would eventually be incorporated into healthy neurones provoking the neurological disease to spread farther. Furthermore, Grey *et al*. [27] found that extracellular vesicles abbreviate the time of aggregation of α-synuclein, which suggests that they provide the catalytic environments for α-synuclein nucleation. In accord to this view, α-synuclein aggregation was accelerated by extracts of extracellular vesicles containing gangliosides [28], a type of lipid that is highly enriched in rafts, that, as mentioned above, is the main target of DDT in the lipid bilayer.

Moreover, extracellular vesicles have strongly been associated with the spreading of toxic β-amyloid protein aggregations in Alzheimer's disease, and therefore to the extended loss of neurons that characterizes the disease [29]. Furthermore, as observed with α-synuclein, the aggregation of β-amyloid was accelerated by its incubation with raft-like liposomes enriched in gangliosides, underpinning the importance of extracellular vesicles in β-amyloid nucleation [30].

Strikingly, epidemiological studies have suggested an etiologic relationship between organochlorine pesticide exposure and neurodegenerative diseases [31-34]. In fact, consistently higher levels of organochlorine pesticides, β-hexachlorocyclohexane, dieldrin, and 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE), a metabolite of DDT, have been found in patients with Parkinson's and Alzheimer's diseases compared to controls. In particular, patients exposed to DDT showed that the pesticide crosses the blood brain barrier, accumulates and persists for a long time in the brain as DDE residue [31]. Notably, Richardson *et al*. [34] also showed that patients with Alzheimer's disease had in their bloodstream four times higher levels of DDE than controls, underpinning the concept that the exposure to organochlorine pesticides should be considered a high environmental risk factor for the development of Alzheimer's disease.

In this review, we propose that exposure to DDT, and probably other organochlorine derivatives, could lead to the development of Parkinson's and Alzheimer's diseases by inducing the formation of extracellular vesicles that would promote the oligomerization and spreading of misfolded proteins among neurons.

Another strong supporting evidence of this hypothesis came from data of individuals chronically exposed to DDT. The serum of these subjects, in fact, contained high levels of the pesticide, about 10 μM [35, 36], the concentration at which DDT was shown to provoke rapid extracellular vesicle formation in our experimental settings [7].

5. Extracellular vesicle formation as a potential mechanism by which DDT could increase cancer risk

Cancer growth and metastasis was earlier interpreted as a process derived by clonal selection of mutated cells that acquired the capacity to proliferate incessantly and to migrate in the surrounding tissue. The discovery of extracellular vesicles has proposed that tumorigenesis can also be induced by tumour-promoting molecules transferred to naive cells by

extracellular vesicles [37]. Like the Trojan Horse, extracellular vesicles could indeed lead to a stealthy dissemination of tumour-promoting molecules by protecting these molecules from immune-recognition and degradation. Given that, agents like DDT that increase the formation of extracellular vesicles could thus in principle enhance the risk of cancer.

Exposure to polyhalogenated aromatic hydrocarbons has been shown to increase the risk of thyroid cancer and may explain part of the increased incidence of thyroid cancer during the past several decades [38, 39]. In particular, as shown in samples from patients that had been exposed to these pesticides decades before, due to their high lipophilicity, in fact, polyhalogenated aromatic hydrocarbons accumulate easily in biological tissues where they stay for long time [31].

For instance, the increased risk of papillary thyroid cancer recurrence in subjects exposed to polyhalogenated aromatic hydrocarbons could be driven by the formation of extracellular vesicles containing miRNAs. As we mentioned above, extracellular vesicle can protect circulating cargo like miRNAs from RNase digestion [16]. MiRNAs are small, endogenous non-coding RNAs that regulate gene expression, and that seem to play a major role in the proliferation of different types of human cancer [40].

In particular, Lee *et al*. [41] showed that one characteristic of the papillary thyroid cancer was the release of exosomes containing miRNA-146b and miRNA-222, which strikingly alter the proliferation of nearby and distant cells.

A nested case-control study among the participants of the Nutritional Intervention Trials in Linxian, China, has clearly shown that the risk of developing liver cancer increases proportionally to the quantity of DDT found in the serum levels of each participant. Whereas, interestingly, there was no statistically significant association between liver cancer and serum concentration of the DDT persistent metabolite and environmental degraded product, DDE [42, 43]. Furthermore, it has been shown that the risk of developing liver cancer was particularly high among people exposed directly, either to high levels of DDT (resulting in a higher ratio of DDT to DDE) or, alternatively, to low levels of DDT when the subjects were unable to efficiently metabolize DDT to DDE [42, 43]. Indeed, the induction of extracellular vesicle formation and the spread of their cargo following the exposure to DDT, could explain the increased risk of developing hepatocellular carcinoma in patients exposed to this pesticide.

As a matter of fact, recent studies provided new insights into the role of miRNA in the pathogenesis of liver cancers [44]. For instance, it has been shown that miRNA 221 enhances tumorigenesis *in vitro* and *in vivo* by regulating several targets. In particular, at a post-transcriptional level, it regulates the expression of p27Kip1, which is a member of the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitors that negatively control hepatocellular carcinoma cell cycle progression [45]. Moreover, Fornari *et al*. [46] have demonstrated that miRNA 221 also affect cell cycle progression through the modulation of CDKN1C/p57, a protein that was shown to correlate to tumour aggressiveness, to an advanced tumour stage, to a poor differentiation, to large-fast-expanding tumours, and to portal invasion.

Furthermore, the correlation between toxic organochlorine pesticides and breast cancer has been highlighted recently in a study by Eldakroory *et al*. [47], in which, samples from tumours and normal adjacent tissues of 70 cancer patients were used to measure the concentration levels of organochlorine pesticides. Strikingly, they found significant higher concentration of methoxychlor, DDT, hexa-chlorobenzene (HCB), and chlordane in the tumour tissue samples compared to the surrounding normal tissues, which suggested that the higher levels of organochlorine pesticides in the tissue specimens of breast cancers were probably responsible for the process of carcinogenesis.

Consistent with the role of extracellular vesicles in DDT increased risk of cancer, Menck *et al*. [48] have recently highlighted the importance of extracellular vesicles in breast cancer. They showed that tumour-derived extracellular vesicles influence tumour cells by enhancing their

invasion in both autologous and heterologous manner. Remarkably, neither the respective vesicle-free supernatants nor the extracellular vesicles originated from benign mammary cells were able to promote tumour metastasis. Importantly, the vesicle-mediated tumour invasion was only observed when the extracellular vesicles were assimilated inside the naive cells.

Note that, the literature cited above represents only a limited summary of the evidence highlighting the ability of DDT to increase the risk of cancer and the role played by extracellular vesicles, and several other types of cancer have been correlated with the exposure to organochlorine pesticides [49], and in many cases with the formation of extracellular vesicles [50].

6. Conclusions

In conclusion, based on the large body of evidence gathered in this review, we would like to propose a new paradigm of toxicity by which polyhalogenated aromatic hydrocarbons, and in particular organochlorine pesticides like DDT, exert their detrimental effect. We have in fact observed that because of their high lipophilicity and chlorination, these compounds are able to enter and to accumulate in cells altering the organization of their plasma membranes, and therefore, promoting extracellular vesicle formation, a mechanism by which cells try to get rid of contaminated lipid rafts. The cells poisoned by these pesticides, not only could be unable to respond efficiently to external stimuli, as it was observed in cell cultures transfected with the TSH receptor, but also, they could increase the delivery of extracellular vesicles and their harmful cargo to “healthy” cells. This new mechanism of toxicity could explain how organochlorine derivatives like DDT could promote the development of neurodegenerative diseases and spreading of cancer.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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