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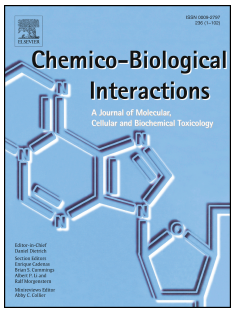

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Effects of pesticide mixtures in human and animal models: an update of the recent literature

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Highlights

- Here we provide an update of our current knowledge on pesticide cocktail effects
- 47% of the compiled studies reported an additive effect
- 36% of the compiled studies described interactions between compounds
- Pesticides interact in various ways but lead mainly to synergic effects
- Mixture effects vary according to the dose and/or physiological target

Keywords: Pesticides, Cocktail effect, Interaction, Synergy, Addition, Antagonism

Abbreviations

Dicarboximide (Dicarbo), neonicotinoid (NEO), organophosphorous (OP), organochlorine (OC), carbamate (CARB), pyrethroid (PYR), azole (AZ), imidazole (IMI), anilopyridine (A-PYR), benzimidazole (BenzIMI), , dithiocarbamate (DITHIOCARB), bi-pyridilium (Bi-Pyr), triazine (TRIA), triazole (TriAz), unclassified (UC): i.e. different from the other chemical

families listed, insecticide (I), herbicide (H), fungicide (F), mixture of insecticides and herbicides (I+H), mixture of insecticides and fungicides (I+F), mixture of fungicides and herbicides (F+H), mixture of insecticides, fungicides and herbicides (I+F+H).

Abstract

This review aims to provide an update on our current knowledge of the various effects of pesticide cocktails. We have collected data from studies conducted in mammalian models *in vitro* and *in vivo* that was published between 2000 and 2014. All ecotoxicological studies were voluntarily excluded. Cocktail effects were classified according to how they had been classified by each author. The frequency of the various cocktail effects and the classes and chemical families of pesticides involved in the observed effects were assessed. When focusing on the function of pesticides (i.e. herbicide, insecticide or fungicide), 46% of the mixtures contained insecticides alone, 15% fungicides alone, and 4.5% herbicides alone. Mixtures with effects associated with neurotoxicity were mainly composed of insecticides, and most studies on the effects of fungicide mixtures (90%) were associated with effects on endocrine regulation and/or reproduction. Dose addition was observed with each kind of mixture except herbicide combinations. In contrast, synergic interactions or greater-than-additive effects were mainly reported for insecticide mixtures. There were few examples of potentiating and antagonistic interactions. We have identified chemical families of compounds specifically involved in synergy, addition, potentiation and antagonism, and those that do not interact when combined. The chemical families identified as being involved in synergy are in agreement with data from another recently published compilation of ecotoxicological studies. For most mixtures investigated, further validation data is still needed from experiments using other compounds and other experimental models but this update provides useful information to help in human health risk assessments.

1. Introduction

There is increasing concern over the health effects associated with the use of pesticides for both agricultural and residential purposes. In recent years, several studies have reported the occurrence of pesticides in a variety of matrices, such as food, water, soil, outdoor and indoor air and house dust, meaning that both general and professional populations are often exposed to compounds from different sources [1-4]. The effects of this combination of pesticides on human health need to be evaluated because the regulatory assessment of pesticide toxicity is currently only performed on selected single compounds.

One of the difficulties in assessing the effect of pesticide cocktails from the literature is the inconsistency of terms used to qualify these effects, which varies between authors. Therefore, we must first clarify that in this review we define the cocktail effects of chemical mixtures to be the result of two distinct situations: (i) where there is no direct interaction between compounds, which may or may not be associated with dose-dependent addition, and (ii) where there is an interaction between compounds. Addition is used to describe situations whereby chemicals do not interact but act together to produce effects without enhancing or diminishing each other's actions [5]. Dose addition is the term usually applied to chemicals that exert their effects through the same target and have similar modes of action. For interacting compounds, the resulting toxicities can be synergic (higher than expected from the additive effect of the doses, greater-than-additive or supra-additive effects are also classed as synergic), antagonistic (lower than expected), or potentiating (when the effect of one compound is increased by another/others). When compounds do not interact and no dose addition is observed, the toxicity of the mixture is either null or equal to that of the most efficient product(s) in the mixture.

Other reviews have already collated information on different aspects of the effects of pesticide cocktails. Carpy et al. [6] examined the available data published between 1985 and

1998 regarding the health risk assessments of the residual concentrations of pesticide mixtures found in human food and drinking water. They reported that both synergy and antagonism occurred within the same organism depending on the organ or target, and that interactions between compounds did not appear to be a common event at these levels. In 2007, Kortenkamp et al.[5] published a review of studies that assessed endocrine disrupter (ED) mixtures in terms of additivity, antagonism or synergy. They concluded that combined effects occur even when all the individual mixture components are present at doses that are below those causing observable effects. To identify the greatest synergic effects of pesticide mixtures, Boobis et al. conducted a critical analysis of the literature from 1990 to 2008 on low-dose synergic effects of mixtures composed of a variety of chemicals (toluene, hydroquinone, pesticides, xylene) [7]. They defined synergy as a mixture response that significantly exceeds that predicted by a non-interaction model. Their search identified 90 studies that in total reported the effects of combinations of 204 different chemicals in mammals. Six of these studies provided useful quantitative estimates of synergy, and from these the authors concluded that the magnitude of synergy at low doses varied from 1.5 to 3.5. From a number of other positive studies, they concluded that the occurrence of synergy was dose-dependent and was observed only at higher doses.

In another review, Hernandez et al. [8] assessed a number of toxicological interactions in pesticide mixtures at the molecular level and their relevance to human health. They reported several examples of cocktail effects, such as the potentiation of the toxicity of some pesticides by others (e.g. malathion by isomalation, pyrethroids (PYRs) by anticholinesterase insecticides, organophosphorous (OP) by organochlorine (OC), carbaryl by OP, and OP by triazines (TRIAs)), the synergy between PYR and carbamate (CARB) compounds, and the antagonism between TRIA herbicides and prochloraz.

Very recently, Cedergreen [9] published a very interesting review of ecotoxicological studies that aimed to identify groups of chemicals that are overrepresented in synergic mixtures and define the molecular mechanisms underlying the observed synergy. Three groups of chemicals were studied, including pesticides, and synergic mixtures were defined as those with a minimum 2-fold difference between the observed and predicted effect of the individual concentrations, using the concentration addition model (CA) as a reference model and including lethal and sub-lethal endpoints. Synergy occurred in 7% of the 136 binary pesticide mixtures, which included mainly cholinesterase inhibitors or azole (AZ) fungicides, both of which are known to interfere with the metabolic degradation of other xenobiotics.

In the present review we provide an update of the recent literature on the impact of pesticide mixtures. We compiled 78 studies published between 2000 and 2014 that were conducted in mammalian model systems (both *in vitro* and *in vivo*). Ecotoxicological data were excluded although ecotoxicology and mammalian toxicity are linked to each other. From these studies, we identified those which had experimentally assessed the associated effects of simultaneous exposure to a combination of two or more pesticides and which had clearly reported the joint toxic effects of the pesticide mixtures. The cocktail effects were grouped into five classes according to the classifications made by the authors of the studies without recalculation of their results: (i) Addition, when authors clearly reported dose addition or additive effects; (ii) Synergy, when terms such as “synergistic effects”, “greater-than-additive effects”, or “supra-additive” were used; (iii) Antagonism, when antagonistic interactions or less-than-additive effects were reported; (iv) Potentiating interactions, when potentiation or potentiating effects were reported; or (v) No interaction, when authors reported that the cocktail effects did not exceed those of the individual compounds. Studies were not included if authors did not clearly state a dose additive effect, if they did not characterize the mixture effects as “cooperative” or “positive”, if the minimum information needed to make an

assessment was lacking, or if the term "cumulative" was used (the excluded studies are presented on Table 6 as additional data). Moreover, we also assessed the various observed effects in terms of the function and chemical family of the constituent pesticides in the mixtures. The experimental model used in each case was also taken into account (i.e. whether the study was conducted *in vivo*, or *in vitro* using cultured animal or human cells) in order to assess the utility of such studies for projecting their results to effects on human health.

2. Materials and Methods

2.1 Study identification

An electronic search using the term "pesticides mixture" was initially performed to obtain a list of relevant articles from PubMed (www.ncbi.nlm.nih.gov). This was supplemented by further searches using various combinations of the following keywords: fungicide, herbicide, insecticide, exposure, combined toxicity, combination, cocktail, cell line model, *in vivo*, *in vitro*, animal model, pesticide model, using the AND operating term to complete the search. When only abstracts were available on pubmed we either asked authors to send us a pdf copy of their article or we obtained a copy from the INIST library of the Centre Nationale de la Recherche Scientifique (CNRS). From this, we compiled a set of 78 experimental studies on the impact of pesticide mixtures that were published between 2000 and 2014. Many studies published prior to 2000 were previously incorporated into the technical report published by the European Centre for Ecotoxicology and Toxicology of Chemicals, Brussels [10]. In this document, the authors assessed studies based on the effects of co-exposure to chemicals (pesticides and other chemicals) at doses relevant to environmental human exposure. In contrast to this report, we did not select articles based upon the doses used.

2.2 Study selection

Studies were excluded if:

- They were not published in English;
- They did not provide sufficient data (e.g. studies that looked at the effect of a mixture but not the individual pesticides);
- They were performed on ecotoxicological models such as insects, amphibians, or nematodes, or fish;
- The minimum information needed for assessment was lacking or the term "cumulative" was used. The studies that were excluded for this reason are listed in the supplementary data (Table 6).

Studies were included in the analysis when they complied with the following inclusion criteria:

- They had studied pesticides, including molecules currently banned;
- They were published in peer-reviewed journals, between 2000 and 2014;
- They had qualified any observed cocktail effects.

2.3 Data extraction

The following general and methodological information was abstracted from each paper: the references; pesticides evaluated; dose, duration, frequency, and manner of exposure (for *in vivo* studies) or cell model (for *in vitro* studies); parameters studied; observed effects; and the authors' conclusions in terms of whether there were synergic, antagonistic, positive or potentiating interactions, and/or whether there were any additive effects (Table 1). We also grouped the data into four categories according to the physiological functions targeted by the pesticide mixtures: (1) those associated with the central nervous system, neurotoxicity, brain or neuronal function and/or neurobehavior; (2) those associated with hematopoietic or

immune parameters and/or inflammation (i.e. bone marrow, lymphocytes, cytokines, etc.); (3) those associated with cell biology (i.e. oxidative stress, genotoxicity, DNA damage, cell viability or proliferation) or metabolism (i.e. general, renal, hepatic, energetic and xenobiotic metabolism); and (4) those associated with parameters of reproduction, reproductive functions, and/or hormone or endocrine regulation.

2.4 Data analysis

The various types and combinations of pesticides (herbicides (H), insecticides (I), fungicides (F) or mixtures of these) were first classified according to the physiological function targeted (see Section 2.3 for details on the four categories used). Then, the percentage of each type of pesticide/pesticide mixture inducing: (i) additive effects, (ii) synergy, greater-than-additive effects, or supra-additive effects, (iii) antagonistic effects, (iv) potentiating effects or (v) no effect; was calculated, including all data that were or not deemed significant by the authors. Data were also segregated according to the model used (animal model, or cultured animal/human cells). The chemical families involved in the observed effects were also identified where possible, and their relative contributions towards the effect of each type of mixture were extracted by taking into account the magnitude of the significance of data reported by the authors.

3. Results and discussion

As explained above in the section 2.2 and in the introduction, 24 studies were excluded and the various remaining 54 studies were classified as shown in Table 2, according to four distinct physiological targets and the type or function of the pesticides assessed in the mixtures: insecticides (I), herbicides (H), fungicides (F), I+H, I+F, F+H, or I+F+H. From a general perspective, insecticide mixtures were the compounds most frequently evaluated in combination. Forty-six percent of the assessed mixtures we compiled from the 54 selected studies, focused on insecticide combinations, 15% on fungicide combinations, 4.5% on herbicide combinations, 12% on I+F mixtures, 10.5% on I+H mixtures, 4.5% on H+F mixtures and 7.5% on I+F+H mixtures.

Mixtures assessed on parameters associated with neurotoxicity were mainly composed of insecticide compounds alone (75%), although insecticide mixtures also affected other physiological categories (Table 2). Most studies related to the impact of fungicide mixtures (90%) were dedicated to parameters associated with endocrine regulation and/or reproduction, with 10% reporting neurotoxic properties. The impact of herbicide mixtures was assessed less often, with two studies associating their effects with cell biology, metabolism or oxidative stress [11, 12] and one with reproduction and or hormonal regulation [13]. Cocktails of I+H and I+F were mainly linked to effects on cell biology, metabolism or oxidative stress (Table 2).

3.1 Types and frequency of cocktail effects in terms of their associated functions

The type and frequency of the cocktail effects reported in the literature were then examined in terms of their associated physiological functions. The type of cocktail was defined here by that reported by authors; further details on the types of interaction, the magnitude of the significance were subsequently analyzed for the results presented in Section

3.2. As shown in Table 3, forty-eight percent of the assessed mixtures were described by authors to induce effects in a dose additive manner. Moreover, 17% of the mixtures did not show any interactions between compounds. The remaining 35% of the mixtures assessed caused effects due to interactions between compounds that were independent of a dose effect and, according to the authors, 71% were reportedly synergic, supra-additive or greater-than-additive and 21% were antagonistic. Few were potentiating interactions (8% of the total reported interactions) and these were observed only *in vitro*. For example, at non-cytotoxic concentrations, a fungicide potentiated the cytotoxicity of an insecticide in Ehrlich ascites tumor cells [14]. Recently, potentiating interactions were also reported between two insecticides and between an insecticide and a herbicide (I+H), where they increased the cytotoxicity of cultured human hepatocytes [15]. Antagonistic effects were shown to occur when combining different insecticides, different fungicides, or a mixture of herbicides and fungicides (Table 3). In rat dopaminergic cells in culture, a mixture of insecticides was found to exert an antagonistic effect on cytosolic calcium concentrations [16], and in human HepaRG liver cells, a mixture of insecticides had an antagonistic effect on parameters linked to apoptosis [17]. Recently, Savary et al. [18] reported the antagonistic effect of an insecticide mixture on the activity of Cytochrome P450 2B6 in cultured HepaRG cells. Three *in vivo* animal studies have also reported antagonistic interactions. In C56BL/6 mice, a herbicide antagonized the impact of a fungicide on neurochemical targets [19], and two insecticides were shown to interact in an antagonistic manner on lipoperoxidation in adult albino male Wistar rats [20]. Oral exposure of one fungicide also antagonized the increase in serum testosterone induced by another fungicide in male rats [21]. Synergic interactions were mainly observed with insecticide mixtures and were not reported for herbicide mixtures or for F+H or I+F+H mixtures, since the few studies conducted on herbicide mixtures did not allow firm conclusions to be drawn regarding additive or non-additive effects [11, 22]. A recent study

[23] showed that some fungicides interact synergistically regarding their anti-androgenic activity in human mammary breast cancer cell lines, according to the dose. A greater-than-additive effect was reported by Pruett et al. [24] on interleukin production in mice exposed to a mixture of a herbicides and insecticides for 7 days. In human peripheral lymphocytes *in vitro*, the combination of I+F also interacted in a possibly synergistic manner [25]. Together the compiled studies observed a dose addition with each kind of mixture except with a combination of herbicides. In addition, some studies reported pesticide mixture effects that were equivalent to the effect of the most active compound alone, inferring that there was no interaction (Table 3). This was the case with some mixtures of insecticides, fungicides, or both insecticides and herbicides or herbicides and fungicides. For example, a mixture of three insecticides did not induce synergistic or additive effects on oxidative stress compared to when they were tested alone both *in vitro* in rat lymphocytes and *in vivo* in adult rats [26, 27]. Similarly for fungicides, Prutner et al. [28] reported that the overall effects of a mixture of fungicides belonging to the same chemical class that shared either the same or opposite mechanisms of action were determined by the most potent compounds in the human adrenocortical carcinoma cell line H295. The impact of a mixture composed of two insecticides and one herbicide was also not different from that induced by the most efficient compound *in vitro* in primary human and mouse hepatocytes [29] as well as *in vivo* on hematopoietic parameters in mice [30]. Combining a herbicide with a fungicide also did not change the effect of the former on neurodegeneration in rats [31].

Overall, the large number of studies concerning insecticides allows us to draw overall conclusions following the analysis of these studies in combination. They have revealed that insecticide mixtures can lead to cocktail effects due to

(i) Dose addition (33 reports of dose addition or an additive effect: 12 in animal models, 11 in cultured human cells and 10 in cultured animal cells);

(ii) Interactions between compounds (24 reports). Seventeen reported synergic, greater-than-additive or supra-additive effects (8 in animal models, 7 in cultured human cells and 2 in cultured animal cells), 5 reported antagonistic interactions (3 in animal models, 1 in cultured human cells and 1 in cultured animal cells) and 2 potentiating interactions (1 in cultured human cells and 1 in cultured animal cells).

(iii) Being the most efficient compound of the mixture (i.e. no interaction with other pesticides in the mixture). This was reported by 12 studies: 6 in animal models, 1 in cultured human cells and 5 in cultured animal cells) (Table 3).

In conclusion, different combinations of insecticides were associated with different effects. Combinations of fungicide compounds led mainly to additive effects. Four studies reported such an effect in cultured human cells, two in cultured animal cells and one in an *in vivo* animal model (Table 3). I+H combinations were associated with synergy (one example), potentiating effects (two examples), addition (one example), and no interaction (two examples) and all studies (except those concluding a potentiating effect) were performed in animal models (Table 3). For I+F combinations, one *in vivo* example was classified as having "synergic or greater-than-additive effects", one with "potentiating effects" in cultured animal cells, and three causing "addition": one from *in vivo* experiments and two from cultured human cells (Table 3). F+H combinations led to synergy (one example *in vivo*), antagonistic effects (one example *in vivo*), or no interaction between the fungicides and herbicides (one example *in vivo* and one in cultured animal cells). Mixing the three classes of pesticides only led to dose addition, as reported by three *in vitro* studies and one *in vivo* experiment (Table 3).

Together these data show that the function of pesticides does not necessarily determine the effects of the mixture, as the same combinations of pesticides has been found to lead to

distinct effects. Therefore, we next focused on whether the chemical family of the pesticide, and where possible the dose and biological target, plays a role in the various types of cocktail effect.

3.2 Types and frequency of cocktail effects in terms of the chemical family, the experimental model, the dose.

This section compares the different chemical families of the pesticides involved in the reported cocktail effects in terms of the physiological targets described by the authors in the compiled studies and the experimental models used (Table 4). Interestingly, in most cases each specific combination was associated with only one studied physiological parameter, for example the impact of mixtures of carbamate (CARB) or organophosphorous and carbamate (OP+CARB) compounds was only reported in studies related to neurotoxicity, and mixtures of OC+TRIA compounds in studies related to immunity or haematopoiesis. The exceptions were mixtures of organoclorine (OC), OP, OP+PYR (pyrethrinoid), OP+OC and azoles (AZ,) which were associated with at least two different physiological parameters. In order to find the parameters affected in each type of cocktail mixture, we decided to overcome the distinction between the studied targets. In the following paragraphs we describe the pesticide cocktails that led to synergic, antagonistic, and additive effects, and in cases of non-interaction. Each sub-section is split into 3 parts: (i) provides a general list of the various mixtures that were described to produce this effect, the physiological parameters involved and the model used in each given situation; (ii) analyzes the combinations of compounds that gave rise to that type of effect only, according to the significance of the data provided by authors; (Table 5a) and (iii) concludes our findings and allows us to identify the chemical combinations of compounds that could be a risk to human health. Moreover the names of all the chemical families their abbreviations and the corresponding compounds discussed in the

manuscript, as well as their functions (insecticide, herbicide or fungicide) are described in table 5b .

3.2.1 *Synergic pesticide mixtures*

3.2.1.1 General list of chemical families with synergic, greater-than-additive or supra-additive effects

The results in Table 4 show that synergy was observed between compounds thought to have both distinct and common mechanisms of action. In the cultured Chinese hamster ovary (CHO) cell line, a mixture of five pesticides (OP+PYR+2TriAZ (triazole)+TRIA (triazine)) induced a greater-than-additive effect (i.e. the predicted effect concentrations were higher than the observed effect concentrations) on induced androgen receptor activity [32]. In mouse neuroblastoma cells, synergy was detected between combinations of OP and pyrethrum [33]. Combinations of OP+CARB, OC+CARB, OP+OC appeared to clearly interact in a synergic manner *in vitro* in human peripheral lymphocytes and AZ+dicarbo (dicarboximide) in human mammary breast cancer cells [23, 34]. In addition, a mixture of TRIA+OC had a much greater-than-additive effect on IL6 production in mice, with a 20% predicted effect for the individual effects of each compound versus 80% when assessed in combination. This was a specific effect since it did not apply to a different biological parameter (the inhibition of cJun activation) [24]. Exposing male Wistar rats to a mixture of pyrethrinoid+neonicotinoid (PYR+NEO) (delthamethrin and thiachloprid) was reported to synergistically increase genotoxicity and cytotoxicity in rat bone marrow cells [35]. A mixture of 2OP and 2OC (chlorpyrifos, monochrotophos, lindane and endosulfan) produced a synergic effect on apoptosis and the proliferative activity of rat bone marrow cells *in vivo* [36]. However, an *in vitro* experimental model in mice splenocytes and thymocytes that combined 1 OP with 1 OC (lindane and malathion) produced an additive effect [37, 38]. The combined effect of PYR+OP (deltamethrin and chlorpyrifos) in male Wistar rats was also synergic and this effect

was shown to be specific for certain physiological parameters (catalase activity)[20]. Moreover, other OP+PYR mixtures (e.g. diazinon and cypermethrin) produced an additive effect on reproductive parameters in mice [39], but when assessed in mouse neuroblastoma cells *in vitro* were found not to interact [40]. Mixtures of anilopyridine (A-PYR) with the phenyl Pyrrole or of Neo+phenylamide produced "some degree of synergy or possible synergy" when assessing their effects on oxidative stress-related gene expression in human neuroblastoma cells [41] and on micronuclei formation in rat bone marrow or human lymphocytes, respectively [25]. Christen et al. [23] reported a clear dose-related synergic interaction between azoles and dicarboximide compounds in a binary mixture in a human mammary breast cancer cell line. The differences in synergy observed at different equi-effective concentrations were suggested to be due to either the different strengths of the competing antagonistic compounds for the ligand binding site of the AR, interactions with distinct sites of the AR, or transactivation. A mixture of pyridine and CARB compounds was also reported to interact synergistically on the nigrostriatal dopaminergic system in mice [42], but no interaction was found when assessing the inflammatory processes linked to the neurodegeneration process in rats [31]. Both studies used comparable doses, with mice injected intraperitoneally twice a week for 6 weeks in the first study [42], and rats injected twice a week for 4 weeks in the second study. The different duration of exposures may explain the differential effects of this mixture.

Synergy was also observed with mixtures of compounds belonging to the same chemical family which supposedly shared a common mechanism of action (Table 4). Only a few examples are described in the following paragraph. Richardson et al. [43] showed that the sequential exposure of rats to a mixture of two OPoxon compounds (oxon metabolite of organophosphorous compounds) resulted in greater-than-additive effects on choline esterase activity at higher concentrations, whereas simultaneous exposure to the same mixture led to

additive effects on this parameters [44]. Dose-dependent synergic interactions were also reported by Moser et al. [45] in pre-weaning and adult Long Evans rats exposed to a mixture of 7 carbamate compounds. The effects were mostly dose additive with mixture ratios based on relative potency factors (i.e. hazard-based mixtures), whereas greater-than-additive, or synergistic effects were noted when the mixture ratios used were based on amounts sold in California (i.e. were exposure based). These results were similar across various endpoints in both adults and pups. The magnitude of the synergy was up to twofold in adults and threefold in pups. In contrast, in the same animal model, Mwanza et al. [44] reported an additive effect of a mixture of carbamate compounds, however they assessed mixtures of only two carbamate compounds and used doses higher than those tested in the studies of Moser et al. Synergy between OC compounds has also been clearly reported by various authors in human cell lines (hepatic, mammary, intestinal) and primary cultured hepatocytes (Table 4). The impact of OC mixtures appears to depend on the targets studied, with a good example provided by Savary et al. [18]. These authors showed that a mixture of OC compounds in cultured human cells led to synergy in terms of a cytotoxic endpoint, addition in terms of CYP3A4 expression, and antagonism in terms of CYP2B6 activity. Moreover, [46] reported that the combination of OC compounds lindane and dieldrine led to either an additive effect on membrane depolarization or an antagonistic effect on basal calcium concentrations in the rat dopaminergic cell line PC12. Christen et al. [23] reported a dose-related synergic interaction between azole compounds in a binary mixture in a human mammary breast cancer cell line. On the other hand, in a human adrenocortical cell line, a ternary mixture of three azole compounds (epoxiconazole, propiconazole and tebuconazole) led to additive effects on the cellular endocrine potential [47]. Moreover, binary and quaternary mixtures of azole compounds induced additive effects on basal concentrations and voltage gated calcium channels in rat dopaminergic PC12 cells [48].

3.*2.1.2 Mixtures that are reported to induce only synergic effects* (Table 5 a)

Mixtures that were found to cause only synergic effects were AZ+Dicarbo, A-PYR+phenylpyrrole and OC+CARB in cultured human cultured cells, PYR+NEO and NEO+Phenylamide *in vivo*, and OP+PYR+2AZ+TRIA in CHO cells. In human peripheral lymphocytes, synergic interactions between OC and CARB were confirmed by authors using the statistical method described by Marking (1977) and DeLorenzo and Serrano (2003) [34]. A joint synergic effect of OP+CARB and OC+OP was also confirmed but these chemical mixtures were also shown to interact in an additive manner [37, 49]. However neither the compounds nor the experimental models assessed were the same between these studies. The joint effects of the mixture of AZ and dicarboxymide (dicarbo) in a human mammary breast cancer cell line were calculated on the basis of the Hill regression model for each pesticide in the mixture, using the concept of concentration addition [23]. On the other hand, Coleman et al. [41] described only a small degree of interaction between A-PYR and phenylpyrrole that was reported but not calculated, so this study has been omitted for the discussion. In an animal model the joint effect of PYR and Neo was shown to be significantly greater than that of the individual compounds [35]. This synergy was also observed in animals at high concentrations (around the mg/kg body weight) so cannot be used to estimate the human risk, and were not proved by mathematical modeling. In addition, the effect of NEO and phenylamide mixtures on micronuclei formation in the bone marrow of male rats has been suggested by authors [25]. In CHO cells, after completing concentration–response analyses of the single pesticides, mixture effect concentrations of OP+PYR+2TriAZ+TRIA were predicted by applying the principle of concentration addition (CA) [32]. For the mixture the predicted effect concentrations were found outside the 95% confidence band for the observed

effect, and were higher than the observed effect concentrations, indicating a greater-than-additive (synergic) effect of the mixture on DHT-induced AR activity [32].

3.2.1.3 Conclusion

Synergic interactions have been clearly reported between two insecticides belonging to the OC and CARB families of compounds (endosulfan and carbofuran) and between two fungicides belonging to the AZ and dicarboximide families (vinclozolin and tebuconazole/vinclozolin+ecoconazole) in human mammary breast cancer cells at low concentrations (µM), suggesting that these mixtures may represent a hazard to human health. However, this needs to be confirmed in other cellular and animal models. The synergic effect of OP+PYR+2TriAZ+TRIA (terbuthylazine, bitertanol, propiconazole, cypermethrin and malathion) was significant and observed at relatively low doses but needs to be confirmed in human cell lines before these results can be projected [32] to human health risk.

3.2.2 Antagonistic pesticide mixtures

3.2.2.1 General list of chemical families involved in antagonistic interactions

Antagonistic interactions were reported between compounds belonging to different chemical families. For example, in rat PC12 cells, when OP (chlorpyrifos) was combined at its lower observed effect concentration (LOEC) with its OP oxon-metabolite (chlorpyrifos-oxon), no additivity effects were observed [50]. This LOEC mixture of chlorpyrifos (0.1 µM) with chlorpyrifos-oxon (1 µM) did not significantly inhibit the depolarization-evoked increase in $[Ca^{2+}]_i$ (94 ± 3%, n = 42, N = 5) compared to control cells, whereas the individual compounds both inhibited it by around 25%. These results suggest that these two compounds may interact in an antagonistic manner through competition for specific targets. In another study [19], authors reported an antagonistic interaction of a mixture of TriAZ and pyridine compounds (triadimefon and paraquat) in male mice orally exposed for 12 weeks to doses ranging from 2

to 12mg/kg body weight per day, doses which far exceed those at which humans can be exposed through food intake. Mixtures of OP and PYR compounds (deltamethrin and chlorpyrifos) given to male albino Wistar rats at doses of 1 and 5 mg/kg over 16 weeks led to synergic effects on catalase activity in brain [20], however the same studies showed that these compounds also interacted in an antagonistic manner in their effects on enzymes involved in lipoperoxidation [20]. The effects of OP+PYR mixtures therefore appear to depend on the target and the constituent compounds. Indeed, a different OP+PYR mixture (diazinon and cypermethrin) was found to exert an additive effect on the reproductive function in male mice [39], whereas the same compounds were shown to not interact when assessed *in vitro* in mouse neuroblastoma cells [40]. Antagonistic interactions have also been reported between compounds belonging to the same chemical family (e.g. mixtures of dicarboximide, OP or OC compounds) (Table 4). Two distinct mixtures of OC compounds (lindane and dieldrine: M1, and endosulfan and metoxychlore: M2) were shown to interact in an antagonistic manner on basal calcium concentrations (M1) or CYP2B6 activity (M2), however this antagonism was not observed on other studied parameters. Both of these studies were performed *in vitro* in cultured animal (M1) or human (M2) cells [18, 46]. As described above, other OC compounds have been shown to interact in a synergic [11, 12, 18] or additive manner [18], or to not interact [51] in cultured human cells. Together these results suggest that the antagonistic interactions between OC compounds may depend upon the constituent compounds and the cell model used. Antagonistic interactions between other OP compounds (isomalathion and malathion) have also been reported in their effects on apoptosis in human hepaRG cells [17]. OP mixtures have also been reported to lead to additive effects either *in vivo* in an animal model or *in vitro* in cultured human cells [17, 52]. Another study of OP mixtures by Ojha et al. reported no interaction in an *in vivo* animal model [53]. It is noteworthy that in each study reported here the compounds were different, supporting the concept that the cocktail effect

may depend in part on the chemical family of the compound but mainly on the compound itself. Mixtures of dicarboximide were reported to interact in an antagonistic manner on serum testosterone production in male rats in only one *in vivo* study [21], however administration of the dicarboximide mixture has rather a cumulative effect on other androgen-sensitive end points in the pubertal male rat exceeding the response expected [21].

3.2.2.2 *Mixtures that are reported to induce only antagonistic interactions* (Table5a)

Mixtures of TriAZ+pyridine compounds have been reported by authors to interact in an antagonistic manner. In male C57BL/6 mice exposed to this F+H mixture containing triadimefon (TDF50) and paraquat (PQ) for 12 weeks (at doses of 25 and 10 mg/kg, respectively), authors observed that PQ prevented the increased levels of activity associated with TDF50, suggesting that there were antagonistic interactions between these compounds *in vivo*. However, these results were not confirmed by statistical modeling [19, 50].

3.2.2.3 Conclusions

For compounds producing effects suggestive of antagonistic interactions, none were clearly identified to interact only in an antagonistic manner.

3.2.3 Potentiating pesticide mixtures

3.2.3.1 *General list of chemical families involved in potentiating interactions*

The potentiating effects of pesticides in mixtures was reported *in vitro* in Ehrlich ascite tumor cells for OC+DTCARB mixtures at very high doses (1.2 and 0.6 mM respectively) [14] and for mixtures of TRIA+OC or OP+OC in HepaRG cells [54] (Table4). Upon repeated long-term exposure in a HepaRG model, the effect of the OC compound endosulfan on cell impedance was potentiated when combined with the TRIA compound triazine, and was potentiated even more intensively with the OP compound chlorpyrifos, in a dose-dependent manner. It is noteworthy that mixtures of TRIA+OC or OP+OC compounds have also been

reported to interact in an additive or synergic manner (as described in the section 3.2.1 above with other chemical compounds (e.g. lindane and malathion [37, 38] or atrazine and dieldrine [24]) and in other experimental models. Thus, the potentiation between TRIA+OC or OP+OC compounds may not be attributed to all compounds belonging to these classes of chemicals and may depend on the constituent compounds and/or the experimental model used.

3.2.3.2 *Mixtures that are reported to induce only potentiating interactions* (Table 5a)

Mixtures of OC and DTCARB compounds appear to have only potentiating interactions in terms of their effects on cytotoxicity. This effect was observed *in vitro* and a mechanism was proposed whereby thiram potentiates the cytotoxicity of endosulfan through excessive glutathione depletion, leading to higher oxidative stress as evidenced by the induction of reactive oxygen species (ROS).

3.2.3.3 Conclusions

Potentiating interactions between endosulfan and thiram should be confirmed at realistic doses. Before making conclusions as to the chemical families involved in this effect, other OC and DTCARB compounds must also be assessed using other models.

3.2.4 Additive pesticide mixtures

3.2.4.4 General list of chemical families with additive effects

The pesticide mixtures reported as having additive effects on physiological parameters are presented in Table 4. In this section we will focus on the chemical families eliciting only additive effects (and not combinations of effects) since (i) the list of mixtures reported to produce additive effects is very long, and (ii) mixtures inducing a combination of effects have been described in the above paragraphs.

3.2.4.4 Chemical families involved only in additive effects (Table 5A)

Mixtures that have been reported to induce only additive effects are shown in Table 5 and include compounds within the same (PYR, IMI) or different chemical families (A-PyR+Dicarb, OP+DTCARB, OP+CARB, PYR+BenzIMI, CARB+IMI+AZ+Dicarbo, 2 Phenyl pyrroles+OP+2AZs+Phenol+Dicarbo+CARB). In human adrenocortical carcinoma cells *in vitro*, the combination of A-Pyr with dicarbo (i.e. cyprodinil and procymidone) was reported to act in a dose additive manner in most cases, although this was not determined using statistical modeling [28]. In this study the authors observed that this effect was dose-dependent and was observed for mixtures where both compounds enhanced estrone biosynthesis but belonged to different chemical families. In an *in vivo* study evaluating the cocktail effects of a mixture of CARB, AZ, IMI and Dicarbo compounds [55] at doses close to the No Observable Adverse Effect Level NOAEL, authors reported that the predicted mixture effects based on dose-additivity were in good agreement with the observed effects. However, an *in vivo* study by Astiz et al. assessing the cocktail effects of a 2OP+DTCARB mixture reported that the simultaneous exposure to more than one compound was likely to have additive effects although these were not calculated [56]. Additive cytotoxic effects on tissues and the physiology of metabolic pathways was reported by authors upon repeated exposure of mice to BenzIMI and PYR (carbendazim with cypermethrin) at doses of 10 to 20 mg/kg /day over 28 days [57]. In a separate study, the predicted effect concentrations of the combination of PYR+2 TriAZ compounds (cypermethrin, bitertanol, propiconazole) affecting the function of sex hormone receptors and aromatase enzyme activity in human and animal cell lines were within the 95% confidence band for the observed effects, suggestive of additive mixture effects. Isobole coefficients were estimated to give values relatively close to 1, supporting the finding of an additive mixture effect [32]. The effects of a binary mixture of OP (chlorpyrifos) with CARB (carbaryl) in rat PC12 cells was also interpreted as being additive without using any statistical approaches [50]. Another study found that a mixture of

eleven pyrethrinoids induced the intracellular sodium concentration in mouse embryonic neuronal cells in primary culture in an additive manner. In this study, the experimental mixture data was compared with the predicted mixture concentration–response curve, and a likelihood ratio test was used to test the hypothesis of additivity using an *F*-distribution [58]. Assessing a binary mixture of PYR in neuronal primary culture from mouse embryos also showed an additive effect on electrophysiological properties *in vitro* [59], where authors compared the calculated and experimental results from binary mixture dose–response curves. A mixture of imidazoles also inhibited adrenal cortisol secretion in a monotonic, additive and predictive manner in the human adrenocortical carcinoma cell line H295R [60]. In stably-transfected human breast cancer cells, the responses of this mixture of 8 imidazoles (composed of "pure" AR antagonists: 2 Phenyl pyrroles+OP+2AZs+Phenol+Dicarbo+CARB) agreed very well with the combined effects predicted by CA.

3.2.4.3 Conclusions

The mixture effect predictions based on dose-additivity were in good agreement with the observed effects for mixtures of CARB+IMI+AZ+Dicarbo, PYR+IMI, PYR+2AZs and 2 Phenyl pyrroles+OP+2AZs+Phenol+Dicarbo+CARB. These data should be confirmed in other experimental models before they can be translated to human health risk but may help in the consideration of the impact of pesticides mixtures.

3.2.5 *Mixtures with an absence of interaction between compounds and no additive effects* (Table 5A)

Mixtures of CARB+bipyridilium, OP+OC+TRIA, or AZ+Dicarbo+DTCARB were not associated with antagonistic, additive, potentiating or synergic effects and were reported to contain chemical families that did not interact when combined (Table 5) meaning that the effects of these mixtures were comparable to those of the most efficient constituents [29-31].

These studies were all performed *in vivo* in animal models. As for all types of observed effects, it is crucial that other pesticides belonging to the cited chemical classes are tested and the various mixtures are assessed in other experimental models in order to confirm these data.

4. General discussion

The aim of this study was to update our knowledge on the effects of pesticide mixtures by compiling studies performed in animal and human models. Taken together, our findings indicate that when combined, pesticides interact in various ways, mainly in a dose additive or synergic manner. Moreover, from our study it appears that the effect of each mixture varies according to the compound itself, the dose and the physiological target within the cell or body [23, 45]. Overall, it seems that some combinations of chemical families of pesticides elicit effects in various categories whereas some are associated with one specific type of cocktail effect. For example, synergy was significantly observed with mixtures of compounds belonging to different chemical families (AZ+Dicarbo and OC+CARB in human cells and OP+PYR+2AZ+TRIA in cultured animal calls), additive effects with mixtures of compounds belonging to different or the same chemical families (PYR+2AZs or 3IMIs in human cells, PYR in animal cultured cells and CARB+AZ+IMI+Dicarbo *in vivo* in animal models). However, in each case these observations are only true for one specific compound from the identified chemical family, for example the effects of the OC+CARB mixture correspond only to mixtures of endosulfan and carbofuran. An exception is PYR +AZ+Dicarbo mixtures, whose effects have been confirmed for more than one compound from each of these chemical families. Thus, it is crucial to evaluate other compounds from the chemical families involved in these effects (AZ, OC Dicarbo, etc) before making general conclusions about the cocktail effects related to chemical families. In addition, the mixtures identified as eliciting the effects in each category should be assessed using other experimental models before their results can

be transferred to human risk assessment. In a recent review, Cedergreen [9] defined five groups of chemicals that may be involved in synergistic interactions observed in ecotoxicological studies: OP, CARB, AZ, PYR and TRIA. These results are in agreement with our data and suggest that the focus should be on these chemical families when assessing the impact of exposure to multiple pesticide residues on human health.

The various cocktail effects discussed in this review could be explained by examining both the metabolic pathways (toxicokinetic parameters) and the cellular targets of individual pesticides (toxicodynamic parameters). Some pesticides could bind to or regulate the activity of efflux ABC transporters or enzymes involved in xenobiotic metabolism, leading to a change in the bioavailability and toxicity of other xenobiotics [61-63]. Moreover, two compounds may synergize by different mechanisms but have the same effect [64]. Individually, pesticides are known to exert specific effects on some cell processes and/or key proteins involved in the regulation of general metabolism, cell growth, differentiation, and survival (Figure 1). Individual pesticides can regulate the mitochondrial respiratory chain, leading to apoptosis and/or increased ROS production[65-68]. The subsequent oxidative stress, which is also part of the detoxifying process, could provoke inflammation and/or alter cell signaling enzymes involved in the control of growth and survival or induce DNA damage. Some pesticides are known to bind to or activate nuclear receptors, thereby altering the cell's general or energetic metabolism. Thus, compounds in a mixture could interact at various cellular levels (Figure 1), and it is important to take into account the multiplicity of these possible sites of interaction to explain the various mixture effects. Moreover, the chronic duration of the exposure to contaminants may add another level of complexity that must also be considered in risk evaluation. In our opinion, the complexity of the interactions involving various cellular or molecular levels is likely to lead to unpredictable effects of mixtures.

5. Conclusion

Studying the impact of pesticide mixtures is of great interest to human health risk assessment processes since everyone can potentially be exposed to various cocktails of these compounds through food intake or occupational exposure. Pesticides can interact in various manners, according to the compound itself and its chemical family, the dose and the targeted parameters, and these variables can therefore lead to various effects. However, we have identified chemical families that appear to be specifically involved in one type of effect such as synergy or addition. These results need to be confirmed using other compounds in both *in vivo* and *in vitro* models to allow firm conclusions to be drawn, but they raise concerns about the safety of these pesticide cocktails since they are comparable to those often found in fruits and vegetables or used by professionals on field crops.

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Legend to Figure 1

Figure 1: Illustration of the various cell targets of pesticides and their effects on cell activity.

Some pesticides can bind to or regulate the activity of efflux ABC transporters or enzymes involved in xenobiotic metabolism, leading to a change in the bioavailability and thus the toxicity of the other co-exposed compounds. Some pesticides are known to directly induce DNA damage. Individual pesticides are also known to exert specific effects on some cell processes and/or key proteins involved in the regulation of general metabolism, cell growth, differentiation and survival. Indeed individual pesticides may interfere with mitochondrial respiratory chain complexes or impair oxidative phosphorylation and induce mitochondrial dysfunction, leading to apoptosis and/or an imbalance in cell metabolic activity. Individual pesticides are also known to induce reactive oxygen species (ROS) production by decreasing antioxidant capacity or through increasing XME activities. The subsequent oxidative stress may interfere with inflammatory responses, leading to changes in the cell's metabolic activity and/or alter cellular signaling enzymes involved in the control of growth and survival (apoptotic pathways) or induce DNA damage. In addition, some pesticides are known to bind to or activate nuclear receptors involved in the regulation of general or energetic metabolism.

When combined, the various cellular targets of pesticides become the various possible sites of interaction. The multiplicity of these interaction sites must also be taken into account when assessing the impact of pesticide mixtures.

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Table 1A In vitro and in vivo studies related to the parameters of central nervous system, to neurotoxicity, to brain, to neuronal function, to neurobehavioral.

Studies	Pesticides/ class/ chemical family	Doses/ Exposure way	Target	Observed effects
Thiruchelvam, M., 2000				
C57BL/6 mice	Paraquat and Maneb	• 5-10 mg/kg paraquat 15-30 mg/kg maneb • IP once a week for 4 weeks	Nigrostriatal dopamine system	No effect of pesticide alone ; effect of the mixture : Sustained decreases in motor activity levels of dopamine and metabolites and dopamine turnover were increased Reductions in tyrosine hydroxylase immunoreactivityv compounds, while having no or marginal effects when administered individually, can produce synergistic effects when given in combination
Richardson JR 2001				
in vitro brain and serum from male Sprague Dawley rats	Azinphos methyl oxon and chlorpyrifos oxon	• Dose response	Inhibition of CHE activity by simultaneous or sequential exposure to the metabolites	Simultaneous exposure to the compounds led to additive effects. Sequential exposure resulted in greater than additive effects at the higher concentrations (other factors such as detoxification enzymes or allosteric modulation, may be involved in the departure from additivity. At lower concentration no departure from additivity
Axelrad JC, 2002				
In vitro : NB2a neuroblastoma cells	diazinon and chlorpyrifos, in combination with a commercial formulation of the compounds and, independently, the components of that formulation	• The compounds were tested in pairs in various proportions • 24 Hours	neurite outgrowth measured by light microscopy and quantitative image analysis.	The combination of chlorpyrifos at a maximum concentration of 10 M and pyrethrum at a maximum of 500 nM produced statistically significant synergism (P 0.02).
Reeves R., 2003				
C56BL/6 male mice	Triadimefon and Paraquat	• 25 and 50 mg/kg • 10 mg/kg • IP twice a week/12 weeks	Behavioral and neurochemical effects	Mixture effect :antagonistic interaction of PQ on TDF induced hyperactivity PQ prevented the increased levels of activity associated with TDF50.
Timchalk C, 2005				
In vivo : Oral gavage, rats males Sprague-Dawley	Chlorpyrifos and diazinon	• 0, 15, 30 and 60 mg/kg • 3, 6, 12, and 24 hours postdosing	Pharmacokinetic, pharmacodynamic assays, ChE (plasma, red blood cells, brain)	Coexposure to CPF/DZN at the low dose of 15/15 mg/kg did not alter the pharmacokinetics of CPF, DZN, or their metabolites in blood. A high binary dose of 60/60 mg/kg increased the

				C(max) and AUC and decreased the clearance for both parent compounds, likely due to competition between CPF and DZN for CYP450 metabolism. the overall choline esterase response was additive
Cicchetti F. 2005				
Rats males Sprague–Dawley and dopaminergic (DA) neuron-glia cultures	Paraquat , Maneb	• 10 mg/kg paraquat • 30 mg/kg maneb IP twice a week/4 weeks	Neuronal degeneration	No interaction PQ with or without MB induces neurodegeneration
Tuzmen MN, 2007				
In vivo : adult males albino Wistar *rats*	Deltamethrin, chlorpyrifos	• Oral administration • in food CPF 1mg/kg Deltamethrin 5mg/kg • 16 weeks	Antioxidant enzymes activities in the brain/lipoperoxidation/acetylcholine esterase	the effects of the combination of PYR and OP on LPO may be due to functional, dispositional, or chemical antagonism, while the effects of the combination on CAT activity may be synergistic.
Jia Z, 2007				
In vitro : Neuroblastoma SH-SY5Y cell,	Endosulfan, zineb	• 100 µM (LC25) • 16h	LDH, apoptosis, necrosis ,	Toxicity of mixture higher than that of individual compounds
Flaskos J, 2007				
In vitro : Mouse NB2a neuroblastoma et C6 glioma cells	Diazinon and cypermerthrine	• Concentrations up to 10 microM of both compounds and their mixture • 24 hours	differentiation of neuronal and glial cell lines	cypermethrin had no additional effect on the inhibition of axon outgrowth by diazinon.
Wolansky MJ, 2009				
In vivo :males Long-Evans rats	11 pyrethroids permethrin, bifenthrin, cypermethrin, esfenvalerate, deltamethrin, β-cyfluthrin , tefluthrin, λ-cyhalothrin , fenpropathrin, resmethrin, and *S*-bioallethrin	• Oral gavage • Acute exposure (1 to 4H) each at the ED30	Motor activity	Dose additivity on motor activity
Ojha A, 2011				
In vivo : mâles Wistar Rats Voie orale, aigu 24, 48 ou 72h, chronique 60 jours, Etude génotoxicité (Comet assay) dans tissus (rate, cerveau, rein, foie)	Chlorpyrifos, methyl parathion, malathion	• accute exposure by oral gavage (24, 48, 72H) at ½ LD50 (77.5mg/kg CPF, 6,5 mg/kg MPT, 687,5 mg/kg MLT), • Chronic exposure by daily oral gavage for 60 days : LD50	DNA damages in various tissues	the damage was not the sum of damage caused by individual pesticide, confirming that these pesticides do not potentiate the toxicity of each other.
Cao Z, 2011				
In vitro : Mouse embryonic Neuronal cells in primary culture	deltamethrin, β-cyfluthrin, cypermethrin, permethrin, bifenthrin, esfenvalerate,	0.002-1 µM	Na influx	Additivity of the mixture: authors compared the actual mixture data with the predicted mixture concentration–response curve, and used a likelihood ratio test to test the hypothesis of additivity

	λ-cyhalothrin, tefluthrin, fenpropathrin, resmethrin, and *S*-bioallethrin.			using an *F*-distribution.
Heusinkveld HJ, 2012				
In vitro : Rat dopaminergic PC12 cells,	Lindane, Dieldrine	• 10 nM to 10µM (calcium) 10 or 100 µM (viability) • 20 min ou 24h	Viability (NR), calcium homeostasis	Differential effect of the mixture antagonistic interaction on the basal ca concentration and additive interaction on depolarization
Moser VC, 2012				
In vivo : adult and preweaning males Long-Evans rats	Carbaryl/propoxur / methiocarb/ methomyl/l Formetanate /oxamyl /carbofuran	• relative potency mixture: 0,3/ 1,3/ 3,7/ 8,6/ 15,9 mg/kg for adult and 0.2/L0.9/2.6/6.0/11. 1 mg/kg for PND17 • environmental mixture: 0.1/0.6/1.4/2.8/6 mg/kg for adult and 0.07/0.4/1.0/2.0/4.2 mg/kg for PND 17 • 40 min oral gavages	Behavioral and motor activity; Choline esterase activity in brain and red blood cells	Different interactive properties for different mixing ratios of these chemicals . In contrast, for the environmental mixture, more obvious greater than additive responses were observed in both age groups for all endpoints.This synergism was generally evident at all but the lowest dose tested in adults and pups; The data were mostly dose additive with mixture ratios based on relative potency factors (i.e., hazard-based mixture), whereas greater-than additive, or synergistic, effects were noted with mixture ratios based on amounts sold in California (i.e., exposure based). These results were similar across endpoints in both adults and PND17 pups. The magnitude of the synergy was up to twofold in adults, and up to threefold in pups.
Scelfo B, 2012				
In vitro : neuronal networks from 16 days old Balb/CIRC murine embryos cultured on Microelectrode array (MEA)	deltamethrin or permethrin alone or in mixture	20/80%, 50/50% ou 80/20% at 10, 100 nM, 1, 10, 100 ou 300 µM	Spontaneous neuronal activity	additive Neurotoxicity : Normalized dose–response curves of single chemicals and binary mixtures were fitted to sigmoidal shape curves with values between 0 and 1 (0–100%) by using five different theoretical models. Subsequently the two classical approaches to mixtures study, CA and IA, have been applied to each of the used theoretical models to compare calculated and experimental results from binary mixtures dose–response curves
Coleman MD, 2012				
In vitro : Glial U251 and neuronal SH-SY5Y cells	pyrimethanil, cyprodinil and fludioxonil	62,5 or 500 µM 48H	Energy metabolism ATP level apoptosis, oxidative stress enzymes expression	oxidative stress-related enzyme gene expression increases appeared to demonstrate some degree of synergy in the presence of the combination of agents.
Mwanza JC 2012				
in vivo in male Long Evans rat	Carbaryl and propoxur	1:1.45 part Pro:carb At 0 3 10 45 and 75 mg/kg	ChEsterase neurophysiological activity	Dose addition on brain ChE Erythrocyte ChE had larger difference between acute and

		Oral gavage Acute or repeated exposures (14 days)		repeated exposure and a slightly greater deviation from dose addition
Heusinkveld HJ, 2013				
In vitro : Rat dopaminergic PC12 cells,	Imazalil, Flusilazole,fluconazole, Tebuconazole, triadimefon, Cyproconazole.	• 0,1 à 100µM individual compound • IC20 binary mixtures, • IC10 quaternary • 20 min or 24h	cell viability and oxidative stress and intracellular calcium levels	Additive effects on basal calcium concentration and on voltage-gated calcium channels
Wang HP, 2014				
In vivo : Wistar Rats	chlorpyrifos and carbaryl I+I	• 1/125, 1/50 1/20 LD50 • 90 consecutive days • orally administered	activities of serum cholinesterase (ChE) as well as acetylcholinesterase (AChE) and neuropathy target esterase (NTE) in nerve tissues	independent effect in brain and spinal cord, and additive effect in the sciatic nerves
Meijer M, 2014				
In vitro : Rat PC12 cells	Chlorpyrifos oxon + chlorpyrifos, or + parathion ethyl and methyl , or + carbaryl	10 µM, 20 min Low doses LOEC	Basal and depolarization evoked ca(2+)i concentration	Chlorpyrifoas and carbaryl → additive effect Chlorpyrifos and oxon or parathion → non additive interaction (competition for voltage gated calcium channels)

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Table 1 B In vitro and in vivo studies related to hematopoietic parameters (bone marrow, haematopoiesis, stem cells) and or immunity human lymphocytes

Studies	Pesticides/ class/ chemical family	Doses/ Exposure way	Target	Observed effects
Olgun S, 2006				
In vitro : Thymocytes (mice C57/Bl6),	Lindane, Malathion, Perméthrine, mixture: Lin + Perm or Lin + MLT	• 50 to 150 µM • 5 and 15 minutes	Oxidative stress	Additive effect on oxidative stress mixture Lin + MLT
Pruett, SB, 2006				
In vivo C57Bl/6xC3HF1 mice	Atrazine/dieldrine	• 100-200mg/kg/10-20 mg/kg • IP • daily for 7 days (Dieldrine) : and one dose on day 7 atrazine	Immunotoxicology parameters	the effect was much greater than additive on IL-6 production (adding the individual effects of atrazine and dieldrin on IL-6 production indicates 20% suppression, whereas the combination yields 80% suppression) and essentially additive for inhibition of the activation of c-JUN (a component of the transcription factor, AP1
Das, PP, 2007				
Human peripheral lymphocytes	Endosulfan/ carbofuran/ Monocrotophos	• LC50 4.18 µM/LC50 5.76 µM/LC50 7.5 µM • 72 heures	Genotoxicity of ten binary mixtures	all the three mixtures showed synergistic toxicities
Demsia G, 2007				
In vitro :human Lymphocytes	Imidacloprid, Métalaxyl,	• 0,1, 1, 5, 10, 50, 100 µg/ml alone • 10, 25, 50, 100, 200 µg/ml in mixture • 72 hours	Cytogenetic end-points such as CBMN and SCE induction in human lymphocytes *in vitro*	Possible synergistic effect
In vivo : male Wistar rats	Imidacloprid, metalaxyl,	• Orally exposure mixture 1:1 (I+M) Imidacloprid 22, 45 or 67% LD50 Matalaxyl 11, 22, 45 % LD50 • 24 Hours	*in vivo* MN formation in rat bone-marrow	Possible synergistic effects
Battaglia CLR, 2010				
In vitro : males C57/BL6 Mice splenocytes	Lindane and malathion, MLT, Piperonyl butoxideBO, Seul ou en mélange	<LC25	Cytotoxicity and cell death	The pesticide mixture Malathion and lindane induced an additive increase in cytotxicity compared with the corresponding individual

				treatment
Sekeroglu V, 2011				
In vivo : male albino Wistar Rats,	**Commercial formulation** Deltamethrin(Decis2,5 EC, 25g/L) and Thiacloprid (Clypso OD 240, 240g/L)	• acute exposure **:** (1 gavages/24h) 37,5% LD50 Deltamethrin (15 mg/kg), Thiacloprid (112,5 mg/kg), Delta + Thia (15+112,5 mg/kg) • chronic exposure (1 gavages per days during 30 days)**:** 7,5% LD50 Delta (3mg/kg), Thia (22,5 mg/kg), Delta + Thia (3 +22,5 mg/kg)	Genotoxicity and cytoxicity in rat bone marrow cells, using mitotic index (MI), micronucleus (MN) and chromosome aberrations (CA) assay	Their results indicate that the mixture of DEL and THIA synergistically increased the cytotoxicity and genotoxicity in rat bone marrow cells
Demur C. 2013				
In vivo C57 Bl6 mice	Chlorpyrifos endosulfan atrazine	DJA In food From gestation until PND 98	Metabolic fingerprint and haematopietic parameters	No interaction
Yaduyanshi SK, 2012				
In vivo :male Park strain- 24h mice	Lindane, Endosulfan chlorpyrifos, monochotophos	• Mixture 0,125 LD50, pesticide alone 0,25 LD50 IP • 24H	Genotoxicity (MN) and mutagenicity (Ames)	Their results showed that mixture caused a highest increase in the frequencies of MN at the dose of 0.125 and 0.25 mg of each pesticide /kg body weight compared to individual pesticides. authors concluded to a synergistic effect

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Table1 C : in vivo and in vitro studies related to parameters of metabolism, biochemical parameters in liver, spleen, kidney oxidative stress, and to general parameters of cytotoxicity, genotoxicity,proliferation.

Studies	Pesticides/ class/ chemical family	Doses/ Exposure way	Target	Observed effects
Payne J, 2001				
In vitro : mammary carcinoma cell line MCF-7	0, p'-DDT/ p, p'-DDE /, b-Hexachlorohexane/p, p' DDT	• 1/10/5/4 nM • 7 days	the induction of cell proliferation	Synergistic effect when taking into account the most efficient compound additive effect according to mathematical modeling IA ou CA
Astiz M, 2009				
In vivo : male Wistar rats,	Dimethoate, Zineb, glyphosate, binary and ternary mixtures	• 1/250 LD50 • IP • 3 times a week/5 weeks	Oxidative stress in liver kidneys brain and plasma	Additive effects for the binary or ternary mixture
Rana I and Shivanandappa T 2010				
In vitro Ehrlich ascites tumor cells	Thiram and endosulfan	• 0.5 to 1.5 mM endosulfan and from 2 to 8 mM thiram • Mixture thiram 0.6 mM and 1.12 mM endosulfan	Glutathione level ROS Cytotoxicty	Very high doses potentiating cytotoxicity
Ojha A, 2011				
In vivo : Adult male albino Wistar rats	Chlorpyrifos, malathion, parathion	• 0,25 LD50 • 2 days • oral gavages	Glutathione homeostasis and oxidative status in liver kidneys spleen and brain .	selected OP pesticides have no interactive toxicity
Rouimi P, 2012				
In vitro : Mouse and human hepatocytes in primary culture	Atrazine, Chlorpyrifos, Endosulfan,	• 0,1, 1, 10 µM • 24h	maccroarrays	No interaction
Dikic D, 2012				
In vivo :male and female Swiss mice	Imazalil (I), Cypermethrin (Cy), Carbendazim (Ca)	• 10mg/ 20mg/kg /20mg/kg respectively • Mixture I+Cy : 10/10 mg/kg I+Ca : 10/20 mg/kg, Cy+Ca : 20/10 mg/kg • Oral daily gavage • 28 days	Liver and biochemical parameters	Additive effect for Ca+ I or Ca+ Cy
Nawaz A, 2013				
In vitro: HepaGR cells	Binary and ternary mixture of endosulfan atrazine and chlorpyrifos	• 1, 10 et 25 µM 50, 75 or 100 µM Equimolar concentration in the mixture • Chronic and acute (2 weeks or 24 hours)	cytotoxicity gene metabolism Real-Time Cell Impedance	Endosulfan potentiate the effect of the other pesticides
Takakura N, 2013				
In vitro : Caco2 and HepG2 cells	7mixtures **C1 :** 3fongicides 2 insecticides	• 1, 3, 10, 30 et 100 µM, • 24h	Cytotoxicity. evaluation of the ombined effects of the two most	For the cocktail composed of dichlorodiphenyltrichloroethane (DDT) and dieldrin, the

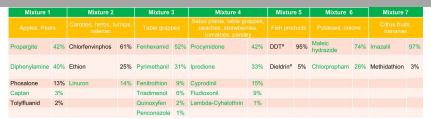
	C2 : 2 insecticides 1 erbicide **C3:** 4 fongicides 1 insecticide 1 herbicide C4 : 2 fongicides 3 insecticides **C5 ;** 2 insecticides (OC) **C6** 1 herbicide un régulateur de croissance **C7** 1 fongicide 1 insecticide		cytotoxic cocktails by comparing the measured effects of the mixtures with the predictions based on additive effects on two concepts-independent action (IA) and concentration addition (CA).	cytotoxicity of the equimolar cocktail proved greater than the additive effect estimated by the two concepts. Furthermore, apoptosis induction was higher in equimolar cocktail than predicted by summing the effects of DDT and dieldrin. Thus, some supra-additive toxicity was found in the DDT-dieldrin cocktail.
Crepet A, 2013				
In vitro : rat and human hepatocytes in primary culture and HepG2, Caco2, ACHN, SH-SY5Y, LS-174T		• 1 to 100µM, • 24 hours	Cytotoxicity, oxidative stress, genotoxicity, apoptosis, PXR activation cellular impedance	OC (mixture 5) greater than additive effect on cytotoxicity.
Josse R, 2014				
In vitro :HepaRG cells	Isomalathion (isoMLT) and malathion (MLT)	• 10, 25, 50 µM MLT, 0,05, 5, 10, 25, 50 µM IsoMLT, • mixture MLT/IsoMLT : 25/0,05, 25/5, 10/10, 25/25 µM, • 24h	Cytotoxicity, viability, genotoxicity, oxidative stress	Additive genotoxic effect and Antagonistic effect on apoptosis
Savary CC 2014		•		
In vitro HepaRG cells	Endosulfan and metoxychlore	24 H or 2 weeks 10 to 100 µM	cytotxicity	A synergistic cytotoxic effect was observed in HepaRG cells, after exposure to each pesticide at 100 *µM* in mixture Additive effect on CYP3A4 antagonistic effect on CYP2B6 activity

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Table 1 D : In vitro and in vivo studies related to parameters linked to reproduction, sexual hormones level, hormone receptor

Studies	Pesticides/ class/ chemical family	Doses/ Exposure way	Target	Observed effects
Mumtaz MM, 2002				
In vitro : HeLa cells cotransfected with an expression vector encoding estrogen receptor and an estrogen-responsive chloramphenicol acetyltransferase (CAT) reporter plasmid	DDT, DDD, aldrin, dieldrine, endrine	• 0 ; 0,001 ; 0,01 ; 0,1 ;1 et 10 µM binary mixture • 18 hours	transcriptional activation of an estrogen-responsive reporter gene in transfected HeLa cells	no detectable estrogenic activity for any of the six organochlorines tested singly. no synergistic interactions among combinations of these pesticides
Greenlee AR, 2004				
In vivo Murin preimplantation embryos	• dicamba/pendimethalin • Dicamba/2,4-D/atrazine • Chlorothalonil/mancozeb/diquat • atrazine/metolachlor/2,4-D/ammonium nitrate • Chlorpyrifos/terbufos/ permethrin • Dicamba/ 2,4-D/MCPP	• 0.03/0.04µg/ml • 0.03/0.01/0.03 • 0.015/0.003/0.0022 • 0.035/ 0.1/0.01/1 • 0.003/0.0001/0.05 • 0.03/0.01/0.0005	effects on mouse preimplantation embryo development, a period corresponding to the first 5-7 days after human conception	Mixtures showed a pattern of injury similar to pesticides tested individually
Valeron PF, 2009				
In vitro : Human mammary epithelial cells (HMEC)	p,p′-*DDT*/ o,p′-*DDE*/ p′,p′-*DDE,* p,p′-*DDD/aldrine/ dieldrine*	• 0.46/1.43/1.08/1.33/0.2/0.035 1x, 100x 500 and 1000x the serum concentration • 96 hours	Cytotoxiccity viability Gene expression	Additive effect of the mixture on cell viability
Blystone, CR 2009				
male Sprague Dawley Rats	Vinclozoline Iprodione	• 0/10/30/50/100 mg/kg/J 50 mg/kg/J • Oral gavage from PND 23 to PNDJ55-57	Male rat development The age at puberty (preputial separation [PPS]), organ weights, serum hormones, and ex vivo testis steroid hormone production	Serum testosterone increased from vinclozolin exposure but iprodione significantly decreased this effect with the effect of iprodione being most prominent when combined with vinclozolin at 60 mg/kg/day
Kjaerstad MB, 2010				
In vitro : human adrenocortical carcinoma cell	imidazoles (econazole, ketoconazole, miconazole, prochloraz) and triazoles (epoxiconazole, propiconazole,	• 4 equimolar mixtures (0,025 à 50 µM) • 48 Hours	endocrine potential (transcriptional	When predicting the effect of the mixture of the

line H295R	tebuconazole)		activation AR, estradiol and testosterone levels	azole fungicides on testosterone production, the shape of the predicted curve was different from the observed effect curve but, the predicted curve was however, close to the observed values suggesting additivity
Perobelli JE, 2010				
In vivo : Rats Lewis males pesticides	Dicofol/dieldrine/endosulfan/permethrine/dichlorvos/mixture	• LOEL/LOAEL/LEL/ • In the diet • 8 weeks	Reproductive toxicity	Additive effect or no interaction according to the studied parameters
Ohlsson , 2010				
In vitro : adrenocortical carcinoma cell line H295R	Prochloraz, ketoconazole, Imazalil	• Single compounds 0,003, 0,01, 0,03, 0,1, 0,3, 1, 3, 10µM, • binary mixture: 1:1 (0,06 or 0,2µM) • Ternary mixture 1 :1 :1 (0,003, 0,009, 0,03, 0,09, 0,3, 0,9, 3 ou 9µM) • 24 hours	secretion of cortisol and aldosterone and the effects on steroidogenic gene expression.	• /Additive effect on the secretion of cortisol
Hass U, 2012				
In vivo : female Han-tac rats	epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone	• Doses closed to NOAEL for single pesticide • 14,6, 29,2, 43,8 mg/kg/j for the mixture • Daily gavage during gestation (7 to 21) and PND1 to 16	Sexual development in rat offspring	Severe mixture effects on gestation length, nipple retention and genital malformations were seen at dose levels where the individual pesticides caused no or smaller effects when given alone. Generally, the mixture effect predictions based on dose-additivity were in good agreement with the observed effects.
Wang D, 2012				
In vivo : Male Sv/129 mice	Diazinon, cis-permethrin,	• 10 and 90 µmol/kg/day respectively • 100 µmol/kg/day mixture (30 mg/kg /day approcximatively • Gavage • 6 weeks	Reproductive toxicity	Diazinon inhibited cis-permethrin metabolism . additif effect of the mixture on the reproductive function
Orton F 2012				
In vitro MDA-kb2 cells human breast cancer cells stably	Fludioxonil; fenhexamid, orthophenyl phenol, imazalil, tebuconazole, dimethomorph, methiocarb, pirimiphosmethyl (8 mix ar agonist) cypridonyl, pyrimethanil, vinclozolin, chlorpropham, linuron (5 mix AR	1.17 nM–150 µM,	Antagonist activity	Widely used pesticides act additively *in vitro* as AR

transfected with a firefly luciferase reporter gene that is driven by an androgen–response element-containing promoter	antagonist) and all pest (mix 13)			antagonists
Overgaard , 2013				
In vivo : females rats Han-tac : WH	epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone	• epoxiconazole 3.75 mg/kg/day (Epo-3.75) or 15 mg/kg/day (Epo-15), mancozeb 6.25 mg/kg/day (Man-6.25) or 25 mg/kg/day (Man-25), prochloraz 8.75 mg/kg/day (Prochlo-8.75) or 35 mg/kg/day (Prochlo-35), tebuconazole 12.5 mg/kg/day (Tebu-12.5) or50 mg/kg/day (Tebu-50) and procymidone 12.5 mg/kg/day (Procymi-12.5) or 50 mg/kg/day (Procymi-50). mixture of the five pesticides in different concentrations; 14.58 mg/kg/day (Pestimix-14.58),29.17 mg/kg/day (Pestimix-29.17) and 43.75 mg/kg/day (Pestimix-43.75). • The pesticides were mixed in the following ratio:epoxiconazole:mancozeb:prochloraz:tebuconazole:procymidone 15:50:35:50:50, • daily by oral gavage (2 ml/kg), from GD 7 to GD 21, and again from the day after delivery to pup day 16	on kisspeptin neurons in the rat hypothalamus	No additive effects
Ermler S, 2013				
In vitro : CHO-K1 cells	albendazole, benomyl, carbendazim, flubendazole,mebendazole, oxibendazole, thiabendazole, albendazole oxyde	• Dose 10-7M	MTT, cytochalasin blocked micronucleus assay	Additive effect of the mixture of all pesticides
Prutner W, 2013				
In vitro : Human adrenocortical carcinoma cell line H295R	cyprodinyl, pyrimethanil, iprodione, procymidone, myclobutanil, tebuconazole, azoxystrobin, kresoxim-méthyl, methomyl, captan	• from 0,01to 100µM • 24h	Estrone production	• Combination of fungicides enhancing estrone biosynthesis and belonging to the same chemical group, --> effect of the more potent compound in the mixture • Combination of fungicides enhancing estrone biosynthesis and belonging to different chemical groups --> a dose additivity cypro + procy • two fungicides having opposing effects on estrone biosynthesis--> effect of the compound inhibiting estrone biosynthesis.

				• no case a synergistic effect was observed
Kjeldsen LS 2013				
In vitro : hamster ovary CHO-K1 cells, using luciferase reporter gene assays	2-methyl-4-chlorophenoxyacetic acid (MCPA), terbuthylazine, iodosulfuron-methyl-sodium, mesosulfuron-methyl, metsulfuron-methyl, chlormequat chloride, bitertanol, propiconazole, prothioconazole,mancozeb, cypermethrin, tau fluvalinate,malathion and the metabolite ethylene thiourea (ETU)	• ER et AR transactivation : mixture 5 5.10^{-12} to 2,5.10 M, Aromatase activity : mixture 5.10^{-8} to 2,5.10^{-4} M The estrogenic and antiestrogenic activities of the pesticides were assessed using the stably transfected MVLN cell line (kindly provided by Prof. M. Pons, France), derived from the human breast adenocarcinoma MCF-7 cell line, carrying an estrogen response element luciferase reporter vector The androgenic and antiandrogenic activities of the pesticides were assessed using the Chinese hamster ovary cell line CHO-K1 Effects of the pesticides on aromatase activity were assessed using the human choriocarcinoma JEG-3 cell line	Effects of the pesticides on ER and AR function After completing concentration–response analyses of the single pesticides, mixture effect concentrations were predicted as described (Birkhoj et al., 2004 and Kruger et al., 2008) by applying the principle of concentration addition (CA). Briefly, this model relies on the assumption that mixture components, which do not interact, differ only in potency and consequently, they can be considered as dilutions of one another.	For Mix3, bitertanol, propiconazole, cypermethrin) the predicted effect concentrations were within the 95% confidence band for the observed effects, suggesting additive mixture effects. Isobole coefficients were estimated to give values relatively close to 1, supporting the finding of an additive mixture effect . For Mix5, (terbuthylazine, bitertanol, propiconazole, cypermethrin, malathion), the predicted effect concentrations were found outside the 95% confidence band for the observed effect, and were higher than observed effect concentrations, indicating an effect more than additive (synergistic) of the mixture on DHT-induced AR activity
Christen V, 2014				
In vitro MDA kb2 cells	dicarboximide and azoles	Dose response 24 H	Anti androgenic activity	Interactions in mixtures follow the CA model. However, a high percentage of synergistic interactions occurred..Additivity and synergy **occur according to the dose** (AZ+AZ and DICARBO + AZ)

Table 2. Classification of results from studies listed in Table 1 (available in the supplementary data) investigating the effects of pesticides mixtures. Values in red show the number of mixtures assessed in each category. Some references appear many times in the table where authors have assessed different mixtures. In brackets the percentage values.

	Mixture of insecticides (I)	Mixture of herbicides (H)	Mixture of fungicides (F)	Mixture of I+H	Mixture of I+F	Mixture of H+F	Mixture of I+F+H
67 mixtures assessed in total	**31 (46%)**	**3 (4.5%)**	**10 (15%)**	**7 (10.5%)**	**8 (12%)**	**3 (4.5%)**	**5 (7.5%)**
Parameters linked to neurotoxicity, brain, central nervous system, or neuronal functions **20 mixtures assessed in total**	**15 (75%)** (Richardson, Chambers et al. 2001; Axelrad, Howard et al. 2002; Timchalk, Poet et al. 2005; Flaskos, Harris et al. 2007; Tuzmen, Candan et al. 2007; Wolansky, Gennings et al. 2009; Cao, Shafer et al. 2011; Ojha, Yaduvanshi et al. 2011; Coleman, O'Neil et al. 2012; Heusinkveld and Westerink 2012; Moser, Padilla et al. 2012; Mwanza, Lyke et al. 2012; Scelfo, Politi et al. 2012; Meijer, Hamers et al. 2014; Wang, Wang et al. 2014)	**None**	**1(5%)** (Heusinkveld, Molendijk et al. 2013)	**None**	**1 (5%)** (Jia and Misra 2007)	**3 (15%)** (Thiruchelvam, Richfield et al. 2000; Reeves, Thiruchelvam et al. 2003; Cicchetti, Lapointe et al. 2005)	**None**

Hematopoietic and immune parameters **8 mixtures assessed in total**	**5 (62.5%)** (Olgun and Misra 2006; Das, Shaik et al. 2007; Battaglia, Gogal et al. 2010; Sekeroglu, Sekeroglu et al. 2011; Yaduvanshi, Srivastava et al. 2012)		**None**	**2 (25%)** (Pruett, Fan et al. 2006; Demur, Metais et al. 2013)	**1 (12.5%)** (Demsia, Vlastos et al. 2007)	**None**	**None**
Parameters of cell biology (proliferation, oxidative stress, DNA damage) or metabolism (detoxification, hepatic, renal, energetic, general) **20 mixtures assessed in total**	**6 (30%)** (Payne, Scholze et al. 2001; Ojha and Srivastava 2011; Crepet, Heraud et al. 2013; Takakura, Sanders et al. 2013; Josse, Sharanek et al. 2014; Savary, Josse et al. 2014)	**2 (10%)** (Crepet, Heraud et al. 2013; Takakura, Sanders et al. 2013)		**5 (25%)** (Rouimi, Zucchini-Pascal et al. 2012; Crepet, Heraud et al. 2013; Demur, Metais et al. 2013; Nawaz, Razpotnik et al. 2013; Takakura, Sanders et al. 2013)	**4 (20%)** (Rana and Shivanandappa 2010; Dikic, Landeka et al. 2012; Crepet, Heraud et al. 2013; Takakura, Sanders et al. 2013)	**None**	**3 (15%)** (Astiz, de Alaniz et al. 2009; Crepet, Heraud et al. 2013; Takakura, Sanders et al. 2013)
Parameters linked to reproduction and/or hormonal regulation **19 mixtures assessed in total**	**5 (26.5%)** (Mumtaz, Tully et al. 2002; Greenlee, Ellis et al. 2004; Valeron, Pestano et al. 2009; Perobelli, Martinez et al. 2010; Wang, Kamijima et al. 2012)	**1 (5%)** (Greenlee, Ellis et al. 2004)	**9 (47.5%)** (Greenlee, Ellis et al. 2004; Blystone, Lambright et al. 2009; Kjaerstad, Taxvig et al. 2010; Ohlsson, Cedergreen et al. 2010; Hass, Boberg et al. 2012; Ermler, Scholze et al. 2013; Overgaard, Holst et al. 2013; Prutner, Nicken et al. 2013;	**None**	**2 (10.5%)** (Prutner, Nicken et al. 2013; Christen, Crettaz et al. 2014)	**None**	**2 (10.5%)** (Orton, Rosivatz et al. 2012; Kjeldsen, Ghisari et al. 2013)

			Christen, Crettaz et al. 2014)				

Table 3. Observed effects of different types of pesticide mixtures. Values in red show the number of mixtures assessed in each category. The same reference may be present at various places in the table since some authors have assessed more than one mixture or one mixture led to various effects according to the dose or chemical target.

Reported effects		Insecticides (I)	Herbicides (H)	Fungicides (F)	I+H	I+F	H+F	I+F+H
Interactions (24 examples) 35%								
Synergic, greater-than-additive effects or supra additive *17 examples*	Animal model studies *8 examples*	(Richardson, Chambers et al. 2001; Tuzmen, Candan et al. 2007; Sekeroglu, Sekeroglu et al. 2011; Moser, Padilla et al. 2012; Yaduvanshi, Srivastava et al. 2012)			(Pruett, Fan et al. 2006)	(Demsia, Vlastos et al. 2007)	(Thiruchelvam, Richfield et al. 2000)	
	Human primary cultured cells or human cell lines *7 examples*	(Payne, Scholze et al. 2001; Das, Shaik et al. 2007; Coleman, O'Neil et al. 2012; Crepet, Heraud et al. 2013; Takakura, Sanders et al. 2013; Savary, Josse et al. 2014)		(Christen, Crettaz et al. 2014)				
	Animal cell lines *2 examples*	(Axelrad, Howard et al. 2002; Kjeldsen, Ghisari et al. 2013)						
Antagonistic effect *5 examples*	Animal models *3 examples*	(Tuzmen, Candan et al. 2007)		(Blystone, Lambright et al. 2009)			(Reeves, Thiruchelvam et al. 2003)	
	Human primary	(Josse, Sharanek et al. 2014; Savary, Josse et al. 2014)						

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	cultured cells or human cell lines *1 examples*							
	Animal cell line *1 examples*	(Heusinkveld and Westerink 2012)						
Potentiating effects *2 examples*	Animal model							
	Human primary cultured cells or human cell lines *1 example*				(Nawaz, Razpotnik et al. 2013)			
	Animal cell lines *1 example*					(Rana and Shivananda ppa 2010)		
Additive effects (33 *examples*) **48%**								

	Animal models 12 *examples*	(Richardson, Chambers et al. 2001; Timchalk, Poet et al. 2005; Wolansky, Gennings et al. 2009; Perobelli, Martinez et al. 2010; Moser, Padilla et al. 2012; Mwanza, Lyke et al. 2012; Wang, Kamijima et al. 2012; Wang, Wang et al. 2014)		(Hass, Boberg et al. 2012)	(Pruett, Fan et al. 2006)	(Dikic, Landeka et al. 2012)		(Astiz, de Alaniz et al. 2009)
	Human primary cultured cells or human cell lines 11 *examples*	(Valeron, Pestano et al. 2009; Crepet, Heraud et al. 2013; Josse, Sharanek et al. 2014; Savary, Josse et al. 2014);		(Kjaerstad, Taxvig et al. 2010; Ohlsson, Cedergreen et al. 2010; Prutner, Nicken et al. 2013; Christen, Crettaz et al. 2014)		(Jia and Misra 2007; Takakura, Sanders et al. 2013))		(Orton, Rosivatz et al. 2012)
	Animal cell lines 10 *examples*	(Olgun and Misra 2006; Battaglia, Gogal et al. 2010; Cao, Shafer et al. 2011; Heusinkveld and Westerink 2012; Scelfo, Politi et al. 2012; Meijer, Hamers et al. 2014)		(Ermler, Scholze et al. 2013; Heusinkveld, Molendijk et al. 2013)				(Orton, Rosivatz et al. 2012; Kjeldsen, Ghisari et al. 2013)
No interaction and no additive effects (12 studies) 17%								
	Animal models 6 *examples*	(Perobelli, Martinez et al. 2010; Ojha, Yaduvanshi et al. 2011)		(Overgaard, Holst et al. 2013)	(Rouimi, Zucchini-Pascal et al. 2012; Demur, Metais et al. 2013)		(Cicchetti, Lapointe et al. 2005)	

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	Human primary cultured cells or human cell lines 1 *example*	(Mumtaz, Tully et al. 2002)						
	Animal cell lines 5 *examples*	(Cicchetti, Lapointe et al. 2005; Flaskos, Harris et al. 2007; Meijer, Hamers et al. 2014)		(Prutner, Nicken et al. 2013)			(Cicchetti, Lapointe et al. 2005)	

Table 4. Chemical families of pesticides involved in the various observed interactions. OP = organophosphorous; OC = organochloride; PYR = pyrethrinoid; AZ = azole; IMI = imidazole; BenzIMI = benzimidazole; TRIA = triazine; TriAz = triazole; NEO = neonicotinoide; A-PYR = anilopyridine; Dicarbo = dicarboximide; CARB = carbamate; DTCARB = dithiocarbamate; Bi-Pyr = bipyridilium; phospho = aminoacid phosphonoglycine, UC = unclassified.

	Neurotoxicity			**Immunity and hematopoiesis**			**Metabolism, cytotoxicity, apoptosis, proliferation, and oxidative stress**			**Reproduction or hormones**		
Test system	Animal model	Cultured human cells	Cultured animal cells	Animal model	Cultured human cells	Cultured anima cells	Animal model	Cultured human cells	Cultured animal cells	Animal model	Cultured human cells	Cultured animal cells
Synergy, greater-than-additive effect or supra-additive	▪ **OPoxon+ OP oxon** (Richardson, Chambers et al. 2001) ▪ **PYR+OP** (Tuzmen, Candan et al. 2007) ▪ **7 CARB** ((Moser, Padilla et al. 2012) ▪ **pyridine + CARB**	▪ **2 A-PYR+1 phenyl pyrrole** (Coleman , O'Neil et al. 2012)	▪ **OP+ Pyrethrum** (Axelrad, Howard et al. 2002)	▪ **TRIA + OC** (Pruett, Fan et al. 2006) ▪ **2 OP+2 OC** (Yaduvanshi, Srivastava et al. 2012) ▪ **PYR+ NEO** (Sekeroglu, Sekeroglu et al. 2011) ▪ **2 NEO+1 phenyla**	▪ **OP +CARB** ▪ **OP+OC** ▪ **OC+CARB** (Das, Shaik et al. 2007) ▪ **2 NEO+1 phenylamide** (Demsia, Vlastos et al. 2007) ▪			▪ **OC** (Takakura, Sanders et al. 2013; Savary, Josse et al. 2014) ▪ **4OC** (Payne, Scholze et al. 2001) ▪ **2 OC** Crepet A., 2013			▪ **AZ+AZ And AZ+DiCarbo** (Christen, Crettaz et al. 2014)	▪ **OP+PYR +2TriAZ** (Kjeldsen LS 2013)

	(Thiruchelvam, Richfield et al. 2000)			**mide** (Demsia, Vlastos et al. 2007)								
Antagonistic interaction	• **Pyridine +TriAz**(Reeves, Thiruchelvam et al. 2003) • **PYR+OP** (Tuzmen, Candan et al. 2007)		• **OC** (Heusinkveld and Westerink 2012) • **OP+OPoxon (**Meijer, Hamers et al. 2014)					▪ **OC** (Savary, Josse et al. 2014) **OP** (Josse, Sharanek et al. 2014)		▪ **2Dicarbo** (Blystone, Lambright et al. 2009)		
Potentiating effect				▪ **OP+OC** (Nawaz, Razpotnik et al. 2013) ▪ **TRIA+OC** (Nawaz, Razpotnik et al. 2013)	▪ **OC+DTCARB**(Rana and Shivanandappa 2010)							
Test system	Animal model	Cultured human cells	Cultured animal cells	Animal model	Cultured human cells	Cultured anima cells	Animal model	Cultured human cells	Cultured animal cells	Animal model	Cultured human cells	Cultured animal cells
Addition	▪ **OPoxon+OP oxon** (Richardson, Chambers et al. 2001) ▪ **OP+CARB** (Wang, Liang et al.		▪ **OC+OC** (Heusinkveld and Westerink 2012) ▪ **OP+CARB** Meijer, Hamers et al. 2014) ▪ **PYR** (Cao,	▪ **OC+TRIA** (Pruett, Fan et al. 2006)		▪ **OP+OC** (Olgun and Misra 2006; Battaglia, Gogal	▪ **2OP+DTCARB** (Astiz, de Alaniz et al. 2009) ▪ **AZ+PYR+benzIMI** (Dikic, Landeka et al.	▪ **OC** (Savary, Josse et al. 2014) ▪ **OP** (Josse, Sharanek et al. 2014)	▪ **OC+OP** (Battaglia BLR, 2010)	▪ **3OC+PYR+OP** (Perobelli, Martinez et al. 2010) ▪ **CARB+AZ+IMI+Dicarbox** (Hass,	▪ **5OC** Valeron PF 2009 ▪ **AZ+Dicarbo** (Christen, Crettaz et al. 2014) ▪ **AZ +AZ and**	▪ **Az /IMI** (Ermler, Scholze et al. 2013) ▪ **PYR+2TriAZ +**

	2013; ▪ **PYR** Wolansky, Gennings et al. 2009 ▪ **OP** (Timchalk, Poet et al. 2005) ▪ **7 or 2 CARB** (Moser, Padilla et al. 2012; Mwanza, Lyke et al. 2012) ▪ **OP+PYR** ((Wang, Kamijima et al. 2012)		Shafer et al. 2011; Scelfo, Politi et al. 2012) ▪ **AZ** (Heusinkveld, Molendijk et al. 2013)			et al. 2010)	2012)			Boberg et al. 2012) ▪ **OP+PYR** (Wang D, 2012)	**Dicarbo+ AZ** (Christen, Crettaz et al. 2014) ▪ **TRIA+ PYR+OP +2AZ** (Kjeldsen, Ghisari et al. 2013) ▪ **3AZ** (Kjaerstad, Taxvig et al. 2010) ▪ **A-PYR+ Dicarbo** (Prutner, Nicken et al. 2013) • **2 IMI +AZ**(Ohlsson, Cedergreen et al. 2010) **2 Phenyl pyrrole+ OP+2AZ+ Phenol+ Dicarbo+ CARB** (Orton, Rosivatz et al. 2012)	**TRIA**(kjeldsen LS, 2013)
Test system	Animal model	Cultured human cells	Cultured animal cells	Animal model	Cultured human cells	Cultured anima cells	Animal model	Cultured human cells	Cultured animal cells	Animal model	Cultured human cells	Cultured animal cells
No interaction	• **CARB+ Pyridine** (Cicchetti, Lapointe et al. 2005) • **OP** (Ojha,		• **OP+ PYR** (Flaskos, Harris et al. 2007) • **CARB+**	▪ **OP+OC +TRIA** (Demur, Metais et al. 2013) ▪		▪ **OP** (Ojha and Srivastava 2014)	▪ **TRIA+OP +OC** (Rouimi, Zucchini-Pascal et al. 2012)			▪ **OP+OC+ PYR** (Perobelli, Martinez et al. 2010) ▪ **DTCARB**	▪ **OC** (Mumtaz, Tully et al. 2002)	• **A-PYR+ TriAZ** (Prutner, Nicken et al. 2013)

	Yaduvanshi et al. 2011)		**Bi-PYR** (Cicchetti, Lapointe et al. 2005) • **OP+OP oxon or OP+OP** (Meijer, Hamers et al. 2014)				▪ **OP** (Ojha, Yaduvanshi et al. 2011)			**+AZ+ Dicarbo** (Overgaard, Holst et al. 2013)		

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Table 5a:Chemical families of pesticides specifically involved in each type of interaction. Mixtures in bold have been proven to induce their specific corresponding effects by mathematical modeling.

	Synergy	Antagonism	Potentialization	Addition	No interaction
Cultured human cells	**AZ+Dicarbo** **OC+ CARB** A-PYR+ phenylpyrrole			**PYR+2TriAZ** A-PYR+Dicarb **3IMI**	
Cultured animal cells	**OP+PYR+2TriAZ+TRIA**		OC + DTCARB	PYR+2AZ OP+CARB **PYR**	
In vivo animal models	PYR+NEO NEO+Phenylamide	TriAZ+pyridine		**CARB+AZ+IMI+Dicarbo** PYR+BenzIMI 2OP+DTCARB	CARB+BI-Pyr OP+OC+TRIA DTCarb+AZ+Dicarbo

Function	Insecticide	Fongicide	Herbicide
Chemical family			
Organophosphorus (OP)	Azinphos Chlorpyrifos Diazinon Dichlorvos Dimethoate Malathion Monocrotophos Parathion Pirimiphos		
Carbamate (CARB) and Dithiocarbamate (DTCARB)	Carbaryl Carbofuran Flubendazole Formetanate Mebendazole Methiocarbe Methomyl propoxur	Benomyl Carbendazime Zinebe Thiram (DTCARB) Manèbe (DTCARB) Mancozeb (DTCARB)	Chlorpropham
Organochlorin (OC)	Aldrine Beta-HCH Dieldrine DDE DDT Dicofol Endosulfan Endrine Lindane		
Azole (AZ) triazole (TriAZ) Imidazole (IMI) Benimidazole (BenzIMI)		Bitertanol **(TriAZ)** Cyproconazole**(AZ)** Epoxiconazole**(TriAZ)** Fluconazole**(AZ)** Flusilazole**(AZ)** Myclobutanil**(AZ)** Propiconazole**(TriAZ)** Tebuconazole**(TriAZ)**	Thibendazole **(BenzIMI)** Carbendazim **(BenzIMI)**

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		Triadimefon**(TriAZ)**	
Pyrethrinoïd (PYR)	Beta-cyfluthrine Bifenthrine Cypermethrine Deltamethrine Esfenvalérate Fenpropathrine Lambda-cyhalothrine Permethrine Resmethrine S-bioallethrine Téfluthrine		
Anilino pyrimidine (A-Pyr)		Cyprodinil Pyrimethanil	
Dicarboximide (Dicarbo)		Captan dimethomorph Iprodione Procymidone Vinclozoline	
Imidazole (IMI)		Benomyl Imazalil ketoconazole Prochloraz	
Neonicotinoïde (NEO)	Imidaclopride Thiaclopride		
Phenyl pyrrole		Fludioxonil	
Triazine			Atrazine terbuthylazine
Unclassified (UC)		Fenhexamide	Glyphosate Linuron Paraquat (Bi-Pyr) Metalaxil

Table 5b : some examples of the various chemical families of pesticides and the corresponding compounds used in this study

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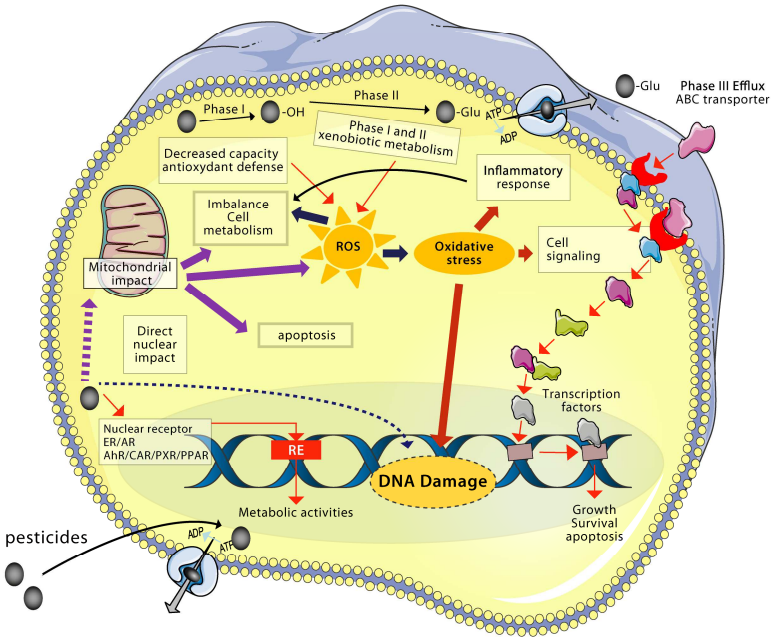
Thiruchelvam M., 2000				
C57BL/6 mice	Paraquat and Maneb	• 5-10 mg/kg paraquat 15-30 mg/kg maneb • IP once a week for 4 weeks	Nigrostriatal dopamine system	No effect of pesticide alone; effect of the mixture. Sustained decreases in motor activity levels for dopamine and metabolites and dopamine turnover increased. Reductions in tyrosine hydroxylase immunoreactivity
Castillo C.G., 2002				
In vivo, male Wistar rats	Endosulfan and methyl parathion and commercial preparation of the mixture	(25 mg/kg) E (2 mg/kg) P • SC 10 days	Neurological renal and hepatic marker functions	When given in combination, the commercial formulation of ES and MP can produce behavioral alterations
De la Rosa, P., 2003				
In vivo female C57 BL/6 mice	3,4-dichloro propionanilide (propanil) and 2,4-dichloro phenoxyacetic acid (2,4-D)	• 14, 50 to 200 mg/kg bw • 2 consecutive IP • extraction bone marrow at day 1, 2, 7 and 14 post treatment	alterations to bone marrow B-cell populations after in vivo exposure	Exposure to the mixture had greater toxic effects than the individual herbicides on bone marrow pre-B and IgM(+) B-cell populations,
Karanth s 2004				
In vivo rat Sprague dawley	Methyl parathion and chlorpyrifos	• Oral exposure gavage. • Effect of concurrent an sequential exposure to Acute exposure to LD 96H of CPF and MPS	Lethality, cholinergic signs and biochemical endpoints	Sequence of exposure to OP that elicits toxicity through a common mechanism of action can markedly influence the cumulative action at the targeted site (AChE and consequent functional toxicity). No information on the nature of interaction
Cory-Slechta D.A., 2005				
In vivo, mice C57/Bl6	Paraquat (PQ) and Maneb (MB) H+F OP+CARB	• Subcutaneous administration • From postnatal days 5-19, 0.3 mg/kg PQ, 1 mg/kg MB • From gestational day 10-17, 1 mg/kg MB, and at weeks 7-8, 5 mg/kg PQ or 15 mg/kg MB for 8 days	Dopamine systems of the nigrostriatal pathway	Cumulative neurotoxicity; effects of PQ and MB were greater those of either pesticide alone (postnatal model)
Jiaz Z., 2007				
In vitro,	Endosulfan, zineb	• 100 µM (LC25)	LDH, apoptosis,	Toxicity of mixture

neuroblastoma SH-SY5Y cells		16 h	necrosis	greater than that of individual compounds
Metzdorff SB 2007				
In vivo Female wistar rats	Vinclozolin, , procymidone flutamide **Flutamide is not a pesticide**	Oral exposure from GD 7 to PND 16 mixture ratio of vinclozolin, flutamide, and procymidone was 0.62:0.02:0.36, and the master mixture contained 22.026 mg vinclozolin, 696.6 mg flutamide, and 12.675 mg procymidon in 600 ml corn oil.	Reproductive organ weight, androgen regulated gene expression in prostates from male rat pups	Additive effects
Gomez J., 2009				
In vivo, male and female TO strain mice	Pesticide formulations of OP: Salut, Selecron, Dursban, Acetllic, Hosthation, Nogos	• 3%, 10% LD50 • Gavage • 7 weeks before premating and preconception	Gestational and litter outcomes	Positive Interaction
Yavuz O, 2010				
In vivo : male Wistar Rats	Tetramethrin a-Cypermethrin, Deltamethrin, Cypermethrin, piperonyl butoxide (BPO)(inhibition Various mixture	• 1/5 LD50 Dermal exposure • 14 days	hematologic and biochemical parameters	their findings showed that although pyrethroids are considered to be of low acute toxicity, they become more toxic when combined with piperonyl butoxide or tetramethrin in certain doses
Sharma H 2010.				
immortalized rat N27 DA neurons	dieldrin and lindane	5 to 25 µM 48H	Cell viability, mitochondria membrane potential, caspase ROS	dieldrin and lindane work cooperatively to induce neurotoxicity. Dieldrin and lindane interacts via different manner: they interact on neuronal gaba receptor (increase ca and OS) L and D interact on mitochondrial membrane potential
Chatterjee S., 2011				
Bone marrow explants In vitro from albino Swiss mice	Alphamethrin, cypermethrine, profenofos, chloropyrofos	• 10% (w/v) alpha, 9% Cyper, 40% Profenofos, 50% Chloropyrofos 25 days	CFU, colony forming assay, cell cycle, apoptosis	Toxicity of the mixture

Aubé M. 2011				
In vitro Mammary cancerous cell line CAMA-1, MCF-7, T47D, MDAMB231 and normal mammary cells CV-1	OC PC,B p,p0-DDE, a-HCH, p,p0-DDT, toxaphene, Aldrin, Dieldrin, Tetra-chlorobenzene, b-HCH, p,p0-DDD, Hexachlorobenzene, Pentachlorobenzene, g-HCH, Mirex	• dilutions 100×10^3 and 50×10^3	Proliferative activity	Differential effects according to the cell line and the estrogenic or anti-androgenic activity of the compounds
Yang J., 2012				
In vivo, male Wistar rats	Dichlorvos, acephate, dimethoate, and phorate	• 1x, 3x, and 9x NOAEL • Drinking water 24 weeks	Oxidative damage in liver	The responses to the mixture did not constitute the sum of the responses produced by each pesticide
Dubey N., 2013				
In vivo, male and female adult Wistar rats	Deltamethrin, fluoride	• 1/100 LD50 and 20 ppm • Daily gavage 28 days	Oxidative stress in liver, biochemical parameters	Positive interaction
Wang H.P., 2013				
In vivo, male Wistar rats	Dichlorvos, deltamethrin	• 1/20, 1/50, and 1/125 LD50 • Daily gavage • 90 days	Metabonomic analysis of the serum and urine	Changes in serum TMAO, alanine, choline, and acetone in this treatment group were higher than in rats treated with either dichlorvos or deltamethrin
Du L., 2013				
In vivo, male Wistar rats	Dichlorvos, acéphate, dimethoate, phorate	• 1x, 3x, and 9x NOAEL • In drinking water, the amount of OP pesticides was adjusted twice a week for the first 8 weeks and then once a week until the end of the experiment • 24 weeks	Metabonomic profiles of rat urine	Joint toxic action
Pant N., 2013				
In vitro, human semen	□ and □-hexachlorocyclohexane (HCH), DDE, DDD, plomb, cadmium	• □-HCH (76.94 µg/L), □-HCH (39.04 µg/L), pp-DDE (53.89 µg/L), pp-DDD (57.49 µg/L), Pb acetate (33.2 µg/L), Cadmium Cl (35.6 µg/L) • 30 min at 96 h	Sperm viability and motility	Positive interaction
Yu Y., 2013				

In vivo, male Sprague-Dawley rats	Dichlorvos/ dimethoate /malathion	• 1/5, 1/10, 1/20 LD50 • Daily by oral gavage from the 15th to the 28th day of lactation	Uterus pathology, neonatal development, reproductive function in offspring	Mixture is more potent than pesticide alone
Taxvig C., 2013				
In vitro, H295R cells; in vivo, pregnant rats	Mix 1 bitertanol propiconazole cypermethrine and Mix 2 bitertanol propiconazole cypermethrine malathion and terbuthylazine	Oral exposure, gavage from GD7 GD21 3 to 50 mg/kg In vitro 1.6 to 100 µM	Hormone level steroidogenesis	Pesticide alone and mixture affect steroidogenesis
Mishra V, 2013				
In vivo : male albino Wistar Rats	Monocrotophos, quinalphos,	• 0.25 LD50 each single pesticide, 1/8^{e} LD50of each pesticide in the mixture • gavage • 2 consecutive days	Redox status of tissues	No synergistic effect compared to single compound
Mwila K, 2013				
In vitro assay : enzymatic detection method using	*In vitro assay : enzymatic detection method using*	*In vitro assay : enzymatic detection method using*		
Du L., 2014				
In vivo, male Wistar rats	Dichlorvos, dimethoate, acephate, phorate	• 1x, 3x, and 9x NOAEL • Drinking water • 24 weeks	Metabonomics in plasma	Joint toxic action at the NOAEL of each pesticide
Baskar R., 2014				
In vivo, albino Swiss mice	Commercial formulation Tatamida (17.8% Imidacloprid), Uthane M-45 (75% Mancozeb)	• 0.5% LD50 • Diet exposure through lactation • Analysis in offspring at PND 28 and 63	Metabolic impact, weight gain, cholesterol, TG, thyroid hormone	Cumulative response of the mixture
Roustan A., 2014				
In vitro, CHOK1 cells	Glyphosate atrazine AMPA DEA	5, 10, 50, and 100 µg/ml 3 H	Cytogenetic potential	Enhanced cytogenetic activities

Figure 1



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