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ORIGINAL

Effects of prenatal and postnatal exposure to chlordimeform on serotonin levels in brain regions of adult's male and female rats

Efectos de la exposición pre y postnatal al clordimeformo sobre los niveles de serotonina en regiones cerebrales de ratas adultas macho y hembra

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Abstract

Introduction: Formamidines pesticides have been described to induce permanent effects on development of monoaminergic neurotransmitters systems. The mechanisms that induce these effects are not known but it has been suggested that these effects could be related to monoamino oxidase (MAO) inhibition. Chlordimeform is a formamidine pesticide which is a very weak inhibitor of MAO although it has been also described to produce neurodevelopmental toxicity.
Objectives and methods: The effects of maternal exposure to chlordimeform on brain region serotonin levels of male and female offspring rats at 60 days of age were evaluated. Maternal and offspring body weight, physical and general activity development were unaffected by the exposure of dams to chlordimeform (5 mg/kg bw, orally on days 6–21 of pregnancy and 1–10 of lactation). Male and female offspring were sacrificed at 60 days of age and possible alterations in the content and metabolism of 5-HT was determined in brain regions by HPLC.
Results: The results showed that this neurotransmitter system was altered in a brain regional-related manner. In male and female offspring, chlordimeform induced a significant decrease in the striatum and prefrontal cortex 5-HT and its metabolite 5-HIAA levels. This effect was with statistical distinction of sex in the prefrontal cortex. In contrast, chlordimeform caused an increase in 5-HT and 5-HIAA content in the hippocampus in male and female offspring with sex interaction. Chlordimeform evoked increases in 5-HT turnover in the prefrontal cortex and hippocampus from females and males respectively but evoked a decrease in these regions from males and females respectively.
Conclusions: The present findings indicated that maternal exposure to chlordimeform altered serotonergic neurochemistry in their offspring in prefrontal cortex, striatum and hippocampus, and those variations show that other mechanisms different from MAO inhibition are implicated.

Keywords: Formamidines; neurodevelopmental toxicity; chlordimeform; rats; serotonin; human risk assessment

Resumen

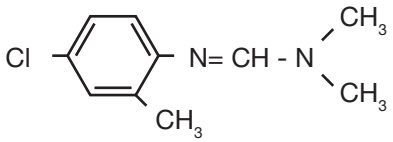
Introducción: Se ha descrito que los pesticidas formamidinicos inducen efectos permanentes en el desarrollo de los sistemas de neurotransmisores monoaminérgicos. Los mecanismos por los que se inducen estos efectos no se conocen, pero se ha sugerido que podrían estar relacionados con la inhibición de la monoamino oxidasa (MAO). El clordimeformo, es un pesticida formamidinico, del que se han descrito efectos neurotoxicos en el desarrollo, aunque es un inhibidor muy débil de la MAO.
Objetivos y métodos: En el presente estudio se evaluaron los efectos sobre los niveles de serotonina en regiones cerebrales de ratas macho y hembra a los 60 días de edad tras la exposición maternal al clodimeformo (5 mg/kg de peso corporal, por vía oral en los días 6-21 de la gestación y 1-10 de la lactancia). El peso corporal de las madres y las crías, y el desarrollo físico y de la actividad general no se vieron afectados por la exposición al clordimeformo. Las crías fueron sacrificadas a los 60 días de edad y las posibles alteraciones en el contenido y metabolismo de 5-HT se determinaron en regiones cerebrales mediante HPLC.
Resultados: El clordimeformo indujo una disminución significativa en el cuerpo estriado y la corteza prefrontal de los niveles de 5-HT y su metabolito 5-HIAA. Este efecto fue estadísticamente influenciado por el sexo en la corteza prefrontal. Por el contrario, el clordimeformo causó un aumento del contenido de la 5-HT y de 5-HIAA en el hipocampo con influencia significativa por sexo. El clordimeformo provoco aumentos en la tasa de recambio de 5-HT en la corteza prefrontal y el hipocampo de hembras y machos respectivamente, sin embargo, provocó una disminución en estas regiones en machos y hembras respectivamente.
Conclusiones: Los presentes resultados indican que las formamidinas y en particular el clordimeformo inducen tras la exposición maternal una alteración permanente del sistema serotoninérgico de forma región y sexo dependiente en la descendencia, las cuales muy probablemente se deban a mecanismos distintos de la inhibición de la MAO.

Palabras clave: Formamidinas; neurotoxicidad en el desarrollo; clodimeformo; ratas; serotonina; evaluación del riesgo para el hombre

Introduction

Chlodimeform [N2-(4-chloro-o-tolyl)-N1. N1-dimethylformamidine] (**Figure 1**) is a member of the formamidine pesticides, widely used as a broad spectrum acaricide, and in agriculture for the protection of fruits, and vegetables[1]. Monoamine oxidase (MAO) inhibition was among the first biochemical actions of the formamidines that were reported[2-3]. Thus, aminergic mechanism of action of chlordimeform was quickly postulated and adopted because neuronal MAO participates in metabolic inactivation of biogenic monoamines which include the neurotransmitters serotonin, norepinephrine, and dopamine. In addition, chlordimeform is an antagonist of reserpine effects[4], alters prostaglandin synthesis[5], has α2 receptor agonist properties[6], and is an endocrine disruptor[7].

Figure 1: Chlodimeform chemical structure (C10H13Cl N2).



Pesticides pose a growing risk to health, with a specific concern about the possible permanent effects that these compounds may have on the development of organisms. Thus, it has been described for formamidine compound amitraz, the induction of permanent alterations on the development of central nervous system (CNS) such as those that affect monoamine neurotransmitter systems, although there is not information about neurodevelopmental effects of other formamidine compounds. The mechanism by which these effects occur is not known but currently it is assumed that the monoaminergic neurotransmitters play a role during development, defined as "morphogenetic"[8-11]. Any change in the levels of catecholamines during development could have a profound effect on brain development, both structural and functional[12]. In this sense, it has been suggested as a possible mechanism of action the inhibition of MAO which may alter the levels of monoaminergic neurotransmitters, although other mechanisms as endocrine disruption on sex hormones that control the expression of enzymes that catalyze the synthesis and metabolism of the monoamine neurotransmitters cannot be excluded[13].

According to all exposed above, we performed a study to establish if maternal exposure to formamidines during gestation and lactation induces permanent alterations on serotoninergic system in adult age. Chlordimeform was chosen because it is the most representative compound in its group which presents a very low inhibition of MAO, allowing us to study more clearly whether the permanent changes observed on levels of serotonin neurotransmitter are due to an alteration of the enzymes that catalyze the synthesis and metabolism of these neurotransmitter rather than inhibition of MAO.

This work focuses its interest in providing new data of formamidines induced neurotoxicity during nervous system development, because new compounds of this family are being developed with therapeutic applications for which these effects are not considered in their risk assessment, which poses a potential health hazard.

Materials and methods

Biological material

All experiments were performed in accordance with European Union guidelines (2003/65/CE) and Spanish regulations (BOE 67/8509-12, 1988) regarding the use of laboratory animals. Six pregnant Wistar rats were housed individually in polycarbonate cages and were assigned randomly to two experimental groups: a chlordimeform treatment group (n = 3) and a control group (n = 3).

Test Chemical and Treatment

Chlordimeform (Sigma, Madrid, Spain) was dissolved in corn oil to provide fast and complete absorption and was administered orally by gavage in a volume of 2 mg/ml. The animals received daily chlordimeform at the dose of 5 mg/kg on days 6 to 21 of pregnancy (GD 6-21) and on days 1 to 10 of lactation (PN 1-10). Control dams received vehicle (corn oil 2.5 ml/ kg) on the same schedules. Dose of chlordimeform was selected based on previous preliminary study that indicated this dose was the higher one that did not cause weight loss or mortality, any reduction of food or water intake as well as did not induce haematological modifications of other clinical histopathological signs of overt toxicity. Moreover we did not see any changes in suckling of maternal caretaking. None of the prenatal or postnatal treatment evoked a significant change in weight of any of the brain regions on PN 60 (data not shown).

Dams were examined daily throughout the gestation and lactation periods for mortality, general appearance and behaviour. The maternal body weights were measured on GD 1, GD 5, GD 6, GD 15 and GD 20. Food and water consumption during pregnancy, length of gestation, litter size and sex ratio were also assessed.

On PN1, all litters were examined externally, sexed and weighed. Litters were organized in groups of twelve pups, six males and six females. Litters were weighed at PN 1, PN 7, PN14 and PN 21. The offspring were weaned on lactation day 21 and were maintained in appropriate conditions, housed individually and without any treatment with full access to food and water until adult age. The study was organized in treated groups of six males and six females randomly selected respectively from the dams' litters exposed to chlordimeform, and control groups of six ma-

les and six females pups randomly selected respectively from the control dams' litters.

At PN 60, male and female rats from control and treated groups (pups from control dams, and pups from dams exposed to chlordimeform, respectively) were sacrificed by decapitation. The brain was removed quickly and the hypothalamus, midbrain, medulla oblongata, cerebellum, brainstem, hippocampus, striatum and prefrontal cortex were rapidly dissected out at 4ºC[14]. Tissues were rapidly weighed and stored at -80 ºC until analysis. All data were collected by experimenters blind to the treatment condition of the offspring.

Determination of monoamine levels

Following sample collections, 300-800 µl of 0.4 M HClO4 containing 0.1% (w/v) $Na_2S_2O_5$ was added to the tissues, and the mixture was homogenized by sonication before neurochemical evaluation was performed. The homogenates were centrifuged for 15 min at 20000 g at 4º C and aliquots of supernatants were taken for analysis of serotonin (5-HT) and its metabolite [5-hydroxy-3-indolacetic acid (5-HIAA)] using a high performance liquid chromatography (HPLC) technique with electrochemical detection [15,13]. Volumes of 200-300 µl of the supernatants (in 0.4 M $HClO_4$) were treated for 3 min at 100º C in a water bath. The samples were then cooled and 30-45 µl of 2 M NaOH were added (final pH: ca. 1.5) and aliquots were injected into a reversed phase HPLC system. For the analysis of the indolalkylamines 5-HT and 5-HIAA, the mobile phase consisted of 0.1 M Na_2HPO_4. $2H_2O$, 0.1 M citric acid (pH 3.5) and 10% (v/v) methanol. Elution was performed at a flow rate of 1 ml/min and the working electrode potential was set at 0.7 V for indolalkylamines.

Peak areas in the sample chromatograms were quantitated by external standard technique using solutions of the indolalkylamines (5-HT and 5-HIAA). 5-HT turnover was calculated as ratio of metabolite to neurotransmitter.

Data analysis

Statistical analysis of data was performed using a Statgraphics software, version Plus 4.1 for windows. Values are expressed as mean ± S.E.M. obtained from 12 animals, six males and six females, in each group (control and treated groups). For values combined for males and females, a two-way ANOVA with treatment × sex interaction was the initial test used. Where a significant treatment × sex interaction was detected, a separate Student's *t* test was carried out for each sex. The results were considered significant at $P < 0.05$. Results significantly different from controls are also presented as change from control (%).

Results

Maternal and offspring body weight, physical and general activity development were unaffected by the exposure of dams to chlordimeform (5v mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation). No differences were found between the body weights of the control and treated dams throughout the pregnancy (data not shown). There were no significant differences in the length of gestation as well as in the litter sizes between dams receiving chlordimeform and those receiving corn oil (control group) (data not shown). Furthermore, no pups were found dead at birth and there were no differences detected in pup weights immediately after birth. Malformation or other overt signs of pesticide toxicity were neither observed. Body weights of pregnant dams and their offspring were unaffected by chlordimeform exposure. In addition, no differences were found in the general maternal behaviour of control and treated dams. Differences were not detected in the body weight of male and female offspring on days 1, 7, 14, 21 (weaning) and at 60 of life (data not shown).

Brain tissues levels of serotonin, its metabolite and the turnover in rat pups at PN 60 are presented in **Table I**. In the hypothalamus, midbrain, cerebellum, medulla oblongata and brainstem, 5-HT, 5-HIAA levels and 5-HIAA/5-HT ratio were not modified by dam exposure to chlordimeform in males and females rat pups at PN 60. In male and female offspring, chlordimeform induced a significant decrease in striatum and prefrontal cortex 5-HT and 5-HIAA levels and an increase of 5-HT and 5-HIAA levels in the hippocampus compared to control animals, displaying in the hippocampus and frontal cortex a significant sex interaction with the treatment effect (**Tables I** and **II**).

In striatum, the reduction of 5-HT content was 15.75% ($P < 0.001$) and 20.92% ($P < 0.001$) for 5-HIAA. In frontal cortex, the reduction of 5-HT content was a 16.99% ($P < 0.001$) and a 31.01% ($P < 0.001$) in males and females respectively and the loss in the content of 5-HIAA was a 31.56% ($P < 0.001$) and a 21.44% ($P < 0.001$) in males and females respectively. In hippocampus the increase of 5-HT content was a 31.05% ($P < 0.001$) and a 19.77% ($P < 0.001$) in males and females respectively, and the increase of 5-HIAA content was a 37.44% ($P < 0.001$) and 13.49% ($P < 0.001$) in males and females respectively. Turnover rate suffered a statistically significant reduction of 18.02% ($P < 0.01$) in males' frontal cortex and 5.08% ($P < 0.001$) in females' hippocampus. However, the turnover rate suffered a significant increase of 4.89% ($P < 0.01$) in males' hippocampus and 13, 92% ($P < 0.001$) in females' frontal cortex (**Tables I** and **II**).

Discussion

The present study shows that prenatal and postnatal exposure to chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation) was not able to induce maternal toxicity since during pregnancy maternal weight gain of treated rats was not modified. In

Table I: Tissue 5-HT and 5-HIAA concentrations in male and female rat pups observed at 60 days of age after the exposure of dams to chlordimeform (5 mg/kg bw orally on days 6 to 21 of pregnancy and 1 to 10 of lactation).

Tissue	5-HT (ng/g)		5-HIAA (ng/g)		5-HIAA/5-HT	
	Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)
HT	2211,69 ± 39,58	2246,49 ± 43,96	1175,98 ± 39,15	1210,17± 43,53	0,53 ± 0,02	0,54 ± 0,03
MB	2605,67 ± 23,42	2619,96 ± 22,86	1721,96 ± 11,30	1725,23 ±13,72	0,66 ± 0,01	0,66 ± 0,01
CB	131,96 ± 7,43	136,73 ± 7,52	116,42 ± 4,06	120,80 ± 5,12	0,90 ± 0,06	0,90 ± 0,08
MO	1204,19 ± 28,25	1206,92 ± 43,62	838,13 ± 17,51	845,56 ± 17,22	0,70 ± 0,02	0,71 ± 0,03
BS	858,80 ± 14,96	869,82 ± 19,58	452,34 ± 9,25	456,68 ± 15,93	0,53 ± 0,01	0,53 ± 0,03
PFC	1172,90 ± 26,90	[b]890,30 ± 30,44	644,05 ± 15,48	[b]471,59 ± 6,84	0,55 ± 0,02	[b]0,53 ± 0,02
ST	848,38 ± 4,72	714,77 ± 5,54*******, [a](-15,75)	726,27 ± 8,55	574,37 ± 26,03*******, [a](-20,92)	0,86 ± 0,01	0,80 ± 0,04
HC	534,01 ± 4,45	[b]669,83 ± 16,23	474,60 ± 6,82	[b]594,50 ± 20,10	0,89 ± 0,01	[b]0,89 ± 0,02

HT: *hypothalamus; MB: midbrain; CB: cerebellum; MO: medulla oblongata; BS: brainstem; PFC: prefrontal cortex;*
ST: *striatum; HC: hippocampus.*
Data represent means ± S.E.M. with values for males and females combined (n=12: 6 males + 6 females).
*Statistical significance is reported for the *P<0.05, **P<0.01 and ***P<0.001 levels compared with the control group.*
[a] *Percentage change from control values.*
[b] *Significant treatment × sex interaction.*

addition, the pesticide did not impair offspring weight; the weight was similar both in pups from treated dams and from control dams. Prenatal and postnatal maternal exposure to chlordimeform did not change the time course of male and female offspring growth and general activity, being these data in agreement with the obtained with amitraz treatment[13]. However, chlordimeform administered during pregnancy and lactation leads to permanent alterations of the serotonergic system in a sex and region dependent way at 60 days of age in rats. Chlordimeform affected the content of 5-HT only in the regions frontal cortex, hippocampus and striatum. The effects observed in our study included a significant decrease of serotonin and its metabolite levels in striatum and frontal cortex and an increase in hippocampus, displaying only in the prefrontal cortex and hippocampus a significant sex interaction with the treatment. Moreover, chlordimeform evoked an increase in 5-HT turnover in prefrontal cortex and hippocampus from females and males respectively but evoked a decrease in these regions from males and females respectively.

Developmental neurotoxicity involves alterations in behaviour, neurohistology, neurochemistry and/or gross dysmorphology of central nervous system occurring in the offspring, as a result of chemical exposure of the mother during pregnancy or lactation. The mechanism through which these permanent effects on serotoninergic system take place is unknown, but monoamine neurotransmitters, such as 5-HT regulate brain development prior to assuming their roles as transmitters in the mature brain[16-18], thus any circumstance that affects these neurotransmitters in the developing brain can alter the final structure and function of that brain. Since the endogenous levels of serotonin are highly regulated by MAO, any change in this enzyme can profoundly affect the developing brain. In this sense, it has been reported that gestational exposure to MAO inhibitors clorgyline and deprenyl produce in offspring at 30 days of age, a significant reduction of serotonergic innervation particularly in the cerebral cortex[19]. Moreover, amitraz, which is a potent MAO inhibitor[2], has been shown to induce permanent alterations of monominergic neurotransmitters systems[13] similar to those induced by chlordimeform through gestational and lactational exposure. Because chlordimeform is a very week inhibitor of MAO, this mechanism could not support the alterations in serotonergic system observed in the present study, and thus in formamidine pesticides, suggesting other mechanisms are implicated.

On the other hand, it has been also described that steroids play a role in the development of catecholamines systems[20-23], and may play a critical role in mammalian brain developmental of both sexes[24]. In this sense chlordimeform has been reported to disruptor different steroids hormones[7], which could also contribute to the permanent effects observed. Moreover, chlordimeform could also affect the neuronal cell replication, differentiation, synaptogenesis and axonogenesis, steroid metabolism, and functional development of neurotransmitter systems, effects that could result in behavioural alterations observed in previous studies after developmental exposure to chlordimeform[25]. The loss of serotoninergic projections could also play an important role in the behavioural alterations. Prenatal exposure to chlordimeform may result in either direct damage or enhanced vulnerability of the neurotransmitter systems to future toxic insult.

Given that the serotoninergic system alteration in the brain regions of our study with chlodimeform (frontal cortex, striatum, and hippocampus) was the same as those affected by amitraz[13] it can be inferred that the mecha-

Table II: Statistical analysis for tissue values with significant treatment × sex interaction

Tissue		5-HT (ng/g)		5-HIAA (ng/g)		5-HIAA/5-HT	
		Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)	Control group	Treated group (pups from treated dams)
CF	Males	1157,41 ± 38,5	960,77 ± 2,78***, [a](-16,99%)	679,37 ± 4,49	464,96± 8,78***, [a](-31,56%)	0,59 ± 0,021	0,4 8± 0,010**, [a](-18,02%)
	Females	1188,40 ± 8,72	819,82 ± 6,68***, [a](-31,01%)	608,73 ± 6,96	478,23 ± 2,84***, [a](-21,44%)	0,51 ± 0,01	0,58 ± 0,007***, [a](13,92%)
HC	Males	536,10 ± 4,09	702,58 ± 5,21***, [a](31,05%)	466,56 ± 3,45	641,25 ± 3,01***, [a](37,44%)	0,87 ± 0,008	0,91 ± 0,010**, [a](4,89%)
	Females	531,91 ± 5,00	637,09 ± 11,10***, [a](19,77%)	482,64 ± 8,04	547,74 ± 2,39***, [a](13,49%)	0,91 ± 0,011	0,86 ± 0,017***, [a](-5,08%)

PFC: *prefrontal cortex. Other tissue values were not evaluated because of the lack of treatment × sex interactions.*
Values are mean ± S.E.M.; control animals (n= 6 males, n= 6 females); treated group (n= 6 males, n= 6 females).
*Statistical significance is reported for the **P<0.01 and ***P<0.001 levels compared with the control group within each sex as determined by one-way ANOVA, followed by the Student's t test.*
[a] *Percentage change from control values.*

nism by which formamidines alter CNS development is similar. Moreover, these brain regions participate in the regulation of learning and memory processes[26-31], thus, it could be considered that these processes could be compromised by exposure during gestation and lactation to formamidines. In addition, the dysfunction in serotonin system is involved in appetite, affective, neuropsychiatric disorders[32-35], among others, which could be also induced by formamidine exposure during development. Further studies are needed to test these functions to confirm that alteration of this neurotransmitter system is the cause of some of these dysfunctions.

Conclusion

The results of this investigation show that formamidines, particularly the chlordimeform, cause developmental neurotoxicity at serotonergic system level. Further studies are required to determine the possible mechanisms through which formamidines induced these effects, specifically the hormonal disruption effects. It is also needed a pathologic examination in the affected regions to determine the effect on the number of neurons, to determine if there is a reduction in innervation. Currently, new molecules with therapeutic application are being developed as the N-hydroxy-N-(4-butyl-2-methylphenyl) formamidine (HET0016) with protective effects against cardiovascular and cerebrovascular diseases. Until now the risk assessment of the family of these compounds has been taken from the standpoint of carcinogenesis. In view of these results it might be appropriate to reconsider the risk assessment of the members of this family based not only on their possible carcinogenic effects but also in the neurotoxic effects during development.The results reported in this study are of great importance and should be incorporated into the risk assessment of pesticides formamidines group.

Compliance with ethical standards

All experiments were performed in accordance with European Union guidelines (2003/65/CE) and Spanish regulations (BOE 67/8509-12, 1988) regarding the use of laboratory animals.

Conflict of interest

The authors declare that there are no conflicts of interest.

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