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RESEARCH ARTICLE

DIAZINON INDUCED NEUROBEHAVIORAL ALTERATIONS AND HISTOLOGICAL CHANGES IN FEMALE WISTAR RAT : PROTECTIVE EFFECT OF PREGNANCY

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ABSTRACT

Diazinon is an organophosphate insecticide with broad spectrum of use especially in agriculture. Organophosphorus components inhibit acetylcholinesterase which causes accumulation of acetylcholine in cholinergic synapses that leading to cholinergic stress. Pregnancy is a period known by remakable increase of steroids neuroactive, molecules with therapeutic opportunities especially in neuroprotection. The aim of this study was to examine the response of pregnancy against the neurobehavior and histological effects induced following the subchronic administration of diazinon. Dose of 10mg/kg b.w was injected daily by intraperitoneal way to pregnant rats between the 7th to 14th day of pregnancy. Virgin female rats were also used as basis of comparaison and obey to the same experimental protocol. Thus, recording of sequences of behavior of elevated plus maze was made in 7th, 14th and 21st day of pregnancy. After delivery which occurs at 21st day and lactation which takes 21 days, we tested the effectivenesse of gabaergic agonist, the clonazepam during the forced swimming test, then the prancreas and thymus sampled after decapitation for the histological study. Ours results showed that application of diazinon neurotoxic product during 07 consecutive days caused increase of anxiety behavior that appears to be irreversible in virgin female rats. For cons, the same treatment associated with pregnancy revealed a healing effect after delivery. Inefficiency of clonazepam treatment was observed in virgin female rats treated with diazinon. However this gabaergic agonist was effective in rats which treated with diazinon during pregnancy. Histological exam showed atrophy of thymus and pancreatic necrosis in virgin femele rats, for cons no histological change observed in rats which treated with diazinon during pregnancy. We conclude that pregnancy can play protective role against the alterations induced by diazinon.

INTRODUCTION

Organophosphate (OP) insecticides which have largely replaced the organochlorine compounds are one of the most widely used estimated to account for about 50% of all insecticides used globally (Casida and Quistad, 2004). Organophosphorus compounds (OPCs) such as diazinon are neurotoxic chemical agent that inhibits acetylcholinesterase (AChE) activity (Delfino *et al*., 2009). This causes accumulation of acetylcholine at cholinergic synapses leading to increased activation of nicotinic and muscarinic receptors. In addition to its inhibition of AChE, it can induce oxidative stress that is important in its toxicity (Amirkabirian *et al*., 2007; Shadnia *et al*., 2007). Recent studies indicate that pesticide intoxication produce oxidative stress by generation of free radicals and induce tissue lipid peroxidation in mammals and other organisms (Shadnia *et al*.,2005; Kovacic, 2003). Thus, oxidative stress is an other mechanism that has been proposed for the toxicity of OPCs in animals and human. Diazinon after malathion is one of the most commonly used OP in the world (Ghafour-Rashidi *et al*., 2007). OP are powerful activators of the HPA axis (e.g. Osicka-Koprowska *et al*., 1984; Smallridge *et al*., 1991) and exposure to these substances may result in persistent neurobehavioral alterations (Richardson, 1995) and histological changes (Dikshith *et al*., 1975). In order to prevent mechanisms of toxicity, particulary oxidative stress, many works showed the efficiency of some natural products with antioxydant properties such as quercetine and/or catechin (Galati *et al*, 2002 ; Uzun *et al*, 2010; Kalender *et al*, 2011). Deficiency and the limited defence mechanisms of animal organism against the potential toxicity induced by neurotoxic product such as diazinon result in persistent neurobehavior alterations and histological changes in virgin female rats such as, increase of anxiety, impairment of spatial memory, brain astrocytoma, hyperlasia of medulla adrenal gland and haemorrhage splenic red pulp (Tayaa *et al*, 2013). Thus, recent studies showed that pregnancy period in female Wistar rats has a positive response against neurotoxicity of toluene (Frih *et al*, 2012 ; Latreche *et al*, 2012) and emotional disorder of lithium-responsive bipolar disorder, type I in women (Paul *et al*, 1999). Paul *et al* (1999) suggests that powerful natural mechanisms preventing the recurrence of

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typical lithium-responsive bipolar disorder, type I may be at work during pregnancy. There is an emerging body of observation suggest that placenta production of hormones and peptides may be one of the important factors involved (Turnbull *et al*, 1974 ; Field, 1984). Changes in various neurohormones and neuropeptides might also play a protective role during pregnancy. These include CRH, ACTH, cortisol, progesterone and progesterone metabolites, estrogens, TSH and prolactin (Paul *et al*, 1999). We hypothesized that pregnancy could provide the body with a protective barrier in response to nerve attacks (stress, inhalation of toxic substances, etc.) and this protection would, a priori, occur via production of neuroactive steroid (NAS) extensively developed during pregnancy (Frih *et al*, 2012). In this context, we developed an experimental approach that vises to evaluate the protective response of pregnancy against the neurobehavioral and histological alterations induced by diazinon, neurotoxic and organophosphorus product.

MATERIAL AND METHODS

Animals and housing

Adult, intact female Wistar rats (Rattus rattus) (n=08) were obtained from Pasteur Institute of Algiers. Rats were group housed in a temperature and humidity controlled room on a reverse light cycle (lights off at 8:00 a.m.) with ad libitium access to water and rats chow in their cage.

Determination of sexual receptivity

Daily (between 10:00 and 11:00 a.m.), females were vaginally masked and paired briefly with a stimulus male (that was conditioned to show consistent, high levels of sexual contact). Sexual receptivity was determined by the response of experimental females to stimulus male investigation. Rats that demonstrated receptive (lordosis) and proceptive behaviors (hopping, darting and ear wiggling) were considered to be in behavioral estrus, while those that exhibited aggressive behaviors (vocalizing, defensive posturing, boxing and avoidance) were not considered in behavioral estrus. Vaginal cytology was used to determine estrous cycle.

After identifying the phases of the rats estrous cycle, we divided them into eight experimental groups (n=08):

V group or Control group: Virgin female rats received an IP injection of olive oil per day from day 7 to day 14.

DZNV group: Virgin female rats received an IP injection of diazinon, from day 7 to day 14.

P group: pregnant rats. Male rats were introduced into the cages for mating regardless of their weight at a rate of one male per female. Fertilization is confirmed by the presence of mucus plug in the vaginal smear, which corresponds to the first day of gestation. The rats received an IP injection of olive oil per day from day 7to day 14.

PDZN group: Pregnant rats treated with diazinon. Gestation was performed in the same way as for the P group and all pregnant rats receiving one IP injection of diazinon per day from day 7 to day 14 of gestation.

V with Clonazepam: virgin rats received clonazepam subcutaneously in a volume equivalent to 2 ml/kg at a dose of 0.25 mg/ml.

VDZN with Clonazepam: virgin rats treated with diazinon received clonazepam

P with Clonazepam: Pregnant rats received clonazepam

PDZN with Clonazepam: Pregnant rats treated with diazinon received clonazepam

Diazinon administration

Diazinon, was obtained from Vapco society (Jordany).The rats received diazinon at dose of 10 mg/kg b.w (1/6 LD_{50}) dissolved in 1 ml/kg of olive oil by intraperitoneal injection for 07 consecutive days exactly between the 7^{th} to 14^{th} of gestation.

Behavior testing

Forced swimming test

Test of Porsolt or forced swimming test. In rats, some behavioral changes occurring may be analyzed in the forced swimming task (FST), which is designed to test the antidepressant profile of drugs. The present study was aimed to analyze in pregnant rats, after delivery, the effectiveness of an agonist GABAergic (clonazepam) those behavioral changes displayed in the FST (Porsolt *et al*, 1979). This approach can we confirm whether the GABAergic pathway is impaired in four experimental groups. Rats were placed in an aquarium of 21° to 22°C water filled to a depth of 35 cm for a 15-min pretest. Injections (saline or clonazepam) were given 23h, 19h and 1 hr before a 5 min test swim. The water depth of 35 cm allowed the rats to swim or float. Clonazepam was administered subcutaneously in a volume equivalent to 2 ml/kg at a dose of 0.25 mg/ml (Da Silva Heaser *et al*, 2007). Saline (0.9%) was also administered subcutaneously in a volume equivalent to 2 ml/kg. The swimming session was videotaped for behavioral analysis. The time of immobility, swimming and climbing are calculated.

Elevated plus maze test

Behavior in the elevated plus maze is also utilized to assess anxiety behavior (File *et al*, 1994). We measured two behavioral variables: the number of entries into open arms and the amount of time spent in open arms. Anxious animals are expected to make fewer entries into open arms and to spend less time in open arms than are non-anxious animals. The plus maze was elevated 50 cm off the ground and consisted of four arms (49 cm long and 10 cm wide). Two arms were enclosed by walls 30 cm high and the other two arms were exposed. As per previous methods, rats were placed at the juncture of the open and closed arms and the number of entries into and the amount of time spent on, the open and closed arms were recorded during a 10 min test. Time spent on the open arms is an index of anxiety and the total number of arm entries is measure of motor activity.

Histological study

After all sessions of behavior testing, pancreas and thymus sampled for the histological exam according to the indications of Martoja R et Martoja M (1967).

Statistical analysis of results

All data are expressed as the mean±SEM (Standard Error of the Mean). All groups showed normal distributions, so a

parametric statistical method; one way analysis of variance (ANOVA) followed by Tukey post hoc test, was used for multiple comparisons. The value of $p<0.05$ or less was considered as the significant difference. Data were analyzed using MINITAB (Minitab® 13.31).

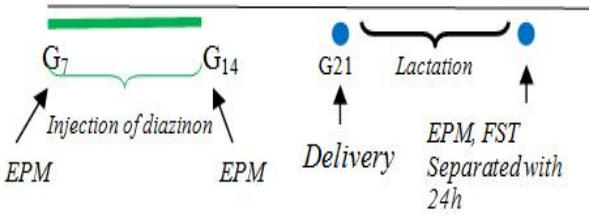

Figure1Schedule of behavior tests

RESULTS

Elevated Plus Maze

Animals were subjected to three test sessions in the Elevated Plus Maze, 7th day (1st session), 14th (2nd session) and after delivery (3rd session). These tests used to measure a number of parameters allow us to assess the degree of anxiety. During the three sessions, the virgin female rats treated with diazinon (VDZN) showed an important decrease in parameters open arms which is very significant ($p<0.01$) in the number of entries and hightly significant ($p<0.001$) in the time of spent (Figure 02a,b) by comparing to virgin group (V). VDZN showed too a highly significant ($p<0.001$) increase in the time of spent in closed arms and a significant increase ($p<0.05$) in the number of entries in closed arms (figure 02c,d). So, we can say that there is a decrease of venturing in the open arms as compared with closed arms. However, the pregnant rats treated with diazinon (PDZN) showed almost the same results in the 1st and 2nd session as compared with (VDZN), but after delivery (AD), this group showed no significant difference with control group (V), that allow us to think to the effect of the final week of pregnancy.

Forced Swimming Test

We found an effectiveness treatment of Clonazepam in the rats of virgin group (Table 01), resulting in a highly significant ($p<0.001$) reduction in immobility time. Clonazepam is also effective in all groups except in VDZN, where the immobility time did not significantly change.

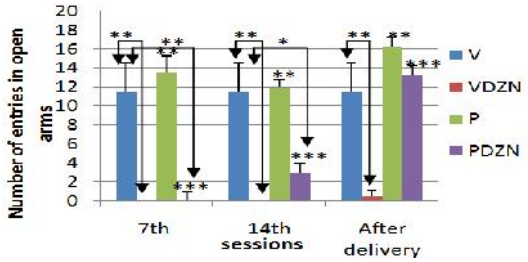

Figure.2a. Number of entries in open arms in EPM (n=08,*p<0.05, **p<0.01, ***p<0.001).

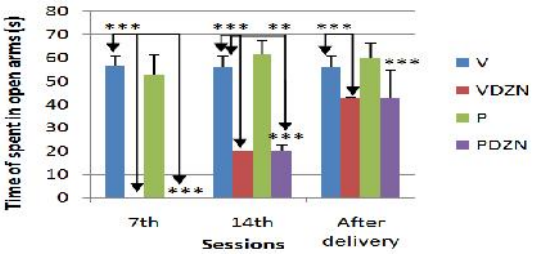

Figure.2b... Time of spent in open arms in EPM.

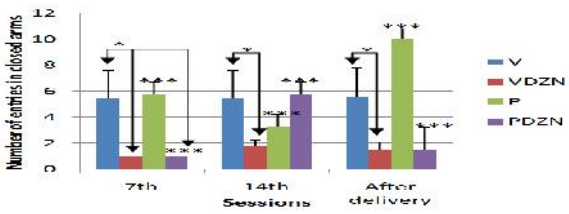

Figure.2c. Number of entries in closed arms

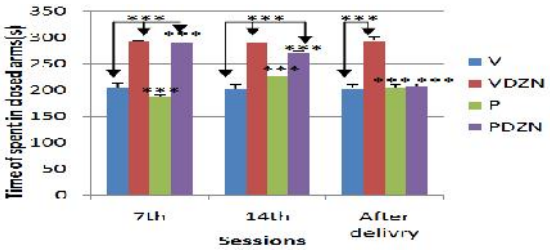

Figure.2d. Time of spent in closed arms

Histological exam

The pancreas of virgin rats treated with diazinon showed necrosis in the islet of Langerhans revealed by the disappearance of the nucleus of its cells (Figure 03b).However the histology of pancreas in the pregnant group treated with diazinon showed no difference with control group(figure 03D). The thymus of virgin rats treated with diazinon showed atrophy revealed by an important decrease in the volume of its lobules (Figure 04b). However, no difference between the group of pregnant rats treated with diazinon (PDZN) and control group (V) (Figure 04D).

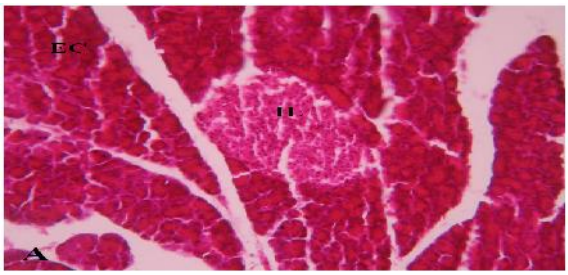

Figure.3a. Pancreas of control group (V) and pregnant group.(200X). colored with hematoxylin-eosine. **EC** : exocrin cells, **IL :** Langerhans Islets

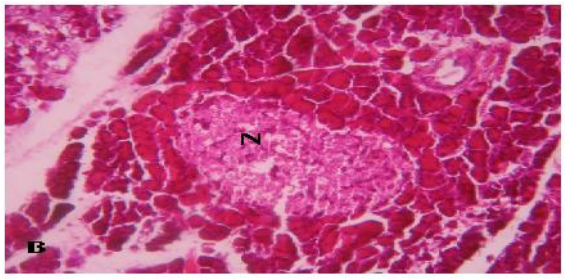

Figure.3b. Pancreas of VDZN group. **N** : Necrosis in Langerhans Islet cells

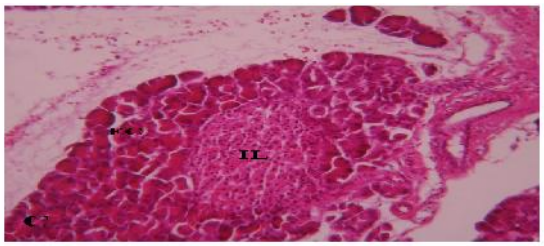

Figure.4a. thymus of control group (V) and pregnant group (P). (100X) colored with hematoxylin-eosine. C : cortex, M : medulla

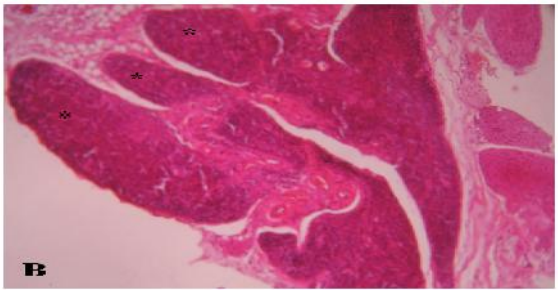

Figure.4b. * Atrophy of lobules thymus in VDZN group

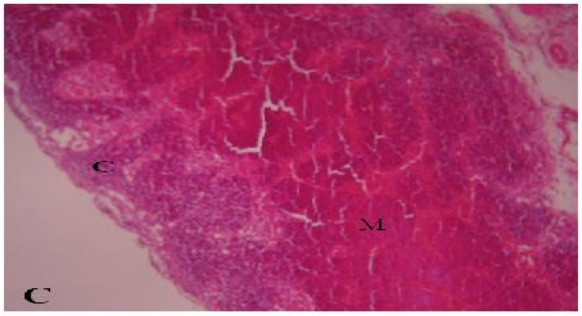

Figure.4c. Thymus with no changes in PDZN group.

study showed decrease of venturing in open arms. The decreased venturing into the open arms in the elevated plus maze is typically interpreted as increase in anxiety (Elliot *et al*, 2004), that reveals the anxiogenic effect of diazinon. We think for example to gabaergic system that its perturbations are involving in anxiety disorder (Mohler, 2006 ; Domschke *et al*, 2008). There is considerable scientific evidence that the GABA system plays a role in anxiety disorders (Zorach *et al*, 2011). It's logical to assume that diazinon perturbs the function of gabaergic system by AChE inhbition. The number of entries in the arms in this device is superior in the rats of control group than in treated group. The number of entries in the arms is usually described as an index of locomotion (Espejo, 1997), therefore, we can conclude that diazinon decrease locomotor activity. Acetylecholine intervens in the control of the muscles through the neuromuscular ending. Excess made at the motor end plate, acetylecholine can inhibit muscle contractions resulting from nerve stimulation (Bocquene, 1996). Diazinon exerts its toxicity by setting its oxygen analogus on acetylcholinesterase (AChE), enzyme neuronal, causing accumulation of acetylcholine endogen in nervous tissue and effector organs (Mayer, 1991). In fact, accumulation of acetylecholine causes nicotinic syndroms that

Table 1 Effet of Clonazepam treatment on the parameters of FST

Rats/Behavior	Climbing time(s)	Immobility time (s)	Swimming time (s)
V without Clonazepam	69.25 ± 5.74	149.25 ± 5.25	91.50 ±8.06
V with Clonazepam	50.00 ± 4.97 b***	132 ± 4.35 b***	118 ±5.74 b***
VDZN without Clonazepam	67.75 ± 3.06	156 ± 2.03	86.25 ± 6.02
VDZN with Clonazepam	65.75 ± 4.65 b*	158 ± 4.97 b*	85 ± 1.26 b*
P without Clonazepam	67.5 ± 6.5	157 ± 5.5	91 ± 8.75
P with Clonazepam	50.25 ± 5.91 b***	133 ± 2.06 b***	118.50 ± 5.80 b***
PDZN without Clonazepam	66.50 ± 6.5	156.75 ± 5.75	90.75 ± 6.25
PDZN with Clonazepam	49.75 ±1.26 b***	132.25 ±2.94 b***	119.75 ± 4.40 b***

(n=08, *p<0.05, **p<0.01, ***p<0.001, b: comparison with *vs* without Clonazepam)

DISCUSSION

The results of our work showed that short terme exposure to diazinon for 07 consecutive days at dose 10mg/kg induced neurobehavior alterations and histological changes including, increase of anxiety and depression, necrosis of pancreas and thymus atrophy which appear to be irreversible in virgin female rats. However, pregnant rats revealed a healing effect preventing the appearance of these changes. In non-pathological form, anxiety is a state of cognitive and behavioral preparedness that an organism mobilizes in response to a future or distant potential threat, so, we can say that anxiety is an acute adaptative response of heightened vigilance and arousal that enables an organism to navigate an unfamiliar environment of unknown danger. In its pathological form, anxiety is a maladaptive state that impairs the ability of an organism to respond optimally to its environment (Leonardo and Hen, 2008).

The Elevated Plus Maze is fluently used for the study of anxiety-related behaviors in rodents (Torres et Escarabajal, 2002). Experience exploits the conflict in rodents, between the fear of open spaces and the desire to explore new environment. The security represented by closed arms, whereas open arms provide exprolatory value. An anxious animal will naturally tend to prefer dark confined spaces and lighting. Based on this principal, anxiety behavior is measured by the degree of avoidance of the open spaces of the labyrinth. A short time spent in the open arms is considered as an index of anxiety (Onaivi *et al*, 1990; Lister *et al*, 1987; Pellow *et al*, 1985). Our

involve muscles fasciculations and cramps, asthenia and growing rapidly reached by the neuromuscular progressing to paralysis of skeletal muscles (Bismuth, 1993). The Forced Swimming Test, test of antidepressants efficacy, respresents a stressful and aversive situation which the rat can't escape, and produces immobility, or behavior despair (Porsolt, 1977 ; Kirby and lucki, 1997). The immobility of animals is interpreted as a lack of will to survive, and is considered a sign of depression in mice. The measurement of immobility time in this test therefore to assess the level depression in mice (Porsolt *et al*., 1977; Petit-Demouliere *et al*., 2005). Clonazepam is gabergic agonist that sets on gabaergic receptor (Tenn et Niles, 1995 ; Haeser, 2007). Our results showed inefficiency of clonazepam treatment in VDZN traduced by an important increase in immobility time. We can say that gabergic receptors are altered. Other studies with diazinon and parathion showed that these OP even subtoxic doses may also produce in the functioning of neurotrophic factors, their receptors and signaling cascade that control cell differenciation and the formation of neural circuits (Jameson *et al*., 2007; Slotkin *et al*., 2007a; Slotkin *et al*., 2007b; Slotkin *et al*., 2008d; Slotkin *et al*., 2010). Heaser *et al*, (2007) showed that oxidative stress in a diabetic model induced by streptozocine causes alteration of gabaergic neuron which could lead to depressive symptoms. So, we can say that oxidative stress induced by diazinon can play role in desctruction of gabaergic receptors. Histology of pancreas in VDZN showed necrosis in Langerhans islets cells. Its has been demontrated that OP poisoning can cause acute pancreatitis via increasing the

pressure in the ducts of the pancreas, which is due to excessive cholinergic stimulation (Hsiao, *et al*., 1996). The pancreas in a sensitive organ and increased pressure can severely damage it tissue (Sahin *et al*., 2003 ; Harputluoglu *et al*., 2003). The molecular mechanism of OP poisoning inducing acute pancreatitis is still undefined. It may be cause obstruction of pancreatic duct and / or the increase of the reactive oxygen species (Dressel, *et al*., 1982 ; Seviallano *et al*., 2003). It has been observed that the obstruction of the pancreatic ducts increase oxidative stress by antioxidant systems weaken enzymatic and non-enzymatic (Gokalp *et al*., 2005). We can say that oxidative stress participated in the necrosis of Langerhans islets. Histology of thymus showed an atrophy. The main response to stress, as in the case of soudain exposure to insectides is usually mediated by rapidly hormonal changes, particulary the catecholamins and glucocorticoids (Jortner, 2008). In addition, stress (Keller *et al*., 1983; Chrousos, 1995; Dominguez-Gerpe and Rey-Mendez, 2001) generate alterations in immune functions. It has been observed that stress leads to involution of the thymus (Clarke et Kendall, 1994; Mic`ic` *et al*., 1997; Dominguez-Gerpe Rey-Mendez, 2001). Decreases migration of bone marrow cells to the thymus (Bomberger and Haar, 1992). It has also been shown that noradrenalin reduces lymphocytes responses in vitro to mitogen stimulation and suppresses cellular immune functions, most likely via adrenergic receptors (Singh *et al*., 1979; Qui *et al*., 1996; Vizi *et al*., 1995; Kurz *et al*., 1997; Vizi and Elenkov, 2002). Apparently, there is an extensive bidirectional communication between the central nervous system (CNS) and the immune system (Webster *et al*., 2002). So, it's logical to say that hyperactivation of HPA axis can play role in thymus atrophy.

The question that arises, why the anxiety disappeared after delivery ? and why Clonazepam was effective in PDZN, and not in VDZN ? and why histological changes were not observed in PDZN ?

Levels of oestrogen and progesteron increase during pregnancy, with declines concentrations in the brain and periphery after birth (Okano et Nomura, 1992 ; Bloch *et al*., 2003). Steroids hormones exert their effects by binding to intracellular receptors, that move to the nucleus and bind to response element in the promoter gene of specific gene (Truss et Beato, 1993). Through this mechanism, steroid receptors become transcription factors that regulate gene expression (Evans, 1988). More recently, research has shown that steroids can also bind to specific neurotransmettors receptors and alter neuronal excitability (Paul et Purdy, 1992 ; Rupprecht, 2003). The steroids molecules that act as neuromodulators in this way are called « Neuroactive Steroid » (NAS) (Paul et Purdy, 1992). The neuroactive steroids are increased during pregnancy and return to pre-pregnancy levels within 6-7 weeks postpartum (Pearson *et al*., 2001 ; Gilbert Evans *et al*., 2005). These steroids neuroactive are represented mainly by pregnenolone and pregnelonoe sulfate (PS), dehydroepiandrosterone (DHEA), dehydroepiandrosterone suflate (DHEA sulfate) and 3-α reduced steroids. Pregnenolone, a derivative of cholesterol by the action of the enzyme cleavage of the cholesterol side chain (cytochrome P450scc) in the mitochondria is the main precursor in the synthesis of NAS (Bicikova *et al*., 2000). Neurosteroids are known to perform growth and neuronal differentiation and to modulate various modes and reactions via neurotransmitter receptors, including those of the acid Gamma-aminobutyric which are considered the most inhibitory receptors important in the CNS (Lambert, 1995). Especially in lesions of the nervous system, the local production of neurosteroids may be stimulated by autocrine or paracrine action remedial responses of neurons and glial cells. The role of neurosteroids has been demonstrated by experiments in cell cultures. When added neurosteroids in a culture medium, they enhance neuronal survival (Bologa *et al*, 1987). and increase the synthesis of specific proteins of myelin by oligodendrocytes called MBP and CNPase (Jung-Testas *et al*., 1997). In pregnant rats, the allopregnenolone protects neurons against toxicity potential by acting, among other things, the $GABA_A$ receptor chloride channels by keeping them open longer (Brussaard et Herbison, 2000 ; Belelli et Lambert, 2005).

According to these data, we can say that pregnancy is involving in the neuroprotection and neurorepair by steroids neuroactive (NAS), in fact, this can explain the desappaerance of anxiety after delivery and the effectiveness of clonazepam in PDZN.
During pregnancy, high levels of allopregnenolone reduce the response of the HPA axis to stress. This is confirmed in the study of Brunton *et al*. (2009) in which the administration of an inhibitor of 5-reductase _, finasteride, causes reactivation of the HPA axis. In the same study, it was shown that the administration of allopregnenolone in non pregnant female rats also suppresses the activity related to adrenal stress. It was reported about estradiol and progesterone that are not directly involved in the suppression of stress-related HPA activity, while the metabolites mentioned above such as allopregnenalone, play a crucial role (Welberg et Seckl, 2001). These data can explain the protection of thymus. The antioxidant properties of steroid hormones have been demonstrated in various cells and tissues. For example 17β-estradiol protects cells against oxidative stress (Ruiz-Larrea, 1995). In addition, it has been well demonstrated that neurosteroids such as estrogens, are able to limit oxidative damage by reducing lipid peroxidation, protein oxidation, the overload of Ca^{+2} in the cytosol and alterations DNA and mitochondria (Farooqui T et Farooqui A, 2011). These data can explain the protection of pancreas toward oxidative stress induced by diazinon.

CONCLUSION

This study showed that pregnancy has a positive response against the toxicity induced by diazinon, thus, cholinergic stress induced by this neurotoxic product leads to oxidative stress and hyperactivation of HPA axis. These phenomenons produce respectively necrosis in pancreas and atrophy in thymus, in addition, the changes in the function of gabaergic neurons that lead to the anxiety, acute depression.However, steroids neuroactive are known by its antioxydant properties, capacity to limit oxidative stress and reduction of HPA axis that confer pregnancy toxico-protection potential.

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