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Oxidative Stress and Apoptosis Biomarkers in Neonate Rats' Brain Exposed to Diquat during Lactation

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ABSTRACT

Background: Toxic chemicals compete with biological macromocules and other small molecules that construct brain structure in their natural function. Interruptions in development of the brain caused by toxic compounds, both before and after birth, can lead to defects that occur quickly after exposure, or much later in life.

Objectives: This study aimed to throw light on the effect of diquat (DIQ) on the development of neonates, brain by determination of oxidative stress and apoptosis markers during lactation period.

Materials and methods: Twenty pregnant female rats from the breeding facility of the Mammalian Toxicology Department, the Central Agricultural Pesticides Laboratory, Agriculture Research Center were included in this study. The lactating dams and their pups were constructed into two experimental groups (10 dams each). The first saved as a control group and received distilled water daily during the breast-feeding period. The second group was intubated with 5.12 mg/kg DIQ as 1/80 of LD_{50} (according to preliminary trails) from the 1st postnatal day (PND1) to the 10th postnatal day (PND10) and served as treated (study) group. Brain samples of neonates were collected after treated period and the 21th postnatal day (PND 21). The oxidative stress biomarkers (SOD, GP_x, GST, GSH and Protein carbonyl) and gene expression of BAX and BCL2 as apoptosis markers were estimated.

Results: The results indicated disruption in both oxidative stress and apoptosis biomarkers as evidenced by increased in GST, GP_x activities, gene expression of BAX and protein carbonyl level. Also, results showed a decline in SOD activity, BCL-2 gene expression and GSH content declined.

Conclusion: The results of this study showed that exposure to DIQ increases the generation of free radicals and can cause oxidative stress and induce neuronal programed cell death

Key words: Diquat, Oxidative Stress, Apoptosis, Gene expression, Lactation, Neonates.

INTRODUCTION

The herbicides of bipyridylium are a group of herbicides. DIQ is mainly seen as an aquatic herbicide, and is also widely used for irradiation in only certain crops; including cabbage, grain sorghum and soy beans. Furthermore, DIQ has some formulations designated for tree and fruit crops targeted sprays, and other areas where there is a need for non-control. Laboratory studies suggest that DIQ by ingestion and dermal absorption is mildly toxic. In addition, serious harmful effects that are not characteristic of paraquat exposure are reported on the central nervous system. Such neurological effects, including nervousness, irritability and restlessness, disorientation and failure to recognize family members or friends are the most obvious symptoms of DIQ injury. Normally, diquat causes kidney damage compared to paraquat [1].

In human breast milk, a wide range of insecticides and toxic chemical pollutants have been identified, because there is widespread concern that increased exposure to pesticides even during the earlier postnatal timespan may endanger the normal development of newborn babies [2]. Completely different toxic substances can be transported from body stores and/or blood to a breastfeeding mother's breast milk and thus lactating young children can be subjected to toxic chemicals that can pose a health risk [3]. The rapid development of the central nervous system occurs both in the uterus and after child's birth and usually requires an appropriate environment. It depends entirely on a complicated relationship between different dynamical factors. Developmental disturbances can be affected by genetic, external and internal factors acting during any of the phases of development [4]. The normal development of the central nervous system requires precise neuronal proliferation, mass migration, differentiation, synaptogenesis, gliogenesis, myelination and programmed cell death [5]. Neurotoxic agents can cause brain injury, particularly if direct exposure actually occurs during most of the early period of growth. If the development of immature embryos in the nervous system is impaired, the effects tend to be long-lasting and possibly permanent [6]. It is progressively

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acknowledged that the developing brain is strongly susceptible to both free radicals and neuronal programmed cell death, which might be the basis of this insecurity to age - dependent injury [7].

For brain development and differentiation, the glutathione system is very crucial because it behaves as a meditator for many mechanisms [8]. Glutathione is proven to be extremely a link between neurological conditions and their metabolism, particularly in the brain, where mitochondrial respiration tends to cause a high oxidative metabolism in the brain. Apoptosis is critical in tissue cell turnover, as well as in normal development and senescence [9].

Apoptosis is a genetically programmed death of cells that induces approximately half of nerve cells to lose during premature development of the brain [10]. The description of the relation between exposure to contaminants and gene transcription neurological disorders confirms the molecular pathways engaged in harmful response and produces insight into how neonatal exposure to neurotoxicants can result in long-term perturbations in the nervous system [11].

Aim of the study: This study aimed to investigate the effect of the herbicide, DIQ on brain development in neonates whose mother exposed to 1/80 LD_{50} (5.12 mg/Kg) of the herbicide during lactation period from the first day of postnatal period to the tenth day of postnatal period.

MATERIALS AND METHODS

Pesticide Used:

Diquat (Roxan, 10% EC) was obtained from Mammalian Toxicology Dept., Central Agricultural Pesticides Lab., Agriculture Research Center, Dokki, Giza, Egypt.

Animals and Experimental Design:

The experimental work was performed in compliance with recommendations for laboratory animal care and use [12]. Twenty pregnant female rats from the breeding facility of the Mammalian Toxicology Department, Central Agricultural Pesticides Laboratory, Agriculture Research Center were included in this study. Animals were fed a well-balanced chow. Pregnant female rats were kept in an air-programmed room under laboratory conditions (temperature of 23 ± 2 °C and a relative humidity of ~ 60 percent at normal light / dark cycle and housed in cages individually). The pups were weighted, counted, sexed and checked for anomalies immediately after delivery (postnatal zero day), and then breast feeding for each corresponding dam. The lactating dams and their pups were constructed into two experimental groups (10 dams each). The first saved as a control group and received distilled water (5ml DW/Kg) daily during the breast feeding period. The second group was intubated with 5.12 mg/kg DIQ as 1/80 of LD_{50} (according to preliminary trails) and served as treated group from the 1st postnatal day (PND1) to the 10th postnatal day (PND10). Brain samples of neonates were collected after treated period and the 21st postnatal day (PND 21).

Ethical approval:

This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use of Central Agricultural of Pesticides Laboratory, Agricultural Research Center.

Biochemical Biomarkers:

The supernatant of brain tissues was prepared by homogenization in sodium phosphate buffer (50 mM, pH 7) containing 0.1 mM of ethylene diamine tetra-acetic acid in 10 % percent (W/V) and centrifugation at 12.000 g for 30-min at 4°C. The supernatant was frozen till used.

Protein content in supernatant was determined by **Bradford** [13] method. The activity of Superoxide Dismutase (SOD) was determined by the method based on the percentage inhibition of pyrogallol auto-oxidation [14]**.** Glutathione peroxidase (GPx) activity was determined depending on the residual of glutathione according to the method of [15, 16]. Glutathione -S-transferase (GST) activity was determined with the method of **Habig and Jakoby** [17]. Protein carbonyl (PC) level was determined according to the method of **Reznick and Packer [18].** Reduced glutathione (GSH) was determined by the method of **Ellman** [16].

Apoptotic Genes Expression Estimation

RNA was extracted from brain by instruction protocol of QIAampRNeasy Mini Kit (Qiagen, Germany, GmbH, Catalogue no.74104) and the corresponding cDNA was synthesized using (Revert Aid Reverse Transcriptase Thermo Fisher, Catalog number: K1622) according to manufacturer's instructions. Real-time polymerase chain reaction (*RT*-PCR) quantification was performed by Strata gene MX3005P instrument using the Quantitect SYBR green PCR kit (Cat. No. 204141) with 25 µl total reaction volume containing: 12.5 µl 2x SYBR Green PCR Master Mix, 1 µl primers, 2 µl cDNA, and 8.5 µl of RNase Free Water. The primers for target and internal reference (β-actin) genes that were obtained from Metabion (Germany) were 5'-

CACCAGCTCTGAACAGATCATGA-3' and 5'-TCAGCCCATCTTCTTCCAGATGGT-3'. They were used for Bax and those for BCL-2 were 5' - CACCCCTGGCATCTTCTCCTT-3' and 5' - AGCGTCTTCAGAGACAGCCAG - 3' [19]. Whereas for ß-actin were 5'-TCCTCCTGAGCGCAAGTACTCT-3 and 5'-GCTCAGTAACAGTCCGCCTAGAA- 3 [20]. Each cycle consisted of denaturing for 5-min. at 94 ˚C, annealing for 30 sec at appropriate annealing temperature and polymerization for 30 sec at 72 ˚C. Dissociation stage was added after the amplification to verify the specificity of the PCR products. Quantitative analysis was performed using the strata gene MX3005P software and variations of gene expression on the mRNA of the different samples were estimated according to the "ΔΔCt" method [21].

Statistical Analysis

Statistical Analysis

Statistical analysis was done using IBM SPSS version 25 software package (SPSS, IBM, and Chicago, IL, USA). Independent *t*-test was used for comparison of the quantitative results. The statistical difference between the group means compared to control group is indicated as follows: *($p \leq 0.05$), ** ($p \leq 0.01$) and ***($p \leq 0.001$).

RESULTS

Data presented in table (1) showed significant inhibition of superoxide dismutase activity ($p \leq 0.05$) in neonate's brain whose mothers' were exposed to DIQ at 5.12 mg/kg b.w. (1/80 of LD_{50}) after 10-days of orally exposure from PND 1 to PND 10. Also, a significant decline in reduced glutathione ($p \leq 0.001$) occurred. On the other hand, results showed significant increases in glutathione peroxidase (GPx), glutathione -S- transferase (GST) activity ($p \leq 0.001$) and protein carbonyl (PC) level ($p \leq 0.01$) of pups' brain that were induced after 10-days of treatment.

Table (1): Diquat and oxidative stress biomarkers in neonates' brain after 10-days of exposure during (PND1 to PND10)

Biomarkers / Treatments	SOD	GP_x	GST	PC	GSH
Control	**4.939** ± 0.31	**5.07** ± 0.065	**35.21** ± 0.88	**7.42** ± 0.36	**1.67** ± 0.03
DIQ (5.12 mg/kg**)**	**3.799** ± 0.15*	**5.46** ±0.044***	**41.607** ±1.75***	**9.95** ±0.32**	**1.50** ±0.01***

The data presented in table (2) indicated that, the effect of DIQ on oxidative stress biomarkers of neonate's brain after lactation period for 21-days. Although the exposure of dams to the herbicide DIQ stopped after 10-days of lactation period. The results in table (2) showed that, SOD activity and GSH-level declined significantly ($p \leq 0.05$). While, the other biomarkers of oxidative stress (GST and PC) increased markedly ($p \leq 0.05$).

Table (2): Diquat and oxidative stress markers in neonates' brain exposed during (PND1 to PND10) at the end of lactation period

Biomarkers / Treatments	SOD	GP_x	GST	PC	GSH
Control	**3.36** ± 0.186	**4.72** ± 0.094	**45.30** ± 1.08	**16.90** ± 0.59	**1.69** ± 0.024
DIQ (5.12 mg/kg**)**	**2.79** ± 0.123*	**4.530** ± 0.070	**51.42** ± 2.22*	**19.11** ± 0.48*	**1.59** ± 0.019*

Gene expression of apoptosis biomarkers as represented by BCL-2 and BAX in table (3) revealed that dams exposed to DIQ at a dose level 5.12 mg/Kg during the lactation period from PND1 to PND10 led to significant increase in apoptosis level as evidenced by increase in BAX expression (*pro*apoptotic marker) to two folds after ten days of exposure while, BCL-2 expression (*anti*apoptotic marker) decreased significantly to 68% of control after 10 days of exposure. The same trend was noticed in the expression of BAX and BCL-2 at the end of lactation period (21 days) although dams were exposed to DIQ during PND1 to PND10.

Table (3): Diquat and gene expression of BAX and BCL-2 in neonates' brain after 10-and 21-days of exposure during lactation period

Biomarkers / Treatments	BAX		BCL-2	
	10-days	21-days	10-days	21-days
Control)	**1.038** ± 0.033	**1.019** ± 0.052	**1.011** ± 0.06	**1.026** ± 0.017
DIQ (5.12 mg/kg**)**	**2.624** ±0.202***	**1.706** ±0.079***	**0.688** ±0.069***	**0.7766** ±0.053***

DISCUSSION

The development of the central nervous system occurs in the utero as well as postnatally and requires an appropriate environment. It depends on a complex relationship between different dynamical factors. Developmental disturbances seem to have genetic and external factors in any development phase [4].

Xenobiotic exposure at an earlier age has sometimes been disclosed to produce a higher severity of changes especially when compared to those in adulthood [22]. Insufficient excretory and underdeveloped xenobiotic–metabolizing mechanisms in newborns may be responsible for such a vulnerability. The rapidly developing nervous system is particularly prone due to higher brain absorption of xenobiotics that impact the regulation of intracellular Ca^{2+} in membranes [23] and may take part indirectly in the control of an antioxidant pathway through the effects on the transmission of mitochondrial permeability [24].

The neonatal brain may also be more susceptible to oxidative stress due to the delay in the expression of superoxide dismutase, catalase and glutathione peroxidase, which has been found to be deficient in samples of white matter tissue of brain tissues [25].

The key biomolecular origins of oxidative stress include disrupted mitochondria and disrupted transmission systems, cyclooxygenase activity, lipoxygenase, docosahexaenoic acid, enzymatic activity of xanthine oxidase, neurotransmitters of catecholamines and amino acids, neutrophils and microglia infiltration, and nitric oxide synthase [26]. It is extremely interesting that many, if not all, of these factors are actively or passively correlated with rises in cytosolic calcium. After production of ROS, it can orchestrate apoptotic and necrotic death pathways, activate metalloproteinases and nitrosylating proteins, destabilize the dignity of the membrane, increase the permeability of the blood brain barrier and cause an abnormal arteriolar reactivity [27].

Redox cycling is a process in which paraquat (PQ^{2+}) receives a single electron from a sufficient source of electron and results in a *mono*cationic radical (PQ^{2+}). The entire process leads to significant free unstable species of oxygen production in organelles-rich enzymes that can precipitate redox cycling, including mitochondria, plasma membrane, and microsome, for example. However DIQ is also a non-electrophilic redox cycling chemical and a potent pro-oxidant that can generate intracellular superoxide anion and other redox products by in vivo frequency reaction [28].

GSH is involved in essential biochemical pathways in cells, i.e. antioxidant protection, biotransformation, intercellular redox homeostasis, cysteine carrier / storage, cell communication, protein activity, gene expression and cell differentiation / proliferation, has been demonstrated. Consequently, GSH balance dysfunction can cause fatal events in cells**.** Cell functions depend on the cellular redox homeostasis especially during the developmental processes ·Cellular functions such energy supply, proliferation, differentiation, maturation and apoptosis are affected by cellular redox homeostasis [29]. GSH depletion-enhanced oxidative stress leading to neuronal degeneration In fact, brain GSH is reduced in some age-related neurodegenerative diseases [30].

The results of this study showed that exposure to DIQ increases the generation of free radicals and can cause oxidative stress. It is possible that glutathione conjugate of DIQ may be focused in the brain because it has been disclosed that oxidative stress impairs the transportation of glutathione conjugate from cells [27]. Increased activity of glutathione peroxidase dependent on selenium and G6PDH at this stage can protect the tissue from extensive damage.

In early postnatal stages, the nervous system (PNS) contains an enormous amount of dead cells [24]. This cell death is apoptotic and occurs during most of the different stages of development, including neurulation, synaptogenesis and the removal of adult nervous system (CNS) neurons. Apoptosis, known as naturally occurring, is essential during growth and

during the development of CNS; BCL-2 is widely expressed [35]. Neurons that do not reach their reasonable new target even during development are likely to suffer from apoptosis due to lack of adequate neurotrophic factors derived from the target [32].

Many studies have shown that the BCL-2 family proteins are correlated with new channel formation in mitochondrial membranes and regulate the release of cytochrome c. The released cytochrome c from mitochondria can lead to the intrinsic apoptotic pathway through the formation of apoptosomes and activation of caspase-9 and thus leads to apoptosis [33]. It has therefore been noted that the counter-regulated production of BCL-2 family proteins determines whether neurons undergo survival or apoptosis [33]

Cytotoxic stress triggers BH3 proteins that inhibit BAX and BAK. As a consequence of oligomers produced by BAX and BAK, the impaired permeability of the outer surface of mitochondria resulted in the liberation of apoptogenic cytochrome c [34]. Cytochrome c binds apoptotic protease-activating factor-1 (APAF-1), inducing its oligomerization, thus activating a caspase-9 initiator. Caspase-9 cleaves and initiates caspase-3 and caspase-7 of the executioner, contributing to apoptosis. By binding BH3-only proteins and thus inactivated BAX or BAK and BCL-2 proteins inhibit previous steps. Apoptosis of cells induced by oxidants was consistently associated with imbalance of GSH/GSSG induced by oxidants. GSSG formation has been followed by loss of mitochondrial integrity, mitochondrial cytochrome c translocation to cytosol and caspase-3 activation [35].

CONCLUSION

The developing brain is strongly susceptible to both free radicals and neuronal programmed cell death which might be the basis of this insecurity to age - dependent neurodegeneration diseases. So, the effect of toxic chemicals on health of lactating young children must be studied in deep to explore the relation of age- dependent diseases.

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