Effects of melatonin in rats in the initial third stage of pregnancy exposed to sub-lethal doses of herbicides


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ABSTRACT

Exposure to the herbicides Parquat (PQ) and Roundup® may cause cell lesions due to an increase in oxidative stress levels in different biological systems, even in the reproductive system. 

Objective: Evaluate the possible changes in reproductive parameters and hepatic, as well as its prevention by simultaneous application of melatonin.

Methods: Thirty-five female rats at the age of 3 months were divided into seven groups: three groups exposed to sub-lethal doses of the herbicides PQ (50 mg/kg) and Roundup® (500 mg/kg) (n = 5, G2, G3 and G4); three groups exposed to herbicides and simultaneous treatment with 10 mg/kg of Melatonin (n = 5, G5, G6 and G7) and control group (n = 5, G1) from the first to the seventh day of pregnancy. On the seventh day of pregnancy, the rats were anesthetized and euthanized, followed by laparotomy to remove their reproductive tissues and liver. Body and ovary weights were taken and the number of implantation sites, corpora lutea, preimplantation losses, implantation rates were counted and histopathology of the implantation sites, morphology of the surface and glandular epithelia of endometrium and hepatic oxidative stress were undertaken.

Results: The present study shows the decrease in body and ovary weight, decrease in the number of implantation sites, implantation rate, in the total number of corpora lutea and increase of preimplantation percentages were observed when compared to the G1: Fig. 1 and Table 1, (p > 0.001 ANOVA/Tukey). The histopathological analysis of the implantation sites showed a disorder of the cytotrophoblast and cell degeneration within the blastocyst cavity in Fig. 4. Morphometry revealed a reduction in surface and glandular epithelia and in the diameter of the endometrial glands (Table 2; p > 0.05 ANOVA/Tukey), whereas in liver, serum levels of thiobarbituric acid reactive substances (TBARS) were found to be significantly elevated (Fig. 2; p > 0.001; p > 0.05 ANOVA/Tukey), and serum level of reduced glutathione (GSH) was significantly lower (Fig. 3; p > 0.001 ANOVA/Tukey). However, treatments with melatonin exhibited improvements in reproductive parameters, as well as reduced lesions in the implantation sites (Fig. 4) and in serum levels TBARS (Fig. 2; p > 0.001 ANOVA/Tukey), serum levels GSH (Fig. 3; p > 0.001; p > 0.05 ANOVA/Tukey).

Conclusions: These results reveal that melatonin is a protective agent against experimentally induced maternal/embryo toxicity with herbicides and favoring normalization of reproductive parameters and hepatic.

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1. Introduction

Exposure to herbicides is a serious public health problem in developing countries, especially those with economies based on agribusiness. The use of these products has grown rapidly in emerging countries, but in most cases there is no effective control over its...
sale and use. In addition, incorrect and discontinuous use of personal protective equipment, the limitations in the monitoring of exposure and the flaws in the diagnosis and treatment of cases of toxicity further aggravate the problem (Forget, 1989; IBGE, 2012).

Among some of the consequences of exposure observed in animals and humans, they are the endocrine imbalance associated with the onset of cancers, infertility, congenital malformations in the genital tract and changes in semen quality (Koifman and Hatagima, 2003), injuries in the embryo-fetal development, in the maturation of physiological systems, anatomic deficiencies, among others (Dent, 2007; Oliveira et al., 2014). Thus, changes during sensitive periods or critical development as the embryonic period can generate major changes, which can manifest itself in later stages of the life cycle or even be translocated to later generations. (Reis Filho et al., 2007). Moreover, herbicides such as Paragquat (PQ) and Glyphosate-Roundup® can promote dysfunction in the pineal gland and can reduce the production of melatonin (MLT) (Bartlett et al., 2011; Seneff et al., 2015) and thus compromise the process of embryo implantation, as well as interfere with pregnancy (Fernando and Rombauts, 2015), and produces reactive oxygen species (ROS) which triggers the lipid peroxidation of the cellular membranes (Peter et al., 1992).

The great interest in the effective treatment for humans and animals intoxicated by herbicides has focused in the impairment or minimizing of cell lesions caused in the several biological systems (Melchiorri et al., 1995; Xu et al., 2007; Serra et al., 2003) including the development of preimplantation embryos in vivo (Hausburg et al., 2005). The use of several compounds, especially those with antioxidant effects, such as MLT, has been underscored (Soares et al., 2008; Maganin et al., 2008; Reiter et al., 2009). Several studies report that MLT stimulates the production of glutathione peroxidase for cell-level defense against oxidative stress (Reiter et al., 1995; Pablos et al., 1996) with the promotion of the stabilization of the cell membrane by making it stronger against oxidative attacks (Garcia et al., 1998), beneficial effect on the processes associated with the development of oocytes, ovulation and early embryonic development (Manjunatha et al., 2009; Vázquez et al., 2010) on endometrial morphology and maintenance of embryo implantation procedure (Dair et al., 2008).

However, the literature does not register MLT effects in female rats submitted to acute toxicity by sub-lethal doses of the associated herbicides PQ and Roundup® and its protective effect on the embryo implantation process. The hypothesis tested was that MLT might act as a protective agent in the mother/embryo interface during the third stage of pregnancy, with an improvement of important pregnancy parameters. The histopathology of the implantation sites, the morphometry of surface and glandular epithelia of the endometrium, the weight of females, ovaries, the number of implantation sites, corpus luteum, implantation rates and pre-implantation losses were assessed.

2. Materials and methods

2.1. Reagents and chemical products

Commercial formulation of glyphosate (Roundup®) made of 360 g/L of glyphosate (N-phosphonomethyl glyline) and 16% (w/v) polyoxyethylene amine (surfactant), Gramoxone® with 200 g/L of PQ (1′,1′-dimethyl-4,4′-bipiridinium) and MLT from Sigma-Aldrich (St. Louis, MO, USA), Dopalem® (ketamine chloral hydrate), Rompun® (xylazin) and Thionembutal® (thiopental) were employed during the experiments.

2.2. Ethical aspects

Procedures involving animals followed recommendations by the Guidelines for the Testing of Chemicals (OECD, 2008), and were approved by the Committee for Ethics in the use of animals of the Universidade Federal Rural de Pernambuco, Brazil, by protocol 063/2013.

2.3. Animals

The experiments were conducted in the Laboratory of Histology of the Department of Animal Morphology and Physiology (DMFA) and at the Research Center (Cenapesq) of the Universidade Federal Rural de Pernambuco. Thirty-five female rats (Rattus norvegicus albinus, Wistar) from the DMFA vivarium were used.

90-day-old females, weight 200 ± 20 g, were kept in a controlled cage (22 ± 2°C, humidity 60 ± 10% and photoperiod of 12 h light/dark) with food and water ad libitum. After 10 days of acclimatization, the females underwent standard vaginal smear to determine the regularity of the estrous cycle. Females with 3 regular estrous cycles were separated randomly and mated to form seven experimental groups (n = 5 each). They were then submitted to treatments and monitored daily with regard to body weight and survival.

2.4. Mating system and verification of copula

The mating system was temporary polygamous, in which a male was maintained in a cage with two females until each mating was verified and was removed afterwards. After mating, a vaginal smear was made every day. The presence of a vaginal plug or sperm cells in the vaginal smear was taken as an indication of effective copulation. Cytological detection of sperm cells was performed daily by the same collector. The collection of vaginal smears was performed using swabs of sterile cotton (Absorve®) and subsequent deposit of biological material in histological slides, which were stained with Harris-Shorr method (Shorr, 1941), then the slides were preserved under glass cover slips using Entellan® mounting medium (EMS, Hatfield, PA, USA). All stained slides were examined using a Leica® DMS500 light microscope (Leica®, Wetzlar, Germany).

2.5. Experimental groups

The experimental groups comprised of: G1 control (treatment with saline solution 0.9% NaCl); G2 exposure to 50 mg/kg dose of PQ; G3 exposure to 500 mg/kg dose of Roundup®; G4 exposure to an associated dose of PQ and Roundup®; G5 exposure to 50 mg/kg dose of PQ plus treatment with 10 mg/kg MLT; G6 exposure to 500 mg/kg dose of Roundup® and treatment with 10 mg/kg MLT; G7 exposure to an associated dose of PQ and Roundup® plus treatment with 10 mg/kg MLT. After exposure to herbicides and treatments with MLT, the rats in their seventh day of pregnancy were anesthetized by intramuscular method with ketamine hydrochloride (80 mg/kg) and xylazine (6 mg/kg) between 9 and 10 h in the morning. They were immediately euthanized with thiopental (40 mg/kg), followed by laparotomy to remove the uterine horns with the implantation sites and the ovaries (Damasceno et al., 2002; Camargo et al., 2009). Histopathological analysis of the implantation sites was performed, coupled to morphometry of the surface and glandular epithelia of the endometrium. The weight of the female rats, ovaries, number of implantation sites, corpus luteum, implantation rate and pre-implantation loss were calculated.

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2.6. Treatment with melatonin

MLT (Sigma-Aldrich) was given intraperitoneally at the start of the evening (18 h), with a 10 mg/kg dose of body weight, from the first to the seventh day of pregnancy. MLT was diluted daily for seven consecutive days in an ethanol/saline 4% solution (Melchiorri et al., 1995).

2.7. Exposure to herbicides

Herbicides were diluted in a saline solution 0.9% for exposure: PQ, at a 50 mg/kg dose of body weight of animal following DL50 100–150 mg/kg (Kimbrough and Gaines, 1970) and 500 mg/kg of Roundup® as from NOEL—dose of 1000 mg/kg of body weight of the animal (Williams et al., 2000) established for glyphosate in teratogenesis assays in rats (EPA, 1996). Herbicides were administered by gavage between 9 and 10 h a.m. from the first to the seventh day of pregnancy and concomitant to the weighing of the females.

2.8. Measurement of lipid peroxidation (LPO)

For oxidative stress analysis by measuring levels of TBARS, samples of the liver were ground in potassium chloride (KCl) 1.15% at a proportion of 10 mL/g till the complete homogenization of the material to analyze the oxidative stress by measuring the levels of TBARS. The homogenate was then transferred to a test tube and 2 mL of the reagent (0.375% thiobarbituric acid and 75 trichloroacetic acid) were added for each mL of the mixture. The tubes in duplicate were sealed and heated in a warm bath at 100 °C for 15 min. The supernatant was separated and absorbance was measured at 535 nm (Buege and Aust, 1978). All doses were performed in triplicate.

2.9. Measurement of glutathione (GSH) concentration

The levels of reduced glutathione (GSH) were quantified by the concentration of non-protein sulphydryl groups in the homogenized. The other half of each homogenized was mixed with an equal volume of 10% (w/v) of trichloroacetic acid (TCA) to precipitate the proteins and then centrifuged at 1000 x g for 10 min. Further, 1 mL of the supernatant was mixed with 1.5 mL of a reaction medium with Tris at 100 mM (pH 8.9) and 0.4 mM of 5,5′-dithiobis-2-nitrobenzoic acid. Incubations were undertaken at room temperature for 5 min and absorbance was measured at 412 nm. Results were compared with a standard curve by α-cistine and corrected for the protein rate of the initial homogenates (Sedlak and Lindsay, 1968). All doses were performed in triplicate.

2.10. Histopathological analysis of the implantation sites

After laparotomy and the removal of the uterine horns, the implantation sites (Teixeira et al., 2004) and the corpora lutea were counted with a stereomicroscope Olympus® SZ40 and pre-implantation losses and implantation rates were calculated (Lemonica et al., 1996). Implantation sites were immersed in buffered formaldehyde 10% and fixed for 24 h. After the fixation, the tissues were washed in a PBS solution, dehydrated in a series of increasing alcohol concentrations (80, 90, 95 and 2 × 100%) and placed in methacrylate historesin glycol (Historesin Leica®). Further, 4 μm cuts, stretched in the water and placed on the laminas, were obtained by microtome Leica® RM 2245 with glass razor. They were dried in a buffer at 60 °C for 1 min and underwent staining with Hematoxylin – Eosin (H.E.) To select the fields to be analyzed, was adopted the following criteria: only the fields with the blastocyst presence in development process for histopathology. The slides were analyzed under a light microscope Leica® DM5000 in duplicaten/animal, and photographed by camera Leica EC3 locked to a microscope for histopathological.

2.11. Morphometric analysis

Morphometric analysis comprised images (2 slides/animal/group) with camera Leica EC3 coupled to a microscope, and the parameters: height of surface and glandular epithelium of the endometrium and endometrial glands diameter were analyzed by Leica Application Suite (LAS) EZ. Ten measurements/field of the parameters under analysis were undertaken for each animal/group. To select the fields to be morphometric ‘analyzed, the following criteria was adopted: select and capture fields on the perimeter along the decidualization zone in each histological sample (slide), ie in the area where there are the luminal epithelium and endometrial glands.

2.12. Statistical analysis

Rates were given as averages ± standard error of average. Differences between groups were determined by the Analysis of Variance (one-way ANOVA), followed by Tukey’s test when a difference was detected. Significance level for rejection of nil hypothesis was fixed at 5% (p < 0.05).

3. Results

3.1. Weight of pregnant rats

Pregnant rats were weighed at the final period of the exposure to herbicides and treatments with MLT. Means were analyzed statistically and revealed that associated and individual exposure of herbicides (p < 0.001) to PQ (p < 0.01) significantly decreased weight when they were compared to control. After MLT treatment, weight decrease was impaired when the female rats were compared to control (p < 0.5) in Fig. 1.

3.2. Analysis of weight of ovaries, number of implantation sites and implantation rates

Associated and individual exposure to herbicides PQ and Roundup® caused lighter ovaries and a decrease in the number of implanted sites when compared to those in control. The association of herbicides provided the lowest ovary weights and the number of implantation sites even though there was no difference between the number of implanted sites between PQ and Roundup® groups. The same behavior for the number of implantation sites was also that for implantation rates. Weight of ovaries among groups treated with MLT was similar to that of control group. The same result occurred for the number of sites implanted and the implantation rate in Table 1.

3.3. Number of corpus luteum and pre-implantation losses

Ovaries of experimental groups evaluated by stereomicroscope showed that individual and associated exposure to herbicides (PQ + Roundup®) also reduced the total number of corpus luteum and increased the percentage of pre-implantation loss when compared to those of control. There was no difference in rates among groups exposed to herbicides Paraquat and Roundup®. MLT enhanced a similar behavior to control in Table 1.

3.4. Measurement of lipid peroxidation (LPO)

The analysis of TBARS levels in the liver tissue after associated or individual exposure to the herbicides PQ and Roundup® revealed high rates, when compared to those of control. The association of
herbicides caused the highest rates in TBARS levels. On the other hand, the groups treated with MLT had rates similar to those of control in Fig. 2.

3.5. Measurement of glutathione (GSH) concentration

In the case of GSH levels, associated and individual exposure of the herbicides PQ and Roundup® decreased when compared to control. Contrastingly, rates similar to those of control were reported among the groups treated with MLT in Fig. 3.

3.6. Histopathological analysis of implantation sites

Fig. 4A represents pregnancy in the uterine horns on the seventh day after intercourse. The histopathological analysis of samples of implantation sites (Fig. 4B) of rats in the control group shows preserved blastocyst with cytотrophoblast, embryoblast, blastocyte cavity and a well-defined deciduous process (Fig. 4C). However, disorganization of the cytотrophoblast and cell degeneration within the blastocyte cavity were reported in samples exposed to associated herbicides (Fig. 4D). Implantation sites in samples with...
Fig. 3. Measurement of glutathione (GSH) concentration in groups G1-G7. Data represent the mean ± SE of GSH levels in experimental groups (n = 5). * Indicates significant difference between control and exposed group **p < 0.001 and recovery when treated with melatonin (MLT) ***p < 0.001” p < 0.01 (ANOVA/Tukey).

Fig. 4. Photographs and photomicrographs of representative uteri collected and implantation sites on the 7th day post coitum of pregnant rats exposed to herbicides Paraquat and Glyphosate-Roundup® and treated with melatonin. Note in (Fig. A) the uterine horns with the implantation sites. (Fig. B) gives an overview of the implantation site (IS). Control, groups revealed preserved structures such as cytotrophoblast (Ct), embryoblast (asterisk), blastocystic cavity (Bc) and a well-defined mature deciduity zone (ZDM) (Fig. C). Note the G7 group (with significant changes) disorder of blastocyst (Bc) and cell degeneration (arrow) of cytotrophoblast within the blastocystic cavity are shown in the group exposed to associated herbicides (Fig. D). After treatment with MLT, highlighted group (associated herbicides) revealed levels of structural organizations similar to those of female rats in control group (Fig. E). Bar = 50 μm. 500 μm.
MLT-treated rats had a structural organization similar to that in rats of the control group (Fig. 4E).

3.7. Morphometric analysis

The morphometric analysis of surface and glandular epithelia and of the diameter of endometrial glands decreased after individual and associated exposure to herbicides PQ and Roundup®. Although the association of herbicides enhanced the lowest rates, the rates of the parameters after MLT treatment were similar to those of control (Table 2).

4. Discussion

Current analysis reveals that acute exposure to sub-lethal doses of the herbicides PQ and Roundup® individual PQ or associated, reduced weight gain of pregnant rats. Similar results were reported for rates exposed to herbicides Atrazine and PQ (Cummings et al., 2000; Costa et al., 2008). Decrease in gain weight is a strong index of systemic toxicity which may lead to the mother’s toxicity and embryo losses (Cummings et al., 2000). Maternal toxicity is one of the causes in embryo-fetal and post-natal development (Khera, 1985; Chahoud et al., 1999), specifically diagnosed by loss of body weight (Chernoff et al., 2008) even though other changes such a decrease in locomotion, diarrhea, piloerection, deaths and reduction in water and food (York et al., 2014) should also be taken into account. The association of herbicides reduced significantly (p < 0.001) weight losses when compared between exposure groups and control, evidencing a possible synergism between herbicides. The synergic effect of PQ during the pre-natal period (Miranda-Contreras et al., 2005) and that of Roundup® between glyphosate and its adjuvant poloxyethylene amine (POEA) (Benachour et al., 2007) would explain current results in the associated group with the greatest weight gain decrease. Further, the herbicides decreased the number of corpus luteum. Similar results were detected in Wistar rats exposed to herbicide PQ (Hamayatkah et al., 2012), in the number of implantation sites, in ovary weight, where the implantation rate decreased and the percentage of pre-implantation losses increased. Similar results were detected in mice exposed to PQ in which embryo development changed a lot and caused a decrease in the percentage of embryos in eight cells and in the number of compact morulas (Hausburg et al., 2005). It has also been verified that the association of the herbicides PQ and Roundup® revealed a synergic action and the highest rates in the parameters under analysis. Similar results involving other herbicide associations (dichlorophenoxyl acetic acid, dicamba and mecoprop) enhanced the same behavior during the implantation of the embryos (Benachour and Seralini, 2009). However, after treatment with MLT, reproductive indicators were similar to those of the control group. MLT has been acknowledged for its benefits in gonads and annexes. MLT produced in the ovaries helps in follicle maturity and in the preservation of the ovule’s integrity prior to and at the moment of ovulation. It is an important agent in the ideal maintenance of reproductive physiology (Reiter et al., 2013).

TBARS levels of the hepatic tissue were high after individual exposure of the herbicides PQ and Roundup®. Suntres (2002) shows that PQ causes the production of EROS which interact with the membranes lipids and thus compromise cell integrity. Roundup® enhances the surplus production of malondialdehyde (MDA) and oxidative stress by producing EROS and, consequently, damage to cell integrity (Catalá and Cerruti, 1997; Rikans and Hornbrook, 1997). The association of herbicides enhanced greater rates of TBARS levels. Synergic activities were also reported in Roundup® and its surfactant POEA (Koller et al., 2012; Kocaman and Topaktas, 2010; Graillot et al., 2012) and PQ (Peng et al., 2007) which explains the synergic effect of the association between the herbicides. There was a decrease in GSH due to the individual exposure of the herbicides PQ and Roundup®, corroborated by similar results reported by Candan and Alagözü (2001) and Cavalli et al. (2013) whereas associated exposure enhanced lower rates for GSH levels and revealed a synergic effect and worsening of hepatic oxidative stress signs. GSH is, therefore, an important endogenous antioxidant produced by the liver whose levels may be altered in several situations of oxidative stress, especially in liver dysfunctions. In fact, it participates in several intra- and inter-cell enzyme processes, which probably explains our results. Similar rates to those of control occurred at TBARS and GSH levels after treatment with MLT. MLT has an antioxidant activity by sequestering the hydroxyl and peroxy radicals (Cagnoli et al., 1995; Reiter et al., 1995, 1997) which stimulate endogenous production of antioxidant molecules such as GSH (Urata et al., 1999), antioxidant enzymes such as glutathione peroxidase which strengthens the antioxidant system (Reiter et al., 1995; Pablos et al., 1996). It also protects cell membrane and makes it more resistant to oxidative attack (Garcia et al., 1998), reduces LPO and increases GSH levels (Melchiorri et al., 1995, 1996). The above foregrounds current results after treatment with MLT.

The histopathological analysis of the implantation sites in female rats of the control group revealed well preserved blastocyst with cytotrophoblast, embryoblast and blastocyst cavity and a well-defined deciduous process. However, there was a disorganization of cytотrophoblast, embryoblast and cell degeneration within the blastocyst cavity after exposure to herbicides, especially to associated herbicides. Lesions may be attributed to the vulnerability of the blastocyst by low concentrations of contaminants, which compromise the process of differentiation of embryoblast and cytotrophoblast and intensify the apoptosis process (Greenlee et al., 2004; Cavieres et al., 2002).

The morphometric analysis of surface and glandular epithelia of the endometrium showed a decrease in the height of the epithelia and in the diameter of the endometrial glands. The association of herbicides enhanced the lowest rates for the parameters under analysis, although rates were similar to those of control after MLT treatment. Embryo growth and development depend initially on the endometrium, in particular, on secretions derived from the endometrial glands that provide the first nutrition to the pre-embryo (Spencer and Bazer, 2004; Burton et al., 2002). Further, biochemical markers, such as integrins, mucins, cytokines, growth factors, immunological endometrial markers and the dynamics of
junctional complexes trigger the endometrial receptivity between the sixth and tenth day of pregnancy (Aboubakr et al., 2004; Preston et al., 2006). Besides being endocrine disrupting agents (Gasnier et al., 2009; Benachour et al., 2012), the herbicides PQ and Roundup® produce reactive oxygen species (ROS) (Hausburg et al., 2005; Williams et al., 2000; Jasper et al., 2012; Benachour and Séralini, 2009; Takenaka et al., 2003) and oxidativestress on the cell, when the production is higher than the cell capacity in degrading them. Consequently, enzyme inactivity, DNA damages, lipid peroxidation and cell death ensue (El-Shenawy, 2009; Hermes-Lima, 2004) which probably explains our morphometric results. In the case of MLT, the protective effect (Maglinh et al., 2008) probably decreases the damages by ROS derived from the herbicides PQ and Roundup®.

In conclusion, MLT treatment acts efficiently against toxic effects on the reproductive system and the embryo, as well as in liver. Moreover, improves fertility indexes such as the prevention of the blastocysts morphological integrity, gain in maternal body weight, ovary weight, number of corpora lutea, viability of the embryo implantation.

References


