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Oxidative stress induced by gibberellic acid in bone of suckling rats

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ABSTRACT

The present study investigates the bone maturity of suckling rats whose mothers were treated with gibberellic acid (GA_3). Female Wistar rats were divided into two groups: group I that served as controls and group II that received orally GA_3 (200 ppm) from the 14th day of pregnancy until day 14 after delivery. In the GA_3 group, an increase in body and femur weights as well as in femur length of pups was noted when compared to controls. Lipid peroxidation was demonstrated by high femur malondialdehyde levels, while superoxide dismutase, catalase, glutathione peroxidase activities, glutathione and vitamin C levels in femur decreased. GA_3 caused a decrease in calcium and phosphorus levels in bone. The calcium concentration in plasma increased and the phosphorus concentration decreased, while urinary levels of calcium decreased and those of phosphate increased. Moreover, plasma total tartrate-resistant acid phosphatase and total alkaline phosphatase increased. Bone disorders were confirmed by femur histological changes.

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1. Introduction

The bone is a connective tissue which constitutes the framework of the body. It is formed and resorbed continuously, starting in the embryo and continuing throughout adult life. Bone disorders are one of the main adverse health effects of several factors, including energy restriction (Fetoui et al., 2006) and xenobiotics intoxication in humans and experimental animals (World Health Organisation, 1992; Satarug et al., 2003; Brzoska and Jakoniuk, 2005). Nowadays, an increasing interest has been focused on bone metabolism effects by many contaminants such as pesticides like dimethoate (Mahjoubi Samet et al., 2005), metals like fluoride (Bouaziz et al., 2004), cadmium (Brzoska and Jakoniuk, 2005) and plant growth regulators (PGRs) (Abdelhamid et al., 1994). PGRs, the endogenous plant hormones, are widely used in agriculture against harmful pests to enhance and control the production of a wide variety of crops (Silverstone and Sun, 2000; Ashikari et al., 2005). In addition, PGRs like gibberellic acid A_3 (GA_3) enhance the growth of fruits (Ben Abdallah et al., 2000; Abdellaoui et al., 2009) and some vegetables (Arous et al., 2001; Chaari-Rkhis et al., 2006). They play an important role in many cellular processes such as promoting stem elongation and overcoming dormancy in seed and buds. They are also involved in parthenocarpic fruit development, flowering, and mobilization of food reserves in grass seed germination (Salisbury and Ross, 1992). PGRs are included in the diet of all herbivorous and omnivorous animals.

Although GA_3 is widely used in agriculture, only a few experiments investigated its possible toxic effects on animals and humans. Therefore, this subject attracted the interest of many researchers recently. Indeed, GA_3 induces toxicity to mammalian systems, particularly the breast, lung (El-Mofty et al., 1994), kidney and liver (Ustun et al., 1992) of adult mice. Moreover, previous investigations of Zalinian et al. (1990) and Bakr et al. (1999) showed that GA_3 induced chromosomal aberrations in human lymphocytes and mice. Furthermore, GA_3 administration by gavage for 22 months induced carcinogenic effect in adult Swiss albino mice (El-Mofty et al., 1994). According to Ozmen et al. (1995) treatment with GA_3 affected sexual differentiation and some physical parameters of laboratory mice. Several studies demonstrated that GA_3 was associated with oxidative stress and cellular damage in many soft organs including heart, kidney, stomach and spleen of adult rats (Celik and Tuluce, 2006) and liver of suckling GA_3-treated rats (Troudi et al., 2010). Furthermore, previous studies of Olson and Hinsdill (1984) reported that PGRs caused increases in the number of splenic plaque-forming cells and circulating white blood cells, hematocrit values and thymus weight in young mice.

Bone, a rigid organ, may be vulnerable to oxidative stress. In fact, production of free radicals is believed to induce bone related diseases by suppressing bone formation and stimulating bone resorption

Abbreviations: GA_3, gibberellic acid; PGRs, plant growth regulators; MDA, malondialdehyde; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; ACP, total tartrate-resistant acid phosphatase; ALP, total alkaline phosphatase; ROS, reactive oxygen species; NPSH, non-protein thiol

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(Ramajayam et al., 2007). The cells have different mechanisms to alleviate oxidative stress and repair damaged macromolecules. The primary defense is offered by enzymatic and non-enzymatic antioxidants which have been shown to scavenge free radicals and reactive oxygen species (ROS). The antioxidant enzymes activities in soft organs of adult rats like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) have been shown to be significantly affected by PGRs (Celik and Tuluce, 2006; Tulice and Celik, 2006; Celik et al., 2007).

To our knowledge, no studies were carried out on oxidative stress and bone disorders in suckling rats whose mothers were treated with GA_3 during late pregnancy and early postnatal periods. Therefore, the present study is undertaken to evaluate bone biochemical parameters, oxidative stress and histopathological changes in the bone of suckling rats following subchronic exposure of adult rats to GA_3 during pregnancy and lactating periods.

2. Materials and methods

2.1. Chemicals

GA_3 in powder was purchased from Sigma Chemical Co. (St. Louis, France). All the remaining chemicals were of high commercially available grade.

2.2. Animals and experimental design

Experiments were performed on male and female Wistar rats weighing 180 ± 10 g purchased from SIPHAT (Tunisia). Food (SICO, Tunisia) and water were available *ad libitum*. After a 1-week adaptation period in a room with controlled temperature (22 ± 2 °C) and lighting (12 h-light/12 h-dark), female rats were mated with males. A sperm-positive vaginal smear was taken to indicate the first day of pregnancy. Twelve pregnant rats were randomly divided into two groups of six each: group 1 that served as controls and group 2 that received 200 ppm of GA_3 (equivalent to 55 mg/kg) through drinking water from the 14th day of pregnancy until day 14 after delivery. The GA_3 dose chosen in our experiment represented 1/100 of LD 50. The day of parturition was considered as day zero of lactation. No delay was observed in the delivery of the treated group. Pups were counted and weighed and each litter was reduced to eight pups (4 males and 4 females if possible) to ensure standardized nutrition and maternal care (Fishbeck and Rasmussen, 1987).

During the course of treatment, daily fluid consumption, body weight gain, and feed consumption were recorded from day 3 until day 14 after delivery. Each lactating rat treated with GA_3 ingested 10.04 mg GA_3/day (Table 1). This dose was similar to the amount released in the air by pulverization (Personal Communication of Plant Biotechnology Laboratory, Science Faculty of Sfax, Tunisia).

On day 14 of lactation at 8:00 am, litters were separated from mothers. Four hours later, dams were anesthetized with chloral hydrate by intraperitoneal way. Oxytocin (10 mIU/ml of 0.9% saline solution) was administrated intraperitoneally and teats were cut off. The expelled milk was aspirated and stored at −20 °C until analysis. Milk tinged with blood was not aspirated, thus avoiding according to Heil et al. (1999) the introduction of blood into the milk collected.

Six dams and forty-eight pups of each group were anesthetized with chloral hydrate by intra-abdominal way. After sacrifice, blood samples were collected in heparined tubes by aortic puncture in dams and by brachial artery in pups. Stomach contents and urine were taken from both control and treated pups and were stored at −20 °C. Plasma samples were drawn from blood after centrifugation at 2200*g* during 15 min and kept at −80 °C until analysis.

Femurs were dissected out and the surrounding muscles and tissues removed. All femur samples were weighed. Some of them were intended for histological examination and the others were stored at −80 °C until biochemical analyses. Bone samples were taken from femoral region and homogenized with 2 ml of 0.1 M Tris–HCl buffer (pH 7.2) using mortar and pestle according to Ramajayam et al. (2007). The homogenates were centrifuged at 10,000*g* for 30 min at 4 °C and the supernatant was used for biochemical estimations. The experimental procedures were carried out according to the general guidelines on the use of living animals in scientific investigations (Council of European Communities, 1986) and approved by the Ethical Committee of Science Faculty of Sfax.

2.3. Biochemical assays

2.3.1. Calcium and phosphorus levels in plasma, urine and femurs

Calcium and phosphorus levels were determined in femurs, after nitric acid mineralization and in plasma and urine using commercial reagents kits (Biocon, Ref 2004, 1904, respectively).

2.3.2. Plasma total alkaline phosphatase (ALP) and acid phosphatase (ACP) levels

The plasma levels of total alkaline phosphatase (ALP) and total tartrate-resistant acid phosphatase (ACP) were determined, respectively, by a colorimetric method (Elitech diagnostics SEES FRANCE, Ref PASL-0500; Biomerieux FRANCE, Ref 746419901).

2.3.3. Protein quantification

The femur protein contents were measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.3.4. Estimation of lipid peroxidation in bone

Lipid peroxidation was determined in femur homogenates by the method of Draper and Hadley (1990) following a reaction of thiobarbituric acid (TBA) with malonyldialdehyde (MDA) formed owing to the peroxidation of lipids. After the incubation of femur homogenates with TBA at 95 °C during 10 min, the pink color produced by this reaction was determined spectrophotometrically at 532 nm.

2.3.5. Antioxidant enzyme activities

2.3.5.1. Catalase activity (CAT). Catalase (CAT) activity was assayed by the method of Aebi (1984). Enzymatic reaction was initiated by adding an aliquot of 20 µl of the homogenized tissue and the substrate (H_2O_2) to a concentration of 0.5 M in a medium containing 100 mM phosphate buffer, pH 7.4. Changes in absorbance were recorded at 240 nm. CAT activity was calculated in terms of µmol H_2O_2 consumed/min/mg of protein.

2.3.5.2. Superoxide dismutase activity (SOD). *Superoxide dismutase* (*SOD*) activity was estimated according to Beauchamp and Fridovich (1971). The reaction mixture contained 50 µl of tissue homogenates in 0.1 M of potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, 13 mM L-methionine, 2 µM riboflavin and 75 µM Nitro blue Tetrazolium (NBT). The developed blue color in the reaction was measured at 560 nm. Units of SOD activity were expressed as the amount of enzymes required to inhibit the reduction of NBT by 50% and the activity was expressed as units per mg of protein.

2.3.5.3. Glutathione peroxidase activity (GPx). Glutathione peroxidase (GPx) activity was measured by the procedure of Flohe and Gunzler (1984). One milliliter of reaction mixture that contained 0.3 ml of phosphate buffer (0.1 M, pH 7.4), 0.2 ml of 2 mM glutathione (GSH), 0.1 ml of sodium azide (10 mM), 0.1 ml of H_2O_2 (1 mM) and 0.3 ml of femur homogenates was prepared. After incubation at 37 °C for 15 min, the reaction was terminated by the addition of 0.5 ml 5% TCA. Tubes were centrifuged at 1500*g* for 10 min and the supernatant was collected. To 0.1 ml of reaction supernatant, 0.2 ml of (0.1 M pH 7.4), and 0.7 ml of 5,50 dithio-bis-(2-nitrobenzoicacid) (DTNB,0.4 mg/ml) were added. After mixing, absorbance was

Table 1
Daily food consumption, water intake and GA_3 quantities ingested by lactating rats: controls and mothers treated with 200 ppm of GA_3 from the 14th day of pregnancy until day 14 after delivery.

Parameters and treatment	Food consumption (g/day/dam)	Drinking water intake (ml/day/dam)	Quantities of GA_3 ingested (mg/day/dam)
Controls ($n=6$)	30.78 ± 0.46	41.47 ± 0.83	–
GA_3 group ($n=6$)	35.21 ± 0.78***	50.22 ± 2.91***	10.04 ± 0.50

GA_3 treated vs controls: ***$p \leq 0.001$.
The values are means ± SE for six rats in each group.

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