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Graphical abstract

Abstract

We investigated the effects of Kalach 360 SL (KL), Glyphosate (G)-based herbicide, on bone tissue in different groups of female *Wistar* rats. Group 1 (n=6) received a standard diet and served as a control, groups 2 and 3 (n=6 each) received 0.07ml (D1: 126 mg/Kg) and 0.175ml (D2: 315 mg/Kg) of KL dissolved in the water for 60 days. The plasma was used to examine the metabolic balance markers (calcium, phosphorus, phosphatase alkaline (PAL), and vitamin D (vit D) and hormonal status (oestrogen and thyroid hormones). As a result, sub-chronic exposure to KL induced a perturbation of bone metabolism (calcium and phosphorus) and hormonal status disturbance. The histological and immunohistochemical study of the thyroid gland revealed a disturbance in morphological structure and thyroid cells function. Moreover, the KL disrupting effect on thyroid function was investigated by measuring changes in plasma levels of thyroid hormones. Free triiodothyronine (FT3) and thyroxine (FT4) were decreased in female rats breast-fed from rats treated with D and D2 of KL. This

effect was associated with an increase in the plasma level of thyroid-stimulating hormone (TSH). Thus, that KL leads to hypothyroidism. Decrease in levels of oestrogen and thyroid dysfunction led to a disruption in the skeletal bone. The histological study and SEM in bone results allowed us to observe, in rats exposed to KL, the thinning and discontinuity of bone trabecular with a significant decrease in the number of nodes (intertrabecular links).In conclusion, KL sub-chronic exposure caused an aspect of osteoporosis.

Abbreviations: AMPA, Aminomethylphosphonic acid; BW, body weight; CAT, catalase; D1, dose 1; D2, dose 2; G, glyphosate; KL, Kalach; LD_{50}, Lethal Dose; LPO, lipid peroxidation; MDA, malondialdehyde; NS, non significant; OCs, osteoclasts; OBs, osteoblasts; PAL, phosphatase alkaline; ROS, reactive oxygen species; SEM, scanning electron microscopy; SOD, superoxide dismutase; FT3, Free triiodothyronine; FT4, thyroxine; TSH, thyroid-stimulating hormone; Vit D, Vitamin D.

Keywords: *Kalach 360 SL; Glyphosate; bone tissue; bone remodeling; osteoporosis; rats.*

1. Introduction

The skeleton is a highly dynamic organ that consists of specialized bone cells, mineralized and unmineralized connective tissue matrices, and spaces that include the bone marrow cavity, canaliculi, vascular canals, and lacunae, containing osteocytes. In adults, bone is constantly being remodeled, first by being tom down (bone resorption) and then by being rebuilt (bone formation) (Baim and Miller, 2009). Normal regulation of bone formation and resorption is a dynamic process, involving a coordinated and delicate balance between osteoclasts (OCs), cells responsible for bone resorption, and osteoblasts (OBs), those responsible for the synthesis of the various constituents of the bone matrix. This process is

known as bone remodeling. Changes in this balance can lead to bone diseases, like osteoporosis, a term used to define the decrease of bone mass per unit volume of anatomical bone. Toxicological studies have shown that bone metabolism is highly sensitive to environmental pollutants (heavy metals, pesticides) which can alter bone composition and mineralization, producing specific bone pathologies (Compston et al., 1999; Rignell-Hydbom et al., 2009; Engström et al., 2012; Mzid et al., 2017).

Human populations throughout the world are exposed daily to low levels of environmental contaminants induced by pesticide residues (Waliszevski et al., 1996), including the G surfactant-based herbicide, namely KL. This compound is one of the most popular herbicides used worldwide notably in Tunisia. KL includes 41.5 % of G as N-(phosphonomethyl) glycine (active ingredient). It contains another 15.5% as a surfactant and 43% of water. This herbicide is manufactured by the Monsanto company, which is a publicly traded agrochemical and agricultural biotechnology producer.

The residue of G-based herbicides and its main metabolites, Aminomethylphosphonic acid (AMPA), are also found in foods, feed and ecosystems (Takahashi et al., 2001; Acquavella et al., 2004; Contardo-Jara et al., 2008). The consequences of the potential effects of these compounds on non-target organisms, such as humans or animals, have been highlighted (Williams et al., 2000; Tomlin 2006; Mangara et al., 2014). Besides, several studies have pointed out that these chemicals can adversely affect endocrine, reproductive, immune and renal systems (Gasnier et al., 2009; Clair et al., 2012; Mink et al., 2012).Recently, we showed that KL is an endocrine disruptor, which can interfere with the female reproductive system, and cause hepatotoxicity and nephrotoxicity (Hamdaoui et al., 2016; 2018; 2019). However, there are no reports related to the effects of such G- based herbicide on bone remodeling and mineral content. Besides, results concerning the effects of KL, G-based herbicide, on bone are very limited. Generally, the bone is a metabolically very

active organ, which accumulates various risk elements and it is usually exposed to a relatively long time.

Therefore, we aim in this study to evaluate the sub-chronic exposure to KL, i.e. low and high doses, on the bone tissue, using histological, histomorphometric, and some biochemical parameters, based on the examination of metabolic balance markers (calcium, phosphorus, PAL and vit D) and hormonal status (oestrogen and thyroid glands).

2. Materials and methods

2.1 Chemicals

The herbicide used in this study was a commercial KL, containing the active ingredient G (360 g/l), isopropylamine salt of n-phosphonomethylglycine (41.5%), surfactant (15.5%) and water (43%). Methyl-methacrylate (MMA), Na_2EDTA- methionin, nitrobluetetrazolium (NBT), riboflavine, H_2O_2 (0.5M), KI solution, acetic acid, trichroloroacetic Acid (TCA), dinitrophenylhydrazine (DNPH), (CuSo4), sulfuric acid, and thiobarbituric Acid (TBA) were purchased from Sigma (St. Louis; MO, USA). All the other chemicals were of analytical grade. They were purchased from a standard commercial supplier.

2.2. Animals

In support of this study, 30 female *Wistar* rats, weighing about 200–220 g, were purchased from the Central Pharmacy (SIPHAT, Tunisia).The animals were used to evaluate the sub-chronic exposure to this herbicide with respect to bone development. They were caged under well-controlled conditions of temperature (22°C), humidity (60%) and 12/12 h light/dark. The experimental protocol was approved by the Ethics Committee in Research and all efforts were made to minimize animal suffering and reduce the number of animals used.

2.3.Preparation of KL solution

The commercial product of KL was purchased from Bioprotection (Arysta Life Science, B.P.80, 64150 NOGUERES France, distributed).

LD_{50} of KL was tested and determined in our laboratory. Indeed, as 10 rats received 0.7 ml of KL and 50% of them died, we considered that KL DL_{50} was 1260 mg of G/kg of BW (Hamdaoui et al., 2018).

2.4.Experimental design

Female *Wistar* rats had free access to a commercial pellet diet (SICO, Sfax, Tunisia) and water *ad libitum*. They were divided into three groups:

- Group 1: composed of 6 rats, which served as a control, received a standard diet.
- Group 2: composed of 12 rats. Each one received, by gavage, 0.07 ml of KL dissolved in 1 ml of water. This dose contained 126 mg of G/Kg (dose 1)
- Group 3: composed of 12 rats. Each one received, by gavage, 0.175 ml of KL, dissolved in 1 ml of water. This dose contained 315 mg of G/Kg (dose 2).

The treatments were conducted for 60 days. Those doses represented about the tenth and one-quarter of LD_{50}, as previously published (Hamdaoui et al., 2016).

Several European studies have examined the level of G found in foods, including products and grains used for human consumption as well as feed for chickens. Traces of the active compound G were found in human urine samples, probably resulting either from occupational use for plant protection purposes or from dietary intake of residues. The acceptable daily intake of a combination of G and certain metabolites (AMPA, etc…) for humans is 1.0 mg/kg (FAO, 2004; 2011). The chronic reference dose for G is 1.75 mg/kg/day (Human-Health Assessment Scoping Document in Support of Registration Review: Glyphosate, 2009). In fact, the effects of G on human health and the environment depend on how much G are present and the length and frequency of exposure. Effects also depend on the health of a person and/or certain environmental factors. Despite the growing and widespread

use of G, evidence of bioaccumulation of G observed in rodent models, as well as increasing concerns for and debates about adverse health outcomes across the population, very few studies have evaluated overall human exposure. Thus, in our study, we evaluate the sub-chronic effects of KL, (G)-based herbicide, on bone tissue in rodent models (Females *Wistar* rats) and the effect of bioaccumulation of G.

Previously, the Lowest Observed Effect Level for systemic toxicity (LOEL) was established for male and female rats (940 and 1183 mg/kg/day respectively) (Rodwell et al., 1980; Draft Human Health Risk Assessment in Support of Registration Review, 2017).

Rats from Group 2 received by gavage 0.07 ml of KL (1/10 DL 50) dissolved in 1 ml of water. This dose contained 126 mg of G/kg (dose 1) while rats from Group 3 received by gavage 0.175 ml of KL (1/4 DL 50), dissolved in 1 ml of water. This dose contained 315 mg of G/kg (dose 2). Both doses were lower than LOEL and carried out over a period of two months.

2.5.Anesthesia

The rats were sacrificed under anesthesia by intramuscular route with a solution of ketamine 50 mg / ml (200 µl) and a solution of midazolam 5 mg / ml (20 µl), as previously published (Hamdaoui et al., 2018).

2.6.Samples preparation

During the whole experiment, the body weight of rats was continuously monitored. The blood samples of rats were drawn in heparinized tubes and centrifuged at 2200 × g for 15 min. The thyroid glands and Femur were collected and weighed. Blood and organ samples were stored at −80°C for subsequent biochemical, mineral and histological analyses.

2.7.Metabolic balance

Calcium, phosphorus, vitamin D (Vit D) and PAL levels in plasma were assayed by colorimetric techniques (Cobas 6000, Roche®) and expressed as mg/L, respectively.

2.8. Hormonal status

2.8.1. *Thyroid hormones and thyroid stimulating hormone analysis*

Six plasma samples were randomly chosen, from each group, for the determination of free T4 and T3 hormones using commercial kits from Immunotech, France (refs: 1363 (FT4); 1579 (FT3). TSH was determined by radioimmunoassay using a rat-specific TSH kit supplied by IBL-Germany (ref.: AHR001).

2.8.2. *Measurement of hormonal ovary (the reproductive gland)*

Since oestrogen restrains the rate of bone remodeling and plays a key role in maintaining bone mass in adult women, Oestrogen was determined using an electrochimiluminescence assay (Architect, Abbot).

2.8.3. *Histological study of thyroid gland*

Thyroids were taken with a piece of trachea, immediately fixed in 10% formalin and then embedded in paraffin. Paraffin sections (4 μm) were cut, fixed on glass slides, stained with hematoxylin and eosin (H&E) and subsequently examined under light microscope. At least four randomly chosen thyroids were examined for each group.

2.8.4. *Immunohistochemical study of thyroid cells function*

Thyroid sections were dewaxed by standard techniques, and then heat treated to retrieve the antigen sites. In order to quench endogenous superoxidases, the sections were treated with 3% hydrogen peroxide indistilled water, at room temperature. Afterward, the sections were incubated for 1 h at room temperature with blocking solution and then with primary antibody overnight at 4°C. The primary antibodies included a rabbit polyclonal anti-calcitonin (reference from CliniSciences: PDR024-S) and a rabbit monoclonal anti-thyroglobulin (reference from CliniSciences: AC-0220A). The reactivity of the antibodies was detected using astreptavidin-peroxidase histostain-SP kit. Positive staining appeared asa brown-yellow color.

2.9.Femur bone

2.9.1. *Bone Samples preparation, inclusion, and histopathology*

For Each group, bone samples were embedded in methyl-metacrylate without previous decalcification at 4°C to maintain enzyme activity. Sections, 7µm thick, were cut dry in parallel to the long axis of the femoral bone core, using a heavy-duty dry microtome (Polycut S Reichert Jung, Germany), equipped with 50 Tungsten carbide knives (Leica Polycut S, Rueil-Malmaison, France). The sections were used for a modified Goldner's trichrome staining. In addition, the decalcified samples of bone specimens were examined with hematoxylin and eosin staining. The following parameters were measured: trabecular bone volume (BV/TV, in %), trabecular thickness (Tb.Th, in µm), and osteoid surface (OS/BS, in %). The latter determination was mainly based on the percentage of endosteal bone surface, presenting features of bone resorption with OCs. The measurements were assessed in the secondary spongiosa, 1 mm under the growth cartilage at a magnification of 200× and 400×.

2.9.2. *Scanning electron microscopy (SEM)*

Scanning electron microscopy (SEM) was performed on the operated femur of treated rats. Briefly, the femur was longitudinally cut, and then immersed in 50% sodium hypochloride to discard organic material. After 3h, bone samples were rinsed for 30 min in distilled water and fixed overnight in 1% osmium tetroxyde dissolved in 0.1 M cacodylate buffer (pH 7.2).Subsequently, the samples were rinsed for 30 min in distilled water, and then dehydrated in ascending series (70%, 95%, and 100%) of ethanol. Finally, the samples were conducted for 1 h in hexamethyl disilazane followed by air drying, using a filter paper. They were SEM analyzed at 20 kV (SEM JEOL JSM-5100, University of Rennes 1, Laboratory of Solid Chemistry and Materials-Biomaterials UMR Rennes, France, Building 10B, France)

2.9.3. *Tissue preparation and oxidative stress determination*

The Femur was dissected out, cleaned and weighed. Some samples were rinsed and homogenized (100 mg/mL) at 4°C in 0.1 mol/L Tris–HCl buffer pH 7.4 and centrifuged at 3000 g for 10 min.

Lipid peroxidation (LPO) in the tissue homogenate was estimated by measuring thiobarbituric acid–reactive substances (TBARS) and it was expressed in terms of malondialdehyde (MDA) content, which is the end-product of LPO (Buege and Aust, 1984, Jebahi et al., 2012). In fact, a sample (1.0 ml) is mixed with 2.0 ml of a solution 15% w/v of trichloroacetic acid (TCA) and 0.375% w/v of TBA prepared in 0.25 N HCI. Other acids can replace TCA which is, however, the most convenient for precipitation of the proteins of the sample. The mixture of the reagent with the sample is heated for 15 minutes in a boiling-water bath, and then cooled and centrifuged at 1500 x g to remove the precipitate of the tissue sample. The MDA concentration of the supernatant can be determined directly from the molar extinction coefficient of the pink pigment or by comparison of the absorption at 535 nm against a standard curve.

2.9.4. *Antioxidant enzyme studies*

Femur samples were rinsed and homogenized (100 mg/mL) at 4°C in 0.1 mol/L Tris–HCl buffer (pH 7.4) and centrifuged at 3000 g for 10 min and the resultant supernatant was used for the determination of the superoxide dismutase (SOD) and catalase (CAT) activities. The SOD activity was assayed by the spectro-photometric method of Marklund and Marklund (1975). CAT activity was assayed calorimetrically at 240 nm and expressed as moles of H_2O_2 consumed per minute per milligram of protein, as described by Aebi (1984). The level of total protein was determined by the method of Lowry et al.(1951), using bovine serum albumin.

2.10. Histological scores analysis

Histological scores of thyroids and femurs of female rats were calculated using Image J 1.48v software (Strissel et al., 2007).

2.11. Statistical analysis

Statistical analyses were performed using the SPSS software package (version 20.0 for Windows; SPSS Inc., Chicago, IL). Quantitative parametric variables were expressed as mean ± SD and compared using Student's t- test when distribution is Gaussian. Non parametric parameters were expressed as median and interquartile ranges and compared using the Mann–Withney's test. Pearson's and Spearman's correlation tests were used to evaluate the associations between continuous variables with a Gaussian distribution and non -Gaussian distribution, respectively. All values are expressed as mean ± SD. Differences were considered significant if $p<0.05$.The final number of animals analyzed used in our study was 6 rats for each group.

3. Results

3.1. Effects of KL (D1 and D2) on body and relative bone weights

The variations in body and relative bone weights of animals subjected to different treatments are illustrated in Table 1. The animal treated with KL showed signs of toxicity from either direct observation or autopsy examination. The LD_{50} is the amount of a material, given all at once, which causes the death of 50% (one-half) of a group of the test animals. The LD_{50} is a way to measure the short-term poisoning potential (acute toxicity) of a material (Canadian Center for Occupational Health and Safety). In our study, mortality of females rats initiated after one month of exposure to KL (D1 and D2).

To the best of our knowledge, G-Based herbicide can induce daily mortality and signs of toxicity (Zhu et al., 2013; Hamdaoui et al., 2016). For that, we multiplied the number of rats in groups 2 and 3 because of the high toxicity of KL. During the experimental period (60 days) of the exposition of KL, 12 deaths occurred. In fact, 6 rats died in each group treated with orally administered KL. The final number of rats in each group was six. A decrease in

relative bone weights of KL-treated group was also recorded (16% and 23%) as compared to the control ($p < 0.05$).

3.2. Phosphocalcic balance finding

As shown in Table 2, the mean values of the calcium levels in Groups 2 and 3 were significantly increased as compared to control (+11% and +12% respectively).Contrarily, a non-significant (NS) decrease of phosphorus (-6%) in group 3 was observed. Statically significant changes were observed in the levels of Vit D (-10% and -11%) in plasma when comparing the KL groups with the control group. Besides, the determination of plasma PAL level, reflecting bone formation, displayed a NS decrease in the KL-treated rats compared to the corresponding controls. Moreover, a decrease of calcium level in urine was determined (-21% and -23%) ($P < 0.05$) (Table 2).

3.3.Hormonal balance finding

3.3.1. *The ovary hormonal levels*

Our finding showed a significant decrease of plasmatic oestrogen levels in the KL-treated rats (-43, -36%), in groups 2 and 3 (Table 2), respectively, when compared to the control group ($p <0.05$).

3.3.2. *Hormonal thyroid variations*

The hormonal thyroid levels FT4 and FT3 in plasma decreased in KL-treated rats (D1 and D2), compared to the control group (FT4: 40.19 % and 52.4 %; FT3: 40.3 % and 50.4 %, respectively) (Fig.1.A and B). In addition, KL (D1 and D2) treatment led to an increase of the thyroid stimulating hormone (TSH) plasma levels (40% and 80%) as compared to the control group (Fig.1 C).

3.3.3. *Histopathological study of thyroid gland*

Thyroid gland of control group displayed a normal aspect (Fig.2.A). However, KL induced a relative increase of the thyroids appearance pertaining to the follicles in rest,

along with a decrease of the number of the active follicles; in addition to the appearance of the macrophage, dilated follicle and flattened epithelium, as shown in Fig.2 (B, C and C1).The histomorphometric study showed a decrease in colloid volume (48% and 55.5%) (Fig.2. D).Our histological finding was in favor of hypothyroidismin KL-treated rats.

3.3.4. *Immunohistochemical finding of thyroid cells function*

In KL-treated animals, both follicular and C cells displayed signs ofdysfunction. Indeed, no positive staining was revealed with the anti-thyroglobulin in the colloid of the thyroid gland, as compared with control group. Following the supplementation with oleuropein and hydroxytyrosol rich extracts, a positive staining appeared as a brown-yellow color in thyroid gland colloids similar to the positive staining. Histomorphometric finding showed a significant decrease in number of positives C cells (Fig.3. D).

We also found a very intense labeling with anti-thyroglobulin protein (Fig.3.A). However, in rats treated with KL, D1 and D2 showed a lower intensity of labeling, with lower intensity in female rats treated with the second dose (Fig.3. B, C). The labeling of calcitonin in the group of control rats was very important (Fig. 3. A1). However, the treatment of female rats with KL either with the dose D1 or D2 caused a disappearance of this marking (Fig.3.B1, C1).

3.4.Effects of treatments on bone oxidative stress parameters: MDA levels and enzymatic Antioxidant Status

MDA levels inbone tissues were increased in KL-treated groups by D1 and D2 (+23% and +31%, respectively) when compared with those of controls (Table 3). The treatment with KL (D1 and D2) caused significant reduction of the activities of SOD (-11% and -25%, respectively) and CAT (-2 % and -9%, respectively) (Table 3).

3.5.Histological bone analyses

After 60 days of treatment, the histological examination on bone, included in MMA without decalcification, cut and stained with Goldner'strichrome, showed a normal and mature bone matrix calcified in the control group (Fig. 4. Aand A1). However, Fig.4, B and B1, exhibited non-spongy trabecular spans with respect to the histology of control rats. These bony traps offer a strong resistance to the bone against a likely crushing. In addition, Fig. 4, C, C1, C2 and C3, also reveal that bone trabeculae were growing rarer. As a result, the bone becomes brittle and more sensitive to fractures. Our histological results indicate a case of osteoporosis, which was confirmed by biochemical results (oestrogen deficiency and hypothyroidism). The osteoporotic aspect could be seen in female rats treated with the increased dose of KL (D2).

3.6.SEM results

Fig.5 displays the SEM images of the endosteal femur. Two distinctive features were recorded. In fact, the endosteums (endocorticals) of control and KL-treated groups showed a homogeneous aspect (Fig.5. A3, B3 and C3). For the cancellous bone (Fig.5. B1, B2, C and C1), compared to control, KL-treated rats displayed fewer and more separated trabeculae. Histomorphometric finding showed an increase in intertrabecular distance in trabecular bone (Fig.5. D).

3.7.Histomorphometric finding

Data obtained from the histomorphometric analysis showed significant changes with respect to the femora in KL-treated animals by D1 and D2 during 60 days compared to controls (Table 4). Exposure to D1 and D2 of KL caused a significant decrease of osteoidsurfaces (OS/BS) by 19% and 24%, respectively, and a significant decrease of trabecular thickness (Tb.Th), trabecular bone volume (BV/TV), and Trabecular number (Tb.N). Besides, an increase in trabecular separation (Tb.Sp) was recorded.

4. Discussion

The present study aimed to evaluate the toxic effect of sub-chronic exposure to KL, G-based herbicide, on bone tissue, by the examination of metabolic balance markers (calcium, phosphorus, PAL and vit D), hormonal status (oestrogen and thyroid glands) and histological evaluation in bone tissue. As part of the biological exploration of the mineral homeostasis, regulating hormones (vit D and oestrogen) in plasma and urine were tested. To our knowledge, our paper represents the first report on the exploration of G-based herbicide in bone tissue of female rats.

Exposure of female rats to D1 and D2 of KL induced a significant decrease in body and bone weights, when compared with controls. Low bone mass can be associated with high or low rates of remodeling with the imbalance between resorption and formation (Lemaire et al., 2004). The contents of calcium and phosphorus in plasma showed a perturbation in KL-treated groups. Our results indicate an increase of calcium and a decrease of phosphorus levels in the KL-treated female rats when compared with controls (Table 2). This could be explained by the nephrotoxicity effect of KL as reported elsewhere (Hamdaoui et al., 2016), since the kidneys play a central role in the homeostasis of these ions (Blaine et al., 2015).This perturbation on homeostasis might, therefore, affect bone mineral density and quality.

PAL, which is an enzyme produced in liver and bone tissue, indicates, at least in part, the bone neoformation and its mineralization (Watts, 1999). We showed a NS decrease in the concentration of PAL in comparison to the control rats (Table 2). This decrease could occur during hepatic affections as reported by Caglar and Kolankaya (2008) and Hamdaoui et al. (2019), in presence of an obstacle to the bile ducts and/or in the osseous affections accompanied by a hypoosteoblastic activity, thus affecting the bone formation.

Our study displayed a decrease in vit D levels in plasma in KL-treated rats during 60 days. This decrease can probably be elucidated by the decrease in the intestinal absorption of this vitamin. Furthermore, the nephrotoxicity and the hepatotoxicity induced by KL could

account for the decrease pertaining to the active form of vit D, after the inhibition of the 25-hydroxylase and 1-α- hydroxylase (Heaneyet al., 2003; Caglar and Kolankaya, 2008; Hamdaoui et al., 2016; 2019).The regulation of bone growth is closely linked to vit D, which is a key factor for bone health. Hence, such deficiency related to vit D may result in bone disorders characterized by defects in mineralization, rickets in children and osteomalacia in adults (Basha et al., 2000; Boonen et al., 2006).

Our results indicate a perturbation of metabolic balance markers (calcium, phosphorus, PAL and vit D), in the KL-treated female rats when compared with controls. This perturbation on homeostasis might therefore affect bone mineral density and quality. The metabolic unbalance markers could be explained by the nephrotoxicity (histological and biochemical findings) effect of KL as reported elsewhere (Hamdaoui et al., 2016).

On the other hand, in KL-treated rats, a deficiency of oestrogen hormone was noted when compared with controls (Table 2). This decrease can be ascribed to the toxicity induced by KL in the female on reproductive systems (Hamdaoui et al., 2018). In fact, oestrogen restrains the rate of bone remodeling and plays a key role in preserving bone mass in adult women by also maintaining a balance between osteoblastic and osteoclastic activity (Manolagas et al., 2002; Manolagas and Parfitt, 2010). Therefore, such oestrogen deficiency can cause loss of bone associated with an increase of bone resorption and a decrease in bone formation, albeit unbalanced. So, the significant decrease in plasma oestrogen levels in groups 2 and 3 could be explained by the severe lesions induced by KL observed in histology of Theca Interna (since these cells are specialized in steroids secretion). Histopathological findings showed a vacuolisation of follicles.We also revealed the formation of vacuoles within or adjacent to cells, oocyte with vacoulation, folliculeatretics and necrosis follicles (Hamdaoui et al., 2018). The damage in ovary tissues could probably be suitable via the direct effect of KL on ovary cells by causing necrosis or via oxidative damage. The oestrogen

deficiency can be explained, probably, by KL toxicity induced in the adrenal glands. In fact, adrenal cortex produces several hormones. The most important are aldosterone (a mineralocorticoid), cortisol (a glucocorticoid), androgens and sex hormones (estrogens) (Turquetil and Reznik, 2019). Since oestrogen is the key modulator of osteoclast formation, and plays an important role in maintaining bone mass in adult women by suppressing bone remodelling and maintaining a balance between osteoblastic and osteoclastic activity (Manolagas et al., 2013) .

This oestrogen deficiency can lead to excessive bone resorption accompanied by inadequate bone formation. Osteoblasts, osteocytes, and osteoclasts express estrogen receptors. In addition, estrogen affects bones indirectly through cytokines and local growth factors. The estrogen-replete state may enhance osteoclast apoptosis via increased production of transforming growth factor (TGF)–beta. Bone tissue is continually being renewed by osteoclast and osteoblast cells during normal physiology, but excessive resorption occurs without adequate new bone formation during postmenopausal osteoporosis (Feng and McDonald, 2011).

Thyroid hormones play a pivotal role in linear development and maturation of the skeleton and they are necessary to achieve peak bone mass (Liote and Orcel, 2000; Bassett and Williams, 2003). G-based herbicides are known to disrupt endocrine functions and have been extensively investigated for their effect on reproduction by both in vivoand in vitro experiments (Stamati et al., 2007; Hamdaoui et al., 2018). However, only a few studies have investigated the toxic effect of G-based herbicides on the thyroid gland.To our knowledge, this study may constitute the first attempt to evaluate the toxic effect on thyroid gland in female rats although several studies have demonstrated the significant relationship between circulating levels of thyroid hormones and exposure to other environmental chemicals (Longnecker et al., 2003; Blount et al., 2007).Our results showed that KL sub-chroic exposure

is able to interfere, directly or indirectly, with the synthesis of thyroid hormones. In fact, plasma levels of thyroid hormones were markedly decreased intreated rats. Consequently, KL (D1 and D2) decreased the thyroid function in female rats (hypothyroidism) and affected the skeletal maturation.

Histological and immunohistochemical analyses of thyroid glands demonstrated that the intoxification of KL induced a morphological disorder and a decrease of the thyroid cells activity. Following KL treatment, thyroids of treated rats presented a relative increase of the appearance pertaining to the follicles in rest, with the decrease of the number of the active follicles, in addition to the appearance of the macrophage, dilated follicle and flattened epithelium as shown in Fig.2 (B, C and C1).Our histological finding is in favor of hypothyroidism of treated rats. It is a condition where in the thyroid gland is not capable of producing enough thyroid hormone. Since the main purpose of thyroid hormone is to "run the body's metabolism", it is understandable that people with such conditions will have symptoms associated with a slow metabolism (Gimeno et al., 1997; Boas et al., 2006; Patrick, 2009).

Our histological and immunohistochemical results of the thyroid gland revealed a disturbance in morphological structure and thyroid cells function. Moreover biochemical results showed a deficiency of thyroid hormone in KL-treated rats. The thyroid hormones, T4 and T3, are key regulators of bone remodeling. So this hypothyroidism observed in our results can cause a reduction in bone mineral density and increases fracture risk, in particular in association with estrogen deficiency (Murphy and Williams, 2004). It has been reported that thyrotoxicosis is an established cause of secondary osteoporosis, and abnormal thyroid hormone signaling has recently been identified as a novel risk factor for osteoarthritis (Monfoulet et al., 2011). Bassett and Williams, (2016) have explained the important role of thyroid hormones in skeletal development and bone maintenance. In fact, T3 inhibits the proliferation and stimulates the differentiation of osteoblastic cell lines and primary calvarial

osteoblasts (Bassett and Williams, 2003). T3 also stimulates osteoclast activity, although it is not clear whether this effect is direct (Kanatani et al., 2004) or is secondary to osteoblast stimulation and mediated by cytokines involved in osteoblast-osteoclast functional coupling (Bassett and Williams, 2016).

Moreover, our results displayed a significant increase of calciumin plasma, which can be explained by the absence of calcitonin in KL-treated groups, as shown by the immunohistochemistry study in the thyroid glands (Fig.3. B1 and C1).It is generally known that hypercalcemia is the result of the absence of calcitonin secretion. The absence of this hormone induced the disturbance of osteoclast activity and acceleration of bone resorption (Williams et al., 2000). Currently, it is well proved that thyroid damage can adversely affect bone metabolism (Tuchendler and Bolanowski, 2014). Our data were in accordance with these findings and revealed that KL, the G-based herbicide, can be a pathogenesis factor by inducing hypothyroidism and bone disorders in female rats.

In this study, KL exposure could probably induce a pro-oxidant event in rat's bone. Infact, an increase in bone MDA content was noted in the KL-treated group when comparedto controls. We hypothesize that KL induces a set of toxicological activities, such as oxidative stress during bone healing. The measurement of MDA, considered as anindex of LPO, and the status of antioxidant enzymes, namely SOD and CAT, were theappropriate indirect ways to assess the pro-oxidant-antioxidant status in tissues. In addition, our results indicated that KL oral administration reduced bone SOD and CAT activities in treated groups when compared to controls. This could be due to the increase in ROS production, as revealed by the high MDA levels after KL treatment. Manolagas (2010) reported that oxidative stress, a shared mechanism of the pathogenesis related to several degenerative disorders associated with aging, including osteoporosis and an increase in ROS was involved in the decreased bone formation.

The treatment with G-based herbicide led to an increase in bone resorption. The femur proved to be a susceptible bone, especially the femoral diaphysis, composed of cortical bone, but also the distal femur, rich in trabecular bone. The histological study and the SEM results revealed that KL induced structural alterations in bone compared to the control rats (Fig. 4 and 5). Besides, we noted in the animals treated with KL an increase of the intertrabecular spaces of the spongy bone. This could result in a reduction of the trabecular density, after the excessive osteoclastic activity, which induced osteoporosis. Previous studies showed that the process of bone remodeling pretreatment might give rise to several toxicological problems, due to exposure to xenobiotics (Misawa et al., 1982; Badraoui et al., 2007; Pesce et al., 2008; Mzid et al., 2017).

In the present study, histomorphometric data were confirmed with SEM observation. Besides, we suggested that osteoblasts' activity was inhibited; however, such activity was stimulated by subchronic exposure to KL (D1 and D2). Recent studies have indicated that pesticides may highly reduce bone density in rats and cause the damage of the skeleton and skeletal malformations as reduction and/or delaying ossification process (Lari et al., 2012; Mzid et al., 2017).

Taking into account the physiological change of the femoral histomorphometric parameter, BV/TV (%), trabecular thickness (Tb.Thμm), trabecularnumber (Tb.N) and osteoidsurfaces (OS/BS %) decreased in KL-treated groups. These findings could be ascribed to the hypo-activity of osteoblasts. In fact, in normal cases, osteoblasts completely fill the resorption cavity. If this does not occur, the bone remodeling is incomplete, which results in decreased wall thickness in trabecular bone. The histomorphometric data showed a significant increase of trabecular separation (Tb.Spμm).

Biochemical, histological and histomorphometric results were correlated to each other, suggesting that subchronic exposure to KL induces osteoporosis and unbalance in the bone remodeling process.

The obtained results previously showed that ovary and kidney are considered as the major organs or sensitive organs for KL toxicity (Hamdaoui et al., 2016; 2018) (histological and biochemical findings). So, subchronic exposure to KL induced endocrine disruption and nephrotoxicity in female rats which can led a metabolic bone unbalance. Herbicides are among xenobiotics that can induce a broad spectrum of toxicological effects and biochemical dysfunctions resulting in serious health problems. All the bone tissue damage observed in histological bone results (Fig.4) in this study are confirmed by SEM observation (Fig. 5). This damage can be the results of endocrine and metabolic balance perturbation that were previously reported. We may consequently claim that KL affects indirectly the bone structure after endocrine perturbation. Future studies shall intend to identify impact of KL on the crystalline fraction of bone tissue made up of hydroxyapatite crystals by using physico-chemical studies (X-ray-diffraction and Fourier Transform Infrared Spectroscopy).

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Compliance with ethical standards:

Our experimental protocol was approved by the Research Ethics Committee and all efforts were made to minimize animal suffering and reduce the number of animals used.

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Figures and tables captions:

Fig.1. Effects of KL (D1 and D2) on plasma levels of thyroid hormones (TSH, FT4 and FT3) of control and treated rats.

Values are expressed as mean ± SD. Significant differences between control and KL-treated groups: * $p < 0.05$; ** $p < 0.01$; *** $P<0.001$.

Fig.2.Histological aspect of the thyroid gland section. Control group (A); rats treated with KL D1 (B); rats treated with KL D2 (C, C1). (D): Histomophometric analysis of colloid area. H&E (200X and 400X). Values are expressed as mean ± SD. Significant differences between control and KL-treated groups: ** $p < 0.01$; *** $P<0.001$.

Symbols indicate:

⟶ : Colloid in non-functional state;

*macrophage

active colloids.

Fig.3. Immunohistochemistry with specific antibody against thyroglobulin and calcitonin protein. For the thyroglobulin, there is a positive immunostaining (White arrow) in the colloid (brown stain) of thyroid gland in control group (A, A1). Negative immunostaining with KL treatment group (D1) (B, B1). Moreover, in the grouptreated with D2 of KL (C, C1) the immunoreactive stains are slightly positive. The immunoreactivity with specific antibody against calcitonin protein in the thyroid gland (A–C1) showed a brown ishcytoplasmic immunoreactive C cells adjacent to the basal membrane of thyroid follicles and in the interfollicular tissue: (White arrow: C cells). Original magnification 200X and 400X.(D):

Histomophometric analysis of positives C cells. Values are expressed as mean ± SD. Significant differences between control and KL-treated groups: ** p< 0.01; *** P<0.001.

Significant differences between KL-treated groups (group 2 vs group 3): +p < 0.05.

Fig.4.Goldner stained sections and SEM micrographs of distal femurs (Epiphysis) from control (A and A1), KL-treated rats orally with the dose 1 (corresponding to 126 mg of G/kg/day) (B and B1), and orally KL-treated rats with the dose 2 (corresponding to 315 mg of G/kg/day) (C, C1, C2 and C3).Histological study showed a thinning and discontinuity of trabecular bone as well as a decreased connectivity and thinning of bone trabeculae continuous trabecular traverse. Original magnification of Goldner stain (200X and 400X);Original magnification of SEM 100×and 500×.

Arrows indicate:

: Continuous trabecular bone

: Disappearance of the nodes (inter-trabecular link)

: Discontinuities of the trabecular spans

Fig.5. SEM micrographs of distal femurs from control (A, A1, A2 and A3), orally KL-treated rats with the dose 1 (corresponding to 126 mg of G/kg/day) (B, B1, B2 and B3), and orally KL-treated rats with the dose 2 (corresponding to 315 mg of G/kg/day) (C, C1, C2 and C3). SEM micrographs of KL-treated rats show decreased connectivity and thinning of bone trabeculae continuous trabecular traverse. (D): Histomophometric analysis of intertrabecular distance in trabecular bone.Original magnification 20×; 100×and 500×.

Values are expressed as means ± SD. Significant differences between control and KL-treated groups: *** P<0.001.

Significant differences between KL-treated groups (group 2 vs group 3): +p < 0.05.

Arrows indicate:

: Thinning and discontinuity of bone trabecular

: Intertrabicular distance

Table 1: Effects of KL (1/10 and 1/4 of DL_{50}) on body weight (g) and absolute and relative bone weights (g/100g BW) in experimental groups:

Values are expressed as mean ± SD,

(FBW) Final body weights

Significant differences between control and KL-treated groups: * $p < 0.05$; ** $p < 0.01$; *** $P<0.001$.

Significant differences between KL-treated groups (group 2 vs group 3) +++ $P<0.001$.

Table 2:Effects of KL (D1 and D2) on some plasma biochemical parameters of control and treated rats.Values are expressed as mean ± SD,

Significant differences between control and KL-treated groups: * $p < 0.05$; ** $p < 0.01$; *** $P<0.001$.

Table 3: Effects of KL on the malondialdehyde (MDA) and antioxidant enzymes activities level in bone of control and treated rats.

Values are expressed as means ± SD,

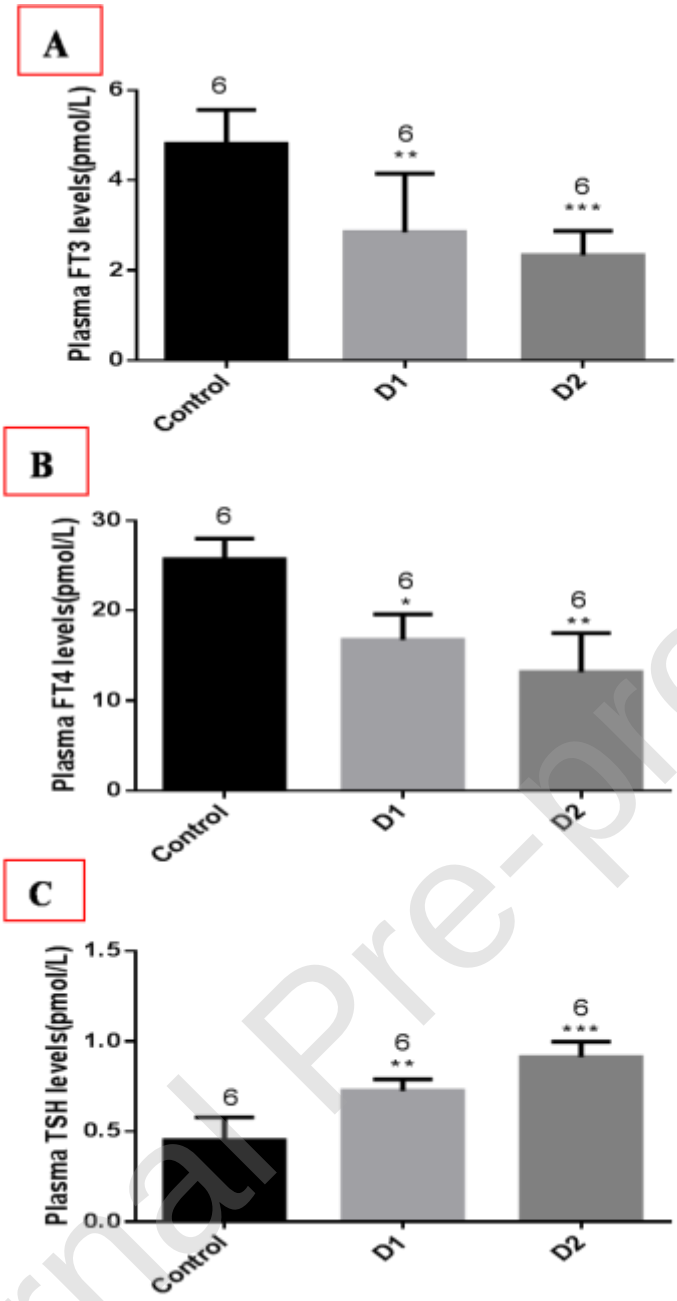
Significant differences between control and KL-treated groups: * $p < 0.05$; ** $p < 0.01$; *** $P<0.001$.

Table 4: Histomorphometric finding in controls and treated groups with KL.

Values were expressed as means ± SD,

Significant differences between control and KL-treated groups: ** $p < 0.01$; *** $P<0.001$.

Fig.1



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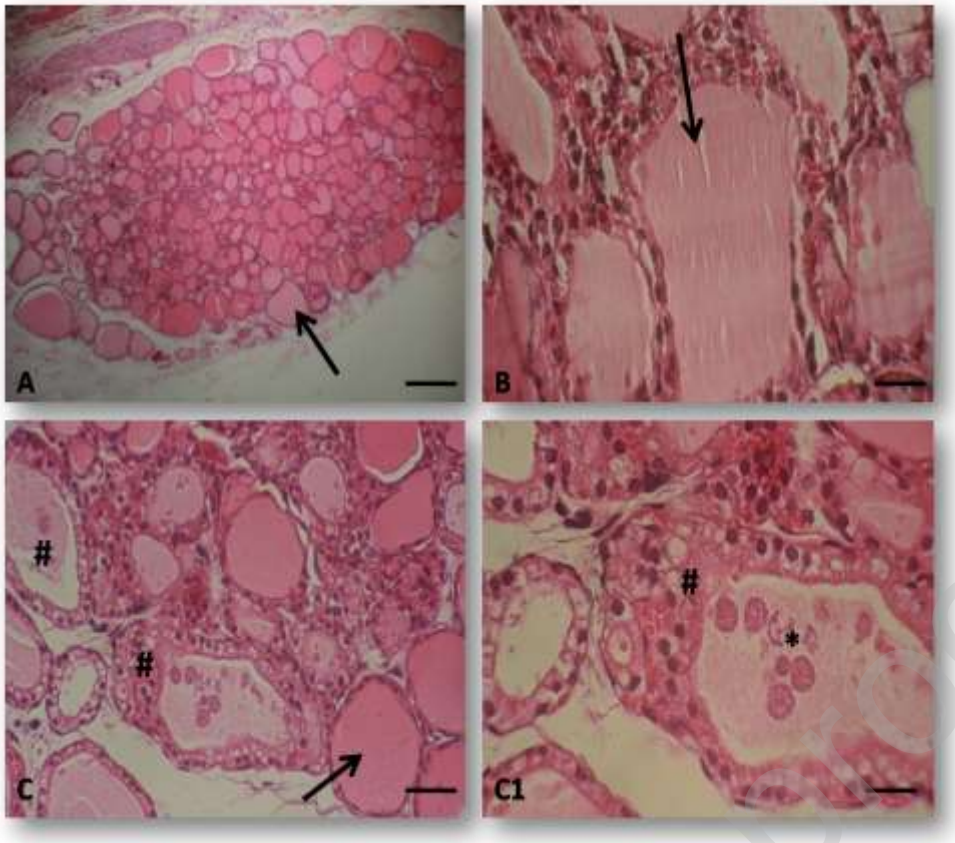
Values are expressed as mean ± SD. Significant differences between control and KL-treated groups: * p < 0.05; ** p < 0.01; *** P<0.001.

Fig.2



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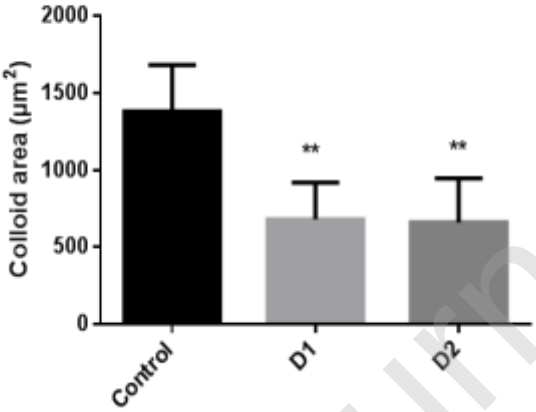

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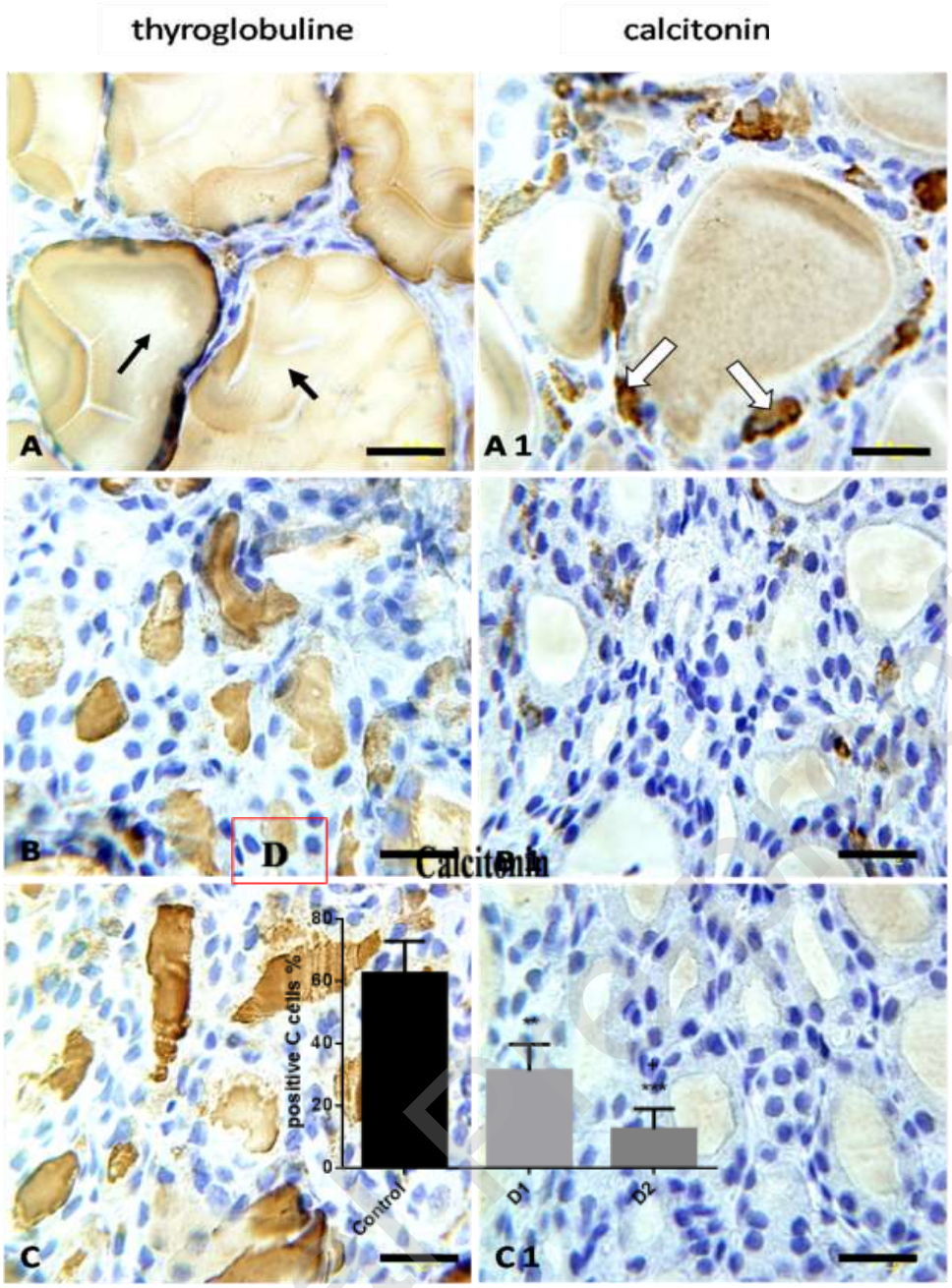
Symbols indicate:

→ **: Colloid in non-functional state;**
*** : macrophage**
#active colloids

Fig.3



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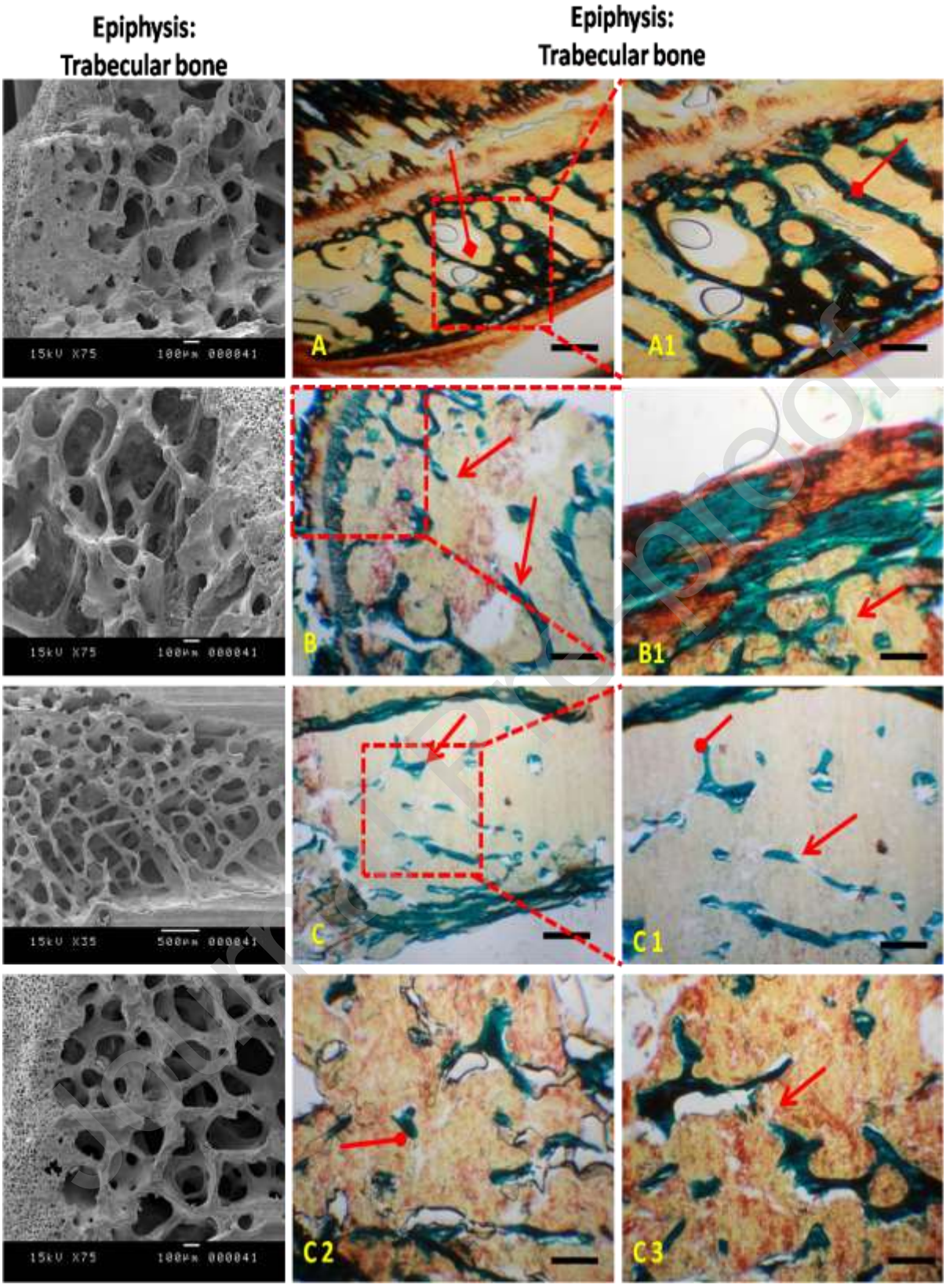
Significant differences between KL-treated groups (group 2 vs group 3): +p < 0.05.

Fig.4


Epiphysis:
Trabecular bone
Epiphysis:
Trabecular bone
15kV X75 100µm 000041
A
A1
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B
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15kV X35 500µm 000041
C
C1
15kV X75 100µm 000041
C2
C3

Fig.4. Goldner stained sections **and SEM micrographs** of distal femurs **(Epiphysis)** from control (A **and A1**), KL-treated rats orally with the dose 1 (corresponding to 126 mg of G/kg/day) (B and B1), and **orally** KL-treated rats with the dose 2 (corresponding to 315 mg of G/kg/day) (C, C1**, C2 and C3**).Histological study showed a thinning and discontinuity of trabecular bone as well as a decreased connectivity and thinning of bone trabeculae continuous trabecular traverse. **Original magnification of Goldner stain (200X and 400X); Original magnification of SEM 100×and 500×.**

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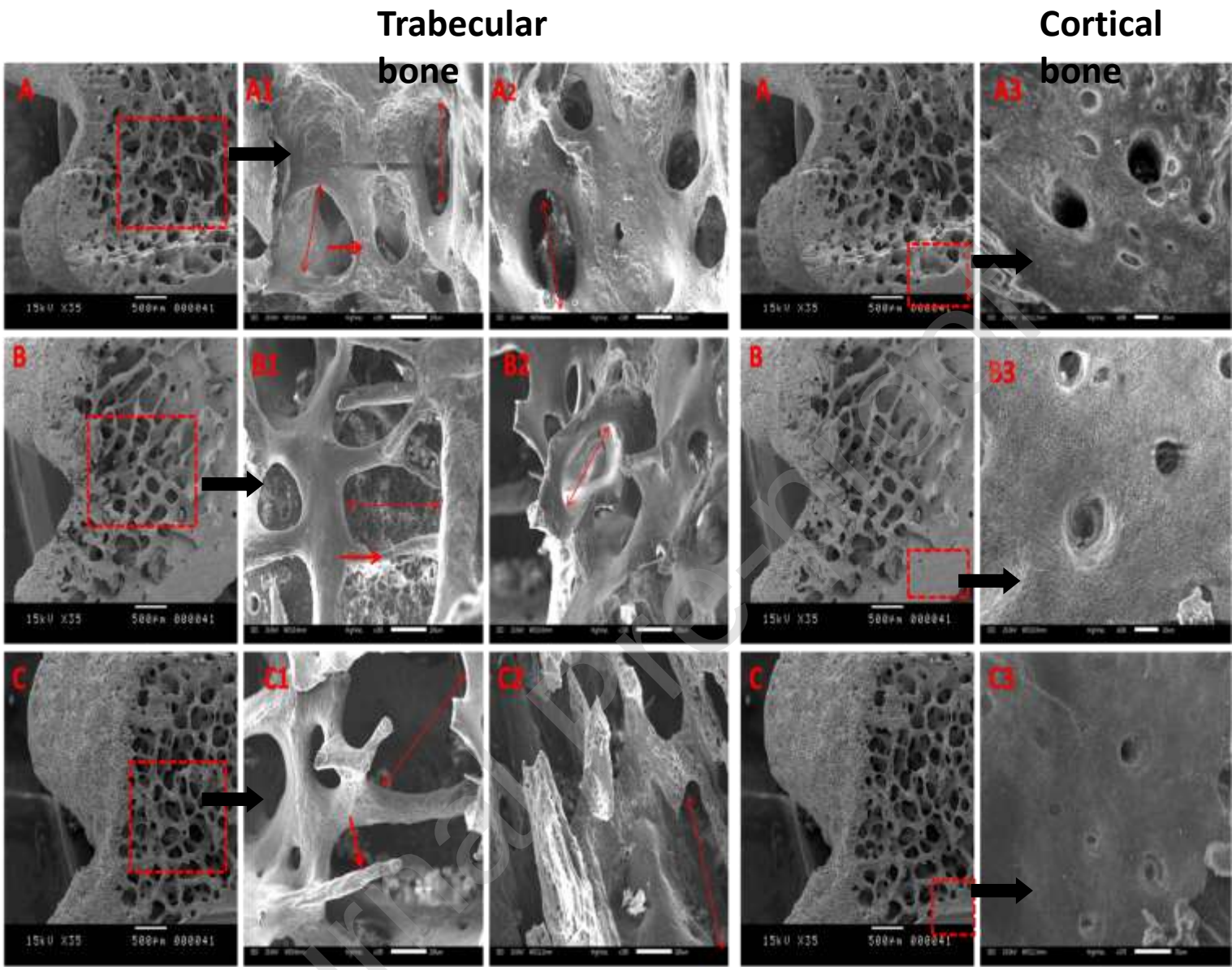
Fig.5


Trabecular bone
Cortical bone
A
A1
A2
A
A3
B
B1
B2
B
B3
C
C1
C2
C
C3

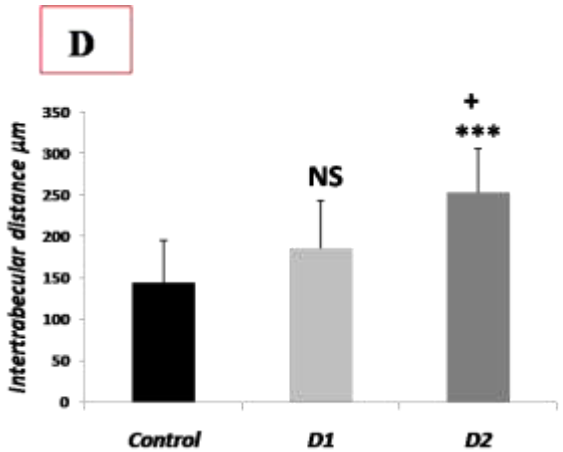

D
Intertrabecular distance µm
350
300
250
200
150
100
50
0
NS
+
***
Control
D1
D2

Fig.5. SEM micrographs of distal femurs from control (A, A1, A2 **and A3**), **orally** KL-treated rats with the dose 1 (corresponding to 126 mg of G/kg/day) (B, B1, B2 **and B3**), and **orally** KL-treated rats with the dose 2 (corresponding to 315 mg of G/kg/day) (C, C1, C2 **and C3**). SEM micrographs of KL-treated rats show decreased connectivity and thinning of bone trabeculae continuous trabecular traverse. **(D): Histomophometric analysis of intertrabecular distance in trabecular bone**. **Original magnification 20×; 100×and 500×.**

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Significant differences between KL-treated groups (group 2 vs group 3): +p < 0.05.

Arrows indicate:

: Thinning and discontinuity of bone trabecular
: Intertrabicular distance

Table 1: Effects of KL (1/10 and 1/4 of DL_{50}) on body weight (g) and absolute and relative bone weights (g/100g BW) in experimental groups:

Parameters & treatments	Group 1 (control)	Group 2 (0.07 ml KL)	Group 3 (0.175 ml KL)
Initial body weights (g)	201 ± 1.67	206.81 ± 5.03	220.63 ± 4.15
Final body weights (g)	211.5 ± 3.72	200.66 ± 2.65 ***	210.83 ± 1.72 *** +++
Absolute bone weights (g)	5.38 ± 0.86	5.04 ± 0.13	4.28 ± 0.74*
Relative bone weights (g/100g of FBW)	0.72 ± 0.09	0.60 ± 0.16	0.55 ± 0.12*

Values **are** expressed as mean ± SD. Significant differences between control and KL-treated groups: * $p < 0.05$; ** $p < 0.01$; *** $P<0.001$. Significant differences between KL-treated groups (group 2 vs group 3) +++ $P<0.001$.

(FBW) Final body weights

Parameters & treatments	Control	Group 2 (126 mg of G /kg/day)	Group 3 (315 mg of G/kg/day)

Table 2: Effects of KL (D1 and D2) on some plasma biochemical parameters of control and treated rats.

Plasma levels (mg/l)			
Calcium	2.55 ± 0.24	2.85 ± 0.10*	2.88 ± 0.07*
phosphorus	1.45 ± 0.69	1.81 ± 0.73	1.35 ± 0.11*
Vit D	49.94 ± 19.02	44.56 ± 13.05*	43.22 ± 13.69*
Oestrogen	33.83 ± 16.58	21.62 ± 8.46	19.4 ± 2.30 *
PAL (UI /L)	83.25 ±17.76	78.5± 18.11 NS	64.66 ± 4.96 NS
Urin levels			18.9 ± 1.28**
Calcium	24.62 ± 6.65	19.21 ± 2.25**	

Values **are** expressed as mean ± SD. Significant differences between control and KL-treated groups: * $p < 0.05$; ** $p < 0.01$; *** $P<0.001$.

Table 3: Effects of KL on the malondialdehyde (MDA) and antioxidant enzymes activities level in the bone of control and treated rats.

Parameters & treatments	Control	Group 2 (126 mg of G /kg/day)	Group 3 (315 mg of G/kg/day)
MDA in bone (nmols of MDA/g tissue)	5.22 ± 0.02	6.44 ± 0.05*	6.86 ± 0.07*
SOD (µmols/mg protein)	12.25 ± 2.07	10.89 ± 1.3*	9.11 ± 0.33***
CAT (µmols of H_2O_2 degraded/min/mg protein)	10.23 ± 0.98	10.02 ± 0.11	9.27 ± 0.13*

Values **are** expressed as **mean** ± SD. Significant differences between control and KL-treated groups: * $p < 0.05$; ** $p < 0.01$; *** $P<0.001$.

Table 4: Histomorphometric finding in controls and treated groups with KL

Parameters & treatments	Control	Group 2 (126 mg of G /kg/day)	Group 3 (315 mg of G/kg/day)

(BV/TV) (%)	22.11 ± 0.62	17.77 ± 1.77 **	16.75 ±1.86***
(Tb.Th) (µm)	65.16 ± 1.72	57.83 ± 1.94 **	57.35 ± 2.71***
(Tb.N) (mm^{-1})	3.36 ± 0.12	2.95 ± 0.27 ***	2.75 ± 0.43***
(Tb.Sp) (µm)	150.16 ± 1.47	171 ± 8.94 ***	173 ± 9.44 ***
(OS/BS) (%)	1.45 ± 0.18	0.55 ± 0.22 ***	0.39 ± 0.17 ***

Values **are** expressed as **mean** ± SD. Significant differences between control and KL-treated groups: ** $p < 0.01$; *** $P<0.001$.