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ORIGINAL ARTICLE

Decreased femoral diaphyseal mechanical strength mainly due to qualitative impairment of cortical tissue in growing rats with stress erythropoiesis

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Abstract Stress erythropoiesis (SE) can be defined as a state of increased red cell production in response to an enhanced secretion of erythropoietin. Under normal conditions, erythropoiesis in the adult occurs in the erythropoietic tissue that is confined to the bone marrow within the skeleton. Hypertrophy of the erythropoietic marrow that occurs during SE must be accommodated in a larger space. The resulting expansion of the marrow space may induce bone resorption and could alter the biomechanical performance of bone. The present study was designed to estimate the effect of sustained SE on diaphyseal structure and biomechanics of rat femur by mechanically testing the diaphyseal stiffness and strength and calculating some indicators of bone material properties. Female Sprague–Dawley rats weighing 100.0±5.2 g were divided in a control (C) and an experimental (E) group. E rats were biweekly injected with 60 mg/kg of phenylhydrazyne during 6 weeks to induce a haemolytic state. SE, in response to haemolysis, was estimated by reticulocyte count and erythrokinetic techniques, which were markedly increased. To assess bone mechanical properties, the right femur was tested in three-point bending test. Sections of the left femur were stained with haematoxylin–eosin. Body growth was not altered by treatment. Diaphyseal bone mass (CSA) was 13% lower in E than in C rats, while the cross-sectional bending moment of inertia (xCSMI) was significantly higher. The "load capacity" extrinsic properties of the femoral diaphysis were about 40% decreased in E rats when compared to C ones, while the bone material quality indicators (elastic modulus and yield stress of cortical bone tissue) were 54 and 38% lower, respectively. The histologic sections of E femora exhibited a marked thinning of cortical bone and the presence of woven bone in the medullary compartment. The results obtained in this study indicate that SE caused the impairment of the diaphyseal bone material properties (elastic modulus and elastic stress) but enhanced xCSMI over the control values. Neither this improvement in diaphyseal cross-sectional design nor the formation of woven bone could offset the impairment in the bone material stiffness. It is thus proposed that the biomechanical impact of SE upon the whole-bone stiffness should have been more determined by the negative effect on bone material quality than by the negative changes in the cortical area.

Keywords Bone biomechanics · Haemolysis · Erythropoiesis · Stress erythropoiesis · Erythrokinetics

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Introduction

Stress erythropoiesis can be defined as a state of increased red cell production in response to an enhanced rate of

synthesis and secretion of erythropoietin (Bozzini et al. 1994), the renal hormone that can be considered as a part of a feedback system that has evolved to adjust the volume of the circulating red cell mass to the tissue oxygen demand (Jelkmann 1992; Koury 2005).

Under normal conditions, erythropoiesis in the adult occurs in the erythropoietic tissue that is confined to the bone marrow within the skeleton. Its topographic distribution can be estimated by making use of in vivo labelling of the marrow compartment with radioactive iron (Van Dyke et al. 1964). The methods are (a) scanning subjects with a whole-body scanner using ^{52}Fe or ^{59}Fe (Anger 1953); (b) taking scintiphotos with the positron scintillation camera using ^{52}Fe (Anger 1963); and (c) using a well counter and ^{59}Fe for assaying individual bones of small animals. Studies performed on the normal rat have shown that the erythropoietic marrow is concentrated in the spine, pelvis and proximal portion of the legs, with relatively little in the distal portion of the legs or the tail (Van Dyke et al. 1964; Bozzini 1965; Bozzini et al. 1974).

Hypertrophy of the erythropoietic marrow due to increased erythroid cell precursors occurs during stress erythropoiesis (Hara and Ogawa 1976), which undoubtedly must be accommodated in a larger space. This erythropoietic reserve space may be gained at the expense of scattered fat cells within the predominantly active red marrow in those species with greater haematopoietic reserve or, alternatively, the available marrow space may be expanded through bone reabsorption (Yoffey 1966; Giuliani et al. 1986). As an example of the latter proposal, increased marrow erythropoiesis in patients with thalassemia syndromes results in the expansion of bone marrow cavities, thinning of cortical bone and, consequently, decreased bone tissues leading to osteoporosis (Mahachoklertwattana et al. 2006).

The *stiffness* (measurable as a load/deformation ratio) and *strength* (assessable as the bone's ability to support load) of a bone (structural properties) are directly related to both its structural geometry and to the determinants of its material strength ("geometric" and "material" properties, respectively) such as the quality and arrangement of its microstructural elements, the mineral density and porosity and other less well-known elements.

Mechanically, the efficiency of a long bone's design depends on both the mass of the diaphyseal compacta and of the distribution of the material around a reference axis (moment of inertia of the cross-section).

The above considerations prompted us to investigate in rats the effects of a pharmacologically induced haemolytic state, which produces an intense stimulation of the rate of erythropoiesis, on the diaphyseal structure and biomechanics of rat femurs by mechanically testing the diaphyseal stiffness and strength and calculating some indicators of bone material properties.

Materials and methods

Female Sprague–Dawley rats weighing 100.0±5.2 g at the onset of the study were divided in two equal groups of ten animals each: control (C) and experimental (E). E rats were biweekly injected subcutaneously with 60 mg/kg of a 2.5% neutralised solution of phenylhydrazyne (PHZ) during 6 weeks to induce a haemolytic state (Erslev and Silver 1975). C rats received saline. Animals were provided with food and water ad libitum. Body weight and food consumption were registered every other day during the experimental period. Other two similar groups of seven animals each were used for haematological determinations. The haematocrit value was determined periodically to assess indirectly the degree of anaemia. Reticulocyte count and erythrokinetic techniques were used to measure the degree of the erythropoietic stimulation. Specifically, iron incorporated into newly formed red cells and the amount of iron present in plasma were measured by injecting 0.2 μCi of ^{59}Fe into a lateral tail vein and determining radioactivity in both blood and plasma samples 3 h later (Bozzini et al. 1970). The blood volume value used for calculation of radioiron utilisation studies was 5% of body weight (Erslev and Silver 1975).

Animals were killed by ether overdose at the end of the experimental period. The femurs were removed, cleaned of adhering soft tissue, weighed and stored at −20°C wrapped in gauze soaked with Ringer's solution in sealed plastic bags. According to Turner and Burr (1993), this is the best method for the long-term preservation of bone samples before testing, causing no change neither in the bending properties of the bone (Sedlin and Hirsch 1986) nor in the Young's modulus (Ashman 1982). Each bone was thawed at room temperature before analysis. Femur growth was estimated directly by taking measurements through the use of digital callipers. To assess cortical bone mechanical properties, the right femur was tested in three-point bending (Hogan et al. 1999). Each bone was placed on two lower supports (11–13 mm span) and central along its length. Load was applied transversally to the bone axis until fracture. The direction of loading corresponded to that assumed to stress bone physiologically in vivo. The test machine (Instron model 4442, Instron, Canton, MA, USA) was operated in stroke control at a rate of 5.00 mm/min. Load/deflection curves showing both the elastic (Hookean behaviour) and plastic (non-Hookean behaviour), separated by the yielding point, enabled graphic determination of the following structural mechanical properties that refer to the whole bone, and thus, reflect the combined effect of bone tissue and shape in addition to tissue material properties (Turner and Burr 1993): (a) *Stiffness* (N/mm), the slope of the force/displacement curve in the linear region of elastic behaviour was calculated from the best-fit linear regression; (b) *Elastic limit* (load at the yielding point; N), the value of the force at the upper extent of the linear

region; (c) *Ultimate load* (load at fracture; N), the value of the force at fracture; and (d) *Elastic absorbing capacity* (EAC; N/mm), the total energy absorbed by the specimen up to the yielding point was calculated as the area under the force/displacement curve.

A representative indicator of bone material properties, the intrinsic bending stiffness of the cortical tissue [*Elastic modulus* (GN m^{-2})] was calculated as the slope of the stress/strain curve. This procedure does not allow an accurate description of bone material properties, but is regarded as suitable for comparative purposes. An estimation of tissue strength was calculated as the *maximum elastic stress* (σ, N m^{-2}), an intensive property of bone material which express the stress in the material when it first begins to sustain permanent deformation.

Using an Isomet low-speed diamond saw (Buehler, Lake Bluff, IL) a 2-mm cross-section slide was cut from the fracture section to perform regularised micromorphometrical determinations of the horizontal and vertical external and internal diameters of the elliptic-shaped fracture sections. This procedure enabled calculation of the amount of diaphyseal bone mass (CSA) and the cross-sectional bending moment of inertia (xCSMI) of the cortical bone fracture section in relation to the horizontal axis (Ferretti et al. 1985).

The left femur was fixed in 10% neutral formalin, decalcified in ethylenediamine tetraacetic acid and processed to be embedded in paraffin. Oriented sections were stained with haematoxylin–eosin.

Results were summarised as means±SD and were considered statistically significant at the level of $P<0.05$. Comparisons between parameters were performed by either Student's *t* test or one-way analysis of variance using GraphPad prism software (GraphPad Software, San Diego, CA, USA).

Results

Mean±SD body weight was 198.81±16.00 and 200.26±16.67 g in C and E rats, respectively, at the end of the experimental period ($P>0.05$). C rats consumed 440.88±64.37 g/100 g body weight, while E rats consumed 428.10±54.97 g/100 g body weight of food ($P>0.05$) during the entire study period. Femur length at autopsy did not significantly differ between C and E rats (30.09±0.95 vs 29.48±1.27 mm, respectively). Body length (nose to ramp) was also not affected by treatment (C=21.34±0.92 cm; E=20.88±1.10 cm). Figure 1a charts the mean haematocrit of rats given biweekly injections of phenylhydrazyne. Injections induced episodes of haemolysis that were revealed by changes in the haematocrit value. Treatment also induced a marked stimulation of red cell production, as revealed by a more rapid clearance of ^{59}Fe from the plasma compartment and a higher utilisation of the isotope in the synthesis of

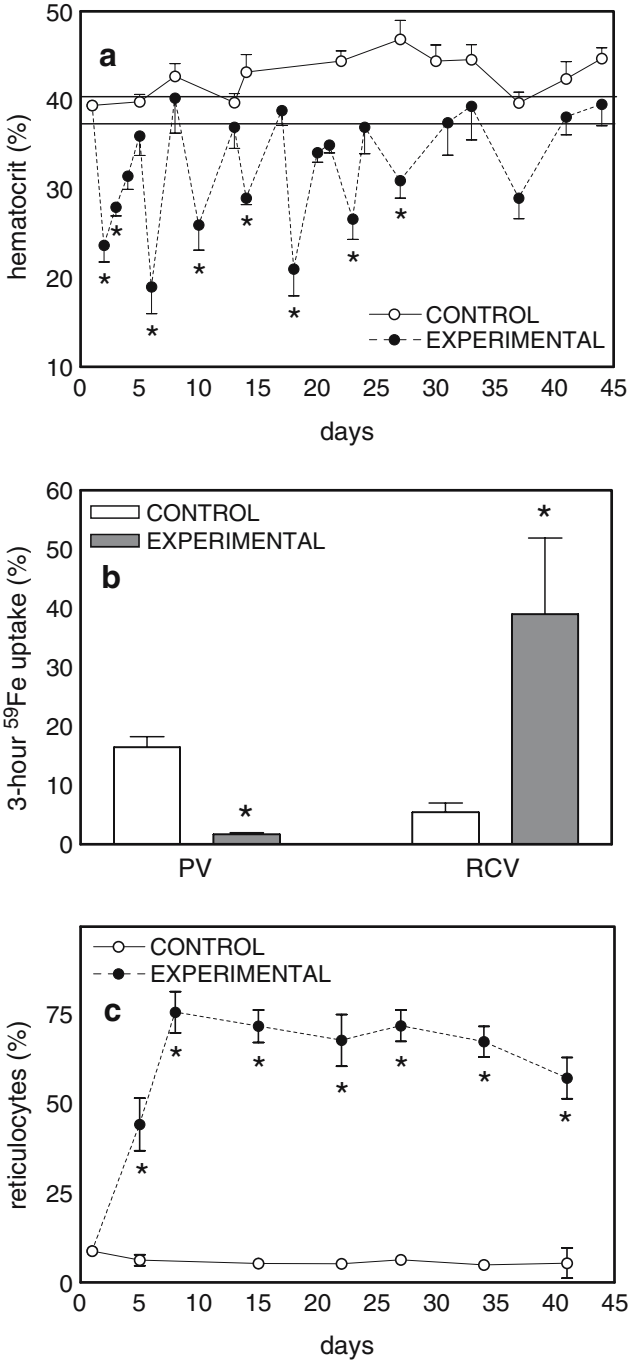

Fig. 1 Haematological parameters in rats with either normal (control) or stress (experimental) erythropoiesis induced by phenylhydrazyne administration. **a** Changes in haematocrit in rats (n=7) during phenylhydrazyne-induced episodes of haemolysis. Biweekly subcutaneous injections of 60 mg/kg of the drug. Values are means±SD. **b** ^{59}Fe (percent of dose) present in both the plasma volume (*PV*) and the total circulating red cell volume (*RCV*) measured at the end of the experimental period. Values are means±SD of seven rats. Values significantly different from control values: *$p<0.05$. **c** Changes in reticulocyte count in rats (n=7) with time during biweekly injections of 60 mg/kg of phenylhydrazyne. Values are means±SD. Values significantly different from control values: *$p<0.05$

haemoglobin in newly formed red cells (Fig. 1b), as well as by an intense reticulocytosis (Fig. 1c).

Changes in cross-sectional geometry of the femur diaphysis are summarised in Table 1. CSA was about 13% lower ($P<0.05$) in E than in C rats. In contrast to this, the CSMI for the PHZ-treated group was significantly higher than the control value. More insights into the details of the cross-sectional geometry results can be gained by considering the outside diameters (OD) and inside diameter (ID) values. Both the horizontal and vertical OD of the E group were significantly higher than those of the C group. The ID results followed a similar but more dramatic trend. The wall/lumen ratio, or cortical wall thickness, was about 53% less in E than in C rats.

The "load capacity" extrinsic properties of the femoral diaphysis are also shown in Table 1. All of them were about 40% decreased in E rats when compared to C ones. The bone material quality indicators, or pre-yield bending stiffness (elastic modulus, E and yield stress of cortical bone tissue, σ), are also shown in Table 1. They were 54 and 38% (respectively) less in E than in C rats.

Data from all the animals studied followed the normally negative association between the modelling-dependent cross-sectional architecture (xCSMI) and bone material quality indicators (elastic modulus, E).

The histologic sections of experimental femora exhibited altered architecture in the diaphyseal zone. The most striking finding was a marked thinning of cortical bone and the presence of woven bone in the medullary compartment.

Discussion

The development of the positron camera more than 40 years ago (Anger 1953) has made it possible to record the distribution of erythropoietic marrow within the skeleton in human subjects and laboratory animals using ^{52}Fe as a positron emisor. As mentioned before, studies performed on the normal rat have shown that the erythropoietic marrow is concentrated in the spine, pelvis and proximal portion of the legs, with relatively little in the distal portion of the legs or the tail (Van Dyke et al. 1964; Bozzini 1965; Bozzini et al. 1974). In humans, when the marrow is called upon to produce red cells at a rate somewhat in excess of normal, hypertrophy of the erythroid marrow occurs at the expense of fat within those areas of the skeleton that contain marrow normally (Van Dyke and Anger 1965). Under such circumstances the gross distribution of erythroid marrow remains normal. When the need for hypertrophy is greater than can be accommodated by replacement of fat, marrow expands into the bones of the extremities. However, this is not the case for the rat. In severely anemic animals, positron pictures indicate no considerable redistribution of marrow within the skeleton during the erythropoietic response (Van Dyke et al. 1964; Bozzini et al. 1974). Therefore, increased density of erythroid elements in the marrow must result in the expansion of bone marrow cavities and consequently decreased bone tissues. The femur of the rat contains red marrow along its entire length, with concentrations at the proximal and distal ends (LoBue et al. 1957): 55 to 65% of the marrow is located in the diaphysis, the remainder being in the epiphyseal region. The bone seems thus appropriate to evaluate the real effect of stress erythropoiesis on its biomechanical properties.

In the present study, stress erythropoiesis was caused in rats by a pharmacologically induced haemolytic state which lasted 43 days. The marked increase in the rate of erythropoiesis in response to treatment was clearly demonstrated by the intense reticulocytosis observed and the erythrokinetic profile that characterises the conditions of

Table 1 Femur diaphysis cross-sectional geometry and extrinsic and intrinsic mechanical properties

Cross-sectional geometry	Control	Experimental	*P*
CSA (mm^2)	7.1380±0.82	6.215±0.75	<0.05
CSMI (mm^4)	11.772±2.11	15.464±1.61	<0.01
Wall/lumen ratio	64.350±11.4	30.113±7.41	<0.0001
Horizontal OD (mm)	3.2516±0.14	4.2466±0.23	<0.0001
Horizontal ID (mm)	2.0600±0.11	3.1000±0.22	<0.0001
Vertical OD (mm)	4.4014±0.32	5.0771±0.35	<0.001
Vertical ID (mm)	2.8380±0.17	3.9633±0.32	<0.0001
Mechanical properties			
Extrinsic			
Elastic limit (N)	36.514±7.47	22.950±6.44	<0.005
Ultimate load (N)	84.351±26.60	47.683±19.88	<0.005
Stiffness (N/mm)	207.62±37.86	126.37±27.15	<0.005
EAC (N/mm)	11.518±5.90	7.136±3.29	<0.05
Intrinsic			
E ($GN\ m^{-2}$)	806.00±134.17	373.57±84.70	<0.001
Stress (N/mm^2)	39.7±11.2	24.8±12.9	

All data are expressed as means±SD.
CSA Cross-sectional area, *CSMI* cross-sectional moment of inertia, *OD* outer diameter, *ID* inner diameter, *EAC* energy absorbing capacity, *E* elastic modulus

increased red cell production, namely, a more rapid clearance of ^{59}Fe from the plasma compartment and a higher utilisation of the isotope in the synthesis of haemoglobin into newly formed red cells. This intense erythropoietic response permitted the haematocrit value to reach almost control values after each haemolytic episode induced by PHZ.

The haemolytic state did no alter neither the overall growth of the animals, as derived from the final body weight, nor the longitudinal skeletal growth, as taken from the final body length, which did not differ significantly between C and E groups. Therefore, no allometric adjustment of the bone geometric and structural variables was necessary. The lack of differences between C and E rats in relation to growth parameters could be explained by the fact that both groups had equal caloric intakes during the experimental period. This would preclude a toxic effect of the administered haemolytic drug.

Stress erythropoiesis has been previously shown to expand marrow space in hypoxic rats (Yoffey 1966; Giuliani et al. 1986) and to induce osteoporosis in thalassemic children (Mahachoklertwattana et al. 2006), findings that predict a biomechanical impact on cortical bone. To fully elucidate the effect of stress erythropoiesis on bone biomechanics, the differences between rats with either normal or increased erythropoiesis were examined for both mechanical properties (extrinsic and intrinsic) and cross-sectional geometry in the present study.

The results obtained in this investigation, which showed an important negative impact of stimulated red cell production on femoral stiffness and strength, enabled the discussion of whether the impaired performance of diaphyseal shafts of treated animals was the result of changes in the amount of cortical bone mass or, in addition, variation in bone material properties was another causal factor.

The mechanical properties of cortical bone were decreased at the end of the study in PHZ-treated rats when compared to controls. The "load capacity" extrinsic properties (stiffness, elastic limit and ultimate load) were significantly decreased, as well as the bone ability to elastically absorb energy in deformation. This effect could have resulted from an impairment of any, or both, the material and geometric properties of the cortical bone. It is well known that in cortical bone, thickness, cortical diameter and porosity affect bone strength.

Concerning the first two factors, the xCSMI and diameter results together suggest that the long-term haemolytic state led to greater radial expansion at both the periosteal and endosteal surfaces. The degree to which the E group diameters exceeded the C group was relatively greater for the ID than the OD (+39–50% vs +15–30%), which further suggests that the cortical wall thickness was lower for E rats. Treatment really gave rise to a significant thinner cortical, as evidenced by the wall/lumen ratio (−53%) and histologic examination. In a general sense, results suggest that the "amount" of cortical bone in the femur diaphysis (as reflected by the CSA) was slightly reduced (−13%), but its "arrangement" or shape was different. In other words, the medullary area of PHZ-treated mice was larger than that of the controls. As the medullary area enlarged, the xCSMI also increased. This enlargement was most likely due to periosteal bone formation, which was coupled with increased endosteal bone resorption. Decreased CSA and cortical width may be considered a kind of biomechanical counterpart of the pathophysiological concept of osteopenia. A similar pattern of bone response was observed in mice with sustained granulocytic stimulation induced by long-term treatment with G-CSF (Lee et al. 1991).

The two indicators of cortical bone material properties assessed in this study, namely, the elastic modulus (which reflects the stiffness of bone tissue) and the stress at the yield point (that is an indirect indicator of bone tissue strength), were significantly reduced by treatment.

What appears to be happening in the femoral diaphysis of rats with induced haemolytic state is an important loss of the quality of bone tissue. Increased periosteal, intracortical and endocortical bone formation rate and turnover probably occur and contribute to increased cortical porosity. The bone as a whole tries to compensate for this, without reaching such compensation. More specifically, the femur diaphysis expands radially to create a higher xCSMI. The cortical tissue is distributed to optimise the xCSMI values of the section to adapt this geometric property to the severe reduction in the intrinsic bending stiffness of the cortical bone. Formation of woven bone in the diaphyseal area may be another attempt to compensate for the imbalance in mechanic requirements caused by the thinning of cortical bone. However, the presence of woven bone fails to restore altered biomechanical response, as it cannot serve as a substitute for cortical bone on account of its poor quality.

The results obtained in this study show that the impairment of the diaphyseal bone material stiffness would reflect changes in the linearly elastic bone behaviour that are known to depend largely on the bone material and geometric properties. The pharmacological-induced haemolytic state impaired bone material properties (elastic modulus and elastic stress), but enhanced the xCSMI over the control values. The improvement in diaphyseal cross-sectional design could not offset the impairment in the bone material stiffness. Therefore, it could be proposed that the sign of the biomechanical impact of stress erythropoiesis upon the whole-bone stiffness should have been more determined by the negative effect on bone material quality than by the negative changes in the cortical bone area concerning the kind of deformation.

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