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Myocyte Cellular Hypertrophy and Hyperplasia Contribute to Ventricular Wall Remodeling in Anemia-induced Cardiac Hypertrophy in Rats

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To determine the effects of chronic anemia on the functional and structural characteristics of the heart, 1-month-old male rats were fed a diet deficient in iron and copper, which led to a hemoglobin concentration of 4.63 g/dl, for 8 weeks. At sacrifice, under fentanyl citrate and droperidol anesthesia, systolic, diastolic, and mean arterial blood pressures were decreased, whereas differential pressure was increased. Left ventricular systolic pressure and the ventricular rate of pressure rise (mmHg/s) were reduced by 9% and 14%, respectively. Moreover, developed peak systolic ventricular pressure and maximal dP/dt diminished 14% and 12%. After perfusion fixation of the coronary vasculature and the myocardium, at a left ventricular intracavitary pressure equal to the in vivo ***measured end diastolic pressure, a 10% thickening of the left ventricular wall was measured in association with a 13% increase in the equatorial cavitary diameter and a 44% augmentation in ventricular mass. The 52% hypertrophy of the right ventricle was characterized by an 11% thicker wall and a 37% larger ventricular area. The 33% expansion in the aggregate myocyte volume of the left ventricle was found to be due to a 14% myocyte cellular hypertrophy and a 17% myocyte cellular hyperplasia. These cellular parameters were calculated from the estimation of the number of myocyte nuclei per unit volume of myocardium*** in situ ***and the evaluation of the distribution of nuclei per cell in enzymatically dissociated myocytes. Myocyte cellular hyperplasia provoked a 9% increase in the absolute number of cells across the left ventricular wall. In contrast, myocyte cellular hypertrophy (42%) was responsible for the increase in myocyte volume of the right ventricle. The proliferative response of left ventricular myocytes was not capable of restoring diastolic cell stress, which was enhanced by the changes in ventricular anatomy with anemia. In conclusion, chronic anemia induced an unbalanced load on the left ventricle, which evoked a hyperplastic reaction of preexisting myocytes, in an attempt to normalize diastolic wall and myocyte stress. (Am J Pathol 1992, 141: 227–239)***

A deficient dietary intake of iron and copper induces hypochromic anemia with alterations in the peripheral circulation and in the loading state of the heart.[1] Peripheral vascular resistance typically diminishes since blood viscosity is reduced, and vasodilation of muscular arteries and arterioles takes place.[2] These events are mediated, respectively, by the decrease in number of circulating red blood cells and the impaired oxygen-carrying capacity of the blood.[3,4] Both systolic and diastolic arterial blood pressures decrease but the latter is affected more than the former, leading to an increase in differential pressure. The combination of these circulatory adaptations results in a volume overload of the left and right ventricular myocardium and a reduction in afterload of the left side of the heart.[5] In addition, cardiac output increases because stroke volume is elevated, and heart rate remains essentially constant.[2] Biventricular hypertrophy develops,[6–8] and myocardial dysfunction and failure may become apparent.[9] Moreover, an increased sympathetic activity has been documented by elevated levels of blood and urinary catechcolamines[10] and decreased concentrations of myocardial norepinephrine.[11] This enhanced adrenergic discharge may contribute to the preservation of ventricular dynamics by impinging on the force-generating capacity of the myocardium.[12] How-

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ever, it is unclear whether the changes in ventricular anatomy with anemia are capable of compensating the alterations in the magnitude of stress on the myocardium. If this phenomenon occurred, the elevation in volume load should be expected to generate an increase in chamber diameter accompanied by a proportional thickening of the wall so that the ratio of wall thickness to chamber radius remains constant.[13,14] Importantly, the cellular mechanisms implicated in the myocardial hypertrophic response with anemia are currently unknown. This is a significant issue because the ability to retain normal cardiac performance in severe hypochromic anemia may depend on the possibility of stimulating a proliferative process in myocytes, which minimizes the abnormal loading state at the cellular level. On the other hand, anemia may be associated with myocyte cell loss, which would have the opposite effect of increasing systolic and diastolic stress on the remaining cells, creating a greater potential for ventricular deadaptation and failure. Therefore, the relationship between cellular growth mechanisms and ventricular function, and their interaction on wall remodeling and cell loading were examined biventricularly 8 weeks after nutritional anemia.

Materials and Methods

Animals

Fifty-one male 1-month-old Wistar rats (Morini Laboratory, Reggio Emilia, Italy) were included in the present study. The rats were housed in wire-mesh cages and maintained on a 12-hour dark-light cycle. All animals were coded and divided into two groups: 29 animals were fed an iron- and copper-deficient diet (anemic) and 22 were exposed to a standard pellet rat chow (control). All rats were sacrificed after 8 weeks.

Diet Composition

Diets were purchased from Piccioni Laboratories (Milan, Italy). The iron- and copper-deficient diet contained 15% protein, 8.5% fat, 73% carbohydrate, 9 ppm of iron, and 6 ppm of copper. The standard diet consisted of 21% protein, 4.8% fat, 61.5% carbohydrate, 200 ppm of iron, and 30 ppm of copper.

Hematologic Profile

Blood (100 μl) was collected on a weekly basis, by puncture of the tail vein. Red blood cell counts (RBC), concentration of blood hemoglobin (Hb), and mean corpuscular volume (MCV) were determined by an automatic analyzer (Coulter Electronics, Hialeah, FL), whereas packed cell volume (PCV) was measured by the microhematocrit method using microcapillaries centrifuged at 14,000*g* (Drumond Scientific centrifuge, Broomall, PA).

Hemodynamic Measurements

Under intraperitoneal anesthesia with fentanyl citrate (5 μg/kg bw) and droperidol (250 μg/kg bw) (Leptofen, Farmitalia Carlo Erba Laboratories, Milan, Italy), the right carotid artery was exposed and cannulated with a microtip pressure transducer (model PR249, Millar Instruments, Houston, TX) while the right jugular vein was cannulated with a PE 50 catheter (Clay Adams, Parsippany, NJ) connected to a P23ID Gould Statham pressure transducer (Gould Inc., Cleveland, OH). Both transducers were connected to a Gould ES1000B electrostatic chart recorder and systemic and venous pressures were monitored and recorded. Mean arterial pressure was obtained from an averaging differential amplifier circuit. The catheters were then advanced into the left and right ventricular chambers, respectively. Left and right peak systolic and end diastolic ventricular pressures were determined. Moreover, left ventricular peak positive dP/dt was evaluated. All these hemodynamic parameters were obtained in the closed-chest preparation. After tracheostomy and mechanical ventilation, a midsternal thoracotomy was performed to expose the ascending aorta and the pulmonary artery. A suture was placed around each vessel, and maximal left and right peak systolic ventricular pressures were obtained by occluding each vessel for 3 seconds.[15] Left ventricular dP/dt was also collected under these conditions.

Fixation Procedure

At the completion of the hemodynamic measurements, the abdomen was opened and the aorta, below the renal arteries cannulated with a polyethylene catheter (PE 200), was connected to a perfusion apparatus. In rapid succession, the heart was arrested in diastole by injecting through the jugular vein approximately 1 ml of cadmium chloride (100 mmol/l), the chest was opened, the right atrium was cut, and the coronary vasculature perfused for 3 minutes with phosphate buffer at a pressure equal to the measured mean arterial pressure. The coronary bed was then fixed by perfusion for 15 minutes with a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde. The left ventricular chamber was filled with fixative and kept at a pressure equal to the *in vivo* measured end diastolic pressure throughout the fixation procedure.[16]

The heart and great vessels were excised. The weights of the right ventricle and the left ventricle inclusive of the septum were recorded, and ventricular volumes were computed by dividing ventricular weight by the specific gravity of muscle tissue, 1.06 g/ml.[17]

Ventricular Size and Wall Thickness

The major intracavitary axis of the left ventricle from apex to base was measured. Subsequently, two adjacent sections, midway between the apex and the base and perpendicular to the longitudinal axis of the ventricle, were obtained and used to measure transverse chamber luminal diameter and the thickness of the free wall. Five equally spaced measurements of the free wall, from the endocardium to the epicardium, were collected from each slice, and their values were averaged. The minimal and maximal equatorial diameters of the left ventricular chamber were similarly determined, and their geometric mean value was computed. These determinations were done at X16 magnification using a dissecting microscope with an ocular micrometer accurate to 0.05 mm. Wall thickness measurements were similarly obtained in the right ventricle. Right ventricular wall area was computed by dividing ventricular volume by wall thickness.[8] This calculation assumes that the ventricular wall may be treated as a thin sheet, and the changes in this value can be interpreted as a index of the variations in ventricular chamber dimensions. The apical slices of both ventricles were used for dry weight determinations.

Light Microscopic Morphometry

For the quantitative analysis of the myocardium, 20 specimens of the right and left ventricles from each heart were postfixed in osmium tetroxide and embedded in araldite. Ten randomly chosen tissue blocks from each ventricle were sectioned at a thickness of 0.75 μm and stained with methylene blue and safranin. Morphometric sampling at a magnification of x1000 consisted of counting the number of myocyte nuclear profiles, N(n), in a measured area, A, of tissue sections in which cardiac muscle fibers were cut transversely. A square tissue area of 10,036 μm^2 was delineated in the microscopic field by an ocular reticle containing 42 sampling points (≠105844, Wild Heerbrugg, Switzerland), and a total of 30 such fields were evaluated in each ventricle of each animal to determine the number of nuclear profiles per unit area of myocardium, N(n)A.[18]

Average nuclear length, Dn, was determined in each ventricle from 50 measurements made at a magnification of x1250 in longitudinally oriented myocytes viewed with a microscope with an ocular micrometer accurate to 0.5 μm. Five blocks from each ventricle, with myofibers sectioned perpendicularly to their length to avoid longitudinal compression, were cut. Sections approximately 2.0 μm in thickness were collected and stained, and 10 measurements of nuclear length were recorded from each tissue section. Only those nuclei in which the nuclear envelope was sharply defined at both ends and the clusters of mitochondria were clearly visible in the areas adjacent to the nuclear edges were measured.[19]

From the estimation of N(n)A and Dn, the number of myocyte nuclei per unit volume of myocardium, N(n)V, was computed using the equation:[18]

$$N(n)_V = N(n)_A / Dn \quad (1)$$

The aggregate number of myocyte nuclei in each ventricle, N(n)T, was then derived from the product of the number of myocyte nuclei per unit volume, N(n)V, and the total ventricular volume, VT:

$$N(n)_T = N(n)_V \times V_T \quad (2)$$

Mean myocyte cell volume per nucleus, $V(m)_n$, was obtained from the volume fraction of myocytes, V(m)V, divided by the number of myocyte nuclei per unit volume of myocardium, N(n)V:

$$V(m)_n = V(m)_V / N(n)_V \quad (3)$$

Electron Microscopic Morphometry

In each animal, 10 blocks from each ventricle with myofibers oriented in the transverse direction were thin sectioned for electron microscopy. Low-power electron micrographs of transverse sections of myocardium, seven from each tissue block, were collected and printed at ×5000 magnification. These micrographs were analyzed morphometrically with a superimposed grid consisting of 140 sampling points and 14 test-line segments, each 150 mm long.

The volume fraction of myocardial components was measured in 3920 low-power micrographs, by counting the fraction of sampling points overlying myocytes, capillaries, and other interstitial structures. The number of myocyte profiles in the sampled area were counted following the criteria described by Gundersen[20] to estimate their numerical density, N(m)A, and cross sectional area.[18]

Length density, length per unit volume of myocytes L(m)V, is numerically equal to their measured numerical density, number per unit area, in transverse myocardial sections:[18]

$$L(m)_V = N(m)_A \quad (4)$$

Therefore, the aggregate length of myocytes in the ventricles, TL(m), was measured from the product of total ventricular myocardial volume, VT, and the length of myocytes per unit volume, L(m)V:

$$TL(m) = L(m)_V \times V_T \quad (5)$$

The average number of myocyte profiles across the ventricular wall, $N(m)_{tm}$, i.e., the number of myocytes that would be traversed by a thin transmural probe inserted perpendicular to the surface of the ventricle, was determined from their numerical density, N(m)A, and previously determined wall thickness, W. This calculation assumes that myocytes tend to be arranged in a hexagonal pattern:[21]

$$N(m)_{tm} = 1.0243\,W \times \sqrt{N(m)_A} \quad (6)$$

An additional sampling of five random fields representative of myocyte cytoplasm were collected from each tissue block and these micrographs were printed at a final magnification of ×40,000 to determine the volume fractions of myofibrils and mitochondria. Two tissue blocks from each ventricle with myofibers longitudinally oriented were used to determine sarcomere length. Five random fields from each of the two tissue blocks were obtained and printed at a final magnification of ×20,000. Mean sarcomere length in myofibrils was determined from 100 measurements in each ventricle.

Diastolic (σD) and Systolic (σS) Midwall Circumferential Stress

Diastolic stress was computed using the Laplace equation,[13]

$$\sigma_D = p \times r/2W \quad (7)$$

where p, r, and W are the diastolic values of ventricular pressure, chamber radius and wall thickness, respectively. In a similar manner, systolic midwall circumferential stress was computed, using the approach previously described[22] and the equation:

$$\sigma_S = p_s \times r_s/2W_s \quad (8)$$

where p_s, r_s, and W_s are the systolic values of ventricular pressure, chamber radius, and wall thickness.

The knowledge of ventricular systolic and diastolic pressures, chamber diameter, and wall thickness in diastole and systole allows a further analysis of diastolic stress in terms of its distributions across the ventricular wall.[16]

$$\sigma_T = \frac{p \times a^3\, 1 + (b^3)/2 \times (R)^3}{b^3 - a^3} \quad (9)$$

where p corresponds to ventricular pressure, R is the radial coordinate, and a and b are the inner and outer radii, respectively.

It should be pointed out, however, that R can be substituted by the number of cells across the wall, N(m)tm, which describes increments in wall thickness, from the endocardium to the epicardium. Thus, equation 10 was integrated with this information to yield stress distributions at the cellular level of organization:

$$\sigma_T = \frac{p \times a^3\, 1 + (b^3)/2 \times [a + (W/N(m)tm \times Cn)]^3}{b^3 - a^3} \quad (10)$$

where W is the ventricular wall thickness, and Cn is the cell layer to be examined with the endocardial surface = O and the epicardial surface = $N(m)_{tm}$. The same procedure was employed for the calculation of the distribution of systolic stress across the wall.

Preparation of Isolated Myocytes

Ten controls and 13 anemic rats were used. Animals were heparinized and killed by cervical dislocation, and the hearts were rapidly removed and immediately washed in ice-cold oxygenated perfusion medium. The aorta was then connected to a stainless-steel needle, and the myocardium was initially perfused in a Lagendorff apparatus at 37°C with an oxygenated calcium-free Krebs-Ringer solution with the addition of 0.8 mmol/l Mg^{++}, at a constant flow of 8 ml/min, to clear the vasculature of blood. Collagenase (0.5% Collagenase type IA, Sigma, St Louis, MO) and 50 μmol/l Ca^{++} were added to the perfusate until the heart became flaccid (20–40 minutes). The left ventricle inclusive of the septum was dissected from the right ventricle, and both ventricles were weighed and minced in the same perfusion medium. The fragments of each ventricle were passed through 400-μm nylon mesh, and the myocytes were isolated by gravity. The supernatant was removed, and the isolated cells were washed twice with collagenase-free Krebs-Ringer solution.

The suspension was adjusted to contain approximately 10–20 × 10^4 cells/ml and applied to a Cytospin 2 centrifuge (Shandon Southern Products, Runcorn, WA) to obtain four monolayers of myocytes from each ventricle. The slides were finally stained with the Romanowsky method. The percentage of rod-shaped isolated cells in the final preparation varied from 65% to 80%. The distribution of myocyte nuclei was counted at a magnification of ×400, and the percentages of nuclei associated with mononucleated and multinucleated cells were mea-

sured. These values in combination with the aggregate number of myocyte nuclei in each ventricle were used to estimate the total number of mononucleated and multinucleated cells in the ventricles.[23]

Data Collection and Statistical Analysis

All morphometric data were collected blindly, and the code was broken at the end of the experiment. Results are expressed as mean ± SD of values determined for the individual animals in each group. Statistical significance was determined using the unpaired two-tailed Student's *t* test. *P* values of less than 0.05 were considered to be significant.

Results

The effects of the iron- and copper-deficient diet on body weight, packed cell volume, blood hemoglobin content, and mean red blood cell volume during the onset and evolution of the anemic state have previously been described up to 7 weeks after the administration of the modified diet.[8] Moreover, the potential differences between a standard rat laboratory diet and a diet supplemented with iron and copper on hematologic profile and gross cardiac characteristics have been examined.[8] Since the addition of iron and copper to the anemic diet resulted in a full preservation of hematologic parameters and ventricular properties, the experimentation in this investigation included only one type of control animals, which were fed a standard laboratory rat chow. In addition, data describing the anemic condition refer exclusively to measurements made at sacrifice. Finally, blood viscosity was not established because it was shown to be decreased by 40% at 7 weeks.[8]

Blood Properties and Arterial Blood Pressure

Table 1 shows that body weight gain was affected by the experimental protocol, −15%. The concentration of blood hemoglobin was reduced by 67%, 8 weeks after the initiation of the anemic dietary regimen. Similarly, PCV decreased 58%, whereas MCV was diminished 24%. The number of RBC per mm^3 of blood was seen to be decreased 43%. These alterations in the hematologic profile were accompanied by 13%, 27%, and 21% reductions in systolic, diastolic, and mean arterial blood pressure, respectively. The greater effect of anemia on diastolic pressure resulted in a 28% increase in differential blood pressure.

In summary, severe hypochromic microcytic anemia increased the preload and decreased the afterload of the heart.

Ventricular Hemodynamics

Measurements of global cardiac function indicated that systolic ventricular pressure decreased by 9% ($P < 0.05$) in the left ventricle (control = 118 ± 12 mm Hg; experimental: 107 ± 12 mm Hg) while this parameter was unaffected in the right ventricle (control = 33 ± 3 mm Hg; experimental = 32 ± 3 mm Hg). End diastolic pressure remained constant in both ventricles (LV: control = 2.33 ± 0.62 mm Hg, experimental = 3 ± 0.87 mm Hg; RV; control = 2.7 ± 1.1 mm Hg, experimental = 1.83 ± 1.34 mm Hg). Peak-developed systolic pressure was reduced only in the left ventricle, −14% ($P < 0.001$) (control = 192 ± 8 mm Hg; experimental = 165 ± 23 mm Hg. Moreover, baseline left ventricular dP/dt diminished by 14% ($P < 0.005$), from 8569 ± 1115 mm Hg/sec in controls to 7403 ± 1512 mm Hg/sec in experimental animals, whereas maximal dP/dt decreased by 12% ($P < 0.05$),

Table 1. *Effects of Anemia on Body Weight, Hematologic Characteristics, and Arterial Pressure*

	Control	Anemia	% Difference
Body weight (g)	344 ± 22	292 ± 34	−15*
Hematologic parameters			
Hemoglobin (g/dl)	13.9 ± 2.1	4.6 ± 0.8	−67*
Packed cell volume (%)	43.4 ± 2.1	18.3 ± 3.0	−58*
Red blood cell count ($\times 10^6/mm^3$)	7.6 ± 0.6	4.3 ± 1.0	−43*
Mean cell volume (fl)	52.8 ± 1.7	40.1 ± 1.7	−24*
Arterial pressures (mm Hg)			
Systolic	110 ± 7.0	96 ± 8.0	−13*
Diastolic	81 ± 2.0	59 ± 8.0	−27*
MAP	91 ± 3.0	72 ± 7.0	−21*
Differential	29 ± 6.0	37 ± 9.0	28**

*,** Indicate values that are statistically significant, $P < 0.0001$ and $P < 0.02$, respectively.
Results are presented as mean ± SD. MAP, mean arterial pressure.

from 16,197 ± 1,597 mm Hg/sec to 14,260 ± 2586 mm Hg/sec. Central venous pressure was similar in controls, 2.3 ± 1.2 mm Hg, and anemic rats, 2.4 ± 1.9 mm Hg.

In summary, chronic anemia did not significantly affect cardiac performance, biventricularly.

Cardiac Weights, Chamber Volume, and Ventricular Thickness

Figure 1 demonstrates that heart weight increased by 46% ($P < 0.0001$) as a result of anemia (Figure 1A), and this overall hypertrophic response was due to a 44% ($P < 0.0001$) and a 52% ($P < 0.0001$) augmentation of the left (Figure 1B) and right ventricle (Figure 1C), respectively. Moreover, the ratios of left ventricular weight and right ventricular weight to body weight increased by 70% ($P < 0.0001$) and 79% ($P < 0.0001$) (data not shown). Measurements of the ratio of tissue dry weight to fixed tissue wet weight were practically identical in control and anemic rats (control: left ventricle = 0.192 ± 0.022, right ventricle 0.194 ± 0.021; anemic: left ventricle = 0.196 ± 0.022, right ventricle 0.191 ± 0.021.

Figure 2 illustrates the effects of anemia on left ventricular cavitary dimensions. Chamber diameter at the equatorial region (Figure 2A), halfway between the apex and the base of the heart, was found to be increased by 13% ($P < 0.001$). Moreover, the major intracavitary axis from apex to base (Figure 2B) expanded by 14% ($P < 0.005$). The combination of these changes in cavitary diameters resulted in a 46% ($P < 0.0001$) enlargement in cavitary volume (Figure 2C). Wall thickness (Figure 2D) at the equator increased by 10% ($P < 0.0001$).

An index of right ventricular dimension was derived from the computation of the changes in wall area.[14,26] Wall area in the right ventricle was found to be expanded by 37% ($P < 0.0001$), from a value of 244 ± 40 mm^2 in controls to a value of 334 ± 57 mm^2 in anemic rats. As explained in the methods section, this computation was used as an approximation of right ventricular chamber size in view of the difficulty in assessing chamber volume directly in this ventricle. Moreover, right ventricular wall thickness increased by 11% ($P < 0.05$) with anemia: control = 0.93 ± 0.08 mm; experimental = 1.03 ± 0.12 mm.

In summary, biventricular hypertrophy with chamber dilation occurred with anemia.

Ventricular Wall Stress

As a result of chamber dilation, circumferential midwall diastolic stress augmented by 30% in anemic rats from a value of 26.10 ± 7.31 dynes/mm^2 in controls to a value of

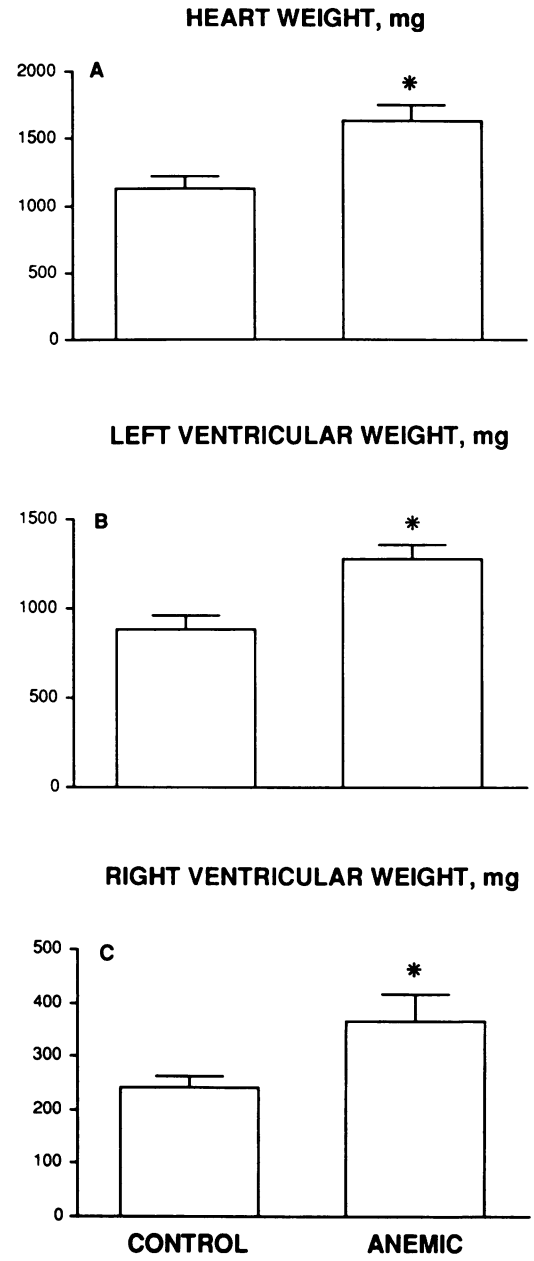

Figure 1. *Bar graph showing the changes in cardiac weights. Values represent mean ± SD. *Indicates a change that is statistically significant different from control value,* $P < 0.05$.

33.80 ± 9.28 dynes/mm^2 in experimental animals, and this elevation was statistically significant ($P < 0.05$). In contrast, the reduction in left ventricular systolic pressure in combination with the thickening of the wall were capable of maintaining systolic stress within control values, counteracting the expansion in cavitary dimension (control = 935 ± 92 dynes/mm^2; experimental = 862 ± 102 dynes/mm^2).

By employing the calculation of right ventricular wall area as an approximation of ventricular chamber size,

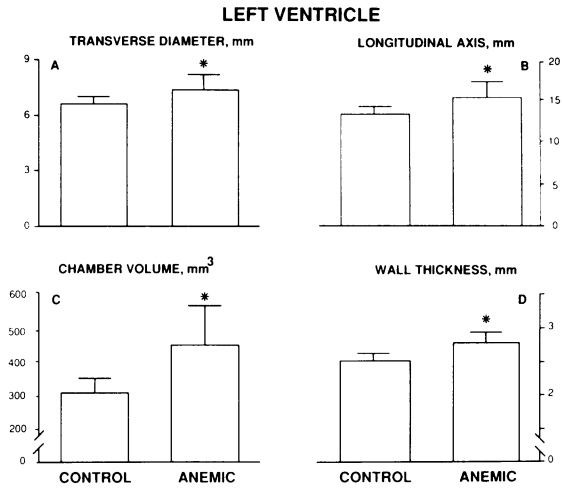

Figure 2. *Bar graph showing the changes in the dimensional characteristics of the left ventricle. See legend to Figure 1.*

cavitary diameters of control and experimental animals were derived. A 17% increase in chamber diameter could be deduced after anemia. Moreover, right ventricular wall thickness increased by 11%, whereas end diastolic ventricular pressure was reduced by 31%. The interaction of these variables led to a decrease in diastolic wall stress on the right side of the heart in spite of ventricular dilation. Similarly, systolic wall stress remained substantially constant in the right ventricle.

In summary, chronic anemia resulted in an elevated left ventricular midwall circumferential diastolic stress.

Myocyte Cell Volume per Nucleus

Table 2 lists the primary data employed for the estimation of myocyte cell volume per nucleus in the left and right ventricular myocardium. The numerical density of myocyte nuclei per mm^2 of tissue decreased significantly in the left and right ventricles of anemic rats. Nuclear length, however, was reduced in the left ventricle only. These changes resulted in a 19% and a 34% reduction in the number of myocyte nuclei per mm^3 of myocardium in the left and right side of the heart, respectively. Moreover, the volume fraction of myocytes in the tissue decreased by 8% and 6% in the left and right ventricles.

Figure 3 illustrates that myocyte cell volume per nucleus (Figure 3A) increased by 14% ($P < 0.0001$) in the left and 42% ($P < 0.0001$) in the right ventricle after anemia. The volume augmentation in left ventricular myocytes was the result of a 5% (NS) decrease in mean cross sectional area (Figure 3B) and a 19% ($P < 0.0001$) increase in average myocyte length per nucleus (Figure 3C). Myocyte cross-sectional area (Figure 3B) and myocyte length per nucleus (Figure 3C) increased by 18% ($P < 0.0001$) and 20% ($P < 0.005$) in right ventricular cells.

Myocyte hypertrophy was accompanied by variations in the surface to volume ratio of these cells. The lateral expansion of myocytes resulted in a 28% ($P < 0.0001$) reduction of this ratio in the right ventricle (control = 329 ± 51 mm^2/mm^3; experimental = 236 ± 32 mm^2/mm^3). The preservation of myocyte diameter in the left myocardium was associated with a constant surface to volume ratio (control = 236 ± 22 mm^2/mm^3; experimental = 224 ± 58 mm^2/mm^3).

In summary, chronic anemia resulted in myocyte hypertrophy, biventricularly. However, the increase in myocyte volume in the right ventricle exceeded that in the left ventricle.

Myocyte Number

Figure 4 illustrates first the total number of myocyte nuclei in the ventricular myocardium of control and experimental animals. This parameter was increased 17% ($P < 0.0001$) in the left ventricle (Figure 4A) of anemic rats. No change was detected in the right ventricle.

From the examination of enzymatically isolated cells, the fractions of nuclei associated with mononucleated and binucleated myocytes was obtained, and their respective values are depicted next in Figure 4. The pro-

Table 2. *Numerical Density of Myocyte Nuclei in the Left and Right Ventricular Myocardium*

	Left ventricle			Right ventricle		
	Control	Anemic	% Difference	Control	Anemic	% Difference
Number of myocyte nuclei per mm^2 of myocardium	542 ± 51	403 ± 32	−26*	667 ± 73	424 ± 75	−36*
Average nuclear length (μm)	13.3 ± 0.8	12.2 ± 0.9	−8**	12.1 ± 0.8	11.6 ± 1.2	−4
Number of myocyte nuclei per mm^3 of myocardium	40,594 ± 2,412	32,931 ± 3,586	−19*	55,077 ± 6,142	36,496 ± 4,637	−34*
Volume % of myocytes	78.7 ± 1.07	72.7 ± 2.85	−8*	77.6 ± 2.29	73.1 ± 1.85	−6*

*,** Indicate values that are statistically significant, $P < 0.0001$ and $P < 0.02$, respectively.
Results are presented as mean ± SD.

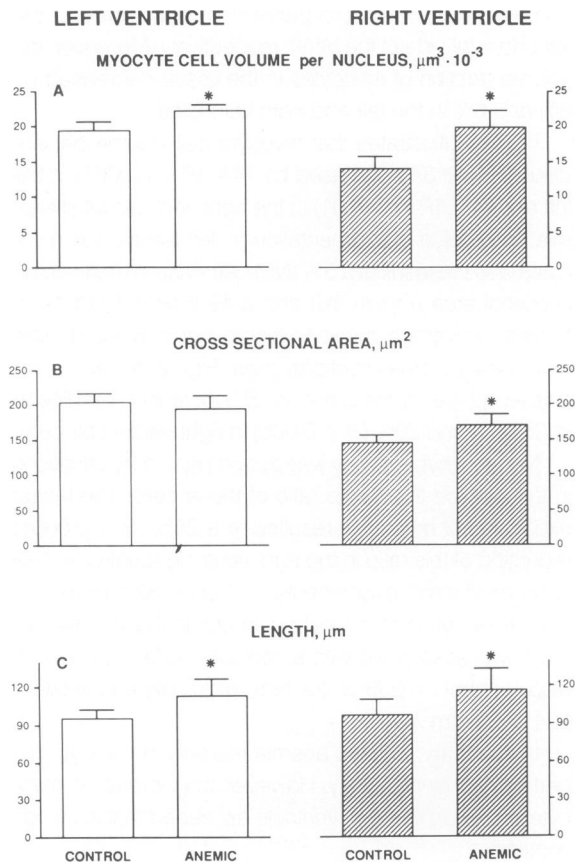

Figure 3. *Bar graph showing the changes in the dimensional properties of myocytes in the left and right ventricles. See legend to Figure 1.*

portion between mononucleated and binucleated cells was seen to remain practically constant in both ventricles of anemic rats (Figure 4B, C). Although not shown, a small fraction of nuclei was observed to be present in trinucleated cells (left ventricle: control = 0.54 ± 0.23%; experimental = 0.68 ± 0.37%; right ventricle: control = 0.56 ± 0.26%; experimental = 0.65 ± 0.60%).

The availability of the number of nuclei per cell and the morphometric estimation of the aggregate number of myocyte nuclei in the ventricular myocardium allowed the derivation of the total number of mononucleated and binucleated myocytes in each ventricle of each animal group (Figure 5). Whereas no variation in the overall number of mononucleated and binucleated myocytes occurred in the right ventricle with anemia, a 17% ($P < 0.0001$) increase in the number of binucleated myocytes (Figure 5B) was found in the left ventricle of experimental rats. Thus, an identical 17% ($P < 0.005$) increase in the total number of myocytes was observed in the left ventricle while the total number of cells in the right ventricle remained essentially constant (Figure 5C).

In summary, chronic anemia led to myocyte cellular hyperplasia in the left ventricle.

Transmural Number of Myocytes and Total Myocyte Length

The morphometric evaluation of the number of myocyte profiles per unit area of myocardium demonstrated that this parameter was substantially unchanged in the left myocardium of anemic rats (control = 3,822 ± 201/mm^2; experimental = 3,733 ± 347/mm^2), whereas a 20% ($P < 0.0001$) decrease was measured in the right myocardium (control = 5,347 ± 447/mm^2; experimental = 4,257 ± 345/mm^2). These values of myocyte numerical density were used to derive the number of myocytes across the ventricular wall, according to equation 6 in the Methods section. This calculation showed a 9% ($P < 0.005$) increase in the transmural number of cells in the left ventricle (control = 159 ± 8; experimental = 174 ± 12). The number of myocytes across the wall was unchanged in the right ventricle (control = 70 ± 7; experimental = 69 ± 9).

The number of myocyte profiles per mm^2 of myocardium was subsequently employed to compute the aggregate length of myocytes in each ventricle. As a consequence of anemia, total myocyte length increased 41% ($P < 0.0001$) and 19% ($P < 0.02$) in the left (control

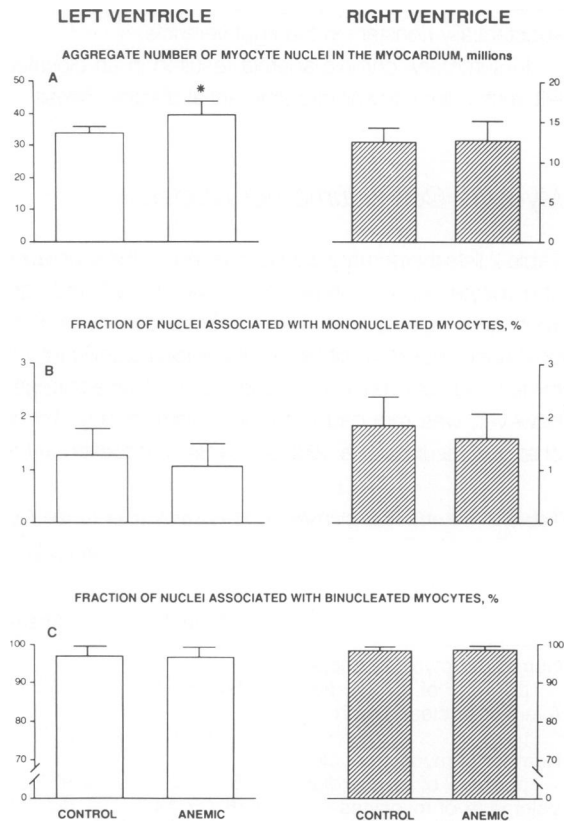

Figure 4. *Bar graph showing the changes in the total number of myocyte nuclei and in the fraction of mononucleated and binucleated myocytes in the left and right ventricles. See legend to Figure 1.*

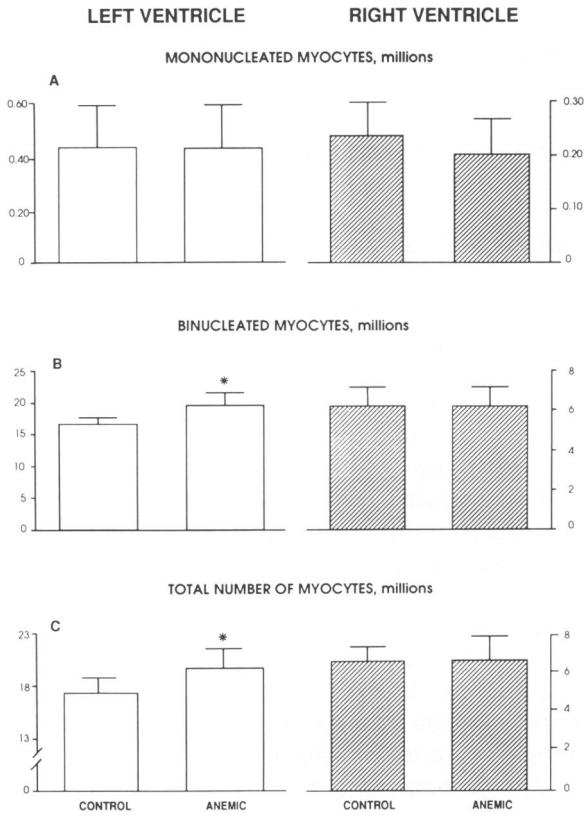

Figure 5. *Bar graph showing the changes in the number of myocyte in the left and right ventricles. See legend to Figure 1.*

= 3184 ± 372 m; experimental = 4490 ± 475 m) and right (control = 1229 ± 145 m; experimental = 1467 ± 260 m) ventricle, respectively. Sarcomere length was found to be comparable in controls and experimental rats, biventricularly (LV: control = 1.97 ± 0.15 μm, experimental = 2.04 ± 0.10 μm; RV: control = 2.01 ± 0.17 μm, experimental = 2.00 ± 0.10 μm). Moreover, the absolute amount of myocyte volume increased by 33% ($P < 0.0001$) in the left ventricle (control = 656 ± 59 mm^3; experimental = 874 ± 80 mm^3) and 42% ($P < 0.0001$) in the right ventricle (control = 176 ± 16 mm^3; experimental = 251 ± 39 mm^3). This difference implies a 27% greater hypertrophic reaction of the myocyte compartment of the right ventricle.

In summary, anemia led to an increase in the number of myocytes across the left ventricular wall and to a significant lengthening of the myoyte compartment, biventricularly.

Myocyte Cell Stress

The availability of the number of myocytes across the left ventricular wall and the values of ventricular pressures and chamber diameter permits the calculation of systolic and diastolic wall stress at the cellular level of organization. These determinations are illustrated in Figure 6. Systolic (Figure 6A) and diastolic (Figure 6B) stresses were seen to be greater in the cells located in the endomyocardium than in the midmyocardium and epimyocardium of both controls and anemic rats. Moreover, diastolic cell stress was higher in anemia throughout the wall. In contrast, no significant difference was observed in systolic cell stress.

In summary, chronic anemia resulted in an elevation of diastolic cell stress, which mostly affected the myocytes located in the inner two-thirds of the left ventricular wall.

Myocyte Volume Composition

Figure 7 shows that anemia resulted in a significant change in the cytoplasmic compartments of left and right ventricular myocytes. In both cases, the mitochondrial fraction (Figure 7A) increased, whereas the myofibrillar component (Figure 7B) decreased leading to an increase in the ratio of mithocondria to myofibrils (Figure 7C), biventricularly. In particular, this ratio increased by

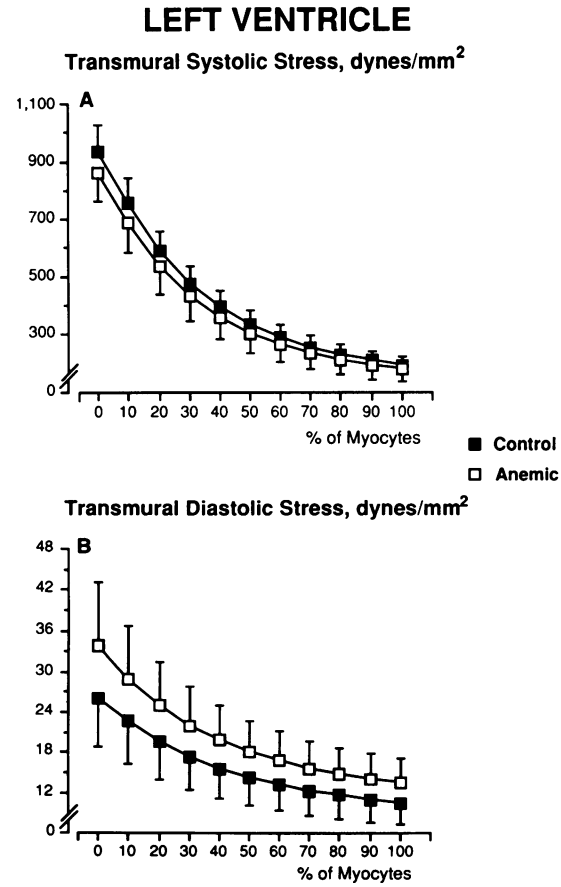

Figure 6. *Bar graph showing the changes in stress on the left ventricular myocytes. See legend to Figure 1.*

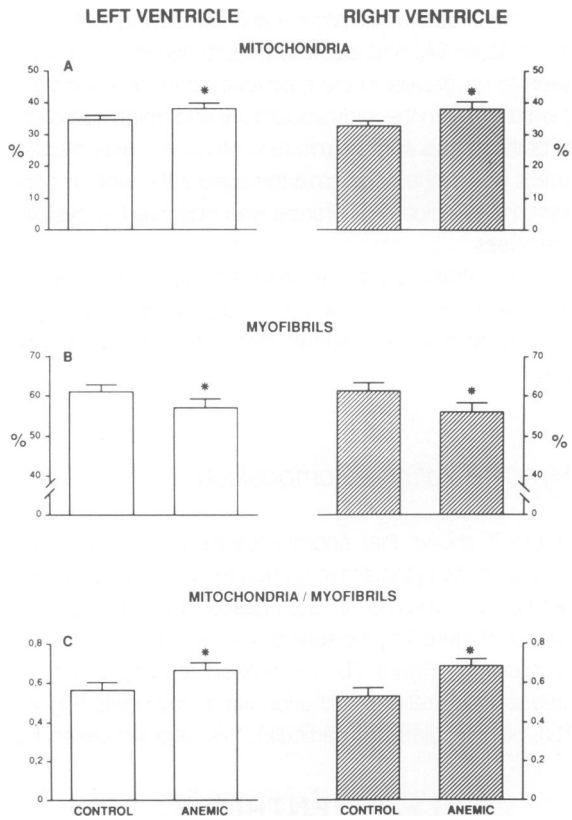

Figure 7. *Bar graph showing the changes in the volume composition of mitochondria and myofibrils in left and right ventricular myocytes. See legend to Figure 1.*

18% ($P < 0.0001$) and 29% ($P < 0.0001$) in the left and right ventricle, respectively. However, both cytoplasmic structures expanded, since hypertrophy of the myocyte population took place in the ventricles.

In summary, anemia induced a disproportional growth adaptation of the mitochondrial and myofibrillar compartments in the hypertrophied myocytes.

Discussion

The findings of the current study indicate that hypochromic microcytic anemia in young adult rats led to eccentric left ventricular hypertrophy with an abnormal elevation in transmural diastolic myocyte stress, which affected mostly the inner two-thirds of the wall. This alteration in the loading state of the myocardium occurred in spite of a proliferative and hypertrophic response of the myocyte population that tended to reduce the magnitude of stress on a cell basis. Moreover, myocyte lengthening resulted in an augmentation in cavitary volume, which contributed to the maintenance of normal left ventricular end diastolic pressure in the presence of an increased preload. At variance with the left ventricle, the compensatory hypertrophic growth adaptation of right ventricular myocytes was capable of normalizing wall stress in the right side of the heart. The increase in number and size of myocytes was characterized by a preferential expansion of the mitochondrial compartment with an elevation in the mitochondrial-to-myofibrillar volume ratio.

Ventricular Function

Data in this investigation show that left ventricular function, 8 weeks after a continuous dietary intake deficient in iron and copper, was characterized by a reduction in left ventricular peak systolic pressure and dP/dt, whereas circumferential midwall diastolic stress was increased. In contrast, right ventricular hemodynamics showed no variation from baseline. Thus, the sustained increase in volume load on the heart generated by anemia provoked a differential effect on the two ventricles. Although these findings are difficult to explain, the greater hypertrophic reaction of the right ventricle in terms of the absolute expansion of the myocyte compartment of the myocardium could account for the variation in contractile performance. Myocyte mass augmented significantly more in the right than in the left ventricle.

Consistent with these observations, increases in volume loads on the two ventricles evoke a greater functional impact on the right than on the left side of the heart.[8,25–28] The right ventricle possesses a smaller mass, thinner wall, and lower number of cells,[14,25,27] leading to a substantially higher degree of stress per myocyte than in the left ventricle.[8] Moreover, the anemic state may influence differently the two ventricles in terms of tissue oxygenation.[29] Indirect evidence of myocardial ischemia in the left ventricle with anemia has been found in the observation that capillary diameter is increased more in the left myocardium than in the right myocardium in spite of a smaller hypertrophic response.[8] Capillary dilation may be interpreted as an attempt to expand the amount of capillary blood accessible for oxygen exchange and simultaneously decrease the diffusion distance for oxygen.[14,30,31] These adaptive changes in capillary properties may reflect a reaction to an ongoing inadequate oxygen tension in the tissue and consequent limited hypertrophy and moderately reduced left ventricular function.

Myocyte Growth and Ventricular Remodeling

Data in the current report demonstrate that different cellular growth processes participate in the adaptation of the ventricular wall in anemia: hypertrophy of right ventricular myocytes, and hyperplasia and hypertrophy of left ven-

tricular myocytes. The addition in parallel of new myocytes accounted for the thickening of the wall in the left ventricle, whereas the lateral expansion of myocytes contributed to the increase in mural thickness of the right ventricle. Moreover, chamber dilation was accomplished by lengthening the myocyte compartment of the myocardium with no change in sarcomere length, biventricularly.

An increasing volume load in the adult heart induces eccentric hypertrophy, in which ventricular chamber volume enlarges without a relative increase in its wall thickness.[13,14] In the compensated form, chamber dilation is accompanied by proportional mural thickening, whereas decompensation takes place when this relationship is not maintained.[13,14,32,33] Observations in this study showing a 46% increase in left ventricular cavitary volume and a 10% augmentation in wall thickness strongly suggest that eccentric hypertrophy with a prevailing expansion in chamber size was present 2 months after anemia. Chamber dilation, which occurred in the presence of a 9% lateral insertion of myocytes, was also documented by a 41% increase in the aggregate length of the myocyte compartment in the ventricle. These values imply that 29% of the overall myocyte lengthening was accomplished by a 19% increase in the average myocyte length per nucleus and an 8% in series addition of newly formed cells to surround a larger cavitary volume. Thus, the 17% myocyte cellular hyperplastic response participated in almost equal proportions to chamber dilation and wall thickening.

The difficulty in analyzing right ventricular chamber volume did not allow a similar characterization of the adaptive mechanisms implicated in the remodeling of the right ventricle in anemia. However, mural thickness expanded by 11% through a 9% increase in myocyte diameter with no change in myocyte number. Since total myocyte length augmented by 19%, chamber dilation with inadequate thickening of the wall may have occurred in the right ventricle as well. Moreover, wall area was found to be significantly increased, further supporting the contention that anemia induced a change in the anatomy of the right ventricle similar to that claimed for the left side of the heart.

The pattern of myocyte growth, which included the fundamental processes of cellular hypertrophy and hyperplasia, may be interpreted as a compensatory response of the myocardium at the cellular level of organization, tending to minimize the effects of the increased volume load on the heart[13] with anemia.[8] The greater myocyte diameter and the parallel addition of cells produced a proportional thickening of the wall, which was capable of compensating for the higher peak systolic wall stress resulting from a larger chamber volume. On the other hand, lengthening of myocytes had the consequence of counteracting the greater end diastolic wall stress[14] by contributing to the enlargement in chamber volume. Chamber dilation appears to be brought about mostly by replication of sarcomere in series,[13] as strongly suggested by the increase in myocyte length with no change in average sarcomere length. Other mechanisms of chamber dilation may involve a spatial rearrangement of myocytes in the ventricle,[34] lateral slippage of muscle fibers within the wall,[35,36] or a combination of both. In spite of these structural adaptations, diastolic myocyte stress remained elevated across the left ventricular wall, although end diastolic pressure was maintained within normal values. The lack of a complete normalization of diastolic stress has been shown in different conditions of volume[37] and pressure[38] overload hypertrophy, and after a segmental[39] or scattered[33] loss of myocytes.

Myocyte Cellular Hyperplasia

The current results demonstrate that myocyte cellular hyperplasia restricted to binucleated cells occurred in the left ventricle with anemia. Although it has been a belief that DNA synthesis in myocytes ceases at day 17 after birth in the rat,[40] recent findings tend to support the concept that proliferation of myocytes in the adult mammalian heart may be induced after a prolonged increase in pressure load on the myocardium.[41,42] The hyperplastic cellular response documented here provides further evidence that fully differentiated adult muscle cells may regain their capacity to multiply. Moreover, myocyte cellular hyperplasia can be elicited *in vitro* by different growth factors and TPA.[43] Importantly, myocyte proliferation has been seen in the senescent rat heart pointing to the possibility that cardiac muscle cells can undergo mitotic division throughout life.[23] Such a potential growth reserve mechanism has been suggested to be operative in the human heart as well.[34]

Observations accumulated so far indicate that nuclear and/or myocyte cellular hyperplasia develop in the stressed ventricle under conditions in which the duration of the overload has been maintained for several months.[24,41,42] In addition, this cellular process has been claimed not to be limited to severe degrees of cardiac hypertrophy.[23,43,44] Thus, the critical heart weight theory, which hypothesizes that a defined magnitude of cellular enlargement has to be obtained before hyperplasia of myofibers begins,[32] has been repeatedly challenged. The present data of a hyperplastic response over a short period of dietary treatment raise additional questions concerning the mechanism responsible for myocyte cell division. However, consistent with recent conclusions, multiplication of myocytes appears to characterize the

phase of transition from functional compensation to the stage of abnormal loading.[23] This possibility seems to be supported by the current results that show that myocyte hyperplasia was restricted to the left ventricle, which exhibited dilated eccentric hypertrophy and elevated wall stress. In contrast, myocyte enlargement typically underlined the hypertrophic growth of the right ventricle in which ventricular dynamics and wall stress were preserved.

Mitochondria and Myofibrils

Findings in this report indicate that the mitochondrial compartment of myocyte cytoplasm grew more than the myofibrillar fraction as a consequence of anemia. Moreover, the increase in the mitochondrial-to-myofibrillar volume ratio was comparable in the two ventricles. These observations are in contrast with previous results in iron-deficient anemia[45] and volume overload hypertrophy.[25,27] In the former case, no change was claimed in terms of the response of myofibrils,[45] whereas in the latter condition a constant ratio of mitochondria to myofibrils was reported.[25,27] There is only one study in which the volume percent of mitochondria was found to be decreased after aortocaval fistula in dogs.[46] The disproportionate expansion of the cytoplasmic structures responsible for energy production and utilization may constitute an adaptive reaction of myocytes to counterbalance the reduced oxygen-carrying capacity of the blood in anemia.

Conclusions

There are several limitations in the current investigation that must be acknowledged. The calculations of systolic and diastolic wall stress are based on the assumption that the ventricle is a thinned-wall sphere, although its configuration did not reflect such a shape. Moreover, these derivations did not take into account the changes in the volume composition of the myocardium. Inhomogeneity and differences between tissues may affect wall and cell stress values. Finally, hemodynamic parameters have been collected under anesthesia, which may also alter ventricular pressure data.

The methodologic approach employed here for the analysis of the changes in cell size and number required multiple calculations and the combination of measurements from tissue preparations and enzymatically dissociated myocytes. These interconnected steps may all have influenced the final results. In addition, the specific density of the various tissue components was not measured, and the isolation procedure may have resulted in the separation of a nonrepresentative sampling of the distinct myocyte populations. The process of myocyte hyperplasia documented here may not necessarily imply that a similar phenomenon may occur in the fully mature adult rat since the young age of the animals may have played a key role in the myocardial response of the left ventricle to anemia.

In conclusion, although these limitations have to be carefully considered, nutritional anemia appears to result in biventricular eccentric hypertrophy with abnormal left ventricular diastolic myocyte stress. Myocyte hyperplasia tends to reduce the magnitude of load on a cellular basis in this chamber.

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