Pergamon

Biochemical Pharmacology, Vol. 47, No. 10, pp. 1759–1766, 1994.
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0006-2952/94 $7.00 + 0.00

0006-2952(94)E0049-Q

ULTRASTRUCTURAL AND CYTOCHEMICAL CHANGES IN THE HEART OF IRON-DEFICIENT RATS

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(*Received* 19 *October* 1993; *accepted* 21 *January* 1994)

Abstract—Male Sprague–Dawley rats aged 3 weeks that were maintained on an iron-deficient diet for 4–5 weeks developed severe anemia with markedly reduced hemoglobin levels (3.94 ± 0.14 Hb g% versus controls 12.9 ± 0.11 Hb g%). Iron-deficiency resulted in marked cardiac hypertrophy (cardiomegaly). On sacrifice, the hearts were processed for light and transmission electron microscopy. The major ultrastructural changes were found in the hypertrophied left ventricle and left papillary muscles. Iron-deficiency caused marked edema in myocytes, sarcomeres were out of register, and degeneration and discontinuities in myofilaments were common. Iron-deficiency resulted in the enlargement of the interfibrillar mitochondria, changes in the matrix and the formation of electron-dense amorphous bodies. The ultrastructural changes in myocytes in response to experimental iron-deficiency were similar to those described by others in cases of experimental ischemia or hypoxia. Mitochondrial changes were also found in the atria of iron-deficient rats. Quantitative cytochemical measurement of succinate dehydrogenase activity was determined and was shown to be substantially reduced in the iron-deficient heart. In severely iron-deficient rats restored to a normal iron-sufficient diet for two weeks, hemoglobin levels recovered, however the myocytes of the hypertrophied left ventricles and papillary muscles continued to show severe degenerative changes.

Key words: iron-deficiency; cardiomyopathy; ultrastructure; rats

Nutritional iron-deficiency develops when dietary intake and gastrointestinal absorption are reduced and become insufficient to meet normal physiological requirements. Consequences of chronic iron-deficiency include the development of anemia and associated severe pathological conditions [1–5]. Iron-deficiency and the subsequent development of anemia are widely considered to be the most prevalent nutritional disorder in humans. It is possible, using controlled diets, to induce experimental iron-deficiency in animals which then develop physiological and pathological disturbances similar to those encountered in iron-deficient humans.

During the course of a study of dietary-induced iron-deficiency in male rats, we have found that one of the major consequences in these animals is the induction of cardiomegaly. Cardiomegaly, which may be associated with cardiomyopathy, has been described in iron-deficient growing male rats [6, 7]. In this present study, we have examined the ultrastructure of cardiac myocytes from iron-deficient cardiomegalic rats to provide added ultrastructural detail of the induced cardiomyopathy to that reported by Sung *et al.* [6].

The present study also includes quantitative cytochemical data concerning succinate dehydrogenase activity in cardiac myocytes in iron-deficient cardiomegalic rats in order to try and correlate changes in mitochondrial activity with ultrastructural changes.

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MATERIALS AND METHODS

Induction of nutritional iron-deficiency in rats. Male Sprague–Dawley rats (21 days old) were maintained on a 12 hr light/dark cycle (lights on at 05.00 hr) and had access to food and distilled water *ad libitum*. The animals were maintained in conformance with the Guiding Principles in the Care and Use of Animals of the American Physiological Society. Iron-deficient groups of rats received a diet low in iron (5 ppm), whereas control rats were given the same diet with added ferrous sulfate. Details of the experimental diet, which is a modification of that described by McCall *et al.* [8], are shown in Table 1. The chemicals for the diet were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The full cream milk powder without vitamin supplement was obtained from Tnuva Food Industries, Ramat Gan, Israel.

At weekly intervals, the animals were weighed and blood samples removed from the tail for hemoglobin determinations. At the end of week 5 of treatment, the iron-deficient animals showed a significant reduction in hemoglobin levels and were analyzed for heart non-heme iron content. A "recovery" group of iron-deficient rats of 5 weeks duration were fed an iron-sufficient diet for a further 2 weeks to determine the degree of restoration of hemoglobin levels and cardiac ultrastructure.

Table 1. Basic iron-deficient diet

Ingredient	Amount (g)
Main mix	
Household sugar (sucrose)	1530
Choline dihydrogen citrate	100
Non-supplemented milk powder	2600
Salt mixture (iron free)	120
Water-soluble vitamin mix	40
Fat-soluble vitamin mix	4
Fat mix	640
Salt mixture (iron-free)	
NaCl	100
$NaIO_3 \cdot H_2O$	0.001
$MnSO_4 \cdot 4H_2O$	5.406
$CuSO_4 \cdot 5H_2O$	1.361
D-glucose*	393.25
Water-soluble vitamin mix	
Pyridoxine hydrochloride	0.25
Pantothenic acid calcium salt	0.60
Aneurine hydrochloride	0.50
Nicotinic acid	0.50
Menadione (Vitamin K_3)	0.05
Folic acid	0.05
4-amino-benzoic acid	0.50
Biotin	0.01
Inositol	10.00
Cyanocobalamin (B_{12})	0.00075
Riboflavin	0.025
D-glucose	487.25
Fat-soluble vitamin mix	
Retinol palmitate (A)	0.40
Ergocalciferol (D_2)	0.05
DL-α-tocopherol	17.50
Soya bean oil	32.50 mL
Fat mixture	
Pig lard	240
Soya bean oil	80 mL

* For the iron-sufficient (Fe^+) control diet 27 g of D-glucose was replaced by 27 g $(NH_4)_2SO_4.FeSO_4.6H_2O$.

Determination of non-heme and hemoglobin contents. The extent of iron-deficiency was established by measuring the hemoglobin level [9] using a commercial kit (Sigma). Heart iron content was determined using a modified procedure of Hallgren and Sourander [10]. The tissue homogenates were heated for 10 min at 90° and then cooled in ice for 10 min to improve cell disruption. Hydrolysis was performed with 2.8 NHCl for 60 min at 90°. After further cooling in ice for 10 min the iron level was determined using 1,10-phenanthroline as an iron-chelator in the presence of 6% sodium acetate and 0.8% hydroxylamine hydrochloride with the absorbance read at 510 nm. Control samples of iron were carried through the standard procedure.

Statistical analysis. All results were analysed using Student's *t*-test. A change was defined as being significant if the difference between the control and iron-deficient groups or control and recovery groups reached a level of significance of $P < 0.05$.

Light and electron microscopy. Rats were anesthetized with ether and following neck dislocation the hearts were rapidly removed and fixed by immersion in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 at room temperature. After weighing the heart, a mid-sagittal cut was made and small blocks, about 1 mm^3, were dissected from the mid-myocardium of the left and right ventricles. Blocks were also dissected from the left and right atria and the left papillary muscle. After 3 hr in the fixative, the blocks were rinsed and stored overnight in 0.1 M sodium cacodylate buffer containing 7.5% (w/v) sucrose. This was followed by postfixation in 1% unbuffered osmium tetroxide (3 hr), dehydration in ascending ethanols, treatment with propylene oxide and embedding in Polarbed 812 epoxy resin (Bio-Rad, Hemel Hempstead, U.K.). Next, 60–90 nm thick sections were cut with a diamond knife on an LKB 'Nova' ultramicrotome, collected on uncoated copper grids and contrast-stained with 1% uranyl acetate in 70% ethanol (5 mins) and 1% lead citrate (5 mins). Electron micrographs were taken on a JEOL 100SX transmission electron microscope at 80 kV. For correlated light microscopy, 1 μm sections cut on the ultramicrotome were stained in 0.1% toluidine blue in 1% borax.

Succinate dehydrogenase enzyme cytochemistry. A mid-sagittal cut was made in the heart and unfixed frozen 8 μm cryostat sections were produced. Succinate dehydrogenase activity was demonstrated cytochemically as described fully by Chayen *et al.* [11]. In brief, the sections were incubated at 37° in a medium containing 30% (PVA) (30 g GO4/140

Table 2. Nutritional and physiological parameters

Parameter	Control (Fe^+) (N = 15)	Iron-deficient (Fe^-) (N = 15)	Recovery (N = 20)
Final body weight, BW (g)	208 ± 1.79	167.4 ± 1.45*	205 ± 2.50
Hemoglobin (g/dL)	12.9 ± 0.11	3.94 ± 0.14*	11.5 ± 0.20
Heart weight, HW (g)	0.86 ± 0.02	1.26 ± 0.05*	1.16 ± 0.50*
Specific heart weight, HW/BW (g/100 g BW)	0.42 ± 0.06	0.75 ± 0.09*	0.57 ± 0.06*
Heart iron concentration (μg Fe/g heart)	47.37 ± 3.13	17.38 ± 2.69*	31.50 ± 2.50*
Serum iron concentration (μg/mL)	6.29 ± 0.38	1.55 ± 0.07*	4.70 ± 0.32
Liver weight (g)	6.20 ± 0.30	10.45 ± 0.05*	8.47 ± 0.40

Fe^+, iron-sufficient; Fe^- iron-deficient.

Values are means ± SEM for the control, iron-deficient and recovery groups.

* Significant differences were determined in experimental groups in comparison with the controls using Student's *t*-test with $P < 0.05$. No significant differences were found in the recovery group (2 weeks after restoration to an iron-sufficient diet) with regard to hemoglobin levels, serum iron concentration or liver weight.

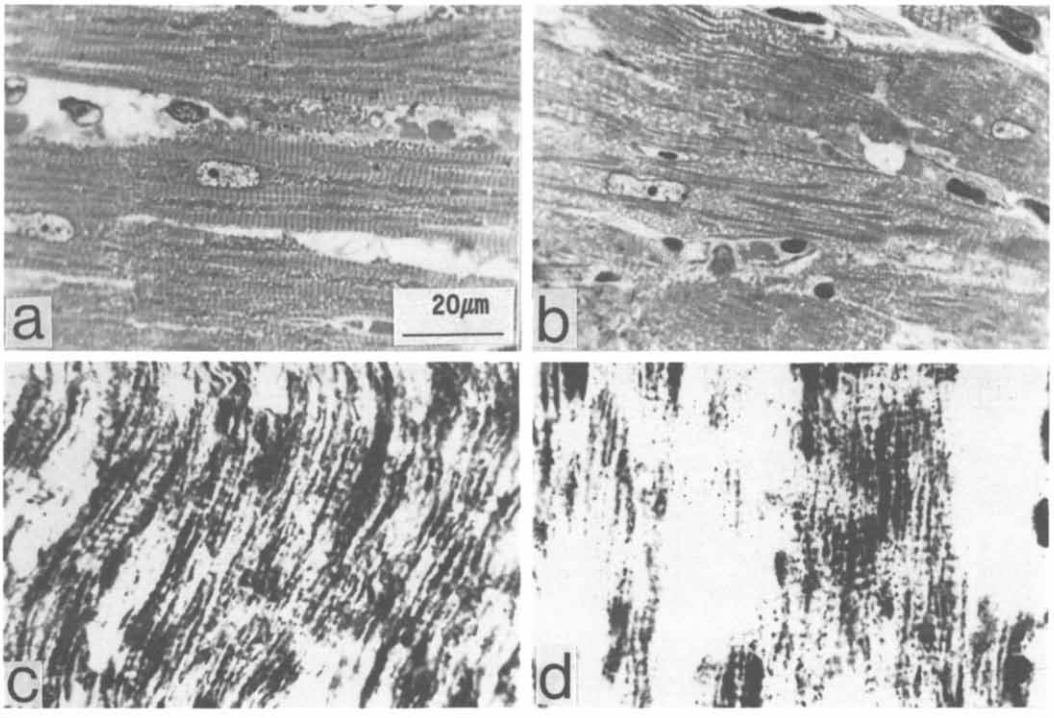

Fig. 1. Light microscopy of myocytes of the left ventricle (a) control (iron-sufficient Fe^+) and (b) iron-deficient (Fe^-) as seen in 1 μm epon sections stained with alkaline toluidine blue. (c) control (iron-sufficient Fe^+) and (d) iron deficient (Fe^-) showing succinate dehydrogenase activity in frozen sections.

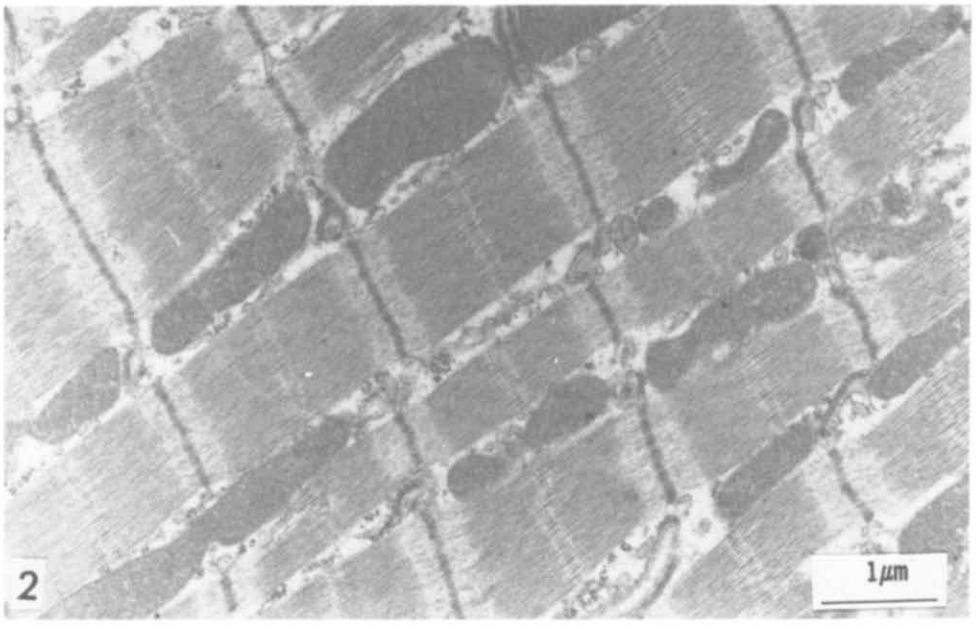

Fig. 2. Details of myocyte of left ventricle of control (Fe^+) rat showing typical orderly-arranged sarcomeres and interfibrillar mitochondria.

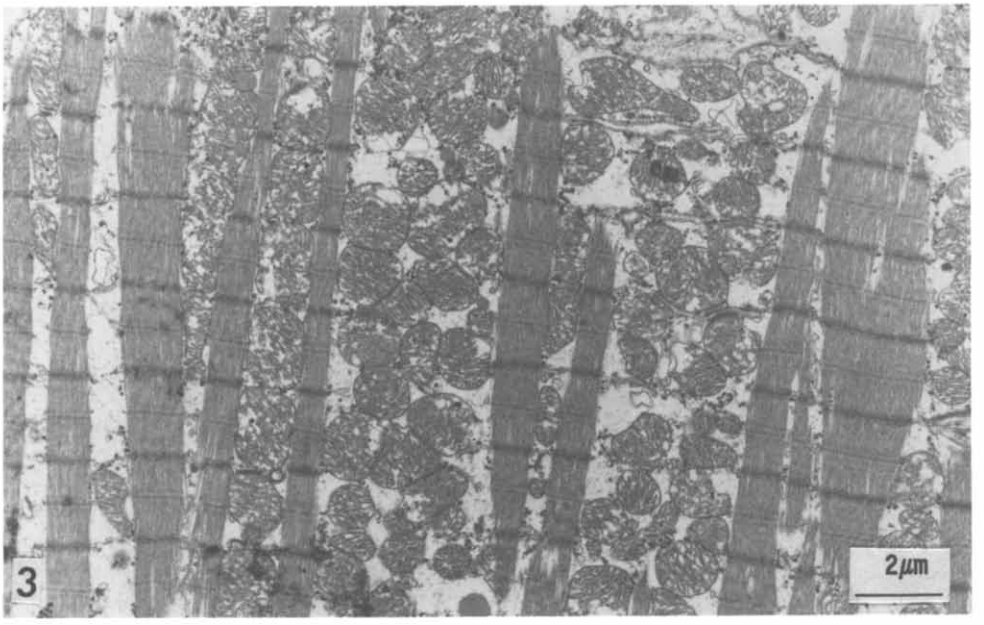

Fig. 3. Details of myocyte of left ventricle of iron-deficient (Fe^-) rat showing signs of edema, loss of contractile material and mitochondrial changes.

PVA in 100 mL, 0.05 M glycyl-glycine buffer, pH 7.8), 3 mg/mL nitroblue tetrazolium, 50 mM sodium succinate and 0.7 mM phenazine methosulfate. (The PVA was purchased from Wacker Chemicals Ltd, Walton-on-Thames, U.K.).

The colored formazan reaction product was quantified in myocytes of the mid-myocardium of the left ventricle by means of scanning and integrating microdensitometry using a Vickers M86 microdensitometer (Vickers Instruments, York, U.K.) as described previously [12]. The instrument settings were: ×40 objective with a scanning spot of 0.5 μm diameter in the plane of the section and light of 585 nm wavelength. The mask size was A5 so that the area scanned for each measurement was 314 μm^2 (diameter of field, 20 μm). Each result represents the mean of 20 fibers (10 in each duplicate). Results are expressed as the mean integrated extinction per

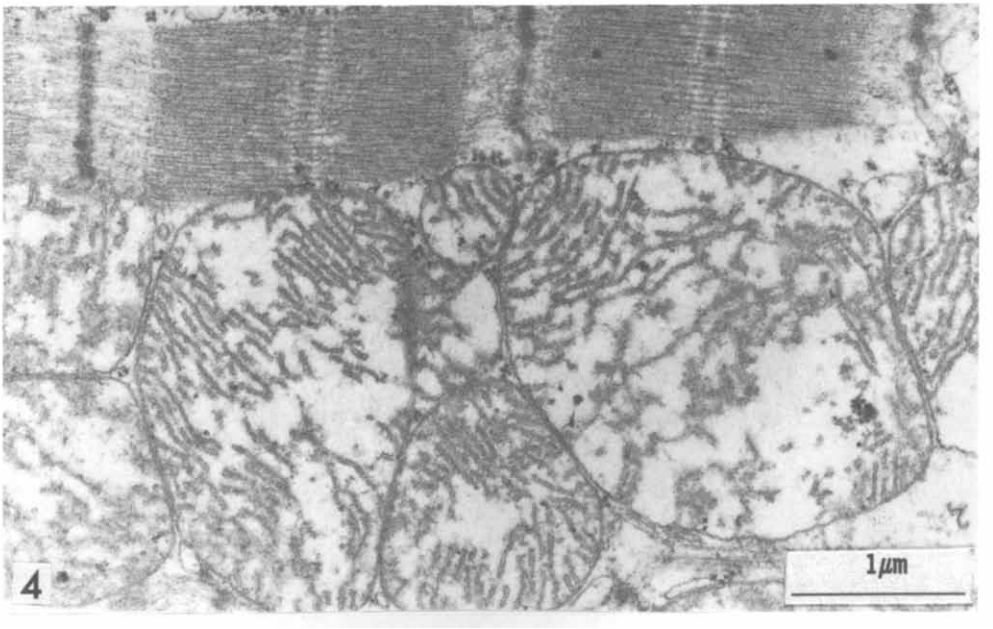

Fig. 4. Details of mitochondria of myocytes of left ventricle of iron-deficient (Fe^-) rat. Note swollen mitochondria with separated cristae and electronlucent matrices.

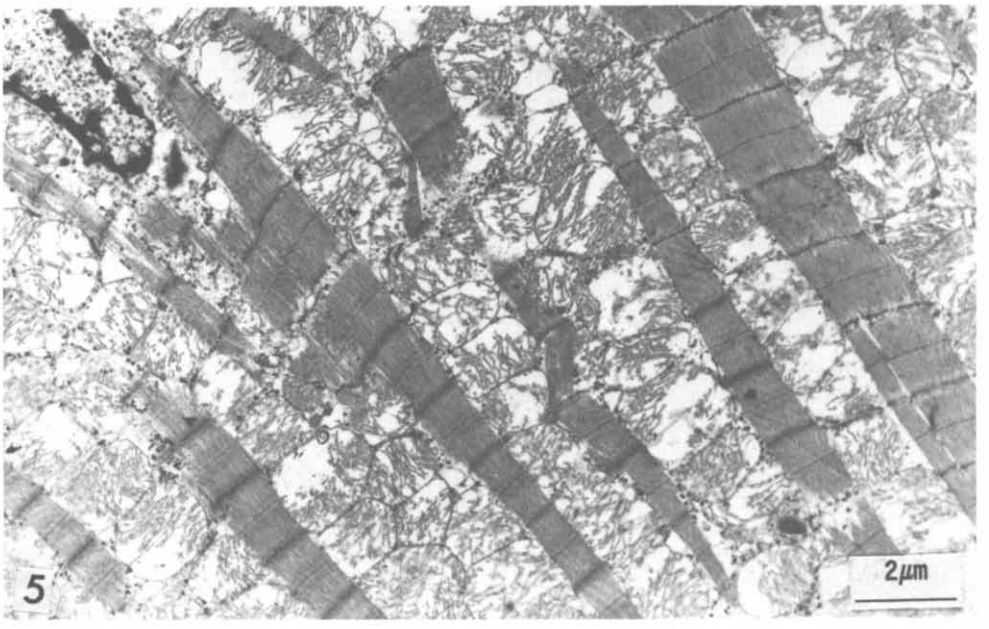

Fig. 5. Part of myocyte of left ventricle of iron-deficient (Fe^-) rat with hypertrophied heart after restoration of an iron-sufficient diet for 2 weeks. The myocyte still shows major degenerative changes with no signs of structural recovery (cf. Fig. 3).

fiber per minute. Under the conditions used the reaction was linear with time over 2.5–10.0 minutes. The measurements were made at 5 min incubation time.

All the chemicals used in the study, unless otherwise specified, were purchased from Sigma.

RESULTS

At the time of sacrifice after 5 weeks of feeding an iron-deficient diet, the iron-deficient rats differed markedly from the controls in both their physical appearance and behavior. The iron-deficient rats were much smaller and weighed considerably less than the iron-sufficient controls (Table 2). The iron-deficient rats showed a generalized lethargy. The body fur in these animals was in very poor condition with substantial focal hair loss. Gross signs of anemia were clearly detectable. In particular, this was seen in the pallid color of the ears and eyes compared

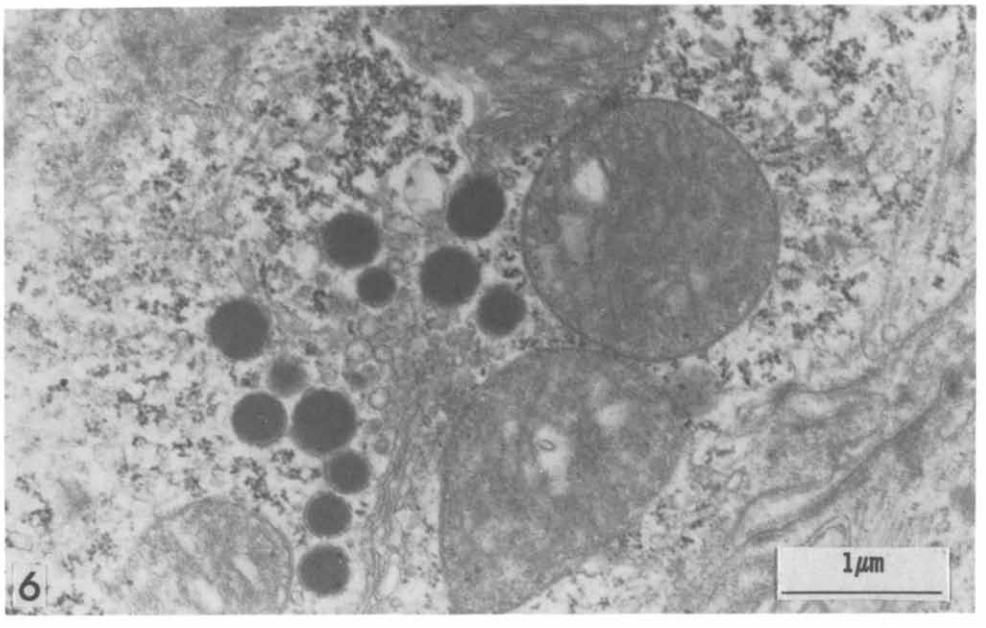

Fig. 6. Detail of myocyte of left atrium of iron-deficient (Fe^-) rat showing development of enlarged abnormal mitochondria.

with the normal ruddy color of the control rats. On opening the body cavities of the iron-deficient rats the anemia was evident, especially the pallid color of the liver, kidneys, spleen and heart. Moreover, the amount of subcutaneous fat and fat surrounding the internal organs was markedly reduced. The nutritional and physiological parameters are presented in Table 2. The anemia was also apparent in the coloration of the blood samples. In the iron-deficient rats the blood was a much paler light-straw color compared with the deeper normal color of the controls. This gross observation correlated well with measured hemoglobin levels (Table 2).

On opening the thoracic cavity, the overall enlarged dimensions of the heart were clearly seen in the iron-deficient rats. We found that the cardiomegaly develops fairly suddenly in weeks 4–5 of the iron-deficient diet (unpublished data). When the heart was removed and cut mid-sagittally, it was clear on gross examination that the major hypertrophy had occurred in the left ventricle and in the left papillary muscles. The degree of hypertrophy was so extreme in most cases as to occlude almost completely the lumen of the left ventricle.

Light and electron microscopy

In 1 μm sections seen by light microscopy of the hypertrophied left ventricle, the myocytes in general appeared smaller and more irregular than those of similar areas of the controls (Fig. 1a and b). At high magnifications, a loss of contractile material and the appearance of clearer areas within the myocytes was evident. At the ultrastructural level, it was found that the myocytes of the left ventricle and left papillary muscle of iron-sufficient control rats showed the typical appearance of normal cardiac myocytes with an orderly arrangement of sarcomeres with typical sarcomeric banding running more or less in parallel in adjacent myofibrils (Fig. 2). The interfibrillar mitochondria were also arranged in an orderly fashion. These were elongated with typical closely-packed cristae and fairly electrondense matrices.

In contrast, the myocytes of the left ventricle of the iron-deficient rats showed extensive interfibrillar edema. Sarcomeres were typically thrown out of register and showed myofilament loss, discontinuities and disorganization (Fig. 3). The interfibrillar mitochondria were grossly enlarged and more pleomorphic than those of the controls. The cristae were more separated and less-closely packed than those of the controls, while the matrices were considerably more electronlucent (Fig. 4). Mitochondria frequently possessed cristae arranged in a circular manner. Amorphous electrondense inclusions were often seen in the matrix of the swollen mitochondria. The myocytes of the hypertrophied left papillary muscle of iron-deficient rats were similar to those found in the left ventricle.

The hearts of iron-deficient rats restored to an iron-sufficient diet for 2 weeks remained grossly hypertrophic with no indication of any amelioration of the degenerative changes caused by the former iron-deficiency. The myocytes of the left ventricles and left papillary muscles continued to show enlarged mitochondria with electronlucent matrices, occasional dense amorphous inclusions, myofilament loss and edema (Fig. 5). Pycnotic nuclei were common in the myocytes. Mast cells were especially prominent in aggregates of the connective tissue between myocytes of these rats.

Table 3. SDH activity in myocytes of the left ventricle of control and iron-deficient rats

	Control (Fe^+)	Iron-deficient (Fe^-)
	N = 6	N = 8
Mean ± SEM	35.0 ± 2.1	20.4 ± 1.3*

SDH, succinate dehydrogenase; Fe^+, iron-sufficient; Fe^-, iron-deficient.

Results are expressed as means integrated extinction/fiber/min × 10^3.

The results are the means of measurements of 20 fibers (10 in each duplicate) ± SEM. *Significant at $P < 0.001$.

Whereas the left and right atria were not markedly hypertrophied in response to the iron-deficiency, mitochondrial changes were seen including mitochondrial swelling and degenerative changes in the cristae (Fig. 6). The secretory granules in the atria of the iron-deficient rats, especially those of the right atria, appeared more abundant and associated with enlarged Golgi bodies, though this has still to be confirmed quantitatively.

Succinate dehydrogenase activity

Succinate dehydrogenase activity, which is found exclusively in mitochondria and is used as a measure of oxidative capacity of myocytes, was markedly reduced in the iron-deficient heart. Even gross examination of sections showed an enormous difference in overall coloration, with the iron-deficient hearts being markedly less colored than the controls. The differences in succinate dehydrogenase activity in myocytes of the left ventricle are shown in Fig. 1c and d. This was confirmed in quantitative densitometry of the colored end-product (Table 3).

DISCUSSION

Forman and Daniels [13] were the first to describe cardiac enlargement as a consequence of experimental nutritional anemia in rats. They interpreted this as a compensatory response to the anemia. Cardiomegaly was also shown to develop in cases of iron-deficiency in rats [14–16]. This was recently confirmed in iron-deficient rats, whose enlarged hearts were shown to have histopathological degenerative changes characteristic of cardiomyopathy by dilatation [7]. Similar lesions were previously described in copper-deficient rats and appear similar to cardiomyopathic lesions by dilatation in humans [17, 18]. Our results confirm the induction of cardiomegaly and cardiomyopathy in iron-deficient rats and we have extended this to the ultrastructural level. We have shown that the major hypertrophy is evident in the left ventricle and left papillary muscles and that the changes induced by the iron-deficiency appear degenerative,

especially the loss of contractile material, damage to sarcomeres and the changes induced in mitochondria.

Iron-deficiency in rats has been shown to impair the respiratory functioning of heart mitochondria including changes in the cytochrome content [19]. The mitochondrial respiratory chain contains many iron-containing heme enzymes (including the cytochromes) as well as non-heme iron [6, 16, 19]. Succinate dehydrogenase activities, which are specific to mitochondria and provide an histochemical measure of mitochondrial activity, are markedly reduced in myocytes of our hypertrophic iron-deficient rat hearts indicating severe mitochondrial dysfunction. This correlates well with reported biochemical data on succinate dehydrogenase and monoamine oxidase activities in the heart of a similar iron-deficient rat model [20]. This also correlated well with our ultrastructural observations where we found major degenerative changes in mitochondria including enlargement, changes in cristae and in the matrix. Our ultrastructural observations confirm results reported by Goodman *et al.* [16], who found enlarged mitochondria with a disrupted appearance in severe hypertrophic iron-deficient rat heart. These mitochondrial changes are similar to those described in cases of experimental ischemia or hypoxia [21, 22] and in the platelets of iron-deficient subjects [23]. It appears that hypertrophied myocardium is extremely vulnerable to hypoxia [24].

The development of amorphous densities in the mitochondrial matrix as seen in our iron-deficient myocytes, is usually considered as an irreversible event associated with the severest effects of ischemia or hypoxia [25–28]. Myofilament loss, disruption, and edema are also found in cases of experimental ischemia or hypoxia [24] It is likely that reduced iron intake and subsequent reduced hemoglobin synthesis with resultant anemia, result in compensatory cardiac hypertrophy with hypoxia. The results presented here correlate well with deleterious changes in the physiological functioning that develop in the hearts of these iron-deficient rats (unpublished data). It would appear that the degenerative cardiomyopathic changes in ventricular myocytes and papillary muscle resulting from iron-deficiency may be irreversible. We were unable to find any indication of repair or correction to the myocytes after restoration for two weeks of an iron-sufficient diet despite the restoration of hemoglobin levels.

The significance of our observations on changes in the ultrastructure of atrial myocyte mitochondria in response to iron-deficiency is still not understood. It is possible these mitochondrial changes result from focal hypoxia consequent to the greatly reduced hemoglobin levels. We do not know if there is any connection between the mitochondrial changes and the synthesis or secretion of atrial natriuretic factors, which were not determined in the present study. It has been reported that acute hypoxia stimulates atrial natriuretic peptide secretion *in vivo* [29] and atrial natriuretic peptides are also released in substantial amounts in humans with dilated cardiomyopathy [30].

Our results add to the accumulating evidence that cardiomyopathy may develop in many instances as a consequence of nutritional deficiency [7]. The causal relationship between the development of cardiomyopathy in its many forms and nutritional deficiency appears clearer in experimental animals than in humans [7]. The cardiomyopathic response of our rats to iron-deficient diets provides a useful animal model for further research on the development of this condition and its long-term consequences, which could involve physiological changes not identifiable with the ultrastructural results present in this study.

Acknowledgements—This study was supported in part by grants from Ross Research Laboratories (U.S.A.), the Paul and Bess Goldings Research Fund for Medical Research (VPR Technion), the A. & E. Blum Fund for Medical Research (VPR Technion 184–188) and the Fund for the Promotion of Research at the Technion (184–197).

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