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Cardioprotective action of sodium gamma-hydroxybutyrate against isoproterenol induced myocardial damage

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Summary. In this study, the effects of graded doses of isoproterenol (IP) on the heart were examined in untreated gerbils and in gerbils anaesthetized with gamma-hydroxybutyrate (GHB), an endogenous metabolite with energy sparing properties. We were interested in the cardioprotective potential of GHB. IP was administered intraperitoneally in doses of 0.1, 0.3, 2.5 and 10.0 mg/kg to different groups of gerbils. Half the gerbils in each treatment group received 500 mg/kg of GHB 30 min before IP, and 250 mg/kg at three subsequent 2-hour intervals. The remaining gerbils in each treatment group received saline at these time points. The animals were sacrificed after 8 hours.

The accumulation of neutral fat droplets in the sarcoplasm was the most consistent effect of IP. The highest dose also produced some scattered myofibre death. The accumulation of fat in the cells could be estimated semi-quantitatively using a histochemical reaction for succinic dehydrogenase, and the volume of fat could be measured more accurately by electron microscopic morphometry. These measurements showed that IP produced a three to five-fold increase in sarcoplasmic fat volume. GHB either abolished or significantly reduced the accumulation of fat and it also completely prevented the myofibre death caused by the highest doses of IP. This cardioprotective effect of GHB was independent of its hypothermic action.

Based on this experience, ultrastructural morphometry of sarcoplasmic fat appears to be a promising method for evaluating cardioprotective measures.

Keywords: gamma-hydroxybutyrate, cardioprotection, isoproterenol, morphometry, electron microscopy

We have been examining the usefulness of morphological techniques for documenting and quantitating the metabolic and fine structural changes in the heart after non-lethal insults caused by exposure to catecholamines. The value of these methods was demonstrated in an earlier study on the myocardial changes found in experimental stroke (Kolin *et al.* 1988; 1989). We now wished to better define the sensitivity of our methods by documenting the changes that occur in the myocardium after exposure to graded doses of isoproterenol (IP) under controlled experimental conditions. Fatty change that is generally accepted as an indicator of non-lethal injury and 'sometimes harbinger of cell death' (Cotran *et al.* 1989) promised to be a sensitive and quantifiable

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Table 1. Isoproterenol induced myocardial damage

Time after IP (h)	IP (mg/kg)	Treatment	*n*	SDH grade (mean ± s.d.)	*P*	Sarcoplasmic fat (mean ± s.d.%)	*P*
8	0.1	Saline	6	2·17 ± 0·68	< 0.05	1.28 ± 0.22	< 0.0005
		GHB	4	1.25 ± 0.50		0.18 ± 0.07	
8	0.3	Saline	5	2.17 ± 1.12	< 0.01	1.42 ± 0.31	< 0.005
		GHB	6	1.50 ± 0.84		0.48 ± 0.30	
8	2.5	Saline	4	2.30 ± 0.51	< 0.05	1.88 ± 0.86	< 0.001
		GHB	4	1.33 ± 1.03		0.52 ± 0.10	
6	10	Saline	5	3.00 ± 0.00	< 0.005	ND	
		GHB	6	1.67 ± 0.98			

ND, Not done.

phenomenon for measuring the degree of myocardial damage.

Previous studies demonstrated that catecholamines cause accumulation of neutral fat droplets in the myocardial fibres (Chappel *et al.* 1959; Ferrans *et al.* 1964; 1970; Kolin & Kvasnicka 1963) and that the volume of the accumulated fat can be quantified by electron microscopic morphometry (Kolin *et al.* 1989; 1991). High doses of IP produce irreversible changes leading to cell necrosis (Chappel *et al.* 1959; Rona *et al.* 1985). We hoped to provide further evidence that our methods could be used to document and measure these effects and that they could also be used to examine the effectiveness of cardioprotective agents. In this study, we examined the cardioprotective potential of the hypothermic hibernation-like state induced by gamma-hydroxybutyrate (GHB), an endogenous metabolite with energy sparing properties (Mamelak 1989). This has been most clearly demonstrated in brain tissue (MacMillan 1978). Tissue protection with GHB has been achieved in brain (Lavyne *et al.* 1983) and gut (Boyd *et al.* 1990).

Materials and methods

All studies were conducted on adult male gerbils weighing between 70 and 90 g that had been maintained on Ralston-Purina LAG Chow and water *ad libitum* under standard laboratory conditions for at least 7 days. There were 8–11 animals in each of four treatment groups. These animals received an intraperitoneal injection of isoproterenol hydrochloride (IP) dissolved in saline in doses of either 0.1, 0.3, 2.5 or 10.0 mg/kg. One-half of the animals in each treatment group received 500 mg/kg GHB (sodium salt) i.p. 30 minutes before the administration of IP and 250 mg/kg of GHB 2, 4 and 6 hours after IP injection. The other animals in each group served as controls and received saline at these time points. GHB was prepared in a saline solution in a concentration of 100 mg/ml. The gerbils were sacrificed under brief halothane anaesthesia 8 hours after the administration of IP. Those gerbils that had received IP in a dose of 10 mg/kg were sacrificed after 6 hours. Additional studies specifically addressed the role of GHB induced hypothermia in the protection of the myocardium. Groups of four gerbils each were treated in the following manner:

(a) Saline (−0.5 h), 0.1 mg/kg IP (0 h), saline (+2 h).
(b) Treated as in (a), but anaesthetized with Nembutal and cooled on ice to 26–28°C.
(c) 500 mg/kg GHB (−0.5 h), 0.1 mg/kg IP (0 h), 250 mg/kg GHB (+2 h).
(d) Treated as in (c), but body temperature maintained at 37°C with heating pads.
(e) Received saline only.

Body temperature was measured with a rectal thermometer. All animals in this part of the study were sacrificed 3 hours after time zero.

The heart was removed from each animal immediately after sacrifice and the left ventricle was cut with a razor to provide two slices 1 mm thick. The first slice was used for unfixed cryostat sections that were stained with nitro blue tetrazolium to demonstrate succinic dehydrogenase activity (Pearse 1972). The staining pattern with this dye changes from fibrillar to granular with increasing myocardial damage (Kakari 1970), and this was graded from 0 to 3 as described previously (Kolin *et al.* 1988).

Tissue cubes 0.5–1.0 mm on each side were excised by razor from the midportion of the left ventricular wall of the second slice and fixed in ice-cold 2% glutaraldehyde–paraldehyde in 0.1 M phosphate buffer at pH 7.4, post-fixed in 1% OsO_4, and embedded in Epon–Araldite resin. Thick sections were made and stained with toluidine blue. Thin sections were then cut from tissue blocks with longitudinally oriented muscle fibres. These sections were examined with a Zeiss EM109 transmission electron microscope and the proportion of the sarcoplasmic

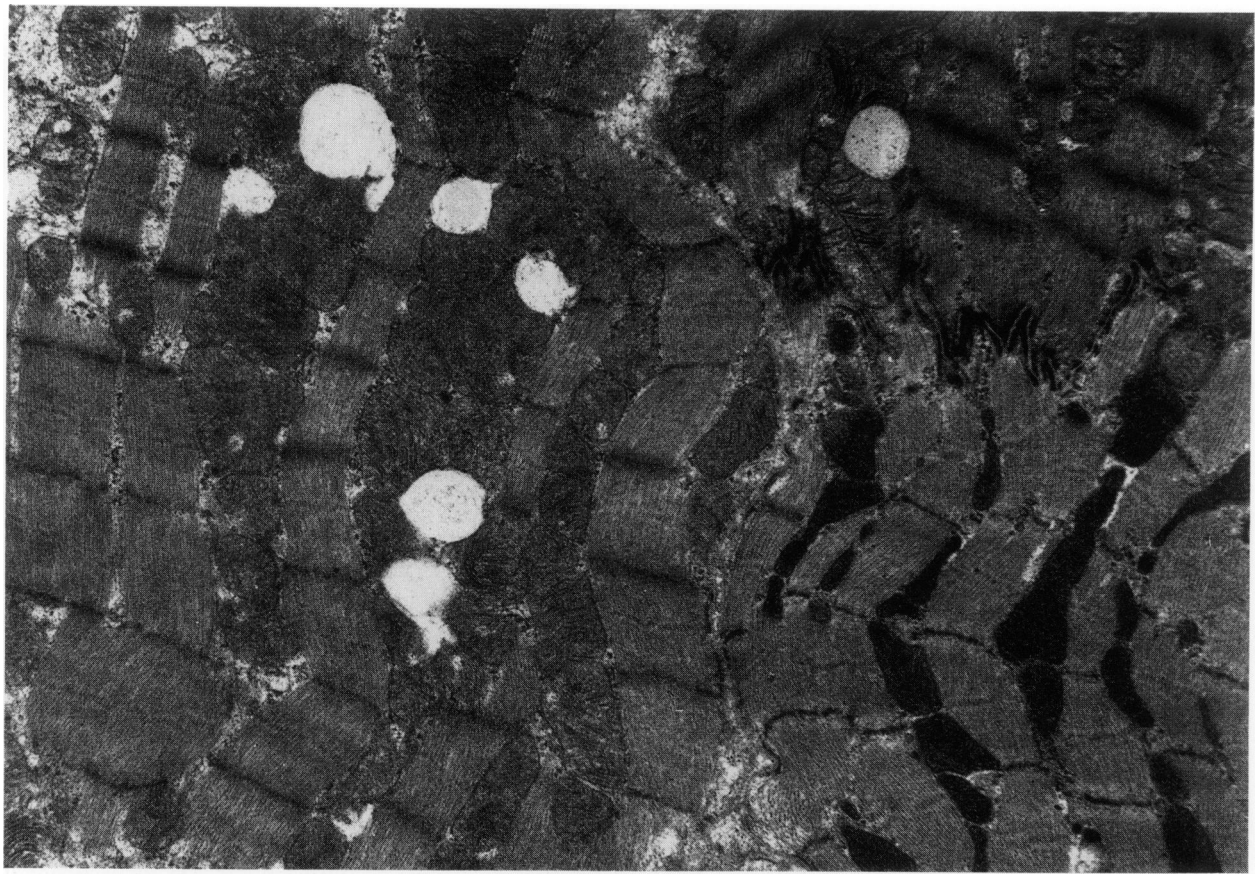

Figure 1. Unprotected myocardium (0.3 mg/kg IP + saline). Large fat vacuoles, and swollen mitochondria in two myofibres, while the right lower corner myofibre appears to be unaffected. × 10000.

volume occupied by fat droplets was determined in each heart in 30 standard selected frames as previously described (Kolin *et al.* 1989). In addition, ultrastructural abnormalities of the mitochondria, contraction bands, myofibrillar disruptions and tears of the sarcolemma were documented. Morphometric fat studies were not done in the 10 mg/kg IP group due to gross irregularities in fat distribution.

An analysis of variance was conducted using the General Linear Models (GLM) procedure of the SAS system to test the difference between the effects of the different doses of GHB and isoproterenol on sarcoplasmic fat content and succinic dehydrogenase reaction pattern. The GLM analysis was followed by Duncan's multiple range test.

Results

Whether by SDH staining or by electron microscopic morphometry (Table 1), a significant increase of neutral fat was observed in myofibres of the left ventricular wall 8 hours after administration of low and intermediate doses of IP. Droplets of fat as large as 1.5 μm could be found in the intermyofibrillary compartment after the administration of IP and they often exerted sufficient pressure to indent adjacent mitochondria (Figure 1). The number and volume of fat globules differed in adjacent myofibres and this created a checkerboard pattern most obvious with higher doses of IP. GHB significantly decreased the accumulation of fat (Figure 2). Other reversible ultrastructural changes induced by lower IP doses such as mitochondrial swelling, contraction bands consisting of 4–10 sarcomeres and intracellular oedema, most obvious in the subsarcolemmal zone, occurred infrequently and irregularly, but were also less common in hearts of GHB treated animals. The highest dose of IP, 10 mg/kg, produced ultrastructural changes that are considered irreversible such as dense intramitochondrial precipitates, ruptures of the sarcolemma and myofibrillar dysruption. Virtually no globules of fat were found in necrotic fibres, while numerous globules were noted in the adjacent surviving muscle cells (Figure 3). These

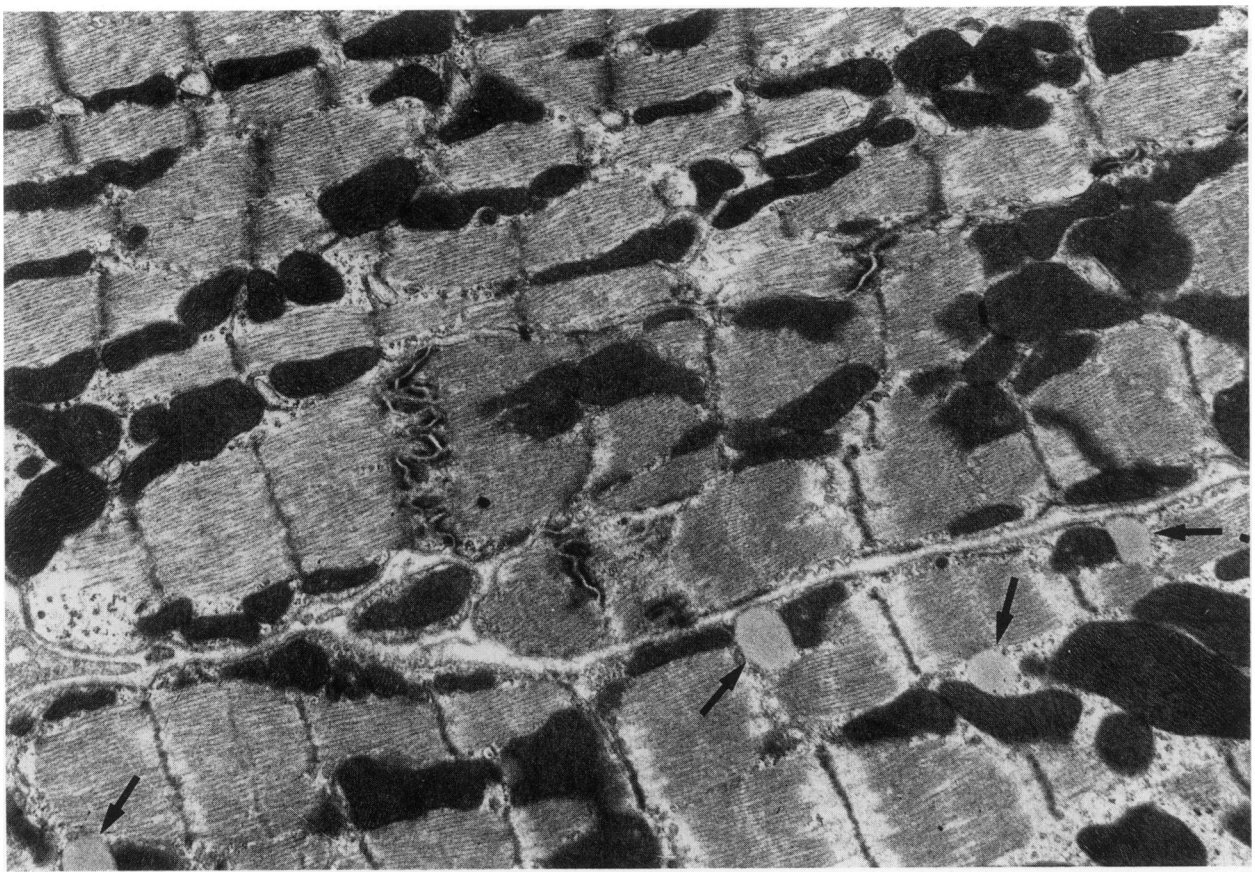

Figure 2. GHB protected myocardium (0.3 mg/kg IP + GHB). Very few globules of fat (arrows), no mitochondrial changes . × 10 000.

irreversible ultrastructural changes were eliminated by treatment with GHB and fat accumulation was markedly reduced (Figure 4). Body temperature fell to between 26 and 28°C in gerbils treated with GHB. Cooling to the same temperature range with barbiturate anaesthesia did not prevent fat accumulation after IP, but GHB effectively prevented the accumulation of fat whether or not cooling was permitted (Table 2).

Discussion

Isoproterenol-induced myocardial injury was selected as a model for testing the cardioprotective potential of GHB, because the experimental procedure is simple and the results are predictable and easily quantified (Chappel *et al.* 1959). Most experimental studies on the effects of catecholamines on the heart have used high, toxic doses that do not duplicate any clinical condition with the possible exception of phaeochromocytoma (Szakacs & Cannon 1958). Isolated necrosis of individual myocardial fibres has been found in patients with intracranial bleeding, prolonged severe shock and other stressful events that may be associated with massive catecholamine release (Chappel *et al.* 1959; Cebelin & Hirsch 1980; Connor 1968; Kolin & Norris 1984). Nevertheless, in most of these conditions the catecholamine discharge does not reach levels that cause irreversible, i.e. lethal, myofibre injury. However, even in absence of necrosis, the myocardial fibres may be sufficiently damaged to become less mechanically efficient and prone to potentially lethal electrical abnormalities. The low isoproterenol doses used in our study may correspond better to these natural conditions.

The accumulation of neutral droplets of fat in the sarcoplasm was the most consistent morphological effect of non-lethal IP toxicity. These were demonstrable either as large irregular granules of lipid-soluble formazans in histochemical dehydrogenase reactions (Kakari 1970) or as well defined droplets that could be quantified by transmission electron microscopy. Other changes such as contraction bands, cellular and mitochondrial swelling, while present, were less amenable to meaningful measurement. The coarsely irregular distribution of contraction bands precluded their quantification and

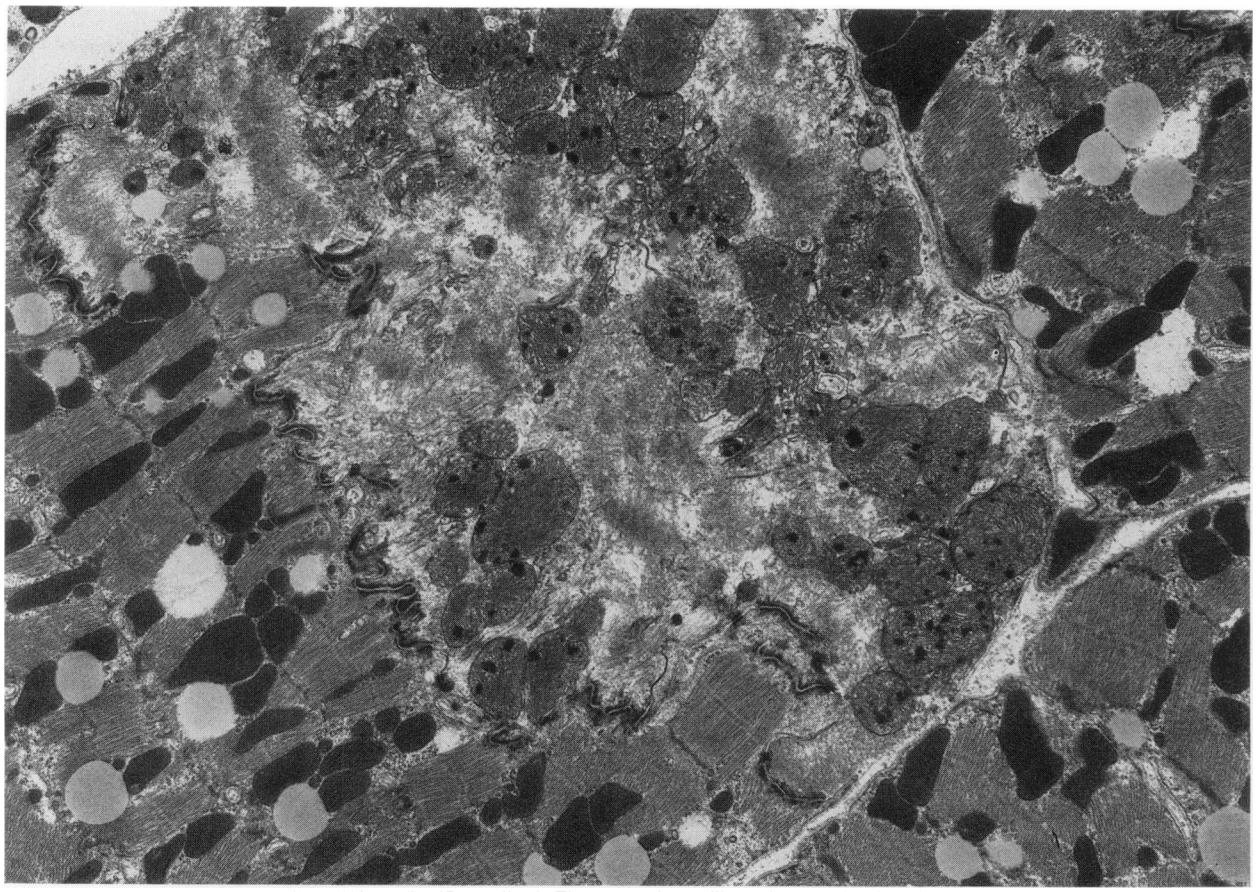

Figure 3. Unprotected myocardium (10 mg/kg IP + saline). The central myofibre shows mitochondrial swelling with dense deposits, myofibrillar disintegration and no droplets of fat. Accumulation of droplets of fat is prominent in the adjacent fibres that do not show any distinct mitochondrial damage. × 5000.

the swelling of the mitochondria was too small to allow sensitive comparisons.

Exposure of the heart to catecholamines increases cardiac work and, as a result, the demand for oxygen may exceed the delivery capacity of the coronary circulation. Fatty acids are the major myocardial energy substrate. Under anoxic conditions, the utilization of fatty acids is impaired and triglycerides are formed when these acids combine with glycerophosphate supplied by anaerobic glycolysis (Buja 1991; Liedtke 1981; Neely & Morgan 1974). The accumulation of neutral fat thus serves as an indicator of impaired myocardial metabolism and of reversible myocardial injury. As the dose of IP was increased, a checkerboard pattern of damaged and undamaged myofibres was produced (Figure 4). Severely damaged myofibres did not accumulate fat so the fat content of these myofibres could, paradoxically, then be less than after exposure to lower doses of IP. Blind biochemical data would not have provided any insights into this phenomenon.

Morphological and morphometric techniques are shown by our study to have a place in the evaluation of cardioprotective agents. They clearly demonstrated the protective effect of GHB against the accumulation of fat as well as against more severe, lethal forms of tissue damage. For low levels of injury the protective effect could be quantified suggesting that our techniques may be useful for assessment of other cardioprotective or conversely cardiotoxic agents.

The reasons for GHB's protective actions remain to be elucidated. Our study concludes that hypothermia is not a major factor in this process, as cardiac protection is observed with GHB under euthermic conditions as well. GHB may act directly at the cellular level to promote the viability of myocardial tissue under anoxic conditions. A protective effect of this nature has recently been demonstrated in the hamster gut and in haemorrhagic shock (Boyd *et al.* 1990, 1992). On the other hand, cardiovascular dynamics and the response to IP may be greatly altered in gerbils anaesthetized with GHB and this may account for the cardiac protection observed. These issues will come under careful scrutiny in future studies.

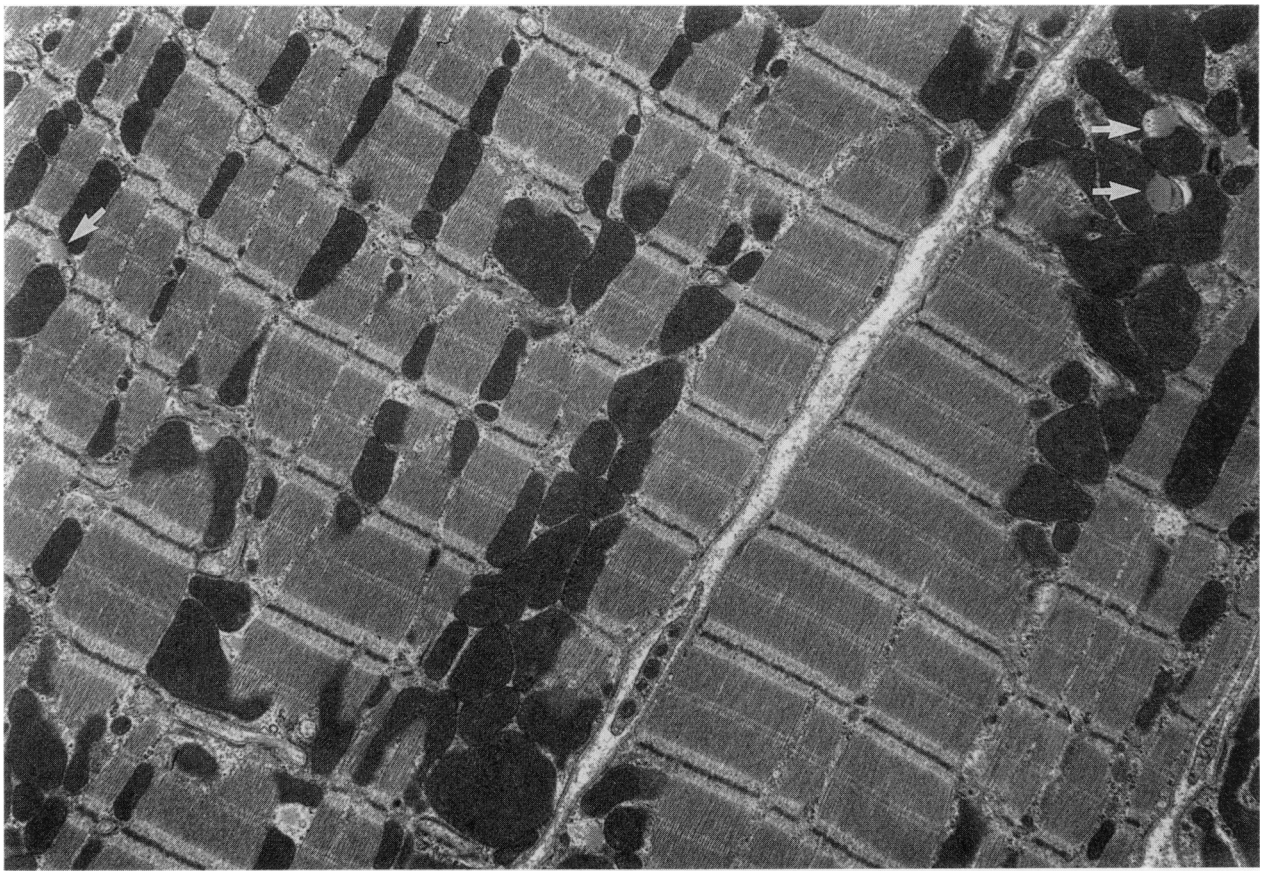

Figure 4. GHB protected myocardium (10 mg/kg + GHB). Occasional lipid droplets (arrows) are found in the otherwise unremarkable myofibres. × 5000.

Table 2. Effect of temperature on isoproterenol induced myocardial damage

Time after IP (h)	IP (mg/kg)	Treatment	Temperature	SDH grade (mean ± s.d.)	Sarcoplasmic fat (mean ± s.d.%)	*P**
3	0.1	Saline	Normothermic	2.2 ± 0.8	1.22 ± 0.25	
3	0.1	Saline	Cooled to 26°–28°C	2.00 ± 0.70	0.99 ± 0.29	
3	0.1	GHB	Hypothermic (27°C)	0.00 ± 0.00	0.09 ± 0.08	< 0.0001
3	0.1	GHB	Heated to 37°C	0.00 ± 0.00	0.12 ± 0.03	< 0.0001
—	—	Saline	Normothermic	0.02 ± 0.45	0.30 ± 0.16	

* Values for sarcoplasmic fat compared with IP and saline treated normothermic groups.

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