Cytoprotection by melatonin and growth hormone in early rat myocardial infarction as revealed by Feulgen DNA staining

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Abstract

OBJECTIVE. To examine the cytoprotective effect of melatonin or recombinant human growth hormone (hGH) on the early phase of a running myocardial infarction in rats by using the Feulgen staining.

METHODS. Rats were subjected to surgical ligature of the left coronary artery or its sham-operation and were studied 1.5–3 h later. Melatonin was administered in the drinking water (100 μg/ml water) for 7 days before surgery. Recombinant hGH (2 IU/kg) was given ip at the time of surgery. Feulgen-stained histological cardiac sections were examined by light microscopy and image analysis.

RESULTS. Infarcted rats receiving vehicle exhibited large, diffuse cardiac lesions with a marked positivity for Feulgen reaction. About 18–20% of the total area recorded became injured 1.5 or 3 h after infarction, respectively. Infarcted rats treated with melatonin or hGH, or the combination of both, and killed 3 h after surgery, showed cardiac sections with scattered lesions and only a few isolated injured muscle fibers. A similar effectiveness of melatonin and hGH, alone or in combination, to decrease injured area by 86–87% and the number of cardiac lesions by 75–80% was observed.

CONCLUSION. A significant cytoprotective effect of melatonin or hGH is demonstrable in an early phase of myocardial infarction in rats.

***Additio**. It is with great sadness we inform that Hugo E. Castagnino MD, PhD, (1938–2002) passed away on August 14. Hugo was a very clever scientist, a dedicated physician and a devoted teacher and friend. Mainly, he was a truly Renaissance man with interests in Music, Fine Arts and History. Hugo was one of the first to propose that growth hormone could be useful for treating cardiac infarction. Unfortunately, he has not survived to see his proposal turned into a promising new approach for the disease.

ORIGINAL ARTICLE

Introduction

One of the most important therapeutic end-points in the treatment of cardiovascular disease is the protection of ischemic myocardium from necrosis occurring in the hours after the onset of ischemia [1,2]. Restoration of blood flow is necessary when treating cardiac ischemia. However, except in very early cases, this is insufficient to prevent the cascade of mediators of cell damage unleashed by ischemia which, on the other hand, is boosted by the deleterious effects of reperfusion. Therefore, any adequate treatment for myocardial ischemia ought to associate, early on, reperfusion with pharmacological inhibition of those intermediaries in damage caused by ischemia/reperfusion, e.g. free radicals. In this way, spreading of the infarct may be avoided more effectively than just with reperfusion alone [1,2].

Melatonin is protective in a series of pathologies in which high production of free radicals is the primary cause of the disease [3]. In the heart, such antioxidant activity of melatonin was first proposed by Reiter and co-workers [4,5] and may explain a number of vivo and *in vitro* cardiac effects of melatonin [6–16].

The present study was undertaken to compare the cytoprotective effect of melatonin on the heart with that of recombinant human growth hormone (hGH), a treatment of demonstrable cytoprotective effectivity in experimental cardiac infarction [2,17,18]. The early morphological changes occurring in the infarcted rat myocardium were assessed by Feulgen staining, a reaction which identifies early permeability changes of nuclear membrane resulting in the spilling of DNA into the cytoplasm [19,20].

Material and Methods

Animals

Adult male Wistar rats (180–250 g) raised in our colony were kept under a 12:12 h light-dark cycle (lights off at 1800 h) with access to food and water ad libitum. Acute myocardial infarction by surgical ligature of the left coronary artery or its sham-operation was performed under diethyl ether anesthesia [21]. Adequate measures were taken to minimize pain or discomfort, in accordance with the principles and procedures outlined in European Communities Council Directives (86/609/EEC).

Melatonin (Sigma Chemical Co., St. Louis, MO) dissolved in ethanol was added to the drinking water at a concentration of 100 μg/ml; the final ethanol concentration was 0.01% for both melatonin-treated and control rats. The water bottles were covered with aluminum foil and were offered to the rats for 7 consecutive days before surgery. Fresh solutions were prepared every 2 days. As reported elsewhere, adult rats drank about 20 ml/day with 90–95% of this total daily water taken up during the dark period [22]. Thus, the melatonin dosage used provided approximately 2 mg of melatonin/day. Nocturnal water consumption did not differ among the experimental groups. Recombinant hGH (Norditropin®, Novo Nordisk, Denmark) (2 IU/kg) or vehicle (0.5 ml saline) was given i.p. immediately before surgery.

All animals were killed 3 h after surgery except for an additional group of 3 untreated rats that were killed 1.5 h after surgery. The hearts were extracted and fixed in 10% formalin for 3 days. Every fixed heart was cut in 4 transversal sections; 5-mm thick sections were embedded in paraffin, mounted, dehydrated and stained with Feulgen. Beta positivity of Feulgen stain is characterized by a reddish hue of cells produced by the spilling of DNA outside the nucleus in damaged tissue. Aldehydes of the DNA base brought about fuchsine recoloring from light green to red in sharp contrast with the back plane, which remains stained light green, like in normal controls [19,20,23,24].

Morphometric analysis

All studies were done by light microscopy. Morphometric measurements were performed using a video overlay-based digitizing interactive tool. The microscopic image was recorded with a color video camera Sony® SSC-DC 309, displayed on the monitor screen and processed with Image-Pro plus 3.0 software (Media Cybernetics, MD, USA).

Each of the 4 sections of the heart represented a different level of depth starting from the base to the apex. The upper (A) and the intermediate (C) levels were used for quantitative analysis. For every level, a circular general diagram including 9 regions (30 areas each). This disposition allowed localization of histological findings and quantification of affected areas. Every area measured amounted 26000 μ^2 regardless of the actual total surface of the area. Total surface analyzed for the general diagram was 780000 μ^2. Two additional diagrams were used for the analysis of the subendocardium (8 regions including 8 areas each) and the subepicardium (4 regions including 10 areas each). All diagrams were independently examined by three observers.

Five rats/group were examined, each heart being analyzed at 2 levels of depth, A and C, with a total of 10 observations per group. Subendocardial and subepicardial areas were examined by separate. Cardiac fibers were classified as δ (normal) or β (injured) by three observers. Presence of small amounts of affected fibers or persistence of muscular striation was considered negative, providing the total number of injured fibers did not surpass 50% of the area considered. Sections were recorded and rated. Results were expressed as total damaged area (μ^2) and as the number of injured areas.

Statistical analysis

Results were statistically analyzed by an analysis of variance (ANOVA) followed by a Student – Newman – Keuls test after logarithmic or square root transformation of data.

Results

Figure 1 depicts the histological appearance of representative left ventricle sections after Feulgen staining. Sham-operated rats showed absence of lesions in all areas examined, that remained uniformly stained with light green (Fig 1, upper left panel). Infarcted, vehicle-treated rats showed diffuse lesions with marked positivity of Feulgen reaction (Fig. 1, upper right panel). A similar picture was found in infarcted rats treated with melatonin (Fig. 1, lower left panel), hGH (Fig. 1, lower right panel) or the combination of melatonin plus hGH (picture not shown), i.e. a few scattered lesions which were limited to isolated injured cardiac fibers, with preserved myofibril striation in most cases.

Table 1 summarizes the effect of the different treatments on total surface and number of injured areas in left ventricle. Three h after cardiac infarction, about 20% of the examined area, or 13.5 out of 30 areas examined, became injured. In an independent group of rats

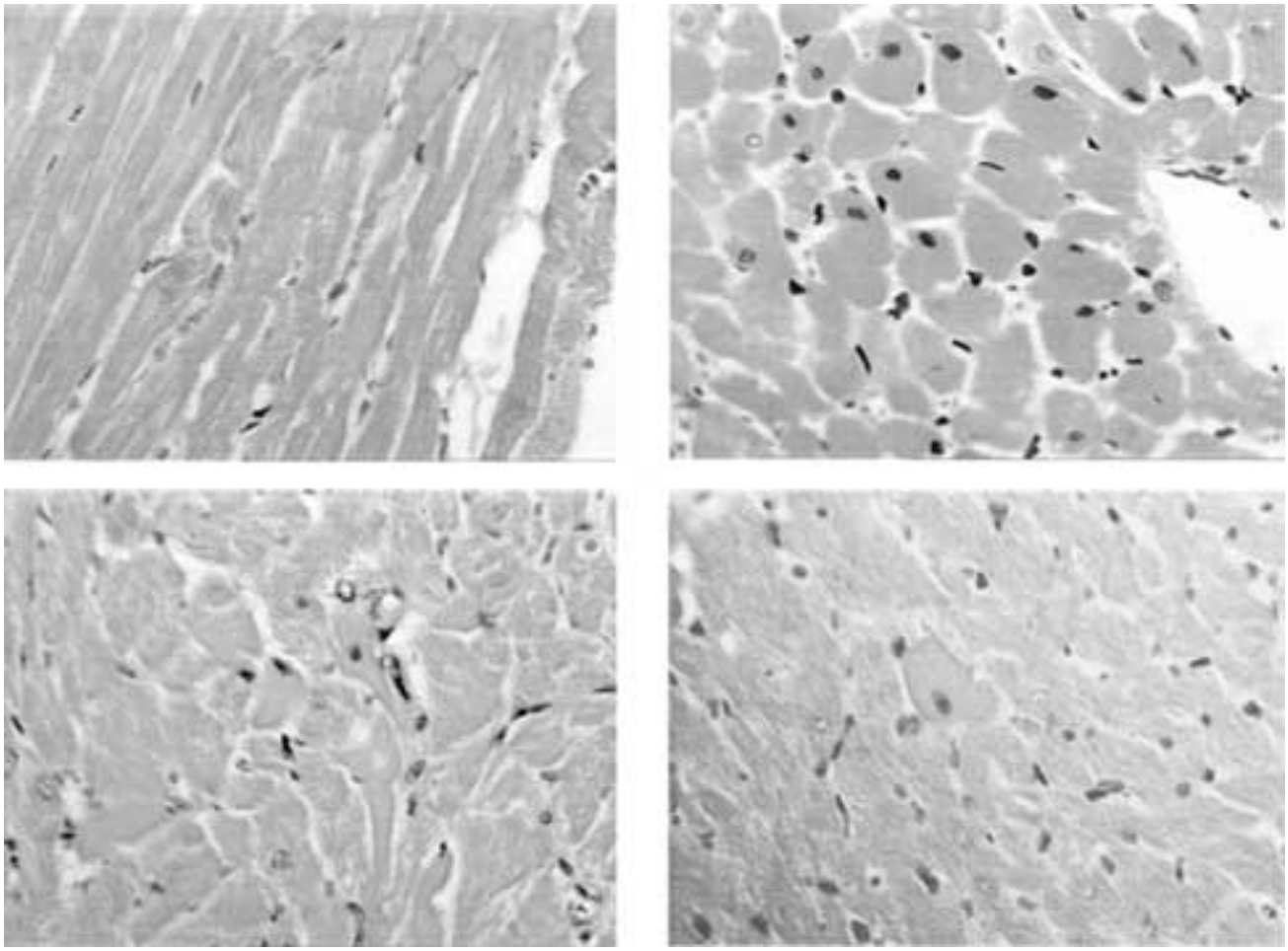

Figure 1. Feulgen staining of left ventricle sections in rats subjected to an acute myocardial infarction or its sham-operation 3 h earlier, as described in Methods. *Upper left panel:* Uniform staining with light green (Feulgen-negative) in a longitudinal section of left ventricle of sham-operated rats (x 250). *Upper right panel:* Feulgen-positive reaction, as characterized by a reddish hue of cells produced by the spilling of DNA outside the nucleus, in a transversal section of left ventricle of infarcted rats injected with vehicle (x 250). *Lower panels:* Small injured areas by ischemia within otherwise preserved left ventricular area of infarcted rats treated with melatonin (*left panel)* and hGH (*right panel*) (x 250). A similar picture was found in infarcted rats treated the combination of melatonin and hGH (picture not shown).

Table 1. Effect of melatonin and growth hormone on total injured area and number of injured areas in the left ventricle of rats subjected to an acute myocardial infarction 3 h earlier, as revealed by Feulgen stain.

Experimental Group	Total injured area (μ^2)	Number of injured areas
Sham-operated + vehicle	0	0
Infarcted + vehicle	170551 ± 117923	13.5 ± 4.9
Infarcted + melatonin	24596 ± 15933	3.4 ± 2.4
Infarcted + GH	25042 ± 18399	2.8 ± 1.8
Infarcted + melatonin + GH	23102 ± 11735	3.1 ± 2.1

Five animals/group were examined, each one comprising 2 levels of depth, i.e., sections A and C (see Methods). Injured areas as indicated by a positive Feulgen staining were calculated as described in Methods and were expressed as μ^2. Thirty areas were examined in each case. Shown are the means ± SD (n= 10 in each group). A one-way ANOVA followed by a *Student-Newman-Keuls test* after log transformation of data (areas) or square-root transformation of data (number of injured areas) indicated that every treatment differed significantly form infarcted, vehicle-treated rats ($p< 0.001$).

studied 1.5 h after infarction, the positive Feulgen reaction was seen in about 18% of examined areas (results not shown).

Treatment with melatonin, hGH or the combination of melatonin plus hGH brought about a comparable decrease of injured area to 13–14% of that seen in untreated infarcted animals, when assessed 3 h after surgery. The number of injured areas was reduced by 75–80% after treatment (Table 1). While in the infarcted rats treated with vehicle, lesions predominated at the subendocardium, in rats treated with melatonin, hGH or both no specific regional predominance of lesions was seen.

Discussion

In the present study we employed the Feulgen reaction at a light microscopical level very early after a cardiac infarction (1.5–3 h) because of several reasons. First, any fuchsinophilic degeneration revealed at this early time would rely mainly on permeability changes of the nuclear membrane of cardiomyocytes. Second, a positive Feulgen reaction would allow disclosure of minor histological details (fading out of muscular striation, presence of nuclear alterations), generally overseen by the simple binary expression of necrotic-savaged areas within affected tissues, as is the case for most other histological procedures [19]. Third, the Feulgen reaction would permit ploidy evaluation [20].

The foregoing results indicate that, 1.5–3 h after infarction, about one-fifth of left ventricle area became Feulgen-positive (predominantly in the subendocardial region). Melatonin, given in the drinking water at a dose of 2 mg/day for 7 days, or recombinant hGH (2 I.U. injected i.p. injection at the time of surgery) were effective to decrease injured area by 86–87% and to reduce the number of lesions by 75–80%, when assessed 3 h after surgery. Although the effect of melatonin or hGH was seen throughout the left ventricle, it was predominant in the subendocardial region, as shown by the disappearance of the subendocardial-subepicardial differences found in infarcted rats treated with vehicle alone.

The effect of melatonin and GH was not additive inasmuch as the combination of both treatments did not result in better cytoprotection than each one by separate. This probably relied on that supramaximal doses of each compound were employed. Further studies employing lower doses of each compound are needed before a conclusive picture on this matter is obtained. It is important to stress that melatonin was given orally while a parenteral administration of hGH was used. Indeed, an orally available, low cost, molecule like melatonin may have a significant clinical utility to reduce the severity of heart damage in situations of ischemia-reperfusion injury [25].

The cardioprotective effects of melatonin or GH on the ischemic myocardium are presumably multifactorial. For example, in the case of melatonin, specific binding sites for melatonin exist in the mammalian heart [26], although there is no information on the subtype of melatonin receptor present [27]. In addition, as above stated, melatonin is a significant free radical scavenger and antioxidant, mainly at pharmacological concentrations, both by directly neutralizing reactive oxygen species as well by augmenting a number of antioxidative enzymes [28].

Therefore, the effect of melatonin on myocardial ischemia at the high pharmacological dose employed is presumably exerted mainly by the free radical scavenging properties of melatonin. Such an activity of melatonin was first proposed in cardiac tissue [4,5] and was used to explain several aspects of *in vivo* and *in vitro* effects of melatonin on heart [6–16].

In the case, of GH, its cytoprotective activity on experimental cardiac infarction and its consequences (i.e., ventricular aneurysms) had been known for some time [2,17,18]. It was proposed that GH acts by preserving the myocardial connective tissue, which displays a complex layout around each myocyte and among neighboring ones [29].This pattern is highly vulnerable to acute coronary ischemia, the ultimate consequence of ischemia being the disappearance of intermyocytic links and the collagen weave that surrounds each cell. GH may act through insulin-like growth factor-1, a mediator of GH activity in several peripheral tissues, since IGF-1 also protected myocardium against death in animal models of infarct and ischemia-reperfusion injury [30,31].The present study further documents the efficacy of hGH to prevent early damage in the ischemic cardiac cells by uncovering a new action of GH, i.e. the protection of the nuclear membrane of cardiomyocytes at a very early stage of infarction.

Cardioprotection against ischemia may be defined as the action by which a cell does not suffer injury to its structure or metabolism after the exposure to chemical or physical stress. How cardiac cells respond to stress is a central problem in cardiovascular biology. Injury produces multiple changes in a cell that ultimately affect protein structure and function. Cells initiate a cascade of events that finally engage essential proteins, the molecular chaperones (e.g., the heat shock family of stress proteins) in decisions to repair or degrade damaged proteins as a defense strategy to ensure survival. Myocardial cell necrosis is a process which progresses by steps; it may be stopped by cardioprotective agents before a point of no return arrives. Therefore, the cardioprotective effects of melatonin or GH on the ischemic myocardium may have therapeutic relevance. It is of interest that the endogenous melatonin secretion is impaired in patients with coronary heart disease [32] and significantly more in those patients with unstable angina and high risk of cardiac infarction [33].

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