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Heart lesions associated with anabolic steroid abuse: Comparison of post-mortem findings in athletes and norethandrolone-induced lesions in rabbits

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Abstract

Among 15,000 forensic post-mortem examinations performed on the coroner's order over a 24-year period (January 1981–December 2004) in the area of Lyon, France (population: 2,000,000), 2250 cases of unexpected cardiac sudden death were identified retrospectively according to WHO criteria. Of these, 108 occurred during recreational sport and 12 occurred in athletes. In the latter category, a history of anabolic steroid abuse was found in 6 cases, whereas pre-existing ordinary cardiac lesions were observed in the 6 remaining cases. To shed light on the possible role of anabolic steroids in the induction of cardiac lesions, an experimental study was conducted in rabbits that were treated orally with norethandrolone 8 mg/kg/day for 60 days, and sacrificed at day 90. The histopathological examination of the heart from treated animals showed coronary thrombosis associated with left ventricle hypertrophy in 3 cases, and lesions analogous to toxic or adrenergic myocarditis in all other treated animals. These findings were very similar to those observed after cardiac sudden death in the 6 athletes with a history of anabolic steroid abuse. In addition, elevated caspase-3 activity in the heart of treated rabbits as compared to controls suggests that apoptosis is involved in the induction of norethandrolone-induced cardiac lesions. These results confirm the cardiotoxic potential of anabolic steroid abuse.

Keywords: Sport; Doping; Anabolic steroids; Norethandrolone; Sudden cardiac death; Apoptosis; Post-mortem examination; Rabbit

Introduction

Cardiac sudden deaths do occur following occasional sport activities and in athletes. An estimated incidence of 3 cardiac sudden deaths per million per year following recreational sport in human subjects with

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pre-existing cardiac lesions has been reported (Tabib et al., 1999), whereas sudden cardiac death in high-level athletes could account for 2–3 deaths/100.00/year (Maron et al., 1986; Corrado et al., 2006). This can be compared to an estimated overall incidence of 0.08 sudden deaths/100.000 in the general population (Corrado et al., 2006). Although unrecognized pre-existing cardiac lesions are obviously likely to play a major role in sudden cardiac deaths, the abuse of doping substances, such as anabolic steroids or cocaine, has been suggested to be involved (Fineschi et al., 2001; Eckart et al., 2004; Haigney et al., 2006; Kindermann, 2006; Di Paolo et al., 2007; Fineschi et al., 2007).

In order to substantiate further the possible role of anabolic steroid abuse in cardiac sudden death, a retrospective analysis of 15,000 forensic post-mortem examinations was conducted. Subsequently, an experimental study in rabbits treated orally with the prototypic anabolic steroid norethandrolone for 60 consecutive days was undertaken in an attempt to reproduce those cardiac lesions observed in post-mortem examinations.

Material and methods

Post-mortem examinations

A total of 15,000 post-mortem examinations were performed on the coroner's order in the Institute of Forensic Medicine at Claude Bernard University (Lyon, France) from January 1981 to December 2004. The cases were analyzed retrospectively to identify unexpected cardiac sudden deaths according to the World Health Organization criteria (WHO, 1969). The procedure used for the pathological examination of the heart was previously described by Tabib et al. (1999). Briefly, the hearts were immediately weighted and examined macroscopically. One sample of the right ventricle, two of the left ventricle and one of the septal crest were taken and fixed using a mixture of ethanol, formaldehyde and acetic acid. After paraffin embedding, 5-mm sections were prepared and stained with hematoxylin–phloxin–saffron (HPS) to study the architecture of cardiac fibers, the aspect of nuclei, the presence of myolysis, necrosis and interstitial fibrosis (either perivascular systematized or reticular non-systematized fibrosis), leukocyte infiltrates and dysplastic lesions in the distal coronary arteries.

Rabbit study

New Zealand White rabbits of both sexes purchased from CEGAV (Saint-Mars d'Egrenne, France) were used throughout. They were 2–3 months of age and weighted approximately 2.5 kg at the start of the study. They were housed singly in an air-conditioned room (temperature: $22 \pm 2\,^{\circ}C$, relative humidity: $55 \pm 15\%$, lighting cycle: 12 h artificial light/12 h dark), and were supplied with nonsupplemented pellet food for rabbits (SAFE, Augy, France) and tap water *ad libitum*. The experimental study plan was approved by the Animals' Ethics Committee of Claude Bernard University (Lyon, France).

Two groups of 6 animals (3 males and 3 females) were used following randomization. One group was treated daily with 8 mg/kg/day norethandrolone (Laphal laboratories, Allauch, France) for 60 consecutive days via the oral route followed by a 30-day treatment-free period. The other group received the same volume of saline daily via the same route for 60 days followed by a 30-day treatment-free period. On day 90, all animals were anesthetized intramuscularly with 400 mg/kg ketamine (Imalgène®, Mérial laboratories, Lyon, France) followed by 32 mg/kg xylasine (Rompun®, Bayer, Puteaux, France) and then their hearts were removed for histopathological examination after thoracotomy. Finally, the animals were sacrificed with 88 mg/kg pentobarbital (Dolethal®, Vetoquinol laboratories, Lure, France) intravenously.

The hearts were immediately cut from the apex to the base to obtain full transverse slices of the mid-section of the biventricular mass. One 5-mm slice from each heart was fixed in a formalin solution (alcohol, formaldehyde and acetic acid), embedded in paraffin, cut in 5-µm sections, which were stained by HPS for histopathological examination. Caspase-3 activity was measured in a second section using the fluorogenic substrate peptide Asp–Glu–Val–Asp–7-amino-4-methylcoumarin (DEVD-AMC, Bachem Biochimie, Voisins Le Bretonneux, France) as previously described (Raisky et al., 2004). Briefly, aliquots containing 80 µg of cytosolic proteins in 50 µl buffer C (5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 10 µg/ml peptidase A and 10 µg/ml leupeptin in 25 mM Hepes, pH 7.5) were dissolved with 225 µl of freshly prepared buffer D (0.1% (w/v) 3-[(cholamidopropyl) dimethylammonio]-1-propane-sulfonate, 10 mM DTT, 1 mM PMSF and 10 µg/ml aprotinin in 25 mM HEPES, pH 7.5) containing 167 µM of substrate, and were incubated for 60 min at 37 °C. Fluorescence was measured using a Perkin–Elmer fluorimeter (excitation at 342 nm, emission at 441 nm). The amount of released 7-amino-4-methyl coumarin (AMC) was calculated by transposing each point onto a scale with purified AMC ($n = 7$–8/group).

The statistical analysis of the results included a comparison of the weight of the heart using Mann and Whitney non-parametric test, a comparison of the percentages of cardiac lesions using Fisher exact test, and finally, a comparison of the mean caspase-3 activity using Tuckey non-parametric test. The threshold of statistical significance was consistently $p < 0.05$.

Results

Post-mortem examinations

Among the 15,000 forensic post-mortem examinations performed from January 1981 to December 2004, cardiac sudden death was retrospectively identified in 2250 cases, of which 1800 had a known pre-existing heart disease. In 120 cases, cardiac sudden death occurred during or immediately after sport, including 108 (90%) in subjects who used to practice recreational sport activities without specific training or medical supervision, and 12 in regular, well-trained and medically controlled athletes. As shown in Table 1, gross and microscopic examination of the heart found 2 types of lesions. In the first group of 6 patients, there were obvious signs of long-standing congenital or acquired cardiopathy without any detectable recent alterations. These lesions did not differ from those commonly seen in the general population. Therefore, it is likely that these patients died of acute worsening of pre-existing cardiac lesions, either previously undetected (patients 1, 4 and 5) or imperfectly characterized (patients 2, 3 and 6). In contrast, the second group of 6 patients had severe acute lesions with unusual anomalies including perivascular interstitial fibrosis, disorganized muscular architecture and dysmorphic myocyte nuclei. Two had hemorrhagic suffusion over the whole myocardium with erythrodiapedesis of the circumferential sub-endocardial floor, while the coronary network remained free (Fig. 1A). In 2 others, there was disseminated myocarditis with various juxtaposed lesions (Figs. 1B and 2A): either areas of latticed myocytic liquefaction necrosis, or areas of edema infiltrated by a few leukocytes (Fig. 2C), or an area of long-standing collagen-rich sclerosis, or more recent sclerosis still tattooed with leukocytes. In the small distal intramyocardial coronary network branches (Fig. 2E), the lumen area was reduced by concentric fibrosis. In another case, lesions of mild congenital dilated cardiomyopathy were associated with a recent coronary thrombosis in a network featuring very mildly sclerotic, non-stenosing young lipid atheromatous plaques (Fig. 1C). A retrospective inquiry confirmed the intake of anabolic steroids in all 6 patients.

Rabbit study

No clinical signs or mortality were seen in control animals. In contrast, 3 rabbits treated with norethandrolone died before the scheduled sacrifice at days 24, 34 and 64, respectively. These 3 rabbits showed major heart lesions of early onset. Their hearts weighted 9, 10 and 8 g, respectively. The mean weight of the treated animals was found to be increased compared to the mean heart weight of the controls (9.17 ± 1.22 g vs. 7.00 ± 0.45 g), but the difference was not statistically significant ($p = 0.08$), presumably because of the small number of animals.

The histopathological examination of the hearts from norethandrolone-treated and control rabbits revealed marked differences. No anomalies were found in the controls, whereas the treated animals presented with multiple myocardial and coronary lesions including disorganized myocyte architecture, misshapen cell nuclei, myolysis, interstitial fibrosis, leukocyte infiltration and coronary lesions with stenosing circumferential intimal sclerosis of the distal network (Table 2, Fig. 2B, D and F).

Table 1. Main characteristic, circumstances of death, heart weight and cardiac lesions at post-mortem examination of 12 athletes with cardiac sudden deaths

Case	Sex	Age (years)	Sport	Circumstances of death	Heart weight (g)	Lesions
1	M	26	Pot-holing	Drowning underground	530	Dilated cardiomyopathy
2	M	38	Soccer	During a match	430	Myocardial contusion scar
3	M	27	Soccer	During a match	340	Arrhythmogenic RV cardiomyopathy
4	M	24	Karting	Championship race	410	Hypertrophic cardiomyopathy
5	M	29	Basket ball	During a match	460	Hypertrophic cardiomyopathy
6	F	19	Rowing	During leisure trip	280	Dispersion of specific sino-atrial node cells
7	M	19	Weight lifting	Training	360	LV apoplexy
8	M	22	PE teacher	Training	520	LV apoplexy
9	M	25	Body building	Training	460	Disseminated myocarditis
10	M	28	Soccer	During a match	380	Disseminated myocarditis
11	M	54	Marathon	End of race	410	Coronary thrombosis and dilated cardiomyopathy
12	M	48	Marathon	Training	430	LV hypertrophy

Long-standing congenital or acquired cardiac lesions were seen in cases 1–6; severe acute cardiac lesions associated with a history of anabolic steroid use in cases 7–12.

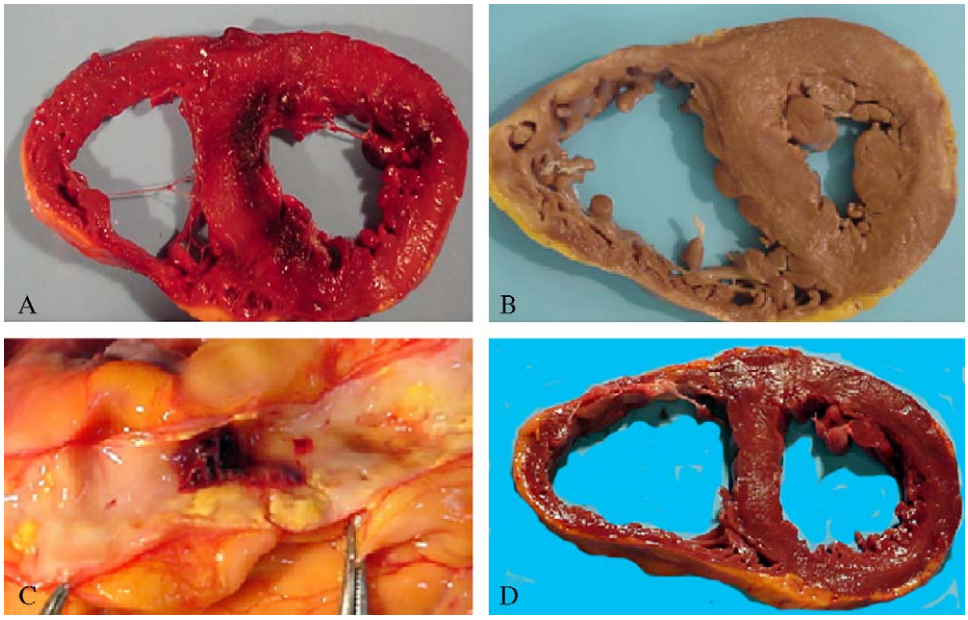

Fig. 1. Macroscopic findings in patients with cardiac sudden death: (A) myocardial apoplexy (patient no. 7); (B) disseminated myocarditis (patient no. 9); (C) coronary thrombosis; (D) dilated myocardiopathy (patient no. 11).

Finally, capsase-3 activity was found to be significantly increased in the heart sample from treated animals as compared to controls: 29.5 ± 8.6 pmol-AMC/min/mg vs. 2.6 ± 1.7 pmol-AMC/min/mg, respectively.

Discussion

In this retrospective study of 15,000 forensic post-mortem examinations in the metropolitan area of Lyon (population = approximately 2 million inhabitants) performed over a 24-year period, 2250 sudden cardiac deaths were identified. In 1800 cases, a pre-existing cardiac disease was known. Among the cardiac sudden deaths, 120 were observed in close chronological relation with either recreational (108 cases) or high-level (12 cases) sport activities. This is in keeping with previously published estimates as regards the incidence of cardiac sudden deaths in the general population and the higher incidence in association with recreational or high-level sport (Maron et al., 1986; Tabib et al., 1999; Corrado et al., 2006; Fineschi et al., 2007). In the latter 12 cases, the post-mortem examination concluded to an acute worsening of pre-existing undetected or ill-diagnosed cardiac lesions in 6 athletes, while markedly different lesions were evidenced in the 6 other patients. The cardiac lesions were reminiscent of those described in adrenergic myocarditis with disseminated sub-endocardial apoplexy of the left ventricle, or in toxic myocarditis with multiple focal areas of necrosis, myolysis and acute or long-lasting scarring fibrosis. Diffuse stenosing sclerosis of the intima in the distal coronary network and dysmorphic myocytic nuclei suggest a long-lasting exposure to anabolic steroids as previously described (Dhar et al., 2005; Giese et al., 2007; Sen-Chowdhry and McKenna, 2006; Turakhia and Tseng, 2007). Interestingly, very similar cardiac lesions were observed in the rabbits treated with norethandrolone. Other cardiac lesions associated with the use of anabolic steroids, such as alterations of myocardial mitochondria and fibrosis with ventricular arrhythmia, have been described (Dhar et al., 2005) but were not observed in this study. It is assumed that the cardiac lesions observed in 4 athletes (1, 3, 4 and 5) could have been evidenced by ECG and echocardiographic monitoring as recommended for the detection of at-risk patients (Beckerman et al., 2004; Sen-Chowdhry and McKenna, 2006; Giese et al., 2007). In contrast, other constitutive anomalies as those seen in patients 2 and 6 can hardly, if at all be detected, except via a pathological examination of the heart. Finally, it is noteworthy that none of these athletes had stenosing atheromatous coronary lesions, which are a common cause of sudden death in the general population (Turakhia and Tseng, 2007), most presumably because of their relatively young age.

As the results of our retrospective analysis of post-mortem examinations suggested a possible role of anabolic steroid abuse in the induction of cardiac lesions resulting in sudden cardiac deaths, an experimental study in rabbits was conducted to confirm this hypothesis. Norethandrolone was selected as this is a prototypic anabolic steroid, the cardiotoxic potential of which has not been extensively investigated.

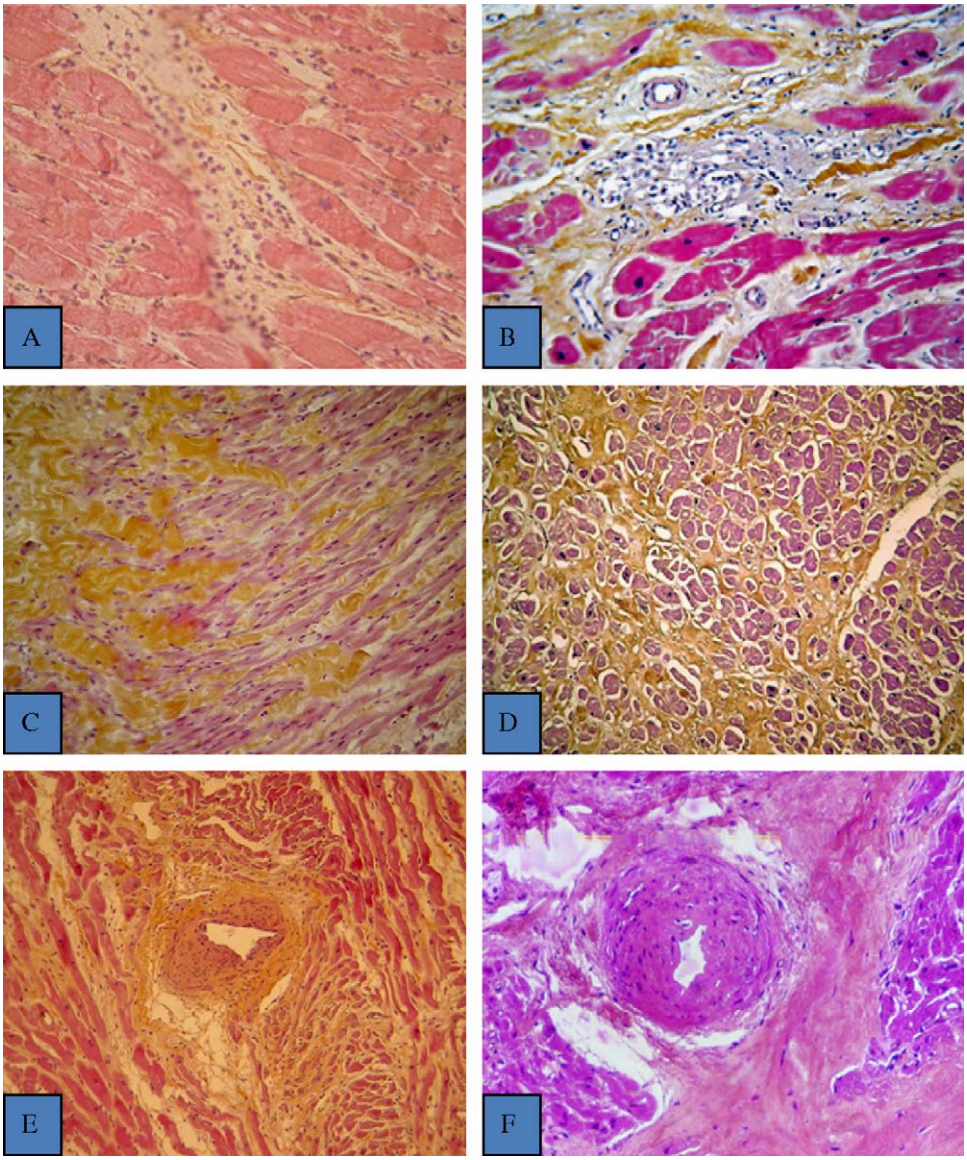

Fig. 2. Comparative histological aspects of observed lesions (hematoxylin–phloxin–saffron (HPS); × 20). Interstitial inflammatory cells: (A) human and (B) rabbit; interstitial reticular fibrosis: (C) human and (D) rabbit; distal coronary network, concentric stenosing intimal sclerosis: (E) human and (F) rabbit.

Table 2. Myocardial and coronary histological lesions in controls and norethandrolone-treated rabbits (0 = no lesions, X = small lesions; XX = medium-size lesions; XXX = large lesions)

	Heart weight (g)	Disorganized architecture	Misshapen nuclei	Myolysis	Focal necrosis	Interstitial fibrosis	Lymphocytes	Dysplasia
Controls ($n = 6$)	9	0	0	0	0	0	0	0
Died D24	9	X	X	X	X	X	X	0
Died D34	10	XX	XXX	XXX	XXX	XX	XX	X
Died D64	8	XX	XX	XX	XX	XXX	XXX	X
Schedule sacrifice D90	8	XX	X	XX	XX	XX	0	0
Scheduled sacrifice D90	10	XX	X	X	X	X	0	0
Scheduled sacrifice D90	10	X	XX	XXX	XXX	XX	0	X

The administration of norethandrolone did not induce ventricular hypertrophy, presumably because of the relatively short duration of exposure (2 months) and the recovery period of 1 month. Indeed, a recent longitudinal study did not find evidence of left ventricular hypertrophy following short-term use of norethandrolone by athletes (Hartgens et al., 2003). In contrast, Thompson et al. (1992) found markedly increased left ventricles following long-term use exceeding the ventricular hypertrophy normally seen in athletes. Left ventricular hypertrophy can account for disturbances in left (Nottin et al., 2006; Krieg et al., 2007) or global (Kasikcioglu et al., 2008) diastolic relaxation resulting in arrhythmias, and potentially in sudden death (Gauthier, 2001). Anabolic steroids have been reported to induce left ventricular hypertrophy restricting the supply of oxygen and energy-rich substrates leading to decreased heart performances in athletes (Tagarakis et al., 2000). Various lesions including misshapen cell nuclei, myolysis, fibrosis and interstitial lesions were found in the heart of norethandrolone-treated rabbits. These lesions of short onset are similar to those seen in toxic myocarditis (Sullivan et al., 1998). They appeared as disseminated foci associated with polymorphic alterations of the myocardial fibers. The anabolic and androgenic effects of norethandrolone can be suspected to account for the induction of these lesions as a variety of cardiovascular complications (Kindermann, 2006) including hypertension, cardiopathy, pulmonary embolism (Liljeqvist et al., 2008), severe and sometimes lethal arrhythmias (Furlanello et al., 2007), atrial fibrillation (Lau et al., 2007) and myocardial infarct (Wysoczanski et al., 2008) have been described following the use and abuse of anabolic steroids. However, very little is known of the cardiotoxic potential of norethandrolone and no cardiac lesions similar to those seen in our norethandrolone-treated rabbits have so far been reported.

An interesting finding is the presence of apoptotic lesions in the heart of norethandrolone-treated rabbits. These lesions consisted of foci of approximately 10 myocardial cells with very dense hyperchromatic nuclei, or irregularly interspersed chromatin within a vesiculous cytoplasm without striations. The same cytonuclear features were observed in some intramyocardial vessels, which strongly supports the induction of apoptotic lesions in these rabbits. Similar results with anabolic steroids have been reported by Zaugg et al. (2001). While the molecular mechanism involved in the induction of apoptosis by anabolic steroids is not elucidated, the heart seems to be one of their specific targets (Sullivan et al., 1998). Receptors binding anabolic steroids have been found in cardiomyocytes from monkeys (McGill and Sheridan, 1981) and humans (Ruizeveld de Winter et al., 1991). Upon binding to these membrane receptors, anabolic steroids could trigger a cascade of events leading to cardiomyocyte death by apoptosis. Anabolic steroids could lead to increased concentrations in polyamines within cardiomyocytes, which in turn would induce anarchic transmembrane transfers of calcium ions resulting in calcium overload detrimental to the normal functioning of the ventricular myocardium (Vicencio et al., 2006). The role of elevated intracellular calcium concentrations in the induction of apoptosis is supported by many studies. Thus, elevated cytosolic calcium concentrations alter the permeability of mitochondrial membranes, which results in the release of pro-apoptotic factors including holocytochrome c, apoptosis-inducing factor and caspase-9, from injured mitochondria (Kroemer et al., 1998).

In our rabbit model, the inter-individual variability of induced cardiac lesions suggests an individual susceptibility to the cardiotoxic effects of norethandrolone: the 3 animals that died before the end of the study were those with the largest myocardial as well as coronary lesions. If these findings hold true for humans, this would account for the relative infrequency of cardiac sudden death in athletes. Interestingly, Fineschi et al. (2001) reported no cardiac lesions in their own series.

In conclusion, sudden cardiac deaths in athletes may have two different causes: common, undiagnosed or otherwise ignored, asymptomatic cardiopathy that may turn lethal during exercise; cardiac lesions induced by doping substances and anabolic steroids in particular. Although our findings that apoptosis is likely to play a key role in the induction of cardiac lesions by anabolic steroids warrant further explorations, they suggest that the development of anti-apoptotic agents to protect the myocardium is worth considering.

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