Int J Legal Med (2008) 122:461–469
DOI 10.1007/s00414-008-0262-2

ORIGINAL ARTICLE

Cardiac oxidative stress determination and myocardial morphology after a single ecstasy (MDMA) administration in a rat model

Daniela Cerretani · Irene Riezzo · Anna Ida Fiaschi · Fabio Centini · Giorgio Giorgi · Stefano D'Errico · Carmela Fiore · Steven B. Karch · Margherita Neri · Cristoforo Pomara · Emanuela Turillazzi · Vittorio Fineschi

Received: 15 November 2007 / Accepted: 30 May 2008 / Published online: 2 July 2008

Abstract Experimental and clinical data indicate that 3,4-methylenedioxy-*N*-methylamphetamine (MDMA) abuse can produce significant cardiovascular toxicity. A mechanism may be a direct toxic effect of redox active metabolites of MDMA. To evaluate the effect of a single MDMA dose on cellular antioxidant defence system and to investigate the morphology in male albino rats, total glutathione (GSH), oxidised glutathione (GSSG), ascorbic acid (AA), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and malondialdehyde (MDAL) were studied. The effects were evaluated at 3, 6, 16 and 24 h after MDMA administration. Antioxidant enzymes activity was significantly reduced: GPx (−24%) and SOD (−50%) after 3 h and GR (−19%) after 6 h from treatment. AA levels decrease (−37%) after 3 h and (−30%) after 6 h; MDAL level increased (+119%) after 3 h; GSH levels decreased after 3 (31.3%) and 6 h (37.9%) from MDMA treatment. GSSG content was not affected by ecstasy administration. Myocardial contraction band necrosis (CBN) was already visible in rats killed at 6 h. After 16 h, macrophagic monocytes around the necrotic myocardial cells were observed, and within 24 h, this infiltrate became more widespread with an early removal of the necrotic material. Calcium deposits were observed within ventricular cardiomyocytes with intact nuclei and sarcomeres. Single administration of MDMA can significantly alter the cellular antioxidant defence system and produce oxidative stress which may result in lipid peroxidation and disruption of Ca^{2+} homeostasis. The depression in Ca^{2+} regulatory mechanism by reactive oxygen species ultimately results in intracellular Ca^{2+} overload, CBN and cell death.

Keywords Ecstasy · Cardiac oxidative stress · Myocardial morphology · Rat model

Electronic supplementary material The online version of this article (doi:10.1007/s00414-008-0262-2) contains supplementary material, which is available to authorized users.

D. Cerretani · A. I. Fiaschi · G. Giorgi
Department of Pharmacology "G. Segre", University of Siena, Siena, Italy

I. Riezzo · S. D'Errico · C. Fiore · M. Neri · C. Pomara · E. Turillazzi · V. Fineschi (✉)
Department of Forensic Pathology, University of Foggia, Ospedale Colonnello D'Avanzo, Via degli Aviatori 1, 71100 Foggia, Italy
e-mail: vfinesc@tin.it

F. Centini
Unit of Forensic Toxicology, University of Siena, Siena, Italy

S. B. Karch
Consultant Forensic Pathologist, P.O. Box 5139, Berkeley, CA 94705, USA

3,4-Methylenedioxy-*N*-methylamphetamine (MDMA) is a semisynthetic entactogen of the phenethylamine family. It is considered a recreational drug and has long had a strong association with the rave culture [18]. The cardiovascular and toxicological consequences of MDMA abuse have not been fully characterised. Acute administration of MDMA increases heart rate, blood pressure and myocardial oxygen consumption in both humans [24, 19] and animals [3], which may ultimately result in hypertension, arrhythmias, cardiac ischaemia and heart failure. MDMA, or the presence of one of its metabolites, leads to a sustained

elevation of circulating monoamines [24] as possible mechanism for MDMA toxicity. Another possible mechanism may be a direct toxic effect of MDMA or its redox active metabolites in cardiac cells. Metabolism of MDMA involves N-demethylation to 3,4-methylenedioxyamphetamine (MDA). MDMA and MDA undergo conversion to the corresponding catecholamine, *N*-methyl-alpha-methyldopamine (*N*-Me-α-MeDA) and alpha-methyldopamine (α-MeDA), respectively, both of which are cathecols that can undergo several step of oxidation to the corresponding orthoquinones and aminochromes with generation of reactive oxygen species (ROS) and reactive nitrogen species that can induced oxidative stress, lipoperoxidation and disruption of cardiomyocytes Ca^{2+} homeostasis [4, 5]. We conducted a study in an in vivo animal model to evaluate: (1) the effect of a single dose of MDMA (20 mg/kg, i.p.) on cardiac cellular antioxidant defence system and to further investigate the mechanisms of the potential toxicity of MDMA in the rat heart of the following parameters: total glutathione (GSH), oxidised glutathione (GSSG), ascorbic acid (AA), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and malondialdehyde (MDAL) as index of lipid peroxidation. (2) We also attempted to describe the cardio-morphological alterations and to discuss the pathogenetic mechanisms that are predictably related to MDMA.

Materials and methods

The experimental procedures followed the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1996) and were approved by the University of Siena Committee for animal experiments.

Animal model and experimental protocol

For the evaluation of oxidative stress, three groups of seven male albino rats (Wistar, Charles River) weighing 200–250 g were used to analyse the effect of MDMA administration (20 mg/kg, i.p.) on rats hearts. After treatment, animals were killed by decapitation at the following times: group 1, 3 h; group 2, 6 h; group 3, control group. Heart samples were used to determine the biochemical parameters of oxidative stress.

Both the histopathological examination of the heart and the plasma concentrations of MDMA/MDA were carried out on 25 rats, each weighing 200–250 g, divided into three groups of seven animals each and treated with MDMA 20 mg/kg, i.p.: group 1 killed 6 h after treatment; group 2 killed 16 h after treatment; group 3 killed 24 h after treatment. One control group of four animals was treated with saline i.p. and killed 24 h after treatment. Plasma samples obtained after the treatments were stored at −80°C until MDMA/MDA gas chromatography/mass spectroscopy (GC-MS) analysis.

Biochemical analysis: oxidative stress evaluation

The hearts of the treated and control animals were immediately dissected and then frozen at −80°C. Concentrations of GSH and GSSG, MDAL and AA levels and of SOD, GPx and GR enzymatic activities were determined as follows.

GSH/GSSG and protein determination

Heart tissue was homogenised in ethylenediaminetetraacetic acid (EDTA) K^+ phosphate buffer, pH 7.4 (1:3, *w/v*) at 0°C. Total GSH was analysed as described by Tietze [35], and GSSG was determined according to Griffith's method [16]. In the remaining aliquot, proteins were assayed according to the method of Lowry [20].

SOD, GPx and GR assessment

To measure cytosolic enzyme activity, the heart samples were homogenised according to Whanger and Butler [37]. GPx activity was measured according to Paglia and Valentine [25]. GR activity was analysed as described by Goldberg and Spooner [15]. Total superoxide dismutase (Cu/Zn superoxide dismutase and Mn superoxide dismutase) was assayed by spectrophotometric method based on the inhibition of a superoxide-induced NADH oxidation according to Paoletti et al. [26]. The cytosolic protein concentration was determined using the Lowry method with BSA as standard [20].

MDAL assessment

The extent of lipid peroxidation in the rat hearts was estimated by calculation of MDAL levels with a high-performance liquid chromatography method and UV detection as described by Shara et al. [33].

AA assay

Heart tissues were homogenised in EDTA–K^+ phosphate buffer pH 7.4 (1:4, *w/v*) at 0°C and analysed as described by Ross [32].

Morphological examination

The hearts of treated and control animals were immediately removed, weighed and fixed in 10% buffered formalin for 48 h then embedded in paraffin (ESM Table 1). Paraffin-embedded tissue specimens of hearts were sectioned at

Table 1 Effect of MDMA (ecstasy) exposure (20 mg/kg, i.p.) on cardiac antioxidant cellular systems

	Control	3 h	6 h
SOD (U/mg protein)	13.65±2.867	9.09±2.176[a]	13.16±3.927
GPx (U/mg protein)	132.52±20.150	97.53±17.258[a]	119.47±21.773
GR (U/mg protein)	12.54±1.229	12.42+3.001	10.17±1.767[a]
MDAL (nmol/g tissue)	1.78±1.092	3.91±0.0991[b]	2.07±1.215
AA (nmol/g tissue)	62.96±10.079	39.23±15.388[a]	43.81±6.928[a]
GSH (nmol/g tissue)	1.82±0.261	1.13±0.316	1.25±0.209[a]
GSSG (nmol/g tissue)	0.55±0.491	0.49±0.211	0.56±0.362

Values are presented as the mean±SD of seven rats.
[a] $p<0.05$ compared with control group
[b] $p<0.01$ compared with control group

4 μm and stained with haematoxylin and eosin, Masson trichrome stain; Von Kossa histochemical reaction was also used. In addition, immunohistochemical investigation of hearts samples was performed with an antibody anti-myoglobin (Dako, Copenhagen, Denmark), anti-troponin I (Abcam, Cambridge, UK) and anti-troponin C (NovoCasta, London, UK). For this study, we used 4-μm-thick paraffin sections mounted on slides covered with 3,aminopropyl-triethoxysilane (Fluka, Buchs, Switzerland). Pretreatment was necessary to facilitate antigen retrieval and to increase membrane permeability to antibodies anti-troponin I and anti-troponin C, boiling in 0.25 M EDTA buffer for 14 min in microwave. The primary antibody was applied in 1:6,000 ratio myoglobin, in a 1:3,000 ratio troponin I and in a 1:6,000 ratio troponin C and incubated for 120/min at 20°C. The detection system utilised was the LSAB+ kit (Dako), a refined avidin–biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules. The positive reaction was visualised by 3,3-diaminobenzidine peroxidation according to standard methods. The sections were counterstained with Mayer's haematoxylin, dehydrated, coverslipped and observed in a Leica DM4000B optical microscope (Leica, Cambridge, UK). Using Leica system, the total area in square millimetres was measured in heart sections. By total screening of the histological area, number of necrotic foci and myocardial cells were counted and their frequency normalised to an area of 100 mm^2. The hearts of animals that died spontaneously were fixed in 10% buffered formalin and examined as the hearts of killed rats. For semi-quantitative analysis, slides

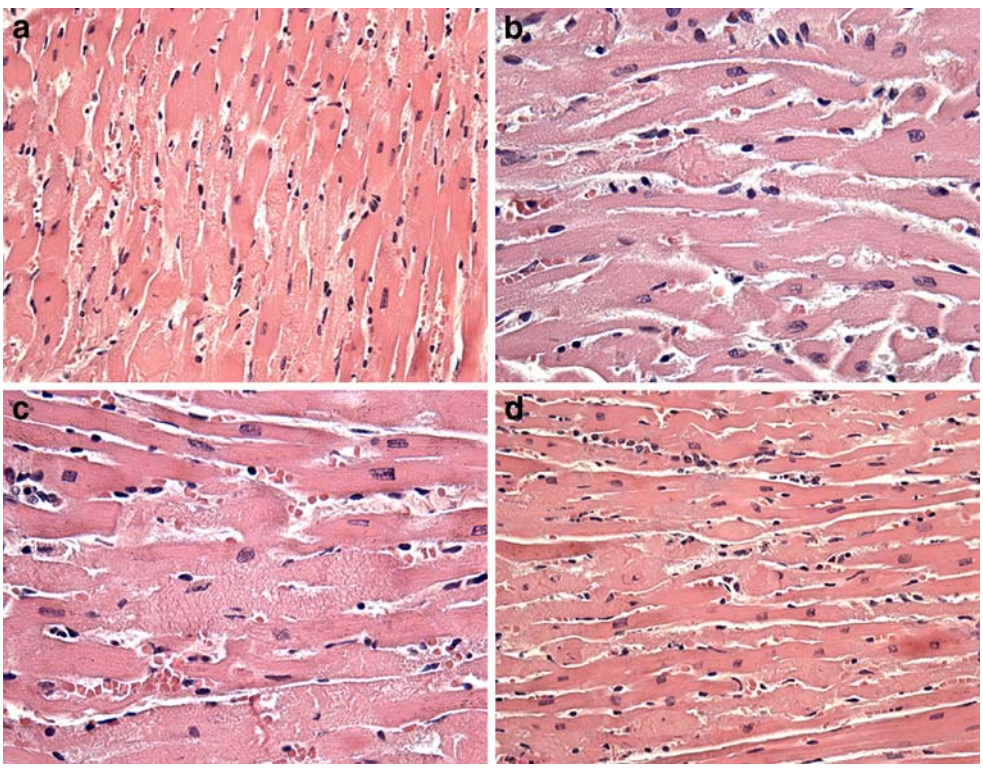

Fig. 1 **A** Early contraction band without macrophage infiltration (**B**) in a rat treated with 20 mg/kg, i.p. of MDMA which was sacrificed at sixth hour (H&E 100× and 250×, respectively). **C** After 16 h, hyper-eosinophilic bands of hypercontracted sarcomeres alternated by clear empty spaces (H&E 60×). **D** A scanty infiltrate of macrophagic monocytes around the necrotic myocardial cells was observed (H&E 60×)

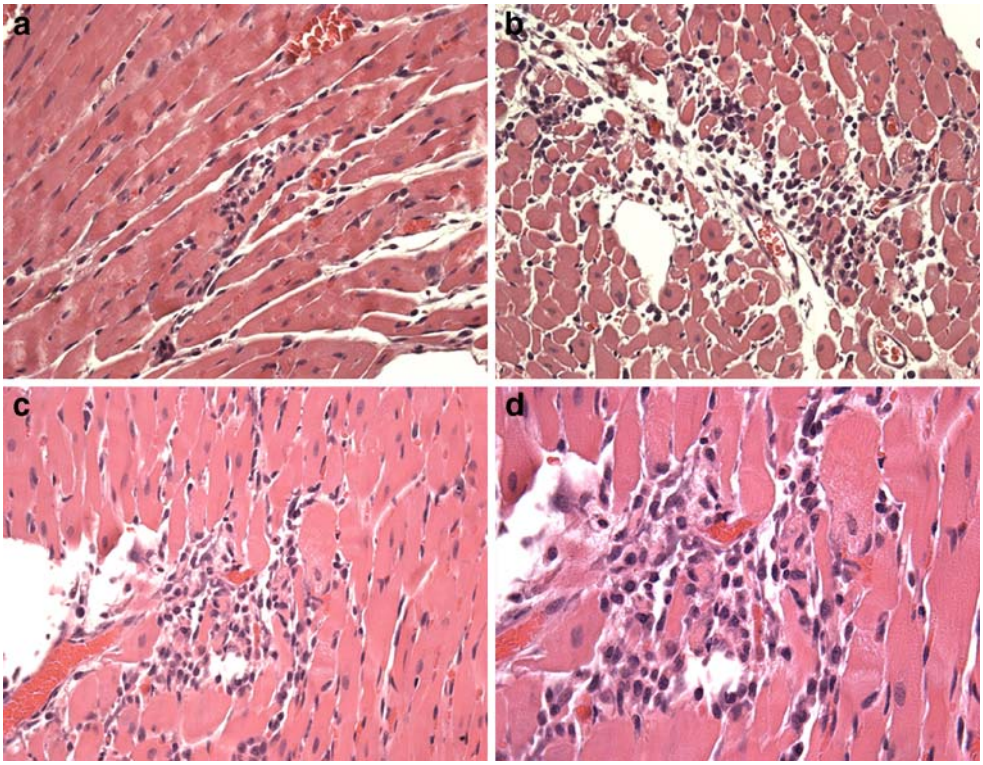

Fig. 2 Rat treated with MDMA 20 mg/kg, i.p. with a survival time of 24 h. **A** Confluent foci of advanced contraction band necrosis plus macrophage reaction (H&E 60×). **B** Monocytes infiltrate became more widespread with an increased number of elements (H&E 60×). **C–D** Early removal of the necrotic material (H&E 60× and 200×, respectively)

were scored in a blinded manner by two observers (MN, IR). Staining pattern within each sample was categorised as absent (++++), weak (+++), moderate (++), intense (+) or strong (−). In cases of divergent scoring, a third observer (VF) decided the final category. The samples were also examined under a confocal microscope, and a three-dimensional reconstruction was performed (True Confocal Scanner, Leica TCS SPE).

GC-MS analysis of MDMA and MDA

MDMA and MDA were analysed according a GC-MS method described by Peters et al. [28]. The analytes were analysed by gas chromatography/mass spectrometry in the selected-ion monitoring mode after mixed-mode solid-phase extraction (HCX) and derivatisation with heptafluorobutyric anhydride. The method was fully validated according to international guidelines [9, 10]. It was linear from 5 to 1,000 μg l^{-1} for all analytes. The limit of quantification was 5 μg l^{-1} for all analytes.

Statistical analysis

Values are presented as means±S.D. The unpaired two-way Student's *t* test was used to compare the results obtained for treated rats with the control group. A value of $p<0.05$ was accepted as indicative of significant difference among groups.

Results

Enzymatic and non-enzymatic antioxidant cellular defence system evaluation

Antioxidant enzymes activity was significantly reduced: GPx (−24%) and SOD (−50%; $p<0.05$) after 3 h and GR (−19%; $p<0.05$) after 6 h from treatment. AA levels decrease (−37%; $p<0.05$) after 3 h and (−30%; $p<0.02$) after 6 h, whereas MDAL level increased (+119%; $p<0.01$) after 3 h; the GSH levels decreased after 3 (31.3%) and (37.9%) 6 h from MDMA treatment, while the GSSG content was not affected after ecstasy administration (Table 1).

Table 2 Frequency and extent of contraction band necrosis in MDMA-induced myocardial toxicity

Rats	Number	Contraction band necrosis	
		Foci	Cardiomyocytes
Group I (6 h)	7	41±32	187±101
Group II (16 h)	7	54±36	93±64
Group III (24 h)	5	43±37	70±13
Group III (spontaneously dead at the fourth hour)	2	130±113	242±129
Group IV (control)	4	3	8

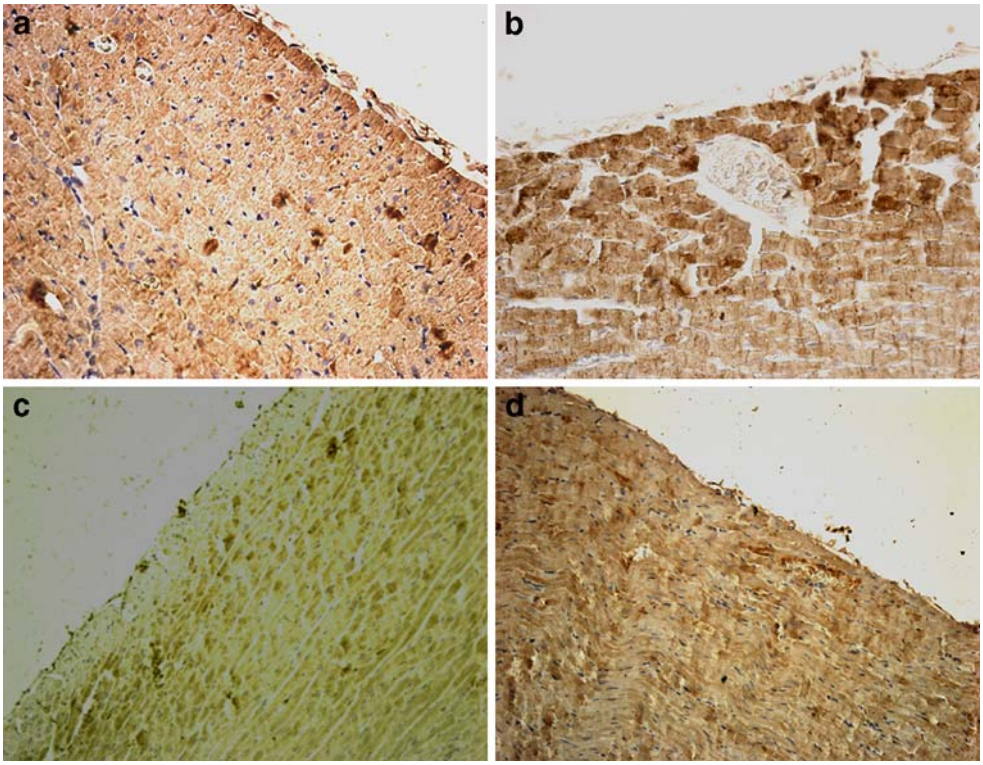

Fig. 3 Low degree of depletion of troponin C at 6 h (**A**), intermediate degree (**B**) and high degree (**C**) of depletion of troponin C at 16 and 24 h, respectively. **D** Control group

Morphologic findings

Contraction band necrosis was clearly visible in rats killed at 6 h. It consisted of scattered foci of hypercontracted myocardial cells which were intensively stained by eosin. Foci of myocardial cells with ruptured myofibrils characterised by hyper-eosinophilic bands of hypercontracted sarcomeres alternated by clear empty spaces were observed.

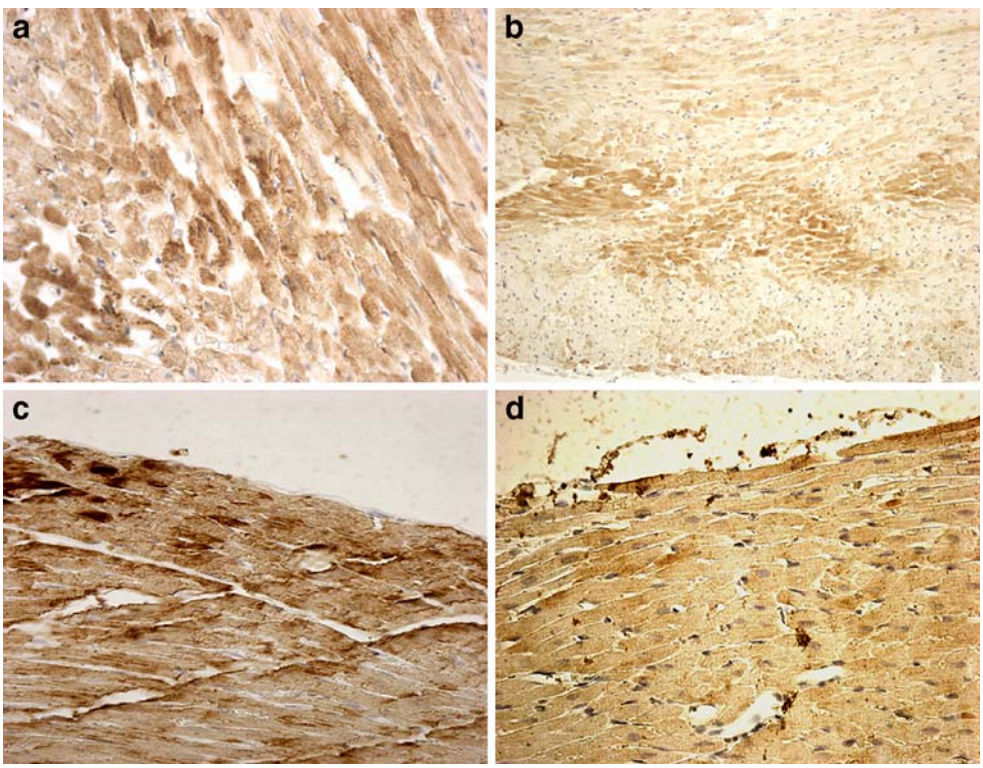

Fig. 4 Intermediate degree of depletion of troponin I at the sixth hour (**A**) and higher degree of depletion of troponin I after 16 h (**B**) and 24 h (**C**). **D** Control group

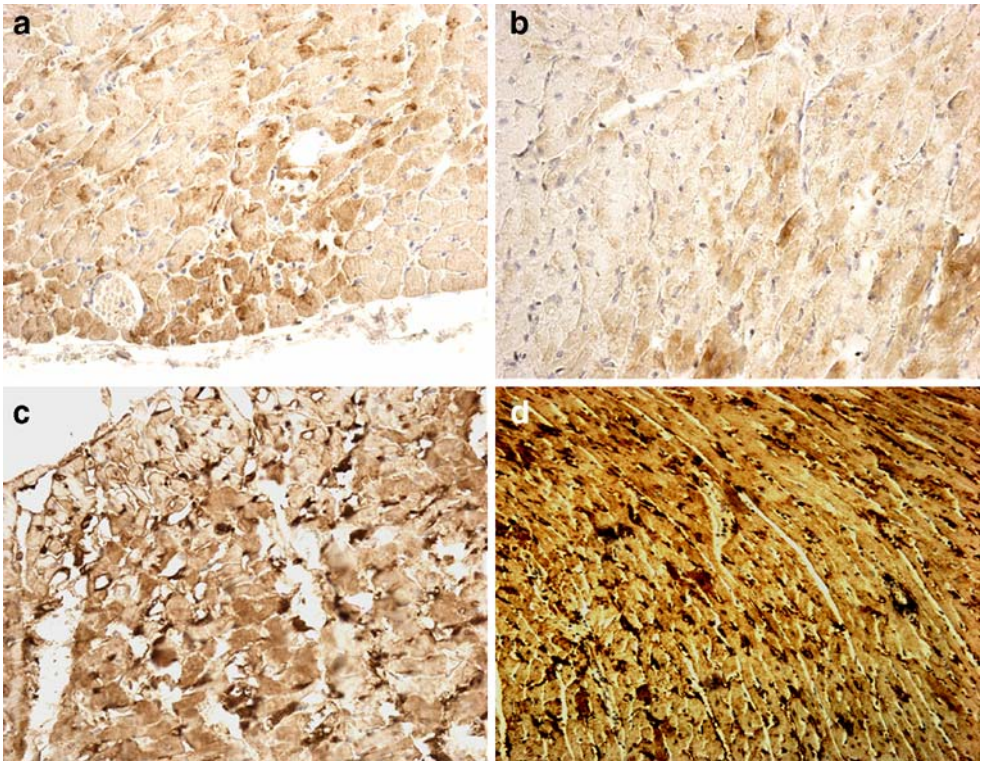

Fig. 5 Depletion of myoglobin was progressively observed at 6 (**A**) and 16 h (**B**); massive myoglobin degree of depletion at the 24th hour (**C**). **D** Control group

By the time 16 h had elapsed, a scanty infiltrate of macrophagic monocytes were seen surrounding the necrotic myocardial cells (Fig. 1). Within 24 h, this infiltrate became more widespread and more intense, and the early removal of the necrotic material had already begun [22, 14, 29, 38] (Fig. 2). The number of necrotic foci and myocardial cells observed in each 100 mm^2 area are reported in Table 2. In control rats group, monocytic infiltrates were absent; in the heart of the rats which died spontaneously, the number of foci and myocytes with contraction band necrosis were significantly more ($p<0.001$) than those found in treated and killed animals. No interstitial haemorrhages within necrotic foci were observed. Occasionally, a small sub-endocardial haemorrhage without relationship to any apparent myocardial necrotic foci was noted. The presence of haematoxynophilic granules within necrotic cardiac myocytes was observed in the rats that died after 24 h. After Von Kossa histochemical reaction, these granules turned out to be positive. The immunohistochemical study of the samples revealed a low degree of depletion of troponin C, troponin I and myoglobin at 6 h; intermediate and high degree of depletion of troponin C, troponin I and myoglobin at 16 and 24 h, respectively (Figs. 3, 4, 5; Table 3).

The confocal laser scanning microscope aspect of the early lesion confirmed the pattern of a fragmentation involvement of the entire cell (pancellular lesion), which

Table 3 Time course of myoglobin and troponins I and C responses in heart specimens

Antibody	Group I (6 h)	Group II (16 h)	Group III (24 h)	Group IV (control)
Anti-myoglobin	+	++	++++	–
Anti-troponin I	+	+++	++++	–
Anti-troponin C	+	++	++++	–

Staining patterns are categorised as absent (++++), weak (+++), moderate (++), intense (+) or strong (−).

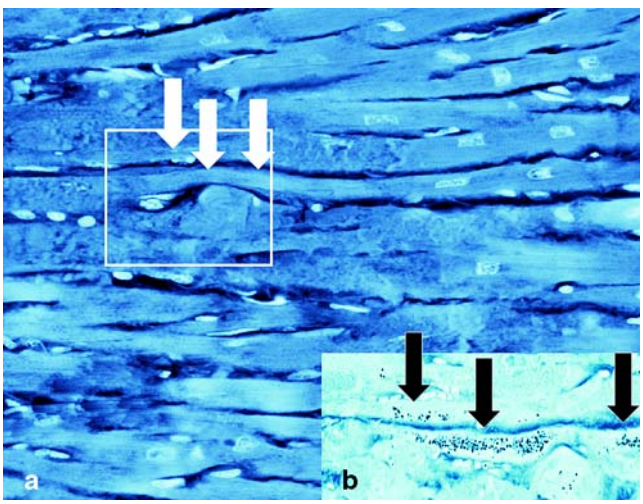

Fig. 6 Confocal laser scanning microscopy: **A** Foci of myocardial cell necrosis (*arrows*) in which broad pancellular lesion are visualised (*insert*). **B** Von-Kossa-positive deposits as round aggregates of granular material (*arrows*) were observed within necrotic ventricular cardiomyocytes

ranges from early breakdown in pathological bands (in the 6-h group) to a total granular disruption (myofibrillar degeneration) especially marked at 24 h. This myocardial cell destruction appeared satisfactory for the purpose of localising Von-Kossa-positive deposits and evaluating their extent. Discrete calcium deposits were observed within ventricular cardiomyocytes with intact nuclei and sarcomeres; they appeared as round aggregates of granular material (Fig. 6). Three-dimensional images of histological sections on confocal laser scanning microscopy were recorded, and the reconstruction of calcium deposits within the myocardial fibres was obtained from the z-lines cut surface (Fig. 7). No calcium precipitates were found in other organs.

MDMA plasma levels

The MDMA plasma levels decreased dramatically with respect to the values detected during the first 6 h after i.p. administration; in rats that died after 4 h, concentrations were similar to those observed at hour six (ESM Table 2).

Discussion

The results of this study demonstrate that even the single administration of MDMA can significantly alter the cellular antioxidant defence system in such a way as to induce oxidative stress. Oxidative stress may result in cellular alterations including a depression in the activity of sarcolemmal Ca^{2+} pump ATPase and Na^{+}–K^{+} ATPase activities. The sum of these changes led to decreased Ca^{2+} efflux and increased Ca^{2+} influx, respectively. Oxidative stress has also been reported to depress the sarcoplasmic reticulum Ca^{2+} pump ATPase and thus inhibits Ca^{2+} sequestration from the cytoplasm in cardiomyocytes [12]. A previous experimental study that investigated the effects of MDMA on the liver demonstrated that increased concentrations of superoxide dismutase, decreased levels of glutathione and increased lipid peroxidation were evident even after a single dose of MDMA [23]. Dose-dependent increases in lipid peroxidation were also observed. One possible mechanism may be a direct toxic effect of MDMA or its redox active metabolites in cardiac cells. Metabolism of MDMA involves N-demethylation to MDA. MDMA and MDA undergo conversion to the corresponding catecholamines, *N*-Me-α-MeDA and α-MeDA, respectively [4, 5]; these metabolites formed in liver cells can reach the heart via the circulation and produce their toxic effects. It may be supposed that if circulating concentrations of the MDMA and MDA metabolites become excessive, then enzymatic, cellular and autoxidative mechanisms could lead to the formation of toxic products such as ROS, orthoquinones and aminochromes, all capable of inducing oxidative stress and lipoperoxidation within cardiac cells. Previous in vitro experiment showed the role of MDMA and MDA metab-

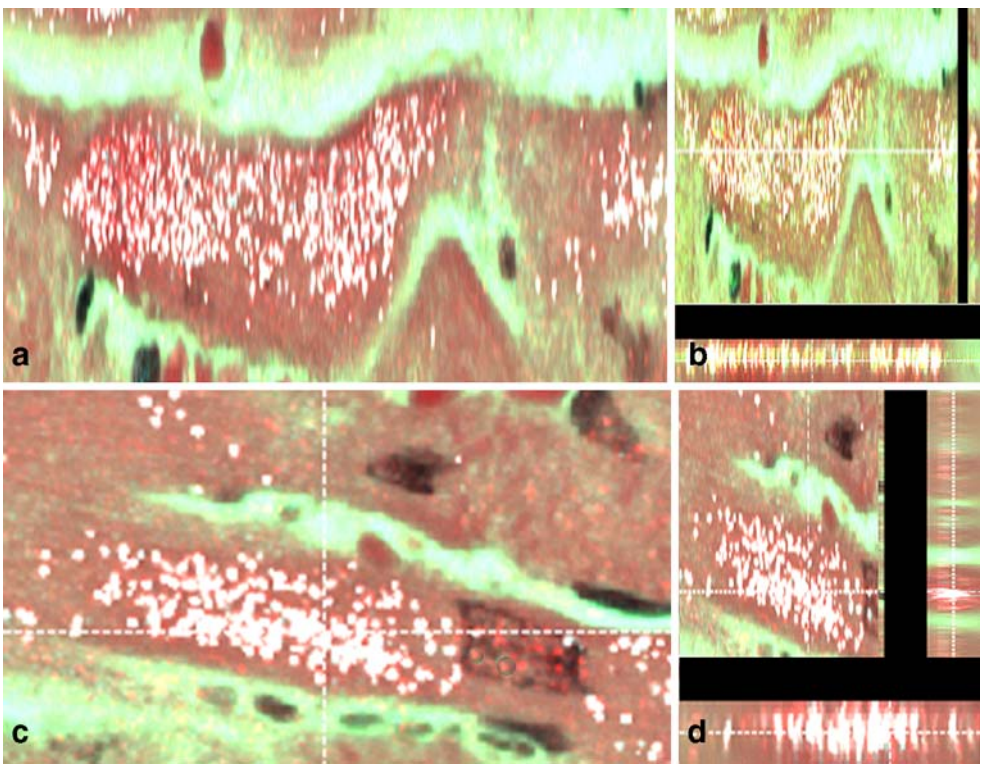

Fig. 7 **A**–**C** Three-dimensional images of histological sections on confocal laser scanning microscopy were recorded, and reconstruction for the purpose of localising of calcium deposits within the necrotic myocardial fibres (**B**–**D** lateral and bottom inserts) was obtained (rotation of 90°) from the z-lines cut surface (*white lines*)

olites (*N*-Me-α-MeDA and α-MeDA) in ecstasy-induced cardiotoxicity [4]; one of the early toxic effects observed in cells exposed to *N*-Me-α-MeDA or to α-MeDA was GSH depletion without the corresponding enhancement in GSSG levels. Our "in vivo" experiments showed a similar behaviour in rat heart after MDMA administration with a loss of GSH level at 3 and 6 h after MDMA treatment without enhancement in GSSG concentrations; it is possible that GSH was used to form GSH adducts. The ability of the GSH conjugates to undergo further redox cycling and produce ROS provides a rationale for the potential role of these metabolites in MDMA-induced cardiotoxicity [2]. GSH is an endogenous antioxidant that plays a crucial protective role against cellular injury; thus, GSH depletion render the cells more exposed to reactive species as ROS, with deleterious effects on cardiomyocytes. We also studied the effects of MDMA on enzymatic cardiac cellular antioxidant system to estimate the enzymatic activity of GPx, GR and SOD. We found that the enzymatic activities of these enzymes were reduced in rat heart after 3 (SOD and GPx) and 6 h (GR) from MDMA treatment. Inhibition of GR and GPx by quinones and aminochromes has been reported [31, 30]. SOD transformed superoxide anion (O_2^-) in H_2O_2, and GPx, GR with GSH are responsible for the elimination of cellular H_2O_2 and organic peroxides. The decreased activity of these enzymes and/or the GSH depletion may compromise this pathway, thereby allowing H_2O_2 to accumulate to toxic concentrations. Hydrogen peroxide through an Haber–Weiss reaction can produce the hydroxyl radical that can initiate the reaction of lipoperoxidation. Compared to controls, we found high levels of MDAL in rat heart after 3 and 6 h from MDMA administration. These levels were more than sufficient to initiate membrane lipid peroxidation and cardiomyocyte injury. Finally, we observed a significant reduction of AA, an important cellular antioxidant; this is likely to be due to the increased requirement of antioxidants caused by oxidative stress, unfulfilled as a result of GSH deficiency.

Furthermore, we still determined the presence of MDMA and MDA in the plasma of samples of rats that had been treated with MDMA; it was present as early at 6 h and still present 24 h after treatment [8–10].

In this study, the myocardial lesion indicates a necrosis of the myocardial cells in a hypercontracted state (tetanic death) characterised by rhexis of the myofibrillar apparatus, anomalous hyper-eosinophilic cross-bands formed by segments of hypercontracted sarcomeres with extremely thickened *Z* lines, as shown ultrastructurally [13]. A high degree of depletion of troponin C, troponin I and myoglobin at 24 h in the killed rats group was demonstrated, confirming the diagnosis of myocardial necrosis. Within chronological limits, the present findings confirm the conclusions of the previous human study:

(a) Ecstasy-related adrenergic overactivity does induce extensive myocardial necrosis; this lesion is the histological hallmark of an acute adrenergic stress linked with malignant arrhythmia.
(b) The absence of any histologically observable myocardial or vascular signs of ischaemia exclude any role for morphological or functional coronary artery obstruction in the genesis of ecstasy-related disorders [1, 7].
(c) Calcium deposits were seen in the cytoplasm of the myocytes in the area of necrosis as granular deposits [36].

Collectively, these results suggest that MDMA may be toxic to the heart through its ability to disrupt cytosolic calcium and mitochondrial homeostasis [6, 34]. The depression in Ca^{2+} regulatory mechanism by reactive oxygen species ultimately results in intracellular Ca^{2+} overload and cell death [21]. Studying the problem is difficult because different experimental animals respond differently both from other models and from humans [11, 17]. In general, the acute effects of MDMA seem to be more or less the same in most animal models, but the models diverge widely when it comes to their ability to produce the changes induced by chronic exposure [27]. Clearly, our study has demonstrated that cellular injury has occurred, but by the very nature of our experimental design, we are unable to assess many of the other issues that we know exist as hyperthermia, dehydration and rhabdomyolysis.

Acknowledgements This work was carried through the financial support of University of Siena, Italy (University Research Plan 2004) and Siniscalco Ceci Foundation, Banca del Monte of Foggia, Italy. We thank Oliviero Brogi, Department of Pharmacology "G. Segre", University of Siena, Italy for his technical assistance.

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