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ORIGINAL ARTICLE

Phenotypic effects of chronic and acute use of methiopropamine in a mouse model

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Abstract

Methiopropamine (MPA) is a structural analogue of methamphetamine and belongs to the category of the novel psychoactive substances. To the best of our knowledge, no experimental study has been performed to evaluate the organ damage evoked by MPA administration in an animal model. Therefore, the main purpose of the present study was to investigate the histological changes in CD-1 male mice following the chronic administration of MPA. MPA-chronically treated mice showed myocardial damage with features consistent with repeated episodes of ischemia and a pattern of kidney damage and gastrointestinal ischemia, with ischemic-necrotic lesions of variable extent. In agreement with the analogies between MPA and methamphetamine, we link organ damage secondary to MPA administration to the vasoconstrictive effect exhibited by both compounds. Chronically MPA-treated mice did not show changes in body weight, food intake, thermoregulation, muscular strength and motor coordination in the accelerod test. However, acute MPA administration significantly increased their heart rate and promoted vasoconstriction, which were associated with the sudden death of a subset of animals (40% of all chronically treated mice). In conclusion, the present study demonstrates that MPA consumption could induce health hazards, highlighting the risk of sudden catastrophic events; therefore, clinicians should be aware of these data and consider MPA screening when no other drug is identified by a urine drug screen.

Keywords Methiopropamine · Novel psychoactive substances · Myocardium · Kidney · Gastrointestinal tract · Mice

Abbreviations

NPSs	Novel psychoactive substances
MPA	Methiopropamine
METH	Methamphetamine
MI	Myocardial infarction

Introduction

In the last 10 years, there has been an increase in the number and types of novel psychoactive substances (NPSs) notified to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Such compounds are defined by EU

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Council Decision 2005/387/JHA as new psychotropic drugs in pure form or in a preparation that has not be scheduled in the 1961 or in the 1971 United Nations Single Convention on Narcotic Drugs/Psychotropic Substances and that may pose a threat to public health comparable to the substances listed in either schedule I, II, III or IV [1]. They are sold through the Internet as "bath salts" or "legal highs" [2], and their market has a rapid turnover, making it difficult for laboratories and institutions to keep up with the appearance of these new compounds. Therefore, little is generally known about NPS's toxicity when they enter the recreational drug scene.

One of them is methiopropamine (MPA), which chemistry directly links to amphetamine and methamphetamine (METH) (http://www.who.int/medicines/areas/quality_safety/4_23_review.pdf); similar to those stimulant drugs, MPA inhibits dopamine and norepinephrine reuptake and, to a lesser extent, serotonin reuptake [3]. The use of MPA in Europe has been recognised since January 2011. MPA's stimulant properties include alertness and an increase in focus and energy, which are appealing to those needing to stay awake and alert for a long time, especially during night hours, either, for instance, to work hard or to go out dancing. This could be sufficient to broaden the Internet market for misuse, especially among younger generations who consider the Internet an easier way to supply their needs. Consuming products containing NPSs can result in harmful behaviours, such as violence, traffic accidents, self-injury or suicide attempts; physical complications, such as liver or kidney injury; and neuropsychiatric symptoms, such as agitation, anxiety, fear, psychosis and panic attack [4]. At present, clinicians should already be aware that NPS abuse could be related to hospital emergencies and even cause death, but there is still limited information on the large amount of substances sold and consumed. In addition, most of them, including MPA, can only be detected by special tests, such as gas/liquid chromatography mass spectrometry; therefore, a toxicological urine screen for routine psychostimulant drugs could be of little help in identifying such cases. Suspicions of NPS intoxication should arise in the presence of patients with unexplained psychiatric, neurological, cardiac or gastrointestinal symptoms. In the scientific literature, the number of reported cases of acute toxicity (hospital admissions and deaths) directly attributed to MPA is very limited. The first was published in 2014, regarding a young woman admitted in the emergency department with palpitations, "chest tightness", nausea and vomiting and a feeling of anxiety and euphoria with agitation and visual hallucinations. These alterations were reversible upon diazepam administration and fluid replacement; MPA was detected in urine at a concentration of 400 μg/l [5]. Another case concerns a young man presenting with paranoid delusion, incoherent speech and auditory and visual hallucinations [6]. In the Identification Of Novel psychoActive substances (IONA) study [7], a patient presented with severe behavioural disturbances associated with psychosis and features consistent with sympathomimetic toxicity (fever, sweating, tachycardia, dilated pupils, increasing auditory and visual hallucinations, agitation, aggression and insomnia) that may be caused or contributed to by MPA, which was detected together with benzodiazepine. Even a case of sudden death has been related to the use of MPA, as there were neither health problems nor other drugs or alcohol detected in the toxicological post-mortem analysis: MPA was detected in the blood (38 mg/l) and urine. The autopsy showed non-specific pulmonary oedema consistent with a fatal cardiac arrhythmia inducing cardiovascular collapse, with the same mechanism of action as METH [8]. However, the authors did not describe any microscopic feature consistent with specific organ damage.

Moreover, MPA's side effects, such as vasoconstriction, cold extremities, "racing heart" and chest tightness, have been widely discussed on informal Internet fora (https://www.erowid.org/chemicals/methiopropamine/methiopropamine_effects.shtml), thereby describing only acute toxicity.

To the best of our knowledge, the literature contains no data on health hazards secondary to chronic MPA consumption, and no experimental studies evaluating its chronic toxicity have been performed. In light of recent scientific evidence highlighting the risk of addiction-like behaviour induced by MPA consumption [9], we evaluated the effects of chronic MPA abuse on body tissues, using a mouse model. Moreover, to better understand the effects of the chronic MPA treatment on mice behaviour, we investigated the effects of chronic MPA on mouse body weight changes and food intake, body temperature, muscle strength and motor coordination. Finally, considering the cardiovascular effects caused by MPA in humans, we studied the effects of acute MPA administration on cardiovascular function (heart rate and vasoconstriction).

Materials and methods

Animals

Sixty male ICR (CD-1®) mice, 25–30 g (Harlan Italy; S. Pietro al Natisone, Italy), were group-housed (eight mice per cage; the floor area per animal was 80 cm^2; the minimum cage height was 12 cm) in a colony room under a constant temperature (23–24 °C) and humidity (45–55%). Food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and tap water were available ad libitum the entire time the animals spent in their home cages. The daylight cycle was maintained artificially (dark between 7 p.m. and 7 a.m.). The present study was performed in accordance with the new European Communities Council Directive of September 2010 (2010/63/EU), a revision of the Directive 86/609/EEC and approved

by the Italian Ministry of Health (licence no. 335/2016-PR) and by the Ethics Committee of the University of Ferrara.

Drug preparation and dose selection

MPA was purchased from LGC Standards (LGC Standards S.r.L., Sesto San Giovanni, Milan, Italy) and initially dissolved in a vehicle composed of absolute ethanol (final concentration was 2%) and Tween 80 (2%) and then brought to the final volume with saline (0.9% NaCl). A solution made of ethanol, Tween 80 and saline was used as the vehicle. MPA and the vehicle were administrated intraperitoneally at a volume of 4 μl/g. The dose tested (10 mg/kg) was chosen based on previous tests and on behavioural and neurological effects reported in human volunteers. In fact, based on the personal experiences of consumers of MPA reported on the Internet (https://www.erowid.org/chemicals/methiopropamine/methiopropamine_dose.shtml), 5 mg is the threshold, up to 15 mg is a light dosage by insufflating and up to 30 mg is a light dosage by oral consumption. When the dosage reaches 60 mg, there is an urge for frequent re-dosing [10]. Using interspecies dose scaling [11], the dose used in this experimental study (10 mg/kg) is equivalent to a high dose of 60 mg/kg in humans.

Experimental setting of the chronic study

The study population was composed of 50 CD-1 male mice that were randomised into four groups (20 animals in group 1, 10 animals in the other groups) and treated for a period of 1 month. Group 1 received MPA (10 mg/kg i.p.) once daily, group 2 received the same dose of MPA (but only on the weekends; 2 days per week, 8 days in total), group 3 received MPA only the day before the sacrifice and group 4 received only the drug vehicle. Successively, another group of 10 animals was treated with the same protocol of group 1 in order to better define the phenotype of the mice dead soon after the beginning of the experiment.

To assess whether chronic MPA administration (30 days) could cause an animal's health and behavioural impairment, we assessed the effects of MPA on mouse body weight changes and food intake, body temperature, muscle strength and motor coordination on accelerod.

The mouse body weight changes and food intake were measured once daily for 30 days.

The three tests (core temperature, grip strength and accelerod) were repeated in a fixed sequence before and 15 min post-drug (MPA or vehicle) administration. Behavioural tests were performed at D1, D3, D7, D14, D21 and D28. Animals were trained before drug administration for approximately 7 days to the specific tasks until their performance became reproducible.

After 30 days, the surviving mice were sacrificed by cervical dislocation and their organs were analysed.

Pathologic evaluation

The following organs were taken and fixed in 4% formalin: brain, heart, liver, kidneys, gastrointestinal tract, tibiae muscles, testis and seminal vesicles. Formalin fixed organs were embedded in paraffin, and 5-μm thick sections were stained respectively with haematoxylin and eosin (H&E), Masson trichrome and Van Gieson stains, briefly as already described [12]. To assess the presence of apoptosis, we performed immunostaining with caspase 3, a cysteine protease that plays a crucial role in the apoptotic pathway. All the slides were evaluated under light microscopy by two observers blinded to the experimental groups. The changes were recorded by photography.

Chronic behavioural studies

Core temperature was evaluated by a probe (1 mm diameter) that was gently inserted, after lubrication with liquid vaseline, into the rectum of the mouse (to about 2 cm) and left in position until the stabilisation of the temperature (about 10 s; [13, 14]. The probe was connected to a Cole Parmer digital thermometer, model 8402.

The effect of MPA administration on muscle strength in mice was evaluated by a grip strength test.

Briefly, the muscle strength of the mouse was measured by the use of a special grid on which the operator clings the mouse and subsequently tried to gently remove it [15]. The grid is connected to a digital dynamometer (ZP-50N, IMADA, Japan), which measures the tensile muscle in g/strength.

Alterations in the motor activity induced by MPA were measured using the accelerod test [13, 16]. In the accelerod test, animals were placed on a rotating cylinder that automatically and constantly (0–60 rotations/min in 5 min) increased velocity. The time spent on the cylinder was measured.

Acute cardiovascular studies

To determine whether the sudden deaths of mice caused by MPA administration could be due to cardiovascular alterations, we undertook a specific study in a group of mice. Cardiovascular changes (heart rate and pulse distension) were detected in awake, freely moving mice, with a non-invasive apparatus (MouseOx PLUS, STARR Life Sciences® Corp. Oakmont, PA; Cambal, [17, 18]). On the neck (not shaved) of the mice, a small collar was equipped with a sensor that detects continuously, with a frequency of 15 Hz, the heart rate (bpm) and the variation of the diameter of the jugular vein (pulse distension in μm). The animals were living free in a cage, without the possibility to feed themselves for the whole

duration of the test. During the first hour, the animal was put in the cage with a "simulation" collar that mimics the same one used to record the data. Subsequently, the "simulation" collar was replaced with a "recording" collar that started to record the heart rate and pulse distension baseline for 60 min. MPA (10 mg/kg, i.p.) or the vehicle was administered to the mice, and the software recorded data for the next 5 h. During the injection of the substance, the software was paused. The test was conducted on 14 mice, which were divided into two groups of seven mice. Each group was treated, respectively, with the vehicle or MPA (10 mg/kg). The effects of the vehicle or MPA treatments were calculated as mean ± SEM of 60 different points, each representing the mean effect per minute.

We considered tachycardia events when the heart rate was almost 200 pulses higher than the average of their basal heart rate.

Data and statistical analysis

Data related to heart rates and pulse distention are expressed as absolute values (bpm, heart beat per minute and μm for the vessels' diameter changes).

The statistical analysis on mouse and food weight changes, core temperature, muscle strength (grip strength test) and motor coordination (accelerod test) was performed using two-way ANOVA followed by Bonferroni's test for multiple comparisons. The analysis of effects induced by treatments on the heart rate and pulse distension (Table 1; mean effect% analysis) was performed with one-way ANOVA, followed by Tukey's test for multiple comparisons. Student's *t* test was used to determine statistical significance ($p < 0.05$) between the two groups (see cardiovascular changes, Table 1). The statistical analysis was performed with the program Prism software (GraphPad Prism, USA).

Results

Pathological findings

Eight of the 20 mice (40%) from group 1 were found dead between 2 and 11 days after the beginning of the experimental period. The mean time elapsed between the beginning of MPA administration and death was 7.25 days. No necropsy was performed on those animals. Conversely, the remaining group

Table 1 Effect of acute administration of MPA (10 mg/kg i.p.) on cardiovascular changes (heart rate and pulse distension) in awake, freely moving mice

MPA 10 mg/kg						
Dead	Heart rate			Pulse distention		
Mice	Basal (bpm)	MPA effect (bpm)	Effect %	Basal (pm)	MPA effect (pm)	Effect %
1	661.2 ± 4.7	862.5 ± 4.03***	+ 30.4	404.5 ± 15.2	137.8 ± 7.3***	− 65.9
2	639.1 ± 11.8	841.4 ± 5.4***	+ 31.6	183.6 ± 5.9	113.5 ± 6.4***	− 38
3	626.4 ± 5.6	829.1 ± 3.7***	+ 32.4	338.6 ± 4.9	203.4 ± 14.8***	− 39.4
Survived		Mean effect %	+ 31.5 ± 0.6##		Mean effect %	− 47.8 ± 9.1##
4	631.2 ± 4.8	658.5 ± 3.9	+ 4.3	248.6 ± 10.1	216.7 ± 8.3	− 15.3
5	691.5 ± 7.2	721.6 ± 6.1	+ 4.2	267.4 ± 6.2	240.8 ± 5.5**	− 10.7
6	702.6 ± 9.8	720.3 ± 20.2	+ 2.5	287.1 + 8.4	228.1 ± 8.2***	− 25.9
7	691.3 ± 4.9	703.5 ± 6.2	+ 1.9	242.5 ± 6.2	213.9 ± 7.5**	− 13.5
Vehicle		Mean effect %	+ 3.2 ± 0.6#		Mean effect %	− 16.4 ± 3.3#
Survived	Heart rate			Pulse distention		
Mice	Basal (bpm)	Vehicle effect (bpm)	Effect %	Basal (pm)	Vehicle effect (um)	Effect %
8	713.5 ± 5.9	700.9 ± 10.5	− 1.7	204.8 ± 6.7	198.1 + 5.6	− 3.0
9	687.6 ± 11.7	697.9 ± 9.5	+ 1.6	285.8 ± 4.8	265.1 ± 7.3*	− 7.9
10	702.7 ± 6.3	688.7 ± 3.7	− 2.0	321.8 ± 5.4	316.4 ± 12.3	− 1.9
11	711.2 ± 7.8	690.9 ± 10.3	+ 3.0	277.1 ± 5.6	286.1 ± 10.8	+ 3.2
12	699.1 ± 9.2	702.8 ± 9.1	+ 0.6	258.9 ± 3.4	288.4 ± 6.9***	+ 10.0
13	702.7 ± 11.2	687.8 ± 5.1	− 2.0	289.1 + 7.1	302.9 ± 7.5	+ 4.8
14	677.5 ± 9.6	680.5 ± 7.3	+ 0.4	258.4 ± 5.9	269.4 ± 9.8	+ 4.3
		Mean effect %	− 0.01 ± 0.7		Mean effect %	1.3 ± 2.2

Data related to heart rates and pulse distention are expressed as absolute values (bpm, heart beats per minute and μm for the vessels' diameter changes). The statistical analysis was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. Student's *t* test was used to determine statistical significance ($p < 0.05$) between two groups

# $p < 0.05$, ## $p < 0.01$ different from the vehicle ((F = 446.4, $p < 0.05$), related to a mild increase in heart rate; and (F = 33.68, $p < 0.05$), related to a mild reduction in pulse distension), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ different from basal (F = 446.4, $p < 0.0001$), related to tachycardia; (F = 33.68, $p < 0.0001$), related to vasoconstriction

1 mice completed the 1-month period of treatment, as well as those mice belonging to the other three groups.

Six of the 12 group 1 mice that completed the treatment (50%) were either found dead or dying on the last day of the experimental period; the others six mice were regularly sacrificed, as well as those mice belonging to the other three groups.

A macroscopic evaluation upon sacrifice of group 1 mice showed that five animals (41.7%) suffered from probable intestinal obstruction (jejunum and ileum were full; colon was empty), one (8.3%) had testicular atrophy, one had axillary lymph node hyperplasia and one had an angiomatous hepatic lesion. Because these alterations were sporadic, they were considered idiosyncratic and no further investigation was performed. No macroscopic anomalies were found in the other groups.

Histologic analysis of the hearts from the six group 1 animals that died or were found dying on the last day of treatment revealed focal lesions consistent with both acute and chronic ischemic myocyte damage. Features of acute damage were represented by small groups of myofibres with increased sarcoplasmic eosinophilia and contraction band necrosis, surrounded by perivascular and interstitial inflammatory cells (Fig. 1c), mostly localised in the sub-endocardial region. In addition, small foci of granulation tissue with oedema and capillary neo-angiogenesis (Fig. 1d) as well as microscopic areas of replacement fibrosis (microscarring) were observed (Fig. 1e), consistent with chronic loss of myocytes. Epicardial coronary arteries were unremarkable.

In order to better define the phenotype of the mice dead soon after the beginning of the experiment, a new set of ten animals was treated with the same experimental protocol and submitted to closer checks. In this way, we have been able to recover for necroscopy analysis four animals. Briefly, the major findings were related to myocardial

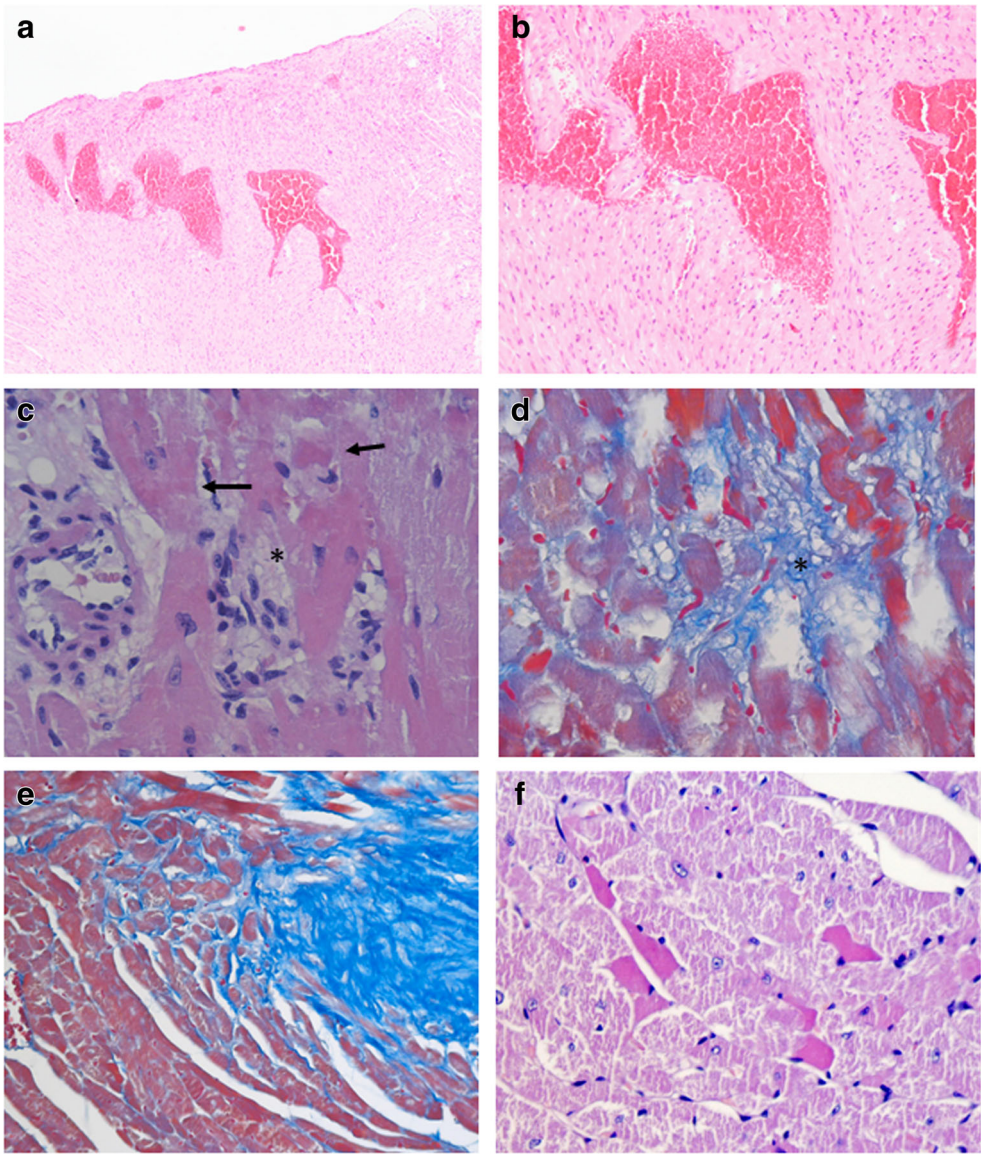

Fig. 1 Myocardial lesions observed in animals treated with MPA (10 mg/kg once daily). **a** Intramyocardial haemorrhage in the myocardium of an animal dead soon after the beginning of the experiment. H&E ×10. **b** Higher magnification showing the myofibers damages caused by bleeding. H&E ×20. **c** Hyper-eosinophilic myocytes with contraction bands (arrows). There are scattered interstitial inflammatory infiltrates (asterisk). H&E ×40. **d** Small focus of granulation tissue (asterisk). Azan Mallory ×20. **e** Myocardial microscarring. Azan Mallory ×10. **f** Sub-endocardial scattered myocytes with sarcoplasmic hyper-eosinophilia. H&E ×20

damages. Indeed, we found important intramyocardial haemorrhages that possibly justify the sudden death of these animals (Fig. 1a, b).

Of the six remaining animals from group 1 that were sacrificed at the end of treatment, only two showed myocardial abnormalities, which were, however, mild and non-specific. There was a slight increase in the interstitial fibrous and cellular component, not associated with myocyte damage (not shown). Isolated myocytes in the sub-endocardial region showed sarcoplasmic eosinophilia with pyknotic nuclei (Fig. 1f). There was neither evidence of hypertrophy nor intracellular vacuolization. All the group 1 animals presented intestinal ischemic lesions (in a variable degree) and kidney anomalies. In two cases, histological sections of the intestinal segment showed ischemic necrosis of the duodenal wall, with separation and thinning of the muscular layer, haemorrhage in the submucosa and mucosal ulceration (Fig. 2b, c). The other sections showed an earlier stage of haemorrhagic ischemia: villi lost their structure and neutrophils migrated into the lamina propria and the epithelium (Fig. 2a). The mesenteric vascular structure did not show any abnormalities. Histological sections of the kidneys showed that the Bowman's capsule developed hyperplasia with large cuboid cells in it, while tubular cells lost their nuclei (a pattern of acute tubular necrosis). Casts and tubular cells went into the tubular lumen and, despite many of the glomeruli appearing remarkably normal, some showed sclerosis and hyalinisation; moreover, haemorrhagic areas were detected (Fig. 2d–f). Van Gieson staining of the kidney sections showed interstitial fibrosis (data not showed). Immunostaining with caspase 3 excluded apoptotic cells in the sections analysed (data not showed).

Histologic features of the organs from group 2 mice were mostly unremarkable, except for the presence of sporadic hypereosinophilic myocytes in 3/10 animals. Organs from both group 3 mice and controls showed no alterations.

Chronic behavioural studies

Mice were given one injection of MPA (10 mg/kg i.p.) or vehicle every day for 30 days, and changes in body weight and food intake were measured daily. Changes in core temperature, muscle strength and motor activity on the accelerod were evaluated once daily, both before (baseline) and 15 min after drug administration.

Chronic administration with MPA (10 mg/kg i.p.) did not affect body weight ($F_{30,\ 434} = 0.1778$, $p = 1$) and food intake ($F_{30,\ 434} = 0.2468$, $p = 1$) in mice.

Similarly, the treatment with chronic MPA, both at baseline and the 15-min post-drug, had no effect on core temperature ($F_{5,\ 84} = 2.22$, $p = 0.0597$), muscle strength ($F_{5,\ 84} = 0.5649$, $p = 0.7266$ and the motor performance on accelerod ($F_{5,\ 84} = 0.9533$, $p = 0.4512$).

Acute cardiovascular studies

Acute administration with MPA (10 mg/kg i.p.) significantly increased heart rate and reduced pulse distension in mice

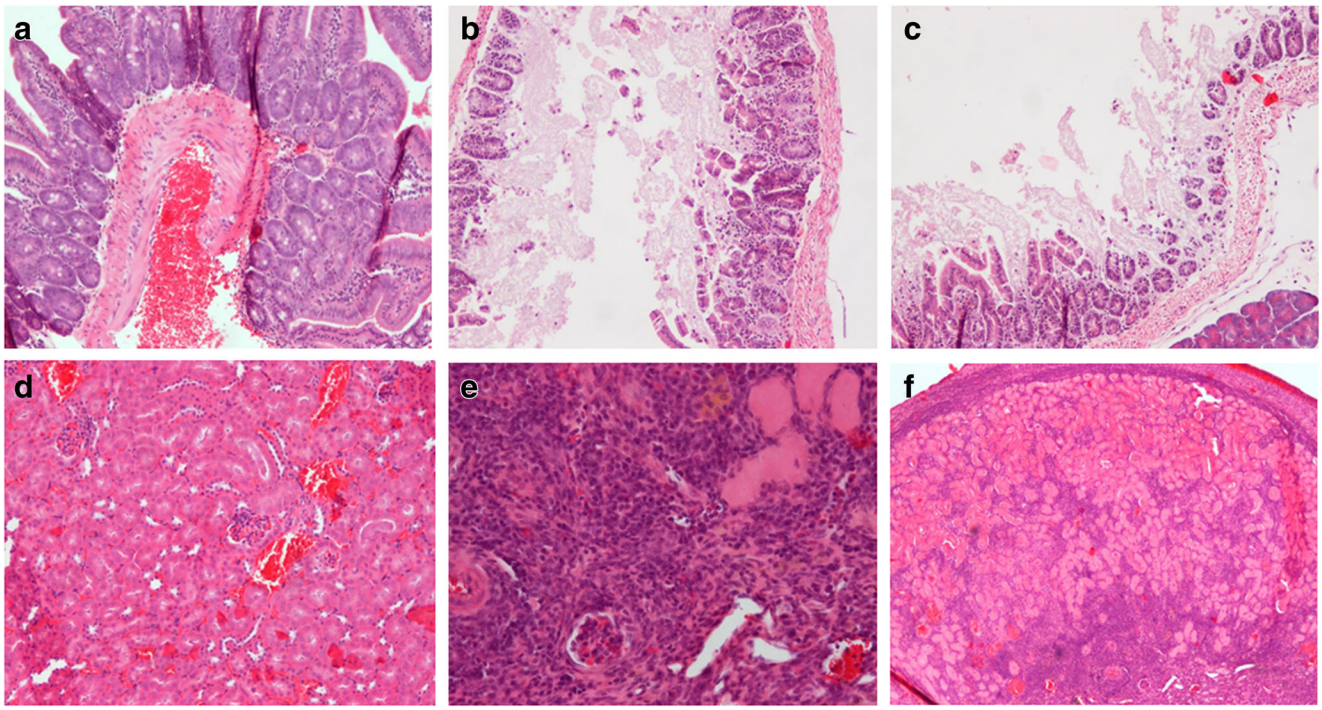

Fig. 2 **a**–**c** Sections of the duodenal wall stained with H&E. Ischemic necrosis, separation and thinning of the muscular layer, haemorrhage in the submucosa and mucosal ulceration. **d**–**f** Sections of the kidneys stained with H&E. Bowman's capsule hyperplasia, acute tubular necrosis, casts, glomerular sclerosis and hyalinisation

(Table 1). Cardiovascular effects appeared 15–20 min after drug administration and lasted for about 60 min. In particular, MPA caused the onset of tachycardia ($F_{2,\ 13} = 446.4$, $p < 0.0001$) and a significant vasoconstriction ($F_{2,\ 13} = 33.68$, $p < 0.0001$) in three out of seven mice (approximately 43% of total treated mice; Table 1). Those mice died after approximately 2 h after the administration of MPA. Other mice treated with MPA (four of seven) reported a mild increase in heart rate ($F_{2,\ 13} = 446.4$, $p < 0.05$) and a mild reduction in pulse distension ($F_{2,\ 13} = 33.68$, $p < 0.05$; Table 1), and the cardiovascular effects disappeared after 60–75 min. All those mice survived.

Treatment with the vehicle did not significantly affect heart rate and pulse distension in mice (Table 1). Only two animals reported changes in pulse distension (mice no. 9 decrease and mice no. 12 increase), possibly related to experimental variability.

Discussion

According to our results, chronic MPA administration results in histologic features of organ damage in a mouse model. To the best of our knowledge, this is the first report providing evidence of organ damage related to prolonged MPA assumption.

We performed this study to assess whether chronic or occasional MPA administration (30 days) could cause an animal's health impairment to evaluate the potential health risk related to human abuse. Therefore, we used doses and treatment schemes that could simulate continuous human consumption.

Behavioural studies show that chronic treatment with MPA does not significantly alter body weight growth and food intake in the mice, thermoregulation, muscle strength and motor activity on the accelerod, highlighting their apparent good health. The lack of facilitation on motor activity in the accelerod test is apparently in contrast with a previous study showing that repeated administration of MPA produces locomotor sensitization in rats, similar to other psychomotor stimulants [9]. The ineffectiveness of MPA in our study could be because mice are well trained in the accelerod test before the experimental section and they remain on the rotating rod for the whole acceleration time (i.e., 5 min). Therefore, with the accelerod test, we can only verify that chronic treatment with MPA does not cause a deterioration in motor skills, but we are not able to evaluate whether it causes an improvement in motor performance. The study clearly demonstrates that acute administration of MPA, similarly to other stimulants [19–23], causes cardiovascular changes in rodents. In particular, we demonstrated that MPA promoted tachycardia and vasoconstriction in mice, and these effects could be the cause of acute deaths of some mice.

We have been able to analyse by necropsy four animals which acutely died and we found evident myocardial damages due to intramyocardial haemorrhages that could be the cause of acute death.

The main finding in mice which completed the 1-month treatment was the presence of multiple areas of both chronic and acute myocardial damage, with morphologic features consistent with ischemic damage: increase in myocyte eosinophilia, features of contraction band necrosis and a pattern of subendocardial "microscarring". Behavioural studies reported that sudden death in mice was possibly associated with cardiovascular alteration (i.e. an increase in heart rate and massive vasoconstriction). Necropsies of the other MPA-chronically treated mice that were sacrificed the last day of treatment showed a slight increase in the interstitial fibrous and cellular component, not associated with myocyte damage, and sarcoplasmic eosinophilia with pyknotic nuclei of some myocytes in the sub-endocardial region.

In three group 2 mice (3/10), who received MPA only on the weekend (8/30 days, not continuously), we found sporadic hypereosinophilic myocytes. Therefore, we can suppose that myocardial lesions were time-dependent and probably even dose-dependent. The "microscarring" pattern is considered highly indicative of ischemic damage, and this is confirmed by its presence only in the treated animals, with a gradient of severity between animals who received different types of treatment. The most important issue related to "microscarring" is that it could be a substrate for arrhythmias, including ventricular fibrillation, one potential explanation for sudden death.

Moreover, in group 1 mice, we discovered that both the gastrointestinal system and kidneys were critically damaged. Histological sections showed features of acute mesenteric ischemia, with varying degrees of intestinal wall necrosis and inflammation, as well as a kidney injury comparable to renal ischemic damage.

The pathophysiological explanation of our findings is related to the pharmacodynamics of MPA, which structure is analogue to amphetamine and METH, with a thiophene ring replacing the benzene ring. Those stimulant compounds show sympathomimetic effects by inhibiting noradrenaline, dopamine and, to lesser extent, serotonin reuptake [3] and stimulating their release, therefore increasing their concentrations in the synaptic cleft. Their effect is not only relegated to the central nervous system (CNS) but also active in the sympathetic system, probably interfering in heart and vessel functions or in gastrointestinal function. The increase in norepinephrine results in arterial vasoconstriction via alpha-1 receptors and increased chronotropy and inotropy via beta-1 receptors. These effects produce tachycardia and hypertension, characteristic of METH use. Depending on the duration and severity of vasoconstriction, the result can be ischemia in various organs. Several adverse outcomes, such as myocardial

ischemia, hypertension, arrhythmias and rhabdomyolysis, are predictable due to the drug's cardiovascular effects on vasoconstriction. Depending on this, epinephrine, norepinephrine, amphetamines and cocaine are drugs known to cause toxic myocarditis, a diagnosis based on both myocyte damage and inflammatory infiltrate at the morphological study of the heart (after performing an endomyocardial biopsy) [24].

For example, there is an established connection between cocaine use and myocardial infarction (MI), arrhythmia, heart failure and sudden cardiac death. Numerous mechanisms have been postulated to explain how cocaine contributes to these conditions. Among these, in addition to coronary artery vasoconstriction, cocaine may lead to MI by accelerated atherosclerosis and initiating thrombus formation [25]. Indeed, of cocaine abuse-related deaths receiving a full autopsy, some cases show coronary artery disease, enlarged heart, myocarditis and contraction band necrosis; in particular, coronary artery disease and ventricular hypertrophy are constantly found, independent of the cocaine concentration [26].

The same findings are showed in METH abuse-related deaths. For example, an autopsy was performed in a female who underwent a traffic accident and died during the hospital stay. Histological examinations revealed contraction band necrosis in the cardiac muscle of the left and right ventricles, as well as congestion and oedema in both lungs. Toxicological screening of urine and heart blood revealed the presence of METH; the stored scalp hair analysis demonstrated METH as well, suggesting that she was a METH abuser at least about 3 months before she died [27].

Similar effects can also be seen in the mesenteric vessels, as demonstrated by the many case reports of METH-induced acute mesenteric ischemia, and in the renal vessels, as described in some reports on cocaine abuse [28]. In such cases, lesions typically attributed to atherosclerotic disease are linked to a drug-induced artery spasm, which may be activated in individuals with or without atherosclerotic lesions. In the same way as cocaine, METH and its analogues likely lead to vasoconstriction and thrombosis, especially in the coronary arteries, via complex mechanisms including the inhibition of nitric oxide synthesis, the enhanced release of endothelin-1, the increased release of fibrinogen and the von Willebrand factor [29]. Regarding METH-induced acute mesenteric ischemia, a METH abuser presented to the emergency department with confusion, hyperthermia and hypotension, complaining of nausea and vomiting. Hours later, after laboratory tests and radiology imaging, the physicians took the patient into the operating room and discovered infarction of the distal half of the jejunum, the entire ileum, colon and rectum, necessitating a total proctocolectomy with the resection of the ileum and jejunum. Microscopic evaluations revealed ischemia, infarction and fibrinoid necrosis of the vessels, consistent with acute ischemia [30]. In the present experimental study, we found the same pattern of intestinal lesions; thus, we can attribute the same mechanism of action on the mesenteric vessels to MPA.

Additionally, independent of its actions in the cardiovascular system, METH can also affect the gastrointestinal system through direct effects on cocaine-regulated and amphetamine-regulated transcript (CART) peptides [31], as well as indirect effects from the release of dopamine and other neurotransmitters.

METH-related kidney damage is usually considered secondary to hypotension or rhabdomyolysis, but even oxidative stress could play a role [32]. In addition, cocaine has nephrotoxic effects, probably related to changes in renal hemodynamic (due to vasoconstriction), oxidative stress and the induction of renal atherogenesis [33]. Acute kidney injury has been described even in numerous case reports discussing 3,4-methylenedioxymethamphetamine (MDMA) users. In this experiment, kidney lesions are probably secondary to vasoconstriction and resulting ischemia, as described among cocaine effects.

Finally, even data regarding other less common sympathomimetic amphetamine-like medications are available and confirm previous findings: phendimetrazine, phenylpropanolamine and bupropion are used as pharmacological options to treat obesity (anorexiants), and the pathological examination of the hearts of subjects who died suddenly after months/years of treatment with these compounds shows myocardial lesions identical to those described in catecholamine myocardial damage and indicative of chronic remodelling [34].

Interestingly, in this experiment, chronic MPA treatment does not induce physiological (body weight changes, food intake and thermoregulation changes) and behavioural (muscular strength and motor coordination) alterations that can justify the sudden death of mice. Otherwise, MPA administration caused the sudden death of mice in which there was a significant increase in heart rate associated with pronounced vasoconstriction. We can hypothesise that in the absence of atherosclerotic lesions, some vasospasms may have occurred that were fatal (dead mice showing important intramyocardial bleeding) or were without any histological consequences (other mice).

Moreover, one of the most important pieces of study data is the significant number (40%) of dead mice in the group of MPA-chronically treated mice, even within the first days of treatment, in comparison with the other groups with no reported deaths. Considering that MPA abuse is certainly increasing, whenever death occurs, only post-mortem histological changes could be helpful to correlate high levels of MPA in a blood or urine analysis to the cause of death. A problem that will constantly arise involves the phenomenon of poly-abuse: together with NPSs, people continue to use classical illicit substances, such as cocaine and METH as well as consume licit drugs acting on the

central nervous system, such as benzodiazepines and antidepressants. Adverse effects in such cases will increase dramatically, especially related to the cardiovascular system, both in acute abuse and chronic use. We should not underestimate that even a wide range of over-the-counter drugs could increase toxicity when combined with NPSs. We should thus consider that it could be difficult to discriminate each drug's effect.

Therefore, the evidence described in this study could be used by forensic practitioners and forensic toxicologists in the evaluation of pathological changes in deaths likely related to drug abuse.

Conclusion

Currently, MPA, whose consumption started over the last 10 years (first detected in 2011), is a recreational synthetic drug popular in many countries due to its psychostimulant effects. Health hazards for users are various and yet not well known. In this study, we revealed a specific pattern of myocardial damage related to the chronic abuse of MPA that could easily be considered the cause of death in the animals that we found dead, and we demonstrated that chronic MPA consumption could induce kidney and gastrointestinal damage.

This type of knowledge is very useful for forensic practitioners, especially because they can associate myocardial lesions, such as the ones we discussed above, to the use or abuse of substances detected by the toxicological exams. In such forensic cases in which cardiotoxicity is the potential cause of death, the relationship with drug abuse could make a difference in formulating the cause of death.

In another point of view, conscious of the cardiotoxicity related to this new drug, that young people could consume unaware of the potential risks related, health practitioners, together with social workers and politicians, should expand knowledge relative to MPA-related increase of cardiovascular risk and unexpected death and plan appropriate prevention.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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