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Binge ethanol and MDMA combination exacerbates HSP27 and Trx-1 (biomarkers of toxic cardiac effects) expression in right ventricle

Javier Navarro-Zaragoza^{ab}, Clara Ros-Simó^c, María-Victoria Milanés^{ab}, Olga Valverde^c, María-Luisa Laorden^{ab}

a Department of Pharmacology, Faculty of Medicine, University of Murcia, Spain

b Instituto Murciano de Investigación Biosanitaria, Murcia, Spain

c Grup de Recerca en Neurobiologia del Comportament (GRNC), Universitat Pompeu Fabra, Barcelona, Spain

Correspondence should be addressed to:

Javier Navarro-Zaragoza

Department of Pharmacology,

Faculty of Medicine.

Campus of Espinardo. 30100 Murcia.

Telephone: +34868889286

Fax: +34868884150

E-mail address: jnavarrozaragoza@um.es

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ABSTRACT

Aims

Oxidative stress caused by exposure to drugs of abuse such as ethanol or 3, 4 methylenedioxymethamphetamine (MDMA) may derive from direct or indirect effects in many organs including the heart. The aim of the present work was to evaluate cardiac sympathetic activity and the expression and activation of two antioxidant proteins: heat shock protein27 (HSP27) and thioredoxin-1 (Trx-1) after voluntary binge ethanol consumption, alone and in combination with MDMA.

Material and Methods

Adolescent mice received MDMA, ethanol or both. Drinking in the dark (DID) procedure was used as a model of binge. HSP27 expression and phosphorylation at serine 82 (pHSP27), Trx-1 expression, tyrosine hydroxylase (TH) and TH phosphorylated at serine 31 (pTH) were evaluated in adolescent mice 48h and 7 days after treatments in the right ventricle. TH, HSP27 expression and phosphorylation and Trx-1 expression were measured by quantitative blot immunolabeling using specific antibodies.

Key Findings

The expression of HSP27, pHSP27, Trx-1, total TH and pTH in the right ventricle was increased after binge ethanol or MDMA alone. In addition, the combination of binge ethanol+MDMA enhanced TH expression and phosphorylation versus their individual administration.

Significance

These results indicate that this combination could produce higher activation of sympathetic pathways, which could trigger an increased cell stress. On the other hand, increased HSP27,

pHSP27 and Trx-1 expression in the right ventricle by ethanol+MDMA could be a protective mechanism to reduce the adverse effects of oxidative stress caused by both drugs of abuse.

Key words

Heat shock protein 27, thioredoxin-1, right ventricle, binge ethanol, MDMA

1. INTRODUCTION

Presently, drug addiction has a serious public health and social impact [1]. Illicit drugs such as 3,4-methylenedioxymethamphetamine (MDMA) are frequently co-abused with other substances of abuse. Up to 70% MDMA users co-abuse alcohol at dangerous levels [2]. Evidence indicates that the combination of MDMA and ethanol could increase the risk of cell tissue and organ injury (i.e., harmful drug-drug interactions) since acute or sub-chronic exposure to each abused substance can itself cause organ damage. For instance, acute and binge exposure to alcohol, a widely-abused substance and legally available, or amphetamines are known to cause oxidative stress and tissue damage in many organs: pancreas, liver, brain, and heart [3,4]. Repeated, binge administration of methamphetamine, which produces similar cardiovascular effects to MDMA, in rats significantly increased the reactive oxygen species (ROS) levels in the left ventricle, resulting in tyrosine nitration of myofilament and mitochondrial proteins [5]. Studies revealed that MDMA is metabolized to catechols that can undergo redox cycling, with the formation of reactive (and unstable) orthoquinones, generating large quantities of reactive oxygen and nitrogen species [6]. The redox active metabolites of MDMA have also been implicated in the toxic effects of MDMA on the heart [7]. In addition, there is evidence that within the myocardium, repeated and long-term alcohol consumption/exposure is associated with the development of oxidative stress (for review [8]). Thus, stimulating the generation of free radicals, oxidative stress may affect neurologic processes implicated in drug addiction, and associated behavior. It suggests that changes in oxidative balance induced by drugs of abuse may contribute not only in their toxicity but also to their addictive effects [9]. Chronic exposure to drugs of abuse often leads to adaptation in antioxidant systems, indicating a need to cope with increased oxidative stress. Increase of oxidants levels compared to antioxidant defense systems leads to oxidation of proteins, phospholipids or DNA, resulting in cell dysfunction and, eventually, in cell death [1]. In this study we have examined two oxidant proteins: heat shock protein (HSP) 27 and Thioredoxin-1 (Trx-1) because both are expressed in different types of stress and also are involved in drug

addiction [10-12]. HSP27, a member of the small-HSP family, highly express in the heart [13], can protect cells during stressful situations. For example, HSP27 is able to protect neurons in oxidative stress induced after acute and chronic alcohol administration [14]. It is known that HSP27 participates in apoptosis regulation by interacting with and inhibiting several steps of the apoptotic signaling pathway [15]. Another important characteristic for HSP27 is that enhances cell resistance to oxidative injuries due to its activity against peroxidation of lipids, oxidation of proteins, and disruption of f-actin. HSP27 indeed has been given a role in depletion of ROS and nitric oxide levels [16]. Trx-1 is a key antioxidant protein which has a main role in oxidative stress. This protein has disulfide reductase activity and is mainly placed in cytosol. Sometimes, also translocate to the nucleus. Trx-1 act protecting cells under several circumstances and through different stress-responsive elements located in the promoter region: the oxidative stress response element, antioxidant responsive element (ARE), cAMP responsive element (CRE), xenobiotics responsive element (XRE) and Sp-1 [17]. Another different sequence in the promoter region (ARE) of Trx-1 is activated by unsaturated aldehydes, being attributed some positive protection in oxidative stress-induced injury [18]. Trx-1 has been identified as essential for life since knockout mice for Trx-1 are lethal as a consequence of embryo growth and morphogenesis failure [19]. In fact, Trx-1 overexpression has been correlated with higher resistance to oxidative stress compared to wild-type mice [20]. These studies show that Trx-1 is a main protein for cellular and organism survival. Since Trx-1 plays important roles in regulating intracellular redox state [21] and immune response [22] overexpression of Trx-1 resists oxidative stress and subsequently suppresses increases of the proinflammatory cytokines induced by methamphetamine [23]. In addition, Trx-1 attenuated ethanol-induced increases in markers of oxidative stress, inflammatory cytokine expression, apoptosis [24] and mitigates gastric mucosal injury in water-immersion restrain stress and HCl/ethanol-induced gastric ulcer models [25]. Taken together these results demonstrated that ethanol and MDMA can stimulate oxidative stress phenomena. However, it has been not well established the response of antioxidant defense system after ethanol or MDMA

alone or their combination. So, this study investigates the effects of binge ethanol intake, MDMA administration or acute co-administration of MDMA and binge ethanol on HSP27, HSP phosphorylated at serine 82 (pHSP27), Trx-1 expression, tyrosine hydroxylase (TH), and TH phosphorylated at serine 31 (pTH), which is an indicator of sympathetic activity, in cardiac right ventricle focusing in how this intake could produce oxidative stress at heart level. In addition, we have performed these experiments in right ventricle because the most of studies have been carried out in left ventricle, although, there are marked differences between the left and right ventricles, including reactivity to stress, hormones, drugs or alcohol, which affects left and right ventricles differently [26,27].

2. METHODS

2.1 Subjects

Adolescent naive male CD-1 mice (n=26) (postnatal day 21) weighing 25–30 g at the beginning of the experiments were used in this study. Mice were purchased from Charles River (France) and housed four per cage during 7 days (quarantine period) until 1 week prior to the beginning of the experiments when mice were individually housed (postnatal day 28). Experiments started 7 days after the individualization (postnatal day 36). Animal rooms were controlled for temperature ($22\pm 1^{\circ}\text{C}$), humidity ($55\pm 10\%$) and photo-period (12:12 L/D). Lights were turned on at 0800 hours and off at 2000 hours. One week prior to the experiment, mice were switched to a reverse light/dark schedule in which lights turned on at 1900 hours and off at 0700 hours. Food and water were available ad libitum except when water was substituted for ethanol for 2 or 4 h per day according to DID procedure, described below. All the animals care and experimental procedures were conducted according to the guidelines of the European Communities Directive 2010/63/EU regulating animal research and were approved by the local ethical committee "Comité Ético de Experimentación Animal del Parc de Recerca Biomedica de Barcelona" (CEEA-PRBB).

2.2 Drinking in the dark (DID) procedure

This procedure was conducted as previously reported [28]. Briefly, food was removed and the water bottles were replaced with 10-ml graduated cylinders fitted with sipper tubes containing either 20% (v/v) ethanol in tap water or only tap water (groups Ethanol and Water, respectively) 3 h after lights were turned off in the animal rooms. During this time, animals were maintained in home cages individually housed (see above). Briefly, DID is a voluntary binge ethanol drinking method that consists of replacing food and water bottles with 20 % (v/v) ethanol solution or water cylinders that remained in place for 2 h. After this 2 h period, food and water bottles were replaced again. This procedure was repeated on days 2 and 3 and fresh fluids were provided each day. During the following week, the DID procedure was repeated again. On day 4, DID procedure

lasted for 4 h. In addition, on day 4, subjects received two injections of a neurotoxic MDMA dose (20 mg/kg, i.p.) or saline (0.1 ml/10g, i.p.), the first injection at the beginning of the 4-h DID procedure and the second two hours later, as previously described [29]. The dose of MDMA was selected in accordance to previous studies [28-31]. Following the 4 h of free access to fluid and immediately after recording fluid intake, ethanol and water cylinders were replaced with water bottles. This procedure has been shown to produce consistent blood ethanol concentrations (BECs) [32]. After that, animals were separated in two groups: 48 h (postnatal day 40), or 7 days (postnatal day 45) after the last MDMA or saline injection (under ethanol absence) (Fig.1). After the sacrifice, the heart was rapidly removed and the right ventricle was dissected, fresh-frozen, and stored immediately at -80 °C until use.

2.3 Western Blotting

Western blot analysis was performed for TH, pTH, HSP27, pHSP27 and Trx-1. Samples were placed in homogenization buffer [phosphate buffered saline, 2% sodium dodecylsulfate (SDS) plus protease inhibitors (Roche, Germany) and phosphatase inhibitors Cocktail Set (Calbiochem, Germany)], homogenized and centrifuged at 6000 g at 4°C. Equal amounts of protein (50 µg/lane) from each sample were loaded on a 10% SDS-polyacrylamide gel (SDS-PAGE), electrophoresed, and transferred onto a PVDF membrane using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad Lab., California, USA). Non-specific binding of antibodies was prevented by incubating membranes with 1% bovine serum albumin (BSA) in Tris buffer saline Tween (TBST: 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20). Blots were incubated overnight with the following primary anti-rabbit antibodies: anti-polyclonal anti-phospho serine 31 TH (1:500 dilution Abcam, USA), polyclonal anti-total TH (1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-total HSP27 antibody (1:750, Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-phospho Ser82 HSP27 (1:400 dilution; Abcam, UK) and rabbit polyclonal anti Trx-1 (1:1000 dilution, Abcam) in TBST with BSA. Following extensive washings with TBST, the

membranes were incubated for 1h with peroxidase-labeled secondary antibodies at room temperature. After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent western blot detection system (ECL Plus, GE Healthcare, UK) and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). Antibodies were stripped from the blots by incubation with stripping buffer (glycine 25mM and SDS 1%, pH2), for 1h at 37°C. We used anti α -tubulin (Cell Signaling, 52 kDa) as our loading control for all the experiments. Quantification of immunoreactivity bands corresponding to total TH (62 kDa), TH phosphorylated at serine 31 (60 kDa), total HSP27, phosphoHSP27 (27 kDa) and Trx-1 (12 kDa) was carried out by densitometry (AlphaImager, Nucliber, Madrid). Experimental and control samples were included in the same blots and relative variations between bands were calculated in the same image.

2.4 Drugs and reagents

Racemic MDMA hydrochloride was purchased from Lipomed, A.G. (Arllesheim, Switzerland), dissolved in 0.9% physiological saline in order to obtain a dose of 20 mg/kg (2 mg/ml) expressed as the salt, and injected in a volume of 0.1 ml/10 g body weight by intraperitoneal (i.p.) route of administration. Ethyl alcohol was purchased from Merck Chemicals (Darmstadt, Germany) and diluted in tap water in order to obtain a 20% (v/v) ethanol solution. Sodium dodecylsulphate, polyacrylamide gel and poly vinylidene difluoride (PVDF) membrane were obtained from Bio-Rad Laboratory (Teknovas, Bilbao, Spain). Reagents: proteases inhibitor (Boehringer Mannheim, Germany); phosphatase inhibitor cocktail set (Cambiochem, German).

2.5 Statistical analysis

Data are expressed as mean \pm SEM. The distribution of all the results was normal which was checked by the Shapiro-Wilk test. Statistical analysis was determined by a two-way analysis of variance (ANOVA). The Newman-Keuls was used as a post hoc test whenever a significant

difference between three or more sample means was revealed by an analysis of variance (ANOVA). Differences with a $p < 0.05$ were considered significant.

3. RESULTS

Water and ethanol consumption was measured for each mouse every day during the DID procedure (Fig. 1). One-way ANOVA for water consumption did not show differences in the total fluid (ml) consumed between water-treated groups (Water \times MDMA vs. Water \times Saline). Regarding the amount of ethanol (g EtOH/kg and ml) consumed, no differences were found between groups (Ethanol \times MDMA vs. Ethanol \times Saline) in none of the days of the DID procedure. Thus, consumption of water and ethanol remain consistent across days in each group.

3.1 Expression of HSP27, and HSP27 phosphorylated at serine 82 after ethanol binge drinking or MDMA treatment

We examined HSP27, pHSP27 and Trx-1 expression to determine the magnitude and severity of cellular stress during binge ethanol exposure or MDMA treatment. Two-way ANOVA analysis for HSP27 48 hours after MDMA or saline injection revealed a significant effect of binge ethanol [$F(1,20) = 15,76$; $P = 0.0008$] and a significant effect of MDMA treatment [$F(1,20) = 21,14$; $P = 0.0002$]. This analysis also showed a significant effect of binge ethanol 7 days after chronic treatment [$F(1,14) = 14$; $P = 0.0314$] and a significant effect of MDMA [$F(1,14) = 10,54$; $P = 0.0059$]. Newman-Keuls post-hoc test revealed that HSP27 expression was significantly increased ($P < 0.01$) 48 h or 7 days after binge ethanol exposure or MDMA treatment (Fig. 2A,B). In addition, we have observed an increased ($P < 0.05$) expression of HSP27 in ethanol+MDMA group versus ethanol or MDMA alone (Fig 2A,B). We also studied the phosphorylation of HSP27 at serine 82 in the right ventricle at different time points. Two-way ANOVA analysis for pHSP27 48 hours after MDMA or saline injection revealed a significant effect of binge ethanol [$F(1,22) = 16,59$; $P = 0.0005$] and a significant effect of MDMA [$F(1,22) = 20,85$; $P = 0.0002$]. Finally two-way ANOVA for seven days revealed a significant effect of MDMA treatment [$F(1,16) = 9,24$; $P = 0.0078$]. As shown in Fig 2C,

post-hoc test showed a significant ($P<0.05$) increased phosphorylation of HSP27 48 h after MDMA injection or ethanol exposure versus the control group (water+saline). There is also significant ($P<0.01$) interaction between the group exposed to binge ethanol and injected with MDMA versus the group treated with MDMA or ethanol alone. pHSP27 expression was also increased ($p<0.01$) seven days after MDMA-treatment versus the group that received water+saline. In addition, there was a significant ($P<0.05$) increase in pHSP27 in the group exposed to binge ethanol and treated with MDMA versus ethanol and MDMA alone suggesting that the exposure to both drugs of abuse induce long-term changes at heart level (Fig. 2D). However, there are no changes in the ratio pHSP27/HSP, 48 h or seven days after ethanol exposure or MDMA administration (Fig. 2E,F).

3.2 Expression of Trx-1 after ethanol binge drinking or MDMA treatment

Analysis of the expression of Trx-1 in right ventricle by a two-way ANOVA test, 48 h after MDMA or saline injection, revealed a significant effect of binge ethanol exposure [$F(1,15)=12.27$, $P=0.0032$] and MDMA injection [$F(1,15)=7.33$, $P=0.0162$]. Similarly, seven days after last MDMA injection we have observed for Trx-1 a significant effect of binge ethanol [$F(1,15)=4.64$, $P=0.0478$] and MDMA injection [$F(1,15)=9.38$, $P=0.0079$]. Post hoc test revealed a significant ($P<0.05$) increase of Trx-1 after binge ethanol exposure or MDMA injection and a significant ($P<0.05$) interaction between ethanol+MDMA when compared with ethanol+saline or MDMA+water 48 h after their administration (Fig. 3 A). Trx-1 expression remains high seven days after ethanol exposure or MDMA administration (Fig. 3 B).

3.3 Ethanol binge drinking and MDMA effects on TH and TH phosphorylated at serine 31

The influence of MDMA and binge ethanol on the immunoreactivity of total TH was examined in the right ventricle 48 h, and 7 days after the last MDMA or saline injection (Fig 4A,B,C,D). Two-way ANOVA analysis for total TH 48 hours after MDMA or saline injection revealed a significant effect of binge ethanol exposure [$F(1,19) = 7,12$; $P = 0.0152$] and a significant effect of MDMA injection [$F(1,19) = 9,84$; $P = 0.0054$]. Newman-Keuls showed that total TH levels were increased

($P < 0.05$) 48 h after ethanol exposure or MDMA treatment (Fig. 4A), whereas these levels are not modified seven days after MDMA injection or ethanol exposure (Fig. 4B). Additionally experiments were performed in the right ventricle to determine whether MDMA or binge ethanol would activate phosphorylation of TH at serine 31 at different time points (Fig 4C,D). Two-way ANOVA analysis for TH at serine 31 48 hours after MDMA or saline injection revealed a significant effect of ethanol exposure [$F(1,19) = 11,18$; $P = 0.0034$] or MDMA injection [$F(1,19) = 13,35$; $P = 0.0017$]. Newman-Keuls post-hoc test demonstrated that 48 h after binge ethanol exposure there was an increased ($P < 0.05$) phosphorylation of TH at serine 31, without changes after MDMA injection (Fig 4C). However, there was a significant ($p < 0.05$) interaction between ethanol+MDMA 48 h after their administration when compared to MDMA alone (Fig 4C). As can be seen in the Fig. 4D we have not observed changes in this parameter seven days after alcohol exposure or MDMA injection.

4. DISCUSSION

CD1 mice were selected for our experiments because this strain is considered to have low preference for alcohol [33]. Thus, previous results have shown differences between CD1 and other strains such as C57BL/6J suggesting changes in ethanol metabolism and palatability [34]. Nevertheless, blood ethanol concentration is reached by about 50-70 mg% (80 mg% is considered as intoxication in humans) [35].

Although, it is known that the anatomy as well as the mechanical and biochemical properties of the right and left ventricle are different (26, 27), only a few studies can be performed in right ventricle. In the present work, studying right ventricle, we have observed an increased expression and phosphorylation of HSP27 seven days after the combination of MDMA and ethanol. However, these parameters were not modified in the left ventricle (45). In addition, there is an increase of sympathetic activity (enhancement of TH phosphorylation) in the left ventricle after ethanol exposure or MDMA injection (45) without any changes in right ventricle. Altogether these results demonstrate, innovatively, different expression of antioxidant proteins and sympathetic activity in right and left ventricles. The HSP27 expression and phosphorylation increased in right ventricle seven days after ethanol and MDMA may contribute to better protecting this heart chamber from drugs-mediated cardiac toxicity.

In addition to the neurobehavioral consequences of addiction, the relationship between addiction and cardiovascular disorders is well established although little is known about the pathophysiology underlying this comorbidity. So that, investigation about the mechanisms involved in the cardiac adaptive changes that occur during drug abuse exposition deserves more attention. One of the mechanisms implicated in cardiac disorders associated with addiction could be the activation of catecholaminergic system which may exert negative effects on myocardial function. TH, the rate-limiting enzyme involved in catecholamines synthesis, is regulated by two different mechanisms: enzymatic phosphorylation and transcriptional regulation [36].

Particularly, phosphorylation of Ser31 and Ser40 enhance TH activity, which stimulates the production and release in catecholamine terminals [36-38]. The present study showed that binge ethanol and MDMA enhanced the expression of TH and TH phosphorylated at Ser31 in right ventricle. But also, that the combination of these two drugs increased the activity of TH versus the consumption of MDMA alone. These data suggest that Ser31 phosphorylation of TH may be an important modulator of TH activity during the consumption of drug of abuse and might be directly related with increasing in cardiac sympathetic activity responsible in part of the cardiac toxicity induced by ethanol or MDMA [39-43].

On the other hand, oxidative stress caused by exposure to drugs of abuse such as ethanol or MDMA may derive from direct or indirect effects in many organs including heart [39-43]. Increase in the levels of oxidants compared to antioxidant defense systems leads to oxidation of proteins, phospholipids or DNA, leading to cell dysfunction and, eventually, to cell death [1]. Notably, oxidative stress is a common pathophysiological factor in the development of cardiac dysfunction; preservation of myocardial function in these situations is crucial and depends on endogenous adaptive response. HSP27 and Trx-1 are keys of the antioxidant system that protects cells from oxidative stress. HSP27 is one particularly interesting small protein as it can protect cardiomyocytes against stress and heart infarction [44]. According to previous data [45], our results demonstrated an increase of HSP27 expression and phosphorylation in the heart. Although a major organ damage has been described when ethanol is associated to MDMA [46], the mechanism implicated in this interaction is not well known. Our results show an increased HSP27 expression and phosphorylation after binge ethanol+MDMA exposure, indicating an exacerbation of the cellular stress induced by ethanol or MDMA alone. These effects can be observed, even seven days after the treatment, suggesting possible long-term effects of the treatment which may favour future compulsive use of drugs [47]. While HSP27 can block actin polymerization, the phosphorylation of HSP27 is related to re-organization of the actin-based cytoskeletal structures [48]. Thus, the vascular protection induced by phosphorylated HSP27 is mediated by its

interaction with cytoskeletal elements such as actin [49]. In this way it has been described a possible role of HSP27 in the protection of cardiac troponin T degradation by μ -calpain, a protease mediating proteolysis of troponines, after morphine withdrawal [50,51].

On the other hand, experimental studies indicated that Trx-1 overexpression protects against cardiac cell apoptosis and is associated with good cardiac function [52,53]. Trx-1 is reportedly rapidly released into the circulation from the damaged cardiomyocytes [54] and is increased with advancing chronic heart failure [55]. Present results demonstrated, for the first time, an enhancement of Trx-1 expression after ethanol or MDMA alone. In addition, our data suggest that increasing Trx-1 expression in the heart by the combination of binge ethanol+MDMA is a biological strategy to minimize myocardial dysfunction induced by both drugs of abuse. In this way, recombinant human Trx attenuated ethanol-induced increases in markers of oxidative stress, inflammatory cytokine expression and apoptosis [24].

In conclusion, our results demonstrated that binge ethanol, MDMA or the combination between binge ethanol and MDMA increased antioxidant proteins such as HSP27 and Trx-1 that protect myocardial alterations, due to an increased sympathetic activity induced by both drugs of abuse. These data could contribute to establish new targets in the treatment of addictive disorders.

Competing Interests

The authors have declared that no competing interests exist

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Figure Legends

Figure 1. Drinking in the dark test (DID test), see methods section.

Figure 2. Western-blotting analysis of HSP27 (A,B), HSP27 phosphorylated (p) at serine 82 (Ser82) (C,D) and pHSP27/HSP27 ratio (EF) in the right ventricle from animals exposed to water (w) or ethanol (e) and sacrificed 48 or 7 days after the last injection of MDMA (m) or saline (s). The immunoreactivity corresponding to HSP27 or pHSP27 is expressed as a percentage of that in the control group defined as 100% value. Data are the means±SEM (n=4-6), *p<0.05, **p<0.01 versus water+saline; +p<0.05, ++p<0.01 versus water+MDMA; #p<0.05, ##p<0.01 versus ethanol+saline.

Figure 3. Western-blotting analysis of thioredoxin-1 (TRX) (A,B) in the right ventricle from animals exposed to water (w) or ethanol (e) and sacrificed 48 or 7 days after the last injection of MDMA (m) or saline (s). The immunoreactivity corresponding to HSP27 or pHSP27 is expressed as a percentage of that in the control group defined as 100% value. Data are the means±SEM (n=5-6), *p<0.05, **p<0.01 versus water+saline; +p<0.05 versus water+MDMA; #p<0.05 versus ethanol+saline.

Figure 4. Western-blotting analysis of total TH (A,B) and TH phosphorylated (p) at serine 31 (Ser31) (C,D) in the right ventricle from animals exposed to water (w) or ethanol (e) and sacrificed 48 or 7 days after the last injection of MDMA (m) or saline (s). The immunoreactivity corresponding to total TH or THpSer31 is expressed as a percentage of that in the control group defined as 100% value. Data are the means±SEM (n= 5-6), *p<0.05 versus water+saline; +p<0.05 water+MDMA; #p<0.05.

Figure 1
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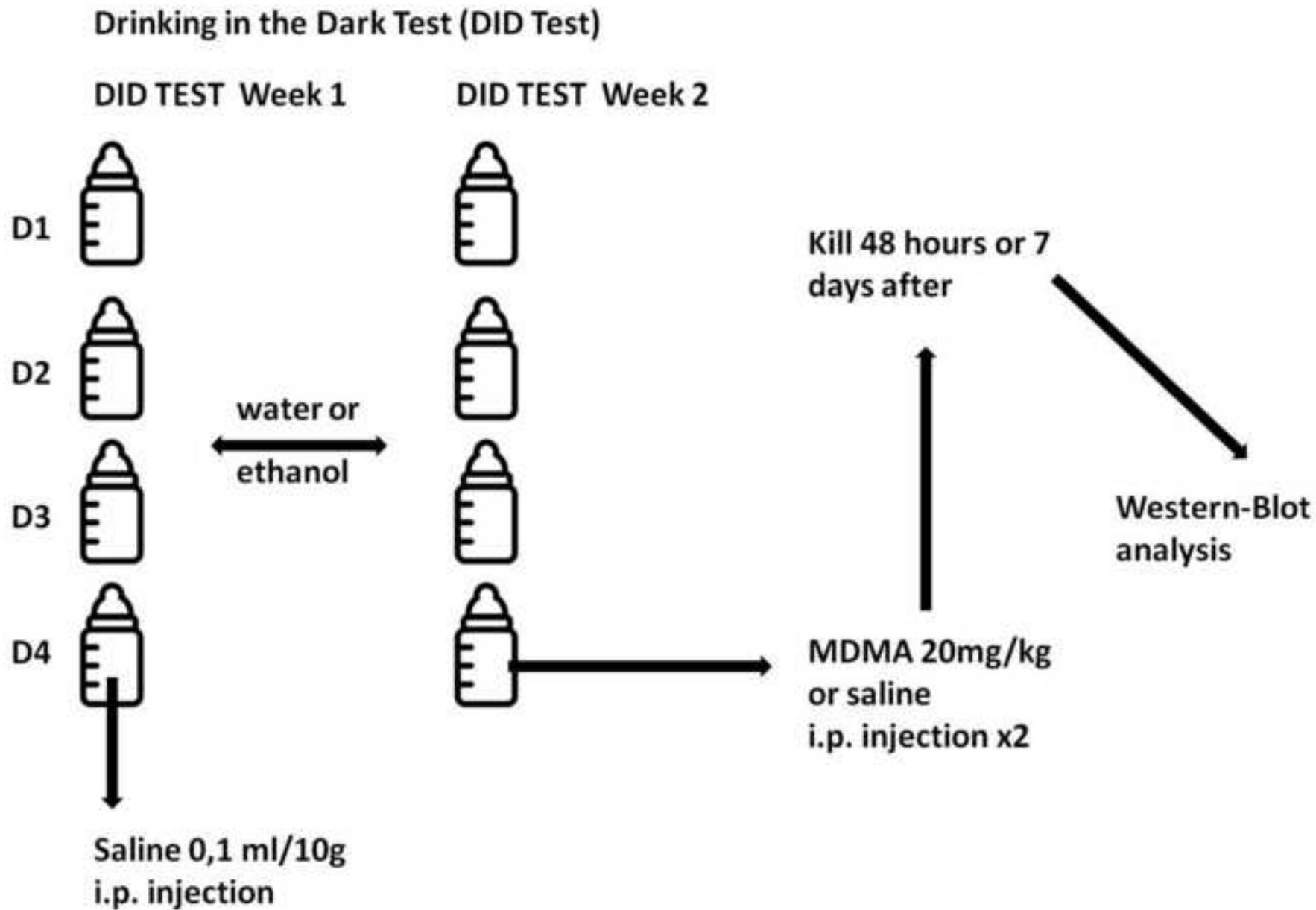


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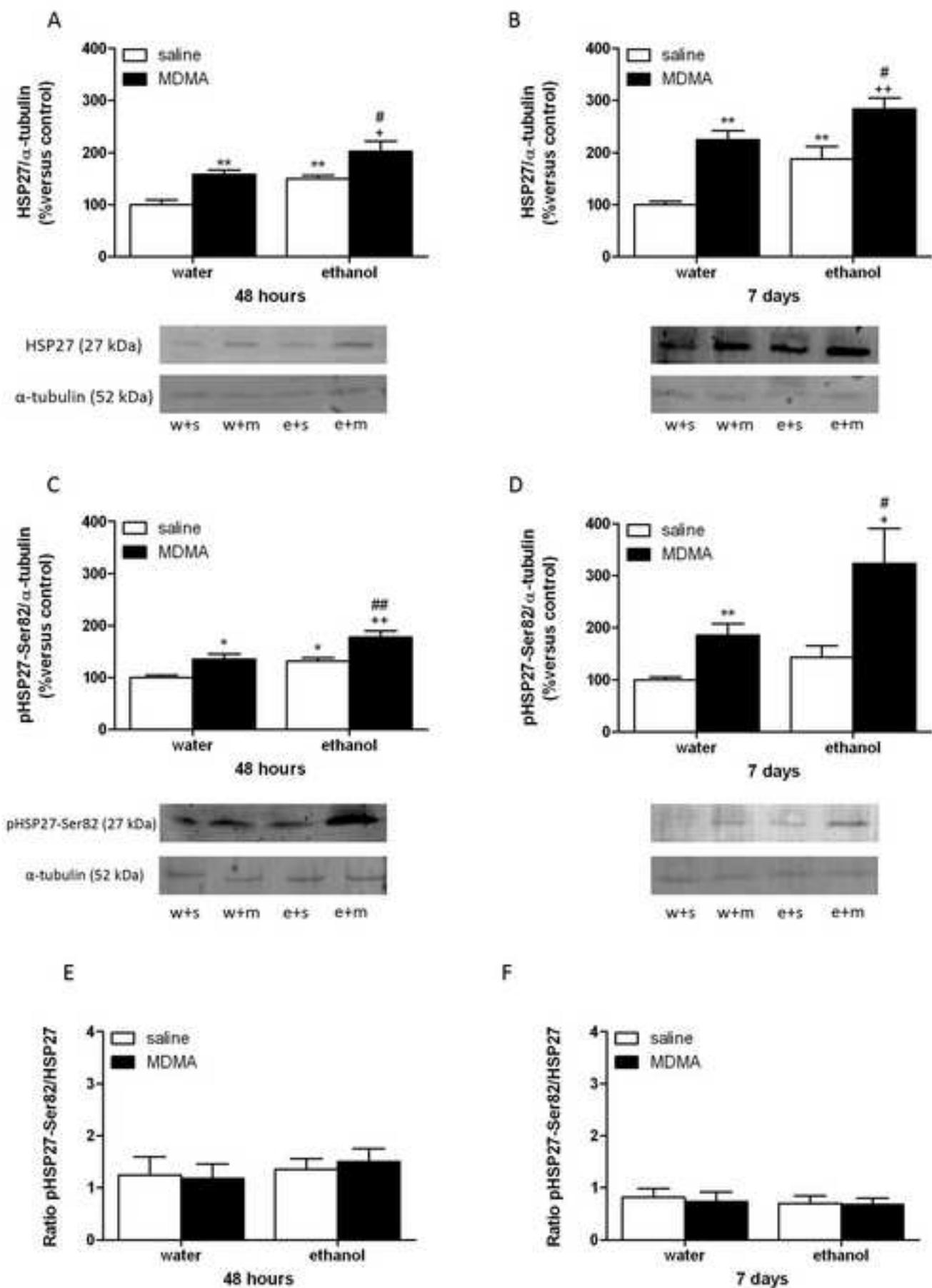


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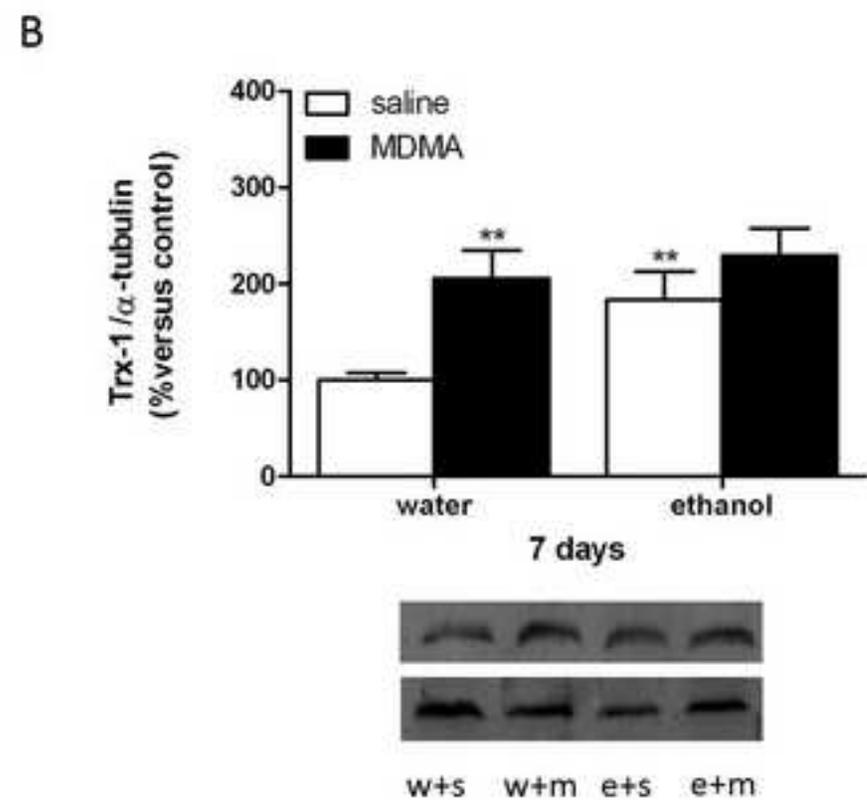
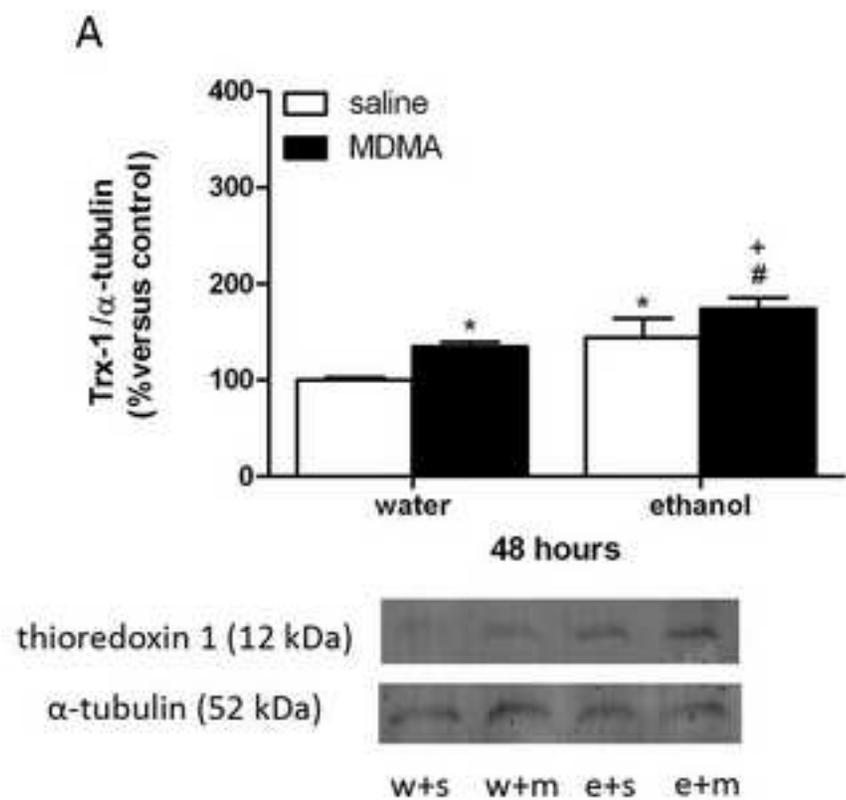


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