Current Neuropharmacology, 2017, *15*, 300-314

REVIEW ARTICLE

The Role of Oxidative Stress in Methamphetamine-induced Toxicity and Sources of Variation in the Design of Animal Studies

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ARTICLE HISTORY

Received: August 24, 2015
Revised: April 21, 2016
Accepted: April 27, 2016

DOI: 10.2174/1570159X14666160428110329

Abstract: ***Background*:** The prevalence of methamphetamine (MA) use has increased in recent years. In order to assess how this drug produces its effects, both clinical and preclinical studies have recently begun to focus on oxidative stress as an important biochemical mechanism in mediating these effects.

***Objective*:** The purpose of this review is to illustrate the variation in the design of preclinical studies investigating MA exposure on oxidative stress parameters in animal models.

***Method*:** The experimental variables investigated and summarised include MA drug treatment, measurements of oxidative stress and antioxidant treatments that ameliorate the harmful effects of MA.

***Results*:** These preclinical studies differ greatly in their experimental design with respect to the dose of MA (ranging between 0.25 and 20 mg/kg), the dosing regime (acute, binge or chronic), the time of measurement of oxidative stress (0.5 h to 2 wks after last MA administration), the antioxidant system targeted and finally the use of antioxidants including the route of administration (i.p. or p.o.), the frequency of exposure and the time of exposure (preventative or therapeutic).

***Conclusion*:** The findings in this paper suggest that there is a large diversity among these studies and so the interpretation of these results is challenging. For this reason, the development of guidelines and how best to assess oxidative stress in animal models may be beneficial. The use of these simple recommendations mean that results will be more comparable between laboratories and that future results generated will give us a greater understanding of the contribution of this important biochemical mechanism and its implications for the clinical scenario.

Keywords: Methamphetamine, drug abuse, oxidative stress, animal model, antioxidants, neurotoxicity.

1. INTRODUCTION

1.1. Methamphetamine and Theories of Toxicity

With the increasing use of methamphetamine (MA) worldwide there has been a growing awareness of the harmful effects of MA exposure, and explorations of the biochemical mechanisms by which such effects are mediated. Its mechanisms of toxicity have been studied extensively and it appears that MA can exert its toxic effect in many ways. Although attempts have been made to explain MA-induced toxicity by its links to excitotoxicity (excessive glutamate release), mitochondrial dysfunction [1], blood brain barrier dysfunction, inflammation and DNA damage [2], oxidative stress has shown to be a promising lead in explaining, at a cellular level, the harmful effects of MA abuse. Fig. (**1**) highlights the cascade of events that occur after MA exposure.

1.2. Oxidative Stress

MA is an amphetamine-type stimulant that crosses the blood–brain barrier easily and stimulates the CNS by acting as a sympathomimetic drug [4]. MA is known to increase the synaptic availability of dopamine (DA) and serotonin (5HT) [5]. These are acute effects of MA however and it is thought that chronic exposure to MA can result in neurotoxicity and long-lasting damage to the dopaminergic axon terminals [6]. Oxidative stress is defined as a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defences which can result in damage [7]. After MA administration the increased DA undergoes auto-oxidization to toxic products known as ROS including hydrogen peroxide (H_2O_2), hydroxyl radicals (OH·) and superoxide radicals ($O_2^{\cdot -}$) (Fig. **2**). If these are not detoxified

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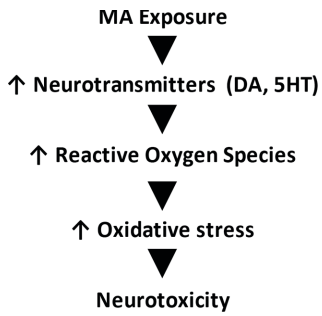

Fig. (1). MA cascade of events. The implications of MA exposure. DA; Dopamine, 5-HT; Serotonin. (Adapted from Halpin, Collins [3]).

by antioxidants or antioxidative enzymes, they may damage proteins, lipids, DNA and RNA [8].

The antioxidant defence system acts as a scavenging system to detoxify these free radicals and prevent or minimise cellular damage. Catalase (CAT), glutathione peroxidase (GPx) and Super Oxide Dismutase (SOD) are scavenger enzymes. However when the system is overloaded these enzymes can be decreased or inactivated by oxidative stress which makes the task of defending against ROS quite challenging [2, 11].

1.3. Neurotoxicity

When the antioxidant defence system fails or is overloaded, the ROS generated can lead to cellular damage by acting on nucleic acids, proteins and phospholipids [12]. Therefore this increased concentration of DA and changes in DA metabolism after MA exposure can switch on the oxidative stress cascade and consequently can lead to the degeneration of dopaminergic terminals [12]. The literature to date has shown that MA-induced neurotoxicity is dependent on dopamine levels and because of this the striatum and nucleus accumbens are the brain regions that have been studied most extensively. These regions have the most robust dopaminergic projections and so are most susceptible to MA-induced oxidative damage [13].

1.4. The Preclinical Picture

The first implications that oxidative stress plays a role in MA-induced neurotoxicity date back to 30 years ago, when it was discovered that the antioxidants vitamin E (α-tocopherol) and ascorbic acid attenuated the depletions of DA and 5HT seen in the striatum after MA exposure [14, 15]. Since then, there has been much interest in this topic, using both animals and humans. Although there have been significant results to date in animal studies, there has been considerable variation between studies and how oxidative stress is measured. The purpose and aim of each study varies but when investigating the effects of MA on oxidative stress it is still unclear which treatment regime, dose, sacrifice time, brain region etc. yields the most significant changes in oxidative stress. Due to this wide range of experimental protocols that exist, the interpretation of these different results is challenging. For this reason, the development of guidelines and how best to assess oxidative stress in animal models may be beneficial. Simple recommendations like these mean that results will be more comparable between papers and that future results generated will give us a greater understanding of the contribution of oxidative stress and its implications for the clinical scenario

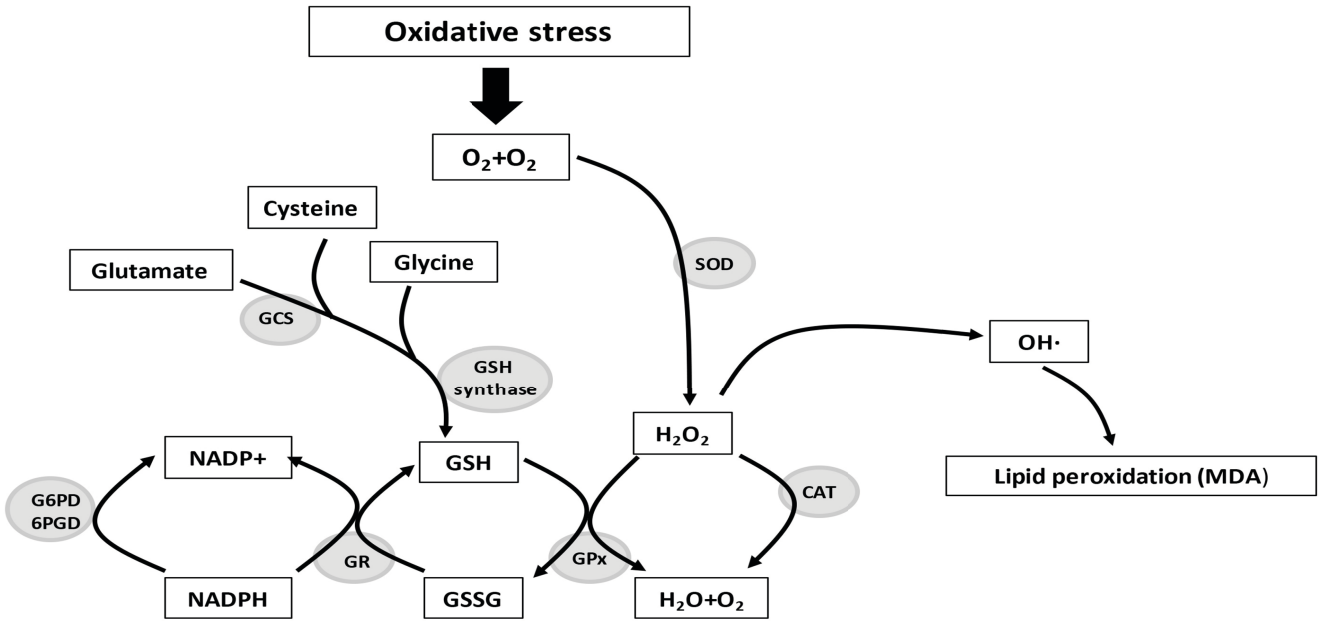

Fig. (2). Oxidative stress pathway. The implications of MA exposure on oxidative stress. SOD; Super Oxide Dismutase, MDA; Malondialdehyde, CAT; Catalase, GSH; Glutathione, GCS; Glutamylcysteine synthetase, GSSG; Oxidised glutathione, GR; Glutathione reductase, GPx; Glutathione peroxidase, G6PD; Glucose-6-phosphate dehydrogenase, 6PGD; 6-phosphogluconate dehydrogenase, NADPH; Nicotinamide Adenine Dinucleotide Phosphate-Oxidase, OH; Hydroxyl Radical, H_2O_2; Hydrogen Peroxide. (Adapted from Halpin, Collins [3], Guo and Chen [9], Isagenix International [10]).

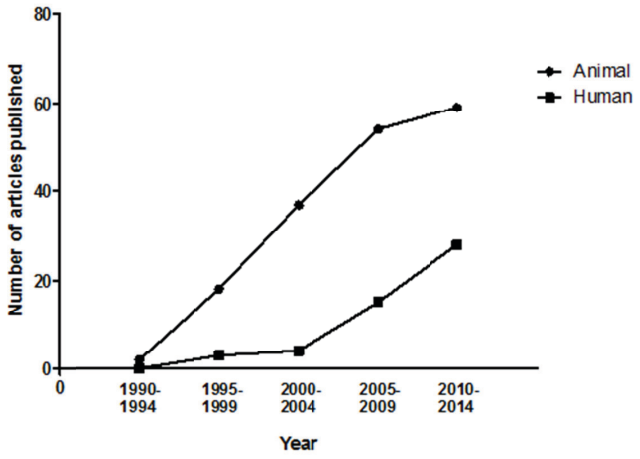

Fig. (3). Papers published. Total number of articles returned when 'Methamphetamine AND Oxidative Stress' was entered into PubMed search engine.

1.5. Aims

The purpose of this review is to review the evidence implicating oxidative stress with methamphetamine exposure in human and animal models and to also propose an optimum protocol for assessing oxidative stress in laboratory animal models exposed to methamphetamine.

1.6. Review Methods

The search terms 'Methamphetamine AND Oxidative Stress' were entered into PubMed search engine. Between 1990 and 2014, 220 articles were published in this area (Fig. **3**). Among these articles 41 studies were relevant in that they involved MA exposure and oxidative stress.

2. HOW TO MEASURE OXIDATIVE STRESS?

In the literature, the methods used for exploring the effects of MA use on oxidative stress in animals vary substantially. By separately focusing on each stage of the experimental design the variables can be categorised accordingly (Fig. **4**).

1) MA drug treatment (Dose, regime, length of exposure, route of administration).
2) Measuring oxidative stress (Time point following administration, brain region(s), parameter/targets to use).
3) Antioxidant treatments (Type of antioxidant, regime, time of exposure, route of administration).

2.1. MA Drug Treatment

2.1.1. Dose

The first documented preclinical studies examining the role of oxidative stress and MA use used doses of 5 and 10 mg/kg [16], but in the intervening years a wide range of MA doses have been employed**.** We previously classified these MA doses into neurotoxic, toxic and pharmacological [17] and Table **1** classifies the MA doses used from these studies accordingly. Although some studies have looked at lower MA doses (pharmacological and toxic), most of the doses employed to date have fallen into the neurotoxic class range (i.e. >5 mg/kg).

The minimum dose of MA used previously has been 0.25 mg/kg [18, 19]. When this dose was employed for 7 days, the authors reported increased carbonyl group formation and lipid damage in all brain regions examined, namely the prefrontal cortex, amygdala, hippocampus and striatum [19],

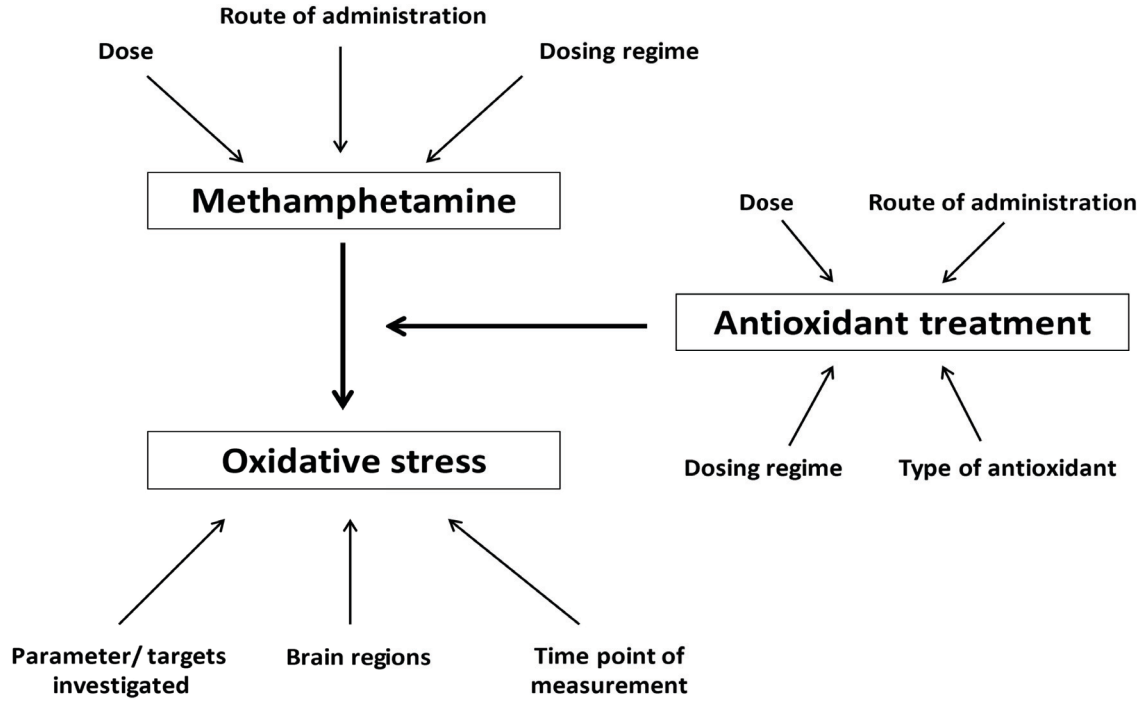

Fig. (4). Experimental variables. Chart depicting the experimental variables that exist in MA oxidative stress studies.

Table 1. Classification of previously used preclinical doses for MA.

Dose Classification	Comments	Dose Range	% of Preclinical Papers
Neurotoxic	Resemble extremely high toxic doses	≥5 mg/kg	64
Toxic	Resemble high pharmacological doses	2-4 mg/kg	12
Pharmacological	Resemble doses used in pharmacological studies	0.25-2 mg/kg	24

The range of doses used in MA oxidative stress preclinical studies (Adapted from McDonnell-Dowling and Kelly [17].

suggesting marked effects on oxidative stress even following a relatively low dose of MA. At the other extreme, the maximum dose of MA has been 80 mg/kg as a "binge" dose (four 20 mg/kg injections in one day) or 20 mg/kg as a chronic dose (20 mg/kg once daily for 10 days) [20]. The total GSH concentration was reduced in the striatum of the MA binge group and the glucose-6-phosphate dehydrogenase (G6PD) activity was reduced in the frontal cortex of the MA chronic group. Although these changes suggest that oxidative stress may be involved in MA-induced neurotoxicity, these changes were only small (slight reductions) and so compared to a low dose of 0.25 mg.kg, there is not a greater effect with this higher dose. The most common MA dose previously employed has been 10 mg/kg with 50% of the MA and oxidative stress studies using this dose [21-24]. At this dose studies have reported many alterations in oxidative stress parameters (Table **2**).

The pharmacological doses (0.25-2 mg/kg) and low toxic doses (2-3 mg/kg) of MA (Table **1**) are the doses that equate to those most commonly abused in the clinical scenario [17]. Therefore it is pertinent that such doses have shown to yield the greatest results for oxidative stress as it allows for comparison of these preclinical studies to what is happening in the human situation.

2.1.2. Dosing Regime

The frequency of MA dosing or the amount of MA injections given on each day varies between each study. The most common frequencies are one dose per day [18, 21, 31] or "binge" dosing with four doses per day (with 2 hour intervals separating the doses) [16, 24, 32, 33] (Table **3**).

The use of multiple injections in one day is generally used as a neurotoxic regime. Rather than trying to achieve a clinical pattern of MA use this method is overloading the protective mechanisms by using a continuous assault of the drug. This method can achieve a higher daily dose without running the risk of giving the animal an overdose or reaching the LD_{50}. Many variations exist for this regime including 2 injections in a day (2 h, 10 h or 12 h apart) [22, 34, 35], 3 injections in a day (2 h or 3 h apart) [26, 36], 4 injections in a day (2 h or 5 h apart) [20, 32] and 5 injections in a day (2 h apart) [37]. However, as seen in the previous section, preclinical studies have found that oxidative stress changes following MA administration can be detected after small doses (0.25 – 0.5 mg/kg, one injection) and therefore the use of a neurotoxic regime may not be causing an increased state of oxidative stress. However, the number of days in which the animal receives the drug can also vary among studies. One day of administration is generally the most common dosing routine and this is combined with multiple injections in a single day. When only one injection is given in a day this is most commonly given for multiple days. Therefore the main strategies that have been previously employed are focusing on either chronic/long term dosing or acute/binge dosing. Chronic dosing (multiple days of dosing) ranges between 4 and 14 days of dosing and Fig. **5** shows all the chronic routines previously used.

Table 3. Dosing routines.

No. Injections/Day	Time Interval Between Injections	% of Papers
1	N/A	30
2	2 h apart	4
2	10 h apart	4
2	12 h apart	4
3	2 h apart	4
3	3 h apart	4
4	2 h apart	35
4	5 h apart	4
5	2 h apart	9

The range of injection routines used in MA oxidative stress preclinical studies.

Table 2. Effects of 10 mg/kg MA on oxidative stress parameters.

Effect	Parameters							
↑	ROS	SOD Activity	MDA	GPx Levers	Protein Carbonyls	CAT Activity	GSSG Cotnent	Uric Acid Content
↓	GSH Levels	GPx Levels						

The range of oxidative stress parameters altered after MA preclinical studies. [22-30].

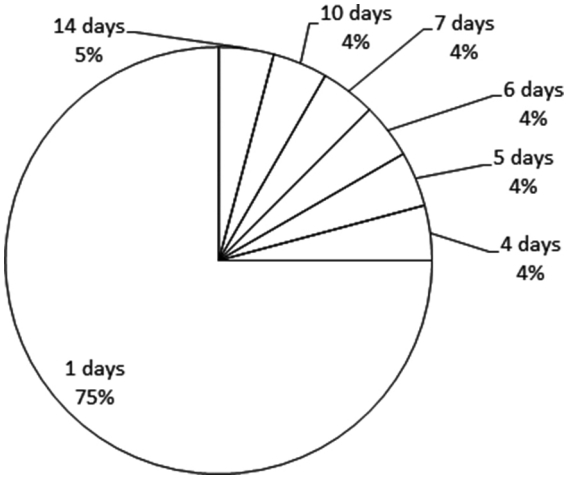

Fig. (5). Drug administration routines. The range of drug administration routines used in MA oxidative stress preclinical studies.

So how do acute, binge, multiple days or chronic dosing compare regarding their effects on oxidative stress? Firstly, looking at acute dosing, da-Rosa, Valvassori [18] showed that acute MA increased carbonyl group formation and Thiobarbituric acid reactive substances (TBARS) generation in the prefrontal cortex, amygdala, hippocampus and striatum at doses 0.5, 1 and 2 mg/kg (by over 100%). A study by Acikgoz, Gonenc [31] found that when given as a single injection for one day at a dose of 5 mg/kg, MA had no effect on TBARS levels, GPx activity or SOD activity in the striatum or prefrontal cortex. However, when administered acutely at 15 mg/kg there was an increase in TBARS levels in both the striatum (by over 150%) and prefrontal cortex (by over 300%) and when given at 10 mg/kg there was an increase in SOD activity in the prefrontal cortex (by over 40%). An acute MA dose of 10 mg/kg increased TBARS levels in the corpus striatum, hippocampus and frontal cortex, increased GSH content in the striatum and frontal cortex, and increased GPx activity in the striatum and frontal cortex (by 30%) [30].

A binge dose could be considered a high MA dose that is given several times in a single day. For example Moszczynska, Turenne [20] administered MA at 20 mg/kg and this was given 4 times in one day at 5 h intervals. In this study, the total GSH concentration was reduced in the striatum of the MA binge group although this reduction was only small (-17%) compared to a 47% increase in total GSH levels after acute MA at 10 mg/kg in the aforementioned study by Flora, Lee [30]. However, in a similar dosing regimen of MA, at only 7.5 mg/kg given 4 times in one day at 2 h intervals, showed significant increases in MDA formation, 4-hydroxynonenal (4-HNE) expression, ROS formation and protein carbonyl expression [38]. The same authors showed that after MA, at 8 mg/kg given 4 times in one day at 2 h intervals, MDA and protein carbonyl content were also increased in the striatum [39]. There are limited preclinical studies that have examined multiple dosing days or chronic dosing (Table **4**). As stated earlier, da-Rosa, Valvassori [19] showed that at 0.25 mg/kg of MA for 7 days increased carbonyl group formation and lipid damage in all brain regions examined. When MA was given chronically at this same dose for 14 consecutive days (one daily injection) the authors also found increased carbonyl group formation and TBARS generation in the prefrontal cortex, amygdala, hippocampus and striatum [18]. Pang, Panee [40] showed that 6 consecutive daily injections of MA at 2.5 mg/kg resulted in higher GSH contents in the striatum, decreased GCS activities in striatum and frontal cortex, decreased activity of GR in the thalamus and increased activity of GR in the striatum and glutaredoxin (Glrx) activity was upregulated in the striatum. At a higher dose of 20 mg/kg for 10 consecutive days (one daily injection) MA only reduced the G6PD activity in the frontal cortex but had no effects on levels of GSH or activities of the other glutathione-related enzymes (GPx, GR, g-GTP (g-glutamyltranspeptidase)) in any of the brain regions (cerebellum, frontal cortex, striatum) [20]. It is interesting to see differences in oxidative stress results after different dosing regimens as although clinically chronic, long-term use is most common, there are a diversity of individual abuse patterns [41].

After looking at all the variations of how MA is given to the animals it is clear that the dose of drug given must be the most important parameter when assessing oxidative stress. The differences in results between studies can be mostly explained by the dose of drug rather than the dosing regime itself. Whether given short term or long term oxidative stress can be detected using various parameters; however when given at low doses (pharmacological doses, Table **1**) the results yielded seem to be more significant and of a greater magnitude compared to higher doses of MA which is suggestive of an inverted U-shaped curve. For example, MA given at 2 mg/kg acutely or 0.25 mg/kg for multiple days seems to have a greater effect on oxidative stress parameters compared to 15 mg/kg acutely or 20 mg/kg for multiple days.

2.1.3. Route of Administration

The route of administration for MA in oxidative stress studies is something that must be considered before beginning the experiment. Among the previous studies, the most common route of administration is intraperitoneal injection (i.p.) with 75% of the studies using this route [16, 27, 33, 35] (Fig. **6**) and subcutaneous injections (s.c.) have been used by a small number of studies (17%). It is unclear why some studies have chosen this different route of administration and this is common between both rats and mice. The dosing routine using s.c. is varied and includes acute and binge dosing but all studies have only looked at one day of dosing. However although it has been reported that behavioural changes after MA exposure are observed regardless of the route of administration it has been shown that with the i.p. route, the drug is absorbed more rapidly [42]. With s.c. injections the absorption is slower but the bioavailability of the drug is 100% compared to i.p. which has a bioavailability of 58% due to hepatic first pass metabolism [42]. As these routes of administration show different pharmacokinetic profiles, then the time point of kill after dosing is very important and must ensure that enough time has elapsed in order for its effects to have occurred, and this point will be discussed later in the review (Section 2.2.1). Further investigation into the different routes of

Table 4. MA treatment regimes.

Species	Dose (mg/kg)	No. Injections/Day	No. of Days of Drug Admin	Route	Refs.
Rats	0.25, 0.5, 1 or 2	1	14	i.p.	[18]
Rats	0.25 or 0.5	1	7	i.p.	[19]
Mice	1, 2, 10, 20	1	1	s.c.	[21]
Rats	2.5	1	6	i.p.	[40]
Rats	2.5	2 - 10 h apart	1	s.c.	[34[
Mice	3	3 - 3 h apart	1	s.c.	[36]
Mice	4	5 - 2 h apart	1	i.p.	[37]
Rats	5	4 - 2 h apart	1	s.c.	[32]
Mice	5 or 10	4 - 2 h apart	1	i.p.	[16]
Rats	5, 10 or 15	1	1	i.p.	[31]
Rats	7.5	4 - 2 h apart	1	i.p.	[33]
Mice	7.5	4 - 2 h apart	1	i.p.	[38]
Mice	8	4 - 2 h apart	1	i.p.	[39]
Mice	10	2 - 2 h apart	1	i.p.	[22]
Mice	10	1	1	i.p.	[27]
Mice	10	3 - 2 h apart	1	i.v.	[26]
Mice	10	1	1	i.p.	[30]
Mice	10	4 - 2 h apart	1	i.p.	[23]
Rats	10	2	5	i.p.	[25]
Rats	10	4 - 2 h apart	1	Unknown	[29]
Mice	10	5 - 2 h apart	1	i.p.	[28]
Rats	10	4 - 2 h apart	1	i.p.	[24]
Rats	15	2 - 12 h apart	4	i.p.	[35]
Rats	20	1/4 - 5 h apart	10	i.p.	[20]

The range of dosing procedures in MA oxidative stress preclinical studies. n=24 papers.

administrations used for oxidative stress would be very beneficial. A comparison of these routes would highlight if there are any differences and if the terminal time point needs to be altered based on the route of administration used. Returning again to the clinical situation then we know that oral use or use through inhalation is how MA is most commonly abused in humans [17] but this has been overlooked in preclinical studies to date and so this should also be included in future research.

2.1.4. Animals

The choice of test subject is an important factor to consider when designing an *in vivo* study to investigate methamphetamine neurotoxicity and oxidative stress, as these parameters may vary between species. To date, only rodents have been investigated with rats and mice been equally represented. In comparison to mice, rats are less susceptible to stress effects [43] and they provide reliable results that can be translated to humans. However, when we compare the results found for oxidative parameters in rats and mice there are no major differences in these parameters and how they are affected (Table **5**).

Another factor to consider is the strain of animal. The main rat strains previously used are Wistar and Sprague-Dawley rats however, Fisher 344 rats have also been used. For Sprague-Dawley rats oxidative stress parameters altered include NO, MDA, CAT, protein carbonyls, GSH, GSSG, GPx, DA and GSH and Wistar rats have shown similar results with altered glutamine, glutamate GABA, protein carbonyls, GSH, DA, MDA and SOD. For mice, the most popular strains that have been employed are C57BL/6 mice, Balb/c AnNCrICrIj mice and Swiss-Webster mice and these have all shown similar results for oxidative stress parameters after MA administration. Therefore it seems that regardless

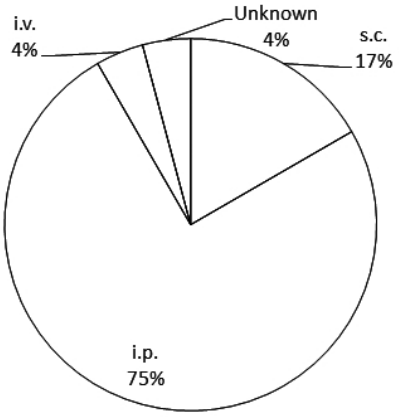

Fig. (6). MA routes of administration. Pie chart depicting the different routes of administration used for MA in MA oxidative stress preclinical studies.

Table 5. Parameters assessed and altered by MA in rats and mice.

Parameter	Rats	Mice
Monoamines	+	+
Protein carbonyls	+	+
Glutathione system	+	+
Scavenger enzymes (CAT/SOD)	+	+
Lipid peroxidation (MDA/4-HNE)	+	+
NO	+	-
Nitroproteins	+	-
p-tyrosine	+	-
2,3 DHBA	+	-
ROS	-	+
Nrf-2 phosphorylation	-	+
BBB integrity	-	+
TNFα	-	+
AP-1	-	+
COX-2	-	+

The range of oxidative stress parameters assessed and altered after MA administration in rat and mice preclinical studies.

of the species and strain of animal used, alterations in oxidative stress measurements are similar between each animal model.

2.2. Measuring Oxidative Stress

2.2.1. Time Point of Measurement

The time point after dosing in which the animal is sacrificed is a crucial part of the experimental design and vital to ensure that the optimal time is selected when most oxidative stress parameters can be assessed. There are many time points of sacrifice that have been used in oxidative stress studies after MA (Fig. **7**) and the most common time point of sacrifice is 24 h after dosing.

This time point has been used after various dosing regimens (acute, binge, multiple dosing days) but it is always 24 h after the last MA injection. When the effects are examined at this time point there have been a wide range of alterations to oxidative stress parameters (Table **6**).

Therefore all these parameters (GSH, Nrf-2, MDA, protein carbonyls, GPx activity and CAT activity) are still measurable 24 h after dosing and again this is regardless of the dosing regimen. Alterations in DA and DOPAC levels, TH activity, TH protein expression, 4-HNE expression, MDA levels, protein carbonyl expression and formation and ROS formation are all still detectable at a sacrifice time point of 3 days after the last MA injection [21, 36, 38]. The studies using this time point looked at acute or binge dosing and the animals only received MA for one day.

Some studies have delayed the point of sacrifice to 1 or 2 weeks after cessation of MA and neurotoxicity was still measurable at these times which suggests long-term damage that is not reversible. After one week Fukami, Hashimoto [33] found that MA given at 7.5 mg/kg, 4 times in one day (2 h intervals), decreased levels of DA in rat striatum and these were still apparent. Hirata, Ladenheim [16] treated transgenic mice with MA at 5 or 10 mg/kg, 4 times in one day (2 h intervals), and sacrificed the animals 2 weeks later. The 5 mg/kg had no effect but the 10 mg/kg decreased 5-HT uptake sites in the striatum. These studies did not look at any oxidative stress parameters per se and although neurotoxicity is still evident after 1 or 2 weeks following dosing it suggests that 24 h may be more suitable for examining oxidative stress parameters such as alterations in TBARS, protein carbonyl, GSH and enzyme levels.

2.2.2. Brain Regions

Among the preclinical studies that have examined the effects of MA on oxidative stress, many brain regions have been assessed (Table **7**). Among these brain regions the striatum has been shown to be the most investigated with 79% of these papers examining this region.

As discussed earlier, this brain region is of most interest as it is the primary target of DA-induced effects. When assessing the striatum many oxidative stress parameters have been altered (Table **8**).

Regardless of the parameter being assessed the striatum is certainly the region that has the most evidence to support the link between oxidative stress and MA use. Other brain regions may be of interest and worth more research to determine their possible association. For example, the dopaminergic tracts in the brain are not limited to the striatum and project to regions such as the hypothalamus, amygdala and the frontal cortex. The prefrontal cortex has shown promising results with a few studies examining oxidative stress in this region. da-Rosa, Valvassori [18] showed increased carbonyl formation and TBARS formation in the prefrontal cortex after chronic MA dosing and Bu, Lv [34] showed decreased 5-HT and DA levels, and evidence of oxidative stress and membrane disruption after binge dosing of MA at 2.5 mg/kg. Bu, Lv [34] also looked at the

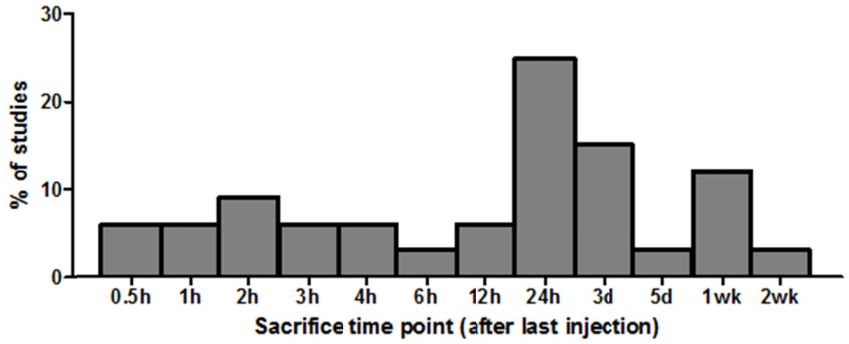

Fig. (7). Time points of sacrifices. The range of sacrificing time points used in MA oxidative stress preclinical studies.

Table 6. Effects of MA on oxidative stress parameters after 24 hours.

Effect	Parameter				
↑	Nrf-2 phosphorylation	GCS activity	MDA	Protein carbonyl	CAT activity
↓	GSH	GPx activity			

The range of oxidative stress parameters altered 24 hours after MA injections in preclinical studies. [25-28, 40].

hippocampus and found similar changes for oxidative stress and membrane disruption. As MA is known to also exert its neurotoxic effects at 5-HT terminals then the projections of the raphe nuclei may also be of interest such as the cortex, hippocampus and hypothalamus.

2.2.3. Parameter/Targets to Use

There are many ways of measuring oxidative stress (Table **9**) and to date the methods used have differed between studies but also in the findings reported. There are only a few human studies that have looked at oxidative stress after MA abuse, which have begun to appear recently in the literature. However among these studies only one study has looked at brain samples and the remaining studies have looked at blood samples. This is important to bear in mind as the use of blood samples may not represent the events occurring in the CNS.

Table 7. Brain regions of interest.

Brain Region	% of Papers
Striatum	79
Hippocampus	38
Cortex	17
Prefrontal cortex	13
Frontal cortex	13
Cerebellum	13
Amygdala	8
Hypothalamus	4
Thalamus	4
NAC	4
Choroid plexus	4
Meninges	4
Brain stem	4

The range of brain regions used in MA oxidative stress preclinical studies.

Table 8. Effects of MA on oxidative stress parameters in the striatum.

Effect	
↑	↓
TBARS	DA
Protein damage	DOPAC
Lipid damage	GCS activity
GSH	DAT density
Protein carbonyls	HVA
GR activity	5-HT uptake sites
Glrx activity	TH activity
MAC1 immunoreactivity	TH expression
COX-2	VMAT2 expression
DA turnover rate	5-HT
4-HNE expression	3-MT
ROS	5-HIAA
SOD activity	
GPx activity	
TNF-α mRNA levels	
GSSG	
Uric acid content	
2,3 DHBA	
p-tyrosine	

The range of oxidative stress parameters altered 24 hours after MA injections in preclinical studies. (18-24, 29-33, 36-40).

Table 9. MA oxidative stress targets.

Mitochondrial complex-I	CAT	GST	g-GTP
ROS	Protein carbonyls	GPx	G6PD
SOD	DA, DOPAC, HVA	DAT	GSH
Tight junction proteins	3-MT, 5-HT	MAC1	ABTS
BBB permeability	5-HIAA	TH	FRAP
ADMA	COX-2 protein	4-HNE	GCS
NOS	Uric acid	AP-1	GGT
Ascorbate	5-HT uptake sites MDA/TBARS Nitroproteins	NF-αB	GR
		TNF-α	Glrx
		Vitamin E	Hydroxyl radicals

The range of parameters and targets used in MA oxidative stress preclinical studies.

Measuring MDA or TBARS levels has shown to be the most common measure in human studies and this has only been measured in blood samples of MA abusers. Solhi, Malekirad [44] took blood samples from people that were regularly using MA (at least once a day for last six months) and found that MDA levels were increased in the blood. However in another study by Walker, Winhusen [45] with people that had used MA for 10.2 ± 7.0 years, they found that there was no difference in TBARS levels; however the authors suggest that this is due to the sample sizes and so this may explain the difference in results. Other parameters that have been used as markers of oxidative stress in humans include oxidative DNA damage using single cell gel electrophoresis in blood samples of MA users and Winhusen, Walker [46] found increased oxidative DNA damage in MA users even after a period of abstinence. In human post-mortem samples (although rare) parameters such as levels of GSSG, GPx, GR, GST, G6PD and CuZnSOD activity have been assessed and MA abusers that were using MA for at least 1 year showed that there MA was associated with increased CuZnSOD activity and GSSG levels in the caudate nucleus [47].

In preclinical studies the same parameters have also been assessed but there are also many others (Table **9**). For example protein carbonyl levels [38], ROS production [22], NOS activity [35], CAT levels [25], SOD activity [31] and COX-2 protein expression [37]. All of these targets have shown significant results which have highlighted a link between MA use and alterations in many oxidative stress parameters. Of all parameters and targets that have been studied preclinically, the most common and perhaps the most promising are those which have focused on the glutathione antioxidant system. This is most likely due to the glutathione antioxidant system being the largest part of the antioxidant defence system and therefore examining this system gives a clear reflection of the oxidative stress levels in the brain. The parameters measured here include total GSH levels, GR activities, GPx activities, G6PD and g-GTP levels [20]. This system has been assessed in over 40% of MA oxidative stress investigations and generally have shown decreased total GSH levels in the striatum [20], increased GPx activity in the striatum and frontal cortex [30], decreased GSH levels [26], decreased GR activity in the thalamus [40] and Glrx activity in the thalamus and striatum [40].

2.3. Antioxidant Treatments

2.3.1. Type of Antioxidant

The use of antioxidant treatments to combat or attenuate the effects of MA neurotoxicity dates back to the mid-1980s. Wagner, Carelli [14] used ascorbic acid as an antioxidant pre-treatment before MA dosing. MA was administered for 4 days at 25 mg/kg and caused a long-lasting depletion of DA and 5-HT however, ascorbic acid pre-treatment (100 mg/kg, 30 min before each MA treatment) attenuated this neurotoxic effect. A wider range of antioxidants were again tested by De Vito and Wagner [15] including ethanol, mannitol and vitamin E and each of these pre-treatments attenuated the depletions of DA and 5-HT in the striatum. Since this time, a number of antioxidants and compounds have been examined to test their effectiveness to attenuate MA neurotoxicity by targeting oxidative stress (Table **10**).

Fig. **8** and Table **11** highlight where in the oxidative stress pathway that these various antioxidants and compounds have had effect and what parameters they have attenuated after MA exposure. The main parameters that have been attenuated include GSH, GPx, MDA, CAT and DA levels. It is important to note that these include compounds which are not known antioxidants and therefore these compounds can also have non-antioxidant mechanisms of action. This section will look at how these treatments have attenuated MA-induced neurotoxicity by focusing solely on the oxidative parameters that have been altered.

N-acetylcysteine is a precursor of glutathione and it acts as an antioxidant. It has been shown to attenuate the MA-induced decrease of DA in the rat striatum [33] and DAT in the monkey striatum [48] when given as a pre-treatment. However this study did not look at any oxidative stress parameters and so it is unclear that at these low doses the antioxidant has restored these parameters to normal levels. N-acetylcysteine as a treatment has also shown to be problematic in that it can have side effects and the bioavailability is very low [26]. Therefore the use of N-acetylcysteine amide which is a modified form of N-acetylcysteine, has been reported as a

Table 10. Antioxidants and compounds previously used.

Antioxidant	Preventative/ Therapeutic	Dose (mg/kg)	Route	Timing	Refs
Caffeic acid	Preventative	100 or 200	i.p.	1 d before MA	[25]
Rottlerin	Preventative	1.5 or 3.0 μ g	Microinfused	Once a day for 5 days and 4-h and 0.5-h before MA	[39]
Lithium	Preventative	47.5	i.p.	Twice a day for 7 d or 14 d	[19]
Valproate	Preventative	200	i.p.	Twice a day for 7 d or 14 d	[19]
N-acetylcysteine amide	Preventative	250	i.p.	30 m before MA	[28]
Sulforaphane	Preventative and Therapeutic	10	i.p.	30 m before MA, 12 h after first SFN and 2 daily SFN for 2 days	[36]
Gastrodia elata Bl	Preventative	500 or 1000	p.o.	Twice a day for 6 d until 90 m before MA	[38]
N-acetylcysteine amide	Preventative	250	i.p.	30 m before MA	[26]
N-acetylcysteine	Preventative	1, 3, 10 or 30	i.p.	30 m before MA	[33]
Phenylbutylnitrone	Preventative and Therapeutic	150	i.p.	With first and third MA	[24]
N-acetylcysteine	Preventative and Therapeutic	150 + 12	i.v.	30 m before MA and continuous infusion over 8.5 h	[48]
Minocycline	Preventative	10, 20 or 40	i.p.	30 m before MA	[49]

The range of dosing procedures for antioxidants and compounds used in MA oxidative stress preclinical studies. n=9 papers.

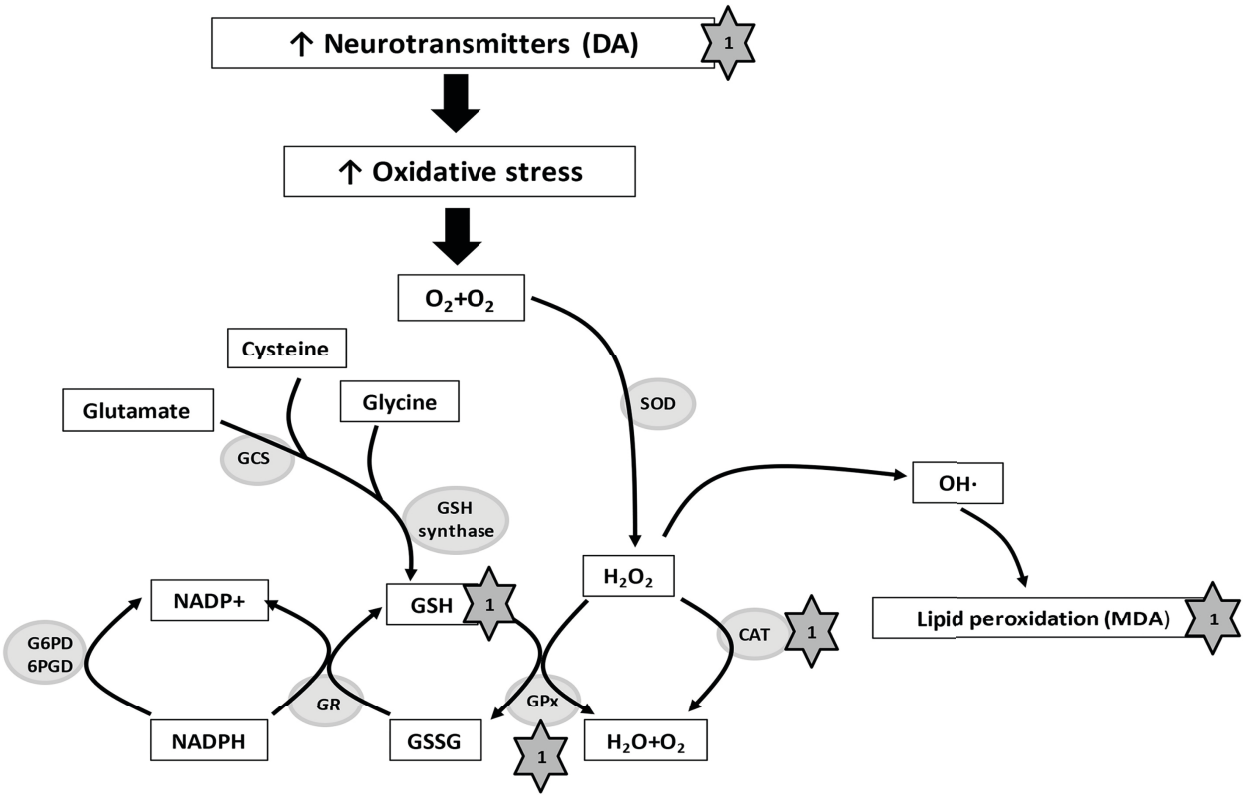

Fig. (8). Treatment targets in the oxidative stress pathway. The implications of treatments on oxidative stress. 1 indicates the parameters that treatments have attenuated. SOD; Super Oxide Dismutase, MDA; Malondialdehyde, CAT; Catalase, DA; Dopamine, GSH; Glutathione, GCS; Glutamylcysteine synthetase, GSSG; Oxidised glutathione, GR; Glutathione reductase, GPx; Glutathione peroxidase, G6PD; Glucose-6-phosphate dehydrogenase, 6PGD; 6-phosphogluconate dehydrogenase, NADPH; Nicotinamide Adenine Dinucleotide Phosphate-Oxidase, OH; Hydroxyl Radical, H_2O_2; Hydrogen Peroxide. (Adapted from Halpin, Collins [3], Guo and Chen [9], Isagenix International [10]).

Table 11. Antioxidants and compounds previously used and their targets in the oxidative stress pathway.

Antioxidant/Compound	Targets/Parameters Altered
Caffeic acid	GSH, GPx, MDA, CAT, PC
Rottlerin	MDA, PC
Lithium	TBARS, PC
Valproate	TBARS, PC
N-acetylcysteine amide	GSH, GPx, MDA, CAT, PC
Sulforaphane	DA, DAT
Gastrodia elata Bl	DA, ROS, MDA, PC
Minocycline	DA, DOPAC, DAT
N-acetylcysteine	DA, DAT
Phenylbutylnitrone	DA

The implications of treatments on oxidative stress parameters. MDA; Malondialdehyde, CAT; Catalase, DA; Dopamine, GSH; Glutathione, GPx; Glutathione peroxidase, DAT; Dopamine Transporter, ROS; Reactive Oxygen Species, TBARS, Thiobarbituric acid reactive substances, PC; Protein Carbonyls, DOPAC; 3,4-dihydroxyphenylacetic acid.

more effective treatment in neurotoxic cases as it can penetrate cell membranes and the BBB [50]. Banerjee, Zhang [26] and Zhang, Tobwala [28] used N-acetylcysteine amide to see if it has the ability to protect against oxidative stress. N-acetylcysteine amide as a pre-treatment at dose of 250 mg/kg was able to restore GSH, GPx, MDA, CAT and protein carbonyl levels and therefore protect against oxidative stress. Caffeic acid is a α-tocopherol protectant in low density lipoprotein and Koriem, Abdelhamid [25] evaluated the antioxidant ability of caffeic acid to see if it could attenuate MA-induced oxidative stress and DNA damage. MA was shown to decrease GSH and GPx levels and also increase MDA, CAT and protein carbonyl levels. Pre-treatment with caffeic acid at 100 or 200 mg/kg restores these oxidative stress parameters to normal levels in the hypothalamus. This again highlights the hypothalamus as a possible region of interest for future investigations. This restoration to normal levels was similar for both doses for all parameters and the restored levels were both comparable to the control animals so was therefore not dose-dependent and the same effect can be seen in the low dose of caffeic acid. Valproate is generally used as a treatment for bipolar disorder but when given at 200 mg/kg da-Rosa, Valvassori [19] showed that valproate prevented MA-induced protein damage and lipid damage (restored TBARS and protein carbonyl levels) in various brain regions. Phenylbutylnitrone is known to work as an oxygen radical spin trapping agent and when given at 150 mg/kg it restored DA levels in the striatum of MA exposed animals to that of control animals [24]. However this study did not look at any oxidative stress parameters and so it is unclear if it has restored antioxidant parameters to normal levels. Although many of these compounds have shown significant results in preventing or attenuating the effects of MA-induced oxidative stress there are very few studies to compare with, each one using a different antioxidant and with a different dosing regimen. In order for these antioxidants to be successful in attenuating or preventing MA-induced neurotoxicity then we must consider how best to deliver these. The next sections will look at how these are administered including dosing regimen, time of exposure and route of administration.

Table 12. Dosing routines.

Dosing Timings	% of Papers
With MA	9
30 m before MA	46
1-h before MA	9
4-h before MA	9
1 d before MA	18
Twice daily for 3 days before MA	9
Twice daily for 6 d before MA	9
Twice daily for 7 d before MA	18
Twice daily for 14 d before MA	18

The range of antioxidant injection routines used in MA oxidative stress preclinical studies.

2.3.2. Dosing Regime

The frequency of antioxidant dosing or the amount of antioxidant injections given on each day varies between each study. The most common frequencies are single daily doses [25, 26, 28, 33] or 1-2 doses for multiple days [19, 38, 39] (Table **10**). The number of days in which the animal receives the antioxidant can also vary among studies. One day of administration is generally the most common dosing routine and this is combined with a single injection in one day. When two injections are given in a day this is most commonly given for several days. Therefore the main strategies that have been previously employed are similar to that of the MA dosing routines and are focusing on either chronic/long term dosing or acute dosing.

Chronic dosing (multiple days of dosing) regimes that have been used ranges from 6 days of dosing to 14 days of dosing and Table **10** shows all the routines previously used. Giving the antioxidant just once has shown to be effective at restoring DA levels, GSH, GPx, MDA and protein carbonyl levels after different MA treatments such as long term MA dosing at 10 mg/kg [25], binge dosing of MA at 10 mg/kg [26, 28] and binge dosing of MA at 7.5 mg/kg [33]. Therefore even with an acute dose of the antioxidant this is still effective at protecting the brain against oxidative stress, albeit this is given as a pre-treatment before the MA administration.

2.3.3. Time of Exposure

Although the antioxidant treatments have been shown to attenuate oxidative stress when given as a pre-treatment we must consider if it can be given as an additional treatment during MA abuse i.e. if a human is abusing MA can the antioxidant treatment combat oxidative stress if administered

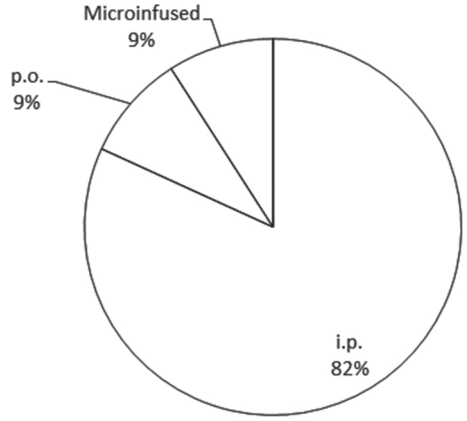

Fig. (9). Antioxidant routes of administration. Pie chart depicting the different routes of administration used for antioxidant treatments in MA oxidative stress preclinical studies.

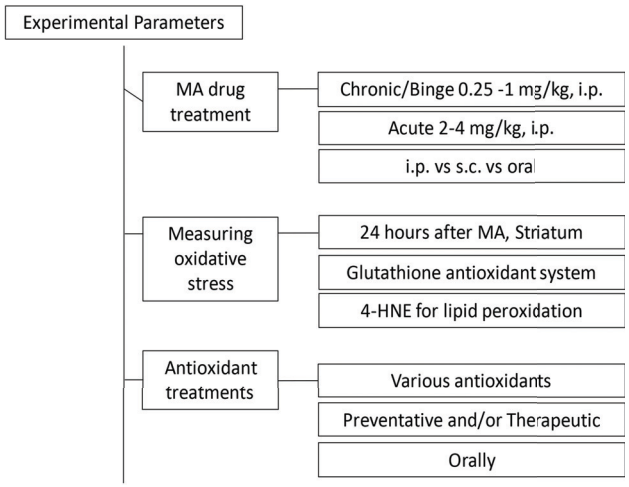

Fig. (10). Recommended experimental variables. Chart depicting the ideal experimental variables for a MA oxidative stress study.

alongside the drug. Some studies have considered this and have tried to examine the therapeutic effects of antioxidants as well as the preventative effects (Table **12**). Chen, Wu [36] used a treatment schedule with sulforaphane to examine the prophylactic and therapeutic effects of this antioxidant in mice. Sulforaphane was given at 10 mg/kg and then 30 min after MA was administered 3 times at 3 mg/kg (3 h intervals). Then sulforaphane was administered again 12 h after the first sulforaphane. Two daily injections (12 h intervals) of sulforaphane were then given for two consecutive days. Treatment with sulforaphane alone did not have any effect on DA and DOPAC levels however sulforaphane attenuated the reduction of striatal DA and DOPAC when given before and during the MA treatment. Although this result supports the idea of antioxidant treatment for therapeutic use it cannot be sure that the pre-treatment of sulforaphane is having the main effect here as we have seen previously that one acute pre-treatment can prevent oxidative stress. Ideally other groups should be included that have just pre-treatment with the antioxidant and also a group that has just dosing with the MA treatment. Also no oxidative parameters have been assessed. More studies are needed to determine the potential therapeutic effects of antioxidants and a comprehensive study of all antioxidant parameters with this treatment regime needs to be performed. A final time-related aspect to consider is after MA exposure and the potential for antioxidants to restore functioning after MA exposure has occurred. This has not been previously investigated.

2.3.4. Route of Administration

The route of administration for the antioxidant in MA oxidative stress studies is something that must be considered before beginning the experiment. Among these studies, the most common route of administration is intraperitoneal injection (i.p.) with 82% of the studies using this route [18, 24, 25, 33] (Fig. **9**).

This choice of administration route is similar to that of the MA administration and this choice is more than likely due to the ease of administration and rapidness of absorption using i.p. rather than how best the drug might be administered. Thinking of how best to administer the antioxidant 2 things need to be considered; is the antioxidant a preventative or therapeutic treatment (i.e. is it before or during the MA treatment) and also how will this be administered clinically. To choose a route of administration one needs to consider that in humans, treatments and drugs are most commonly taken by ingesting the drug in tablet or powder form. Therefore, if a treatment becomes available to attenuate the toxic effects of MA then one needs to ensure that it can be given orally and that it will have the same beneficial effects. The use of an oral route of administration is rare (Fig. **9**). To our knowledge, only one preclinical study has used p.o. (oral) to deliver the antioxidant. Shin, Bach [38] gave the methanol extract of gastrodia elata Bl (antioxidant) as a pre-treatment to MA at 500 or 1000 mg/kg twice a day for 5 days until 90 m before MA treatment. They found that gastrodia elata Bl inhibited MA-induced pathologic oxidative changes. Further investigation into the comparison of the different routes of administrations used would be very beneficial and would tell us if there are any differences and also as there are no s.c. studies, this route should also be included in a comparison study.

3. CONCLUSIONS

The previous sections have focused on the range of experimental parameters that exist in a MA oxidative stress study. From looking at the various study designs that exist it is clear that there is a large diversity among these studies and so the interpretation and comparison of these results is challenging. Our recommendations for the experimental parameters in a MA oxidative stress study are outlined in Fig. **9** and this acts as a guideline for conducting these studies. For these studies, the dose, route of administration, duration and frequency of exposure are all pivotal to the study design (Fig. **10**).

The recommended preclinical study investigating the effects of MA exposure on oxidative stress in a rodent would involve using a dose of 0.25 to 1 mg/kg for multiple days (binge or chronic dosing) or a dose of 2 to 3 mg/kg for one day (acute dosing). These are doses that have not only

yielded the greatest results for oxidative stress so far but they are considered clinically relevant doses and so it allows for comparison to what is happening in the human situation. MA should be administered *via* oral gavage to again mimic the clinical scenario but a comparison study of different routes of administration (i.p., s.c. and gavage) would be of great benefit to see how these may differ when it comes to measuring the oxidative stress parameters. Multiple exposure durations of MA should be investigated that look at each possible clinical pattern of exposure that may exist for example a long-term chronic user, a recreational user, an infrequent user and also a user that has become abstinent from the drug.

The recommended measurements of oxidative stress in a preclinical study investigating the effects of MA exposure would involve a sacrifice time of 24 hours after the last MA exposure. However, it is worth noting that we previously showed at 1 or 2 weeks after cessation of MA, neurotoxicity was still measurable at these times. As mentioned earlier oxidative stress parameters have not been measured in this field after 24 hours and this may be of interest to see are these changes reversible or is long-term damage observed. Therefore, a study investigating sacrifice times may reveal some interesting findings. The main brain region of interest would be the striatum but there is sufficient evidence to show that other brain regions may be of interest such as the hippocampus, hypothalamus, amygdala and frontal cortex. Although TBARS/MDA levels have shown the most consistent results to date both clinically and preclinically however these assays are known to be problematic in that they are unreliable, non-specific and are quite labour intensive. Positive results can be produced in this assay for lipid peroxidation with practically any antioxidant (or superfluous relic) [51]. For this reason, in general oxidative stress assays (regardless of the insult given) 4-HNE is more commonly used as it provides more reliable results yet this has been overlooked previously. Other parameters that have not been previously investigated include 8-Hydroxydeoxy guanosine which is a biomarker of ROS-induced DNA damage and 8-nitroguanine levels that are used to evaluate oxidative DNA damage and these are quite common in other oxidative stress studies. These targets and parameters as well as the glutathione antioxidant system necessitate more investigation in future studies.

The use of antioxidant treatments is still in its early days and therefore optimisation of administration of these could be beneficial. Regardless of the antioxidant selected there are many results. For these studies, the dose, route of administration, duration and frequency of exposure are again all pivotal to the study design (Fig. **10**).

The recommended preclinical study investigating the effects of MA exposure on oxidative stress in a rodent would involve administering a suitable antioxidant dose *via* oral gavage. The oral route of administration of the antioxidant is a very important factor as this allows a more realistic extrapolation to the human situation. Multiple exposure durations of the antioxidant should be investigated to see the potential of the compound as a preventative treatment (pre-treatment before MA) or as a therapeutic treatment (alongside MA or after MA) and which it may be better suited.

The use of these recommendations mean that results will be more comparable between papers and that future results generated will give us a true understanding of what might be happening in the clinical scenario.

LIST OF ABBREVIATIONS

3-MT	=	3-methoxytyramine
4-HNE	=	4-hydroxynonenal
5-HIAA	=	5-hydroxyindoleacetic acid
5-HT	=	Serotonin
6PGD	=	6-phosphogluconate dehydrogenase
ABTS	=	2,2'-azino-bis (3-ethylbenzthiazoline- 6-sulfonic acid)
ADMA	=	Asymmetric Dimethylated L-arginine
BBB	=	Blood Brain Barrier
CAT	=	Catalase
DA	=	Dopamine
DOPAC	=	3,4-dihydroxyphenylacetic acid
G6PD	=	Glucose-6-phosphate dehydrogenase
GCS	=	Glutamylcysteine synthetase
GGT	=	Gamma-glutamyltransferase
Glrx	=	Glutaredoxin
GPx	=	Glutathione peroxidase
GR	=	Glutathione reductase
GSH	=	Glutathione
GSSG	=	Oxidised glutathione
GST	=	Glutathione-stransferase
HVA	=	Homovanillic acid
MA	=	Methamphetamine
MDA	=	Malondialdehyde
NOS	=	Nitric Oxide Synthase
ROS	=	Reactive Oxygen Species
SOD	=	Super Oxide Dismutase
TBARS	=	Thiobarbituric acid reactive substances

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This research is supported by a PhD research scholarship from the College of Medicine, National University of Ireland, Galway, Ireland. The funding source was not involved in this work.

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