

Glutathione Trisulfide (GSSSG) in Neuroprotection

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Patent Application Serial No. 63/023,686, filed on May 12, 2020. The entire contents of the foregoing are hereby incorporated by reference.

TECHNICAL FIELD

Described herein is the use of glutathione trisulfide (GSSSG) in neuroprotection, e.g., in neurodegenerative diseases and to reduce the risk of ischemic injury. The methods can be used, e.g., to reduce risk of injury to brain, spinal cord, and peripheral nerves from ischemia or low blood flow states possibly caused by surgery, trauma, and other conditions that decrease/impair blood flow and or oxygen delivery to the nervous system.

BACKGROUND

Delayed paraplegia is a devastating complication of ischemic spinal cord injury (SCI), which can occur after thoracic and/or abdominal aortic surgery and trauma to the spinal cord. While the incidence of ischemic SCI is reported to be around 3%, more than 80% present with delayed onset of symptoms (Ullery et al., 2011). Although mechanisms of delayed paraplegia are incompletely understood, studies suggest critical roles of motor neuron apoptosis (Kakinohana et al., 2011) and recruitment of microglia and bone marrow-derived macrophages (BMDM) in ischemic stroke (Denes et al., 2008) and SCI (Bell et al., 2013; Donnelly et al., 2011; Kigerl et al., 2007).

SUMMARY

Provided herein are methods for the treatment, or reduction of risk, of a disorder associated with neurodegeneration in a subject. The methods include administering a therapeutically or prophylactically effective amount of a composition prepared using crystals of Glutathione Trisulfide (GSSSG) to a subject in need thereof. In some embodiments, the methods include comprising preparing the composition comprising GSSSG by dissolving a crystalline form of GSSSG in saline at pH 3-6. Also provided

are compositions comprising GSSSG for use in a method of treatment, or reduction of risk, of a disorder associated with neurodegeneration in a subject, e.g., compositions prepared by dissolving a crystalline form of GSSSG in saline at pH 3-6. .

In some embodiments, the disorder is post-ischemic neuronal death.

5 In some embodiments, the disorder is a chronic cerebral degenerative disease, e.g., multi-infarct dementia, Alzheimer's disease, Parkinson's disease, or Lewy body dementia.

In some embodiments, the methods include administering an effective amount of a composition comprising GSSSG within a few minutes to hours after a traumatic injury
10 occurs.

In some embodiments, the methods include administering an effective amount of a composition comprising GSSSG before a scheduled thoracic and/or abdominal aortic surgical procedure.

In some embodiments, the methods include administering an effective amount of
15 a composition comprising GSSSG hours to days before a scheduled thoracic and/or abdominal aortic surgical procedure.

In some embodiments, the methods include administering an effective amount of a composition comprising GSSSG 2-24 hours, and/or 1, 2, 3, 4, 5, 6, and/or 7 days before the scheduled thoracic and/or abdominal aortic surgical procedure.

20 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting.
25 All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

FIGs. 1A-B are graphs showing BMS (A) and survival rate (B) of mice subjected to SCI after preconditioning with GSSSG or DMSO alone.

FIG. 2 is a graph showing that polysulfide, but not Na₂S, protected SH-SY5Y cells from MPP⁺-induced cell death. N=4 each. *, **, ***, ****P<0.05, 0.01, 0.001, 0.0001 vs. vehicle; P<0.01 Control vs. MPP⁺ in each treatment.

DETAILED DESCRIPTION

Persulfide (R-S-SH) and polysulfide (R-S-Sn-S-R) are molecules that contain sulfane sulfur which is a sulfur atom with six valence electrons but with no charge, and possess protective effects against oxidative stress (Akaike et al., 2017; Ida et al., 2014). These molecules can release H₂S and, therefore, antioxidative or protective effects of these molecules seem to be mediated by both H₂S and sulfane sulfur. Glutathione trisulfide (GSSSG) is one of major polysulfide species in mammal tissues that consists of GSSG, a metabolite of glutathione, with an additional sulfane sulfur atom.

Until recently, methods for manufacturing GSSG compounds included the use of toxic gases or risked the production of toxic gases, producing a compound that was not stable or not suitable for pharmaceutical use. EP 3560947 describes a method of manufacturing GSSSG in a stable crystal form. However, the efficacy of this crystal form of GSSSG *in vivo* for neuroprotection has not been described.

The current study examined the beneficial effects of the crystal GSSSG in neuroprotection, including against neurofunctional impairment after SCI in mice. Specifically, the effects of GSSSG preconditioning prior to SCI onset were examined. Patients often undergo aortic surgery after a certain period (e.g., 1 week) of diagnosis depending on conditions, providing an opportunity to use a treatment as described herein to reduce their risk of post-surgical complications. In addition, the results confirmed a protective effect of GSSSG against 1-methyl-4-phenylpyridinium (MPP⁺)-induced neuronal (SH-SY5Y cell) death. MPP⁺-poisoning is an *in vitro* model of Parkinson's disease, demonstrating that the crystal GSSSG can be used in neurodegenerative disease as well.

The results herein demonstrated the beneficial capacity of GSSSG crystal in neuroprotection *in vivo*. The present results showed the effects of GSSSG preconditioning on neurofunctional preservation after SCI; the drug can also be administered after onset of ischemia due to its antioxidative effects.

5 **Methods of Treatment**

The methods described herein include methods for the treatment, or reduction of risk, of disorders associated with neurodegeneration in a subject, e.g., a mammalian subject, e.g., a human or non-human veterinary subject. In some embodiments, the disorder is post-ischemic neuronal death. In some embodiments, the disorder is a chronic
10 cerebral degenerative disease (e.g., multi-infarct dementia, Alzheimer's disease, Parkinson's disease, or Lewy body dementia). Generally, the methods include administering a therapeutically effective amount of a composition comprising a crystalline form of GSSSG as described herein, to a subject who is in need of, or who has been determined to be in need of, such treatment.

15 As used in this context, to "treat" means to ameliorate at least one symptom of the disorder associated with neurodegeneration. The conditions that can be treated using a method described herein can be associated with loss of motor control, paralysis or paraplegia. Administration of a therapeutically effective amount of a compound described herein can result in improved motor control, reduced paralysis or paraplegia.

20 In addition, the methods can result in a reduction in risk of developing loss of motor control, paralysis or paraplegia. Subjects who are at risk of developing loss of motor control, paralysis or paraplegia can include those who have suffered a traumatic injury as well as those who are about to undergo thoracic and/or abdominal aortic surgery. These methods can include administering an effective amount of a GSSSG
25 composition as described herein within a few minutes to hours after a traumatic injury occurs, and/or before, e.g., hours to days before, a scheduled thoracic and/or abdominal aortic surgical procedure.

An "effective amount" is an amount sufficient to effect beneficial or desired results. For example, a therapeutic amount is one that achieves the desired therapeutic
30 effect. This amount can be the same or different from a prophylactically effective

amount, which is an amount necessary to prevent onset of disease or disease symptoms. An effective amount can be administered in one or more administrations, applications or dosages. The compositions can be administered one from one or more times per day to one or more times per week; including once every other day. In some embodiments, the GSSSG is administered every day for at least 2, 3, 4, 5, 6, or 7 days prior to a scheduled thoracic and/or abdominal aortic surgical procedure. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the therapeutic compounds described herein can include a single treatment or a series of treatments.

Dosage, toxicity and therapeutic efficacy of the therapeutic compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds which exhibit high therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in

humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Pharmaceutical Compositions and Methods of Administration

The methods described herein include the use of pharmaceutical compositions comprising GSSSG as an active ingredient, wherein the compositions are prepared using a crystalline form of GSSSG as described in EP 3560947, by dissolving the crystalline GSSSG in a buffer, e.g., saline, at pH 3-6. A method for producing the crystal form of glutathione trisulfide dehydrate can comprise precipitating a crystal of glutathione trisulfide dihydrate in an aqueous solution in which glutathione trisulfide is dissolved, and collecting the precipitated crystal of glutathione trisulfide dihydrate.

Pharmaceutical compositions typically include a pharmaceutically acceptable carrier. As used herein the language “pharmaceutically acceptable carrier” includes saline, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Supplementary active compounds can also be incorporated into the compositions.

Pharmaceutical compositions are typically formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, administration.

Methods of formulating suitable pharmaceutical compositions are known in the art, see, e.g., *Remington: The Science and Practice of Pharmacy*, 21st ed., 2005; and the books in the series *Drugs and the Pharmaceutical Sciences: a Series of Textbooks and Monographs* (Dekker, NY). For example, solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium

hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use can include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration. For example, the GSSSG can be provided in a kit in a crystalline form with a sterile buffer (e.g., saline) at pH 3-6 for use in dissolving the crystals to prepare a solution for injection.

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EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1. Preventive effects of GSSSG against neurofunctional deficit after SCI

10 To elucidate the molecular mechanisms responsible for the delayed paraplegia, we recently developed and thoroughly characterized a murine model of SCI in which mice exhibit delayed paraplegia with minimum operative mortality (Kakinohana et al., 2011). Briefly, under anesthesia and mechanical ventilation via endotracheal intubation, SCI was induced by placing the first clip on the aortic arch between the left common
15 carotid artery and the left subclavian artery and the second clip on the origin of the left subclavian artery. The completeness of the occlusion was ascertained by an immediate and sustained loss of any detectable pulse pressure in the femoral artery pressure tracing. After 5 min of ischemia, the clips were removed, and the chest was closed in layers. At 10 minutes of reperfusion, the arterial catheter was removed, incisions were closed, and
20 animals were allowed to recover from anesthesia. Temperature of erector spinae muscle was monitored and maintained at 37.5°C during whole surgery until recovery from anesthesia. In sham-operated mice, whole surgical procedure was performed as described, but no clips were applied. Motor function was quantified serially at pre-SCI, 24, 48, and 72 h after spinal cord ischemia by the Basso Mouse Scale (BMS) (Basso et
25 al., 2006; Kakinohana et al., 2011). The maximum deficit is indicated by a score of 0. Although BMS score <6 (0 to 5) indicates paraplegia, BMS score >6 (6 to 9) indicates ability to walk.

To examine preventive effects of GSSSG against neurofunctional deficit after SCI, mice were subjected to preconditioning with GSSSG treatment before induction of
30 SCI. Briefly, GSSSG was ground using an agate mortar, dispersed in DMSO using a

sonication water bath and administrated at 200 mg/kg IP daily for 4 days. Control mice were given DMSO alone. Mice were subjected to SCI at 24h after the last administration of GSSSG or DMSO alone.

5 The results showed that all mice treated with DMSO alone exhibited paraplegia after SCI while preconditioning with GSSSG prevented motor functional deficit and paraplegia (Fig. 1A). Preconditioning with GSSSG did not change survival rate of mice after SCI (Fig. 1B).

Example 2. Protective effects of GSSSG in a model of neurodegeneration

10 The effects of GSSSG on 1-methyl-4-phenylpyridinium (MPP+)-induced neuronal (SH-SY5Y cell) death were evaluated. MPP+-poisoning is an *in vitro* model of Parkinson's disease.

SH-SY5Y cells were incubated with or without MPP+ (2 mM) in DMEM/F12 (20%FBS) with or without drugs at 37°C for 24h. Cell viability was measured using the crystal violet assay.

15 The results, shown in Fig. 2, demonstrated that polysulfide, but not Na₂S, protected SH-SY5Y cells from MPP+-induced cell death.

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OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.