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Protamine sulfate induces mitochondrial hyperpolarization and a subsequent increase in reactive oxygen species production

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List of abbreviations: ADP = Adenosine diphosphate; ATP =Adenosine triphosphate; CytOx = Cytochrome c oxidase: PS = Protamine sulfate; ROS = Reactive oxygen species

Abstract

Protamine sulfate (PS) is widely used in heart surgery as an antidote for heparin albeit its pharmacological effects are not fully understood and applications are often accompanied by unwanted side effects. Here, we show the effect of PS on mitochondrial bioenergetics profile resulting in mitochondrial reactive oxygen species (ROS) production.

Polarographic measurements were performed in parallel to membrane potential and ROS measurements by FACS analyzer using tetramethylrhodamine ethyl ester (TMRE) and MitoSOX^{TM} fluorescent dyes, respectively. PS inhibited intact rat heart mitochondrial respiration (stimulated by ADP) to 76% ($p < 0.001$) from the baseline of 51.6 ± 6.9 to 12.4 ± 2.3 nmol O_2 min^{-1} ml^{-1}. Same effect was found when respiration was priorly inhibited by antimycin A (101.0 ± 8.9 vs. 38.0 ± 9.9 nmol O_2 min^{-1} ml^{-1}, $p < 0.001$) and later stimulated by substrates of cytochrome oxidase (CytOx) i.e. ascorbate and tetramethyl phenylene diamine (TMPD); suggesting that PS exerted its effect through inhibition of CytOx activity. Furthermore, the inhibition of mitochondrial respiration by PS was concentration-dependent and accompanied by hyperpolarization of the mitochondrial membrane potential ($\Delta\psi_m$) i.e. 18 % increase at 50µg/ml and a further 3.3 % increase at 250µg/ml PS compared to control. This effect was associated with a strong consequent increase in the production of ROS i.e. 85 % and 88.6 % compared to control respectively. We propose that this excessive increase in ROS concentrations results in mitochondrial dysfunction and thus might relate to the “protamine reaction” contributing to the development of various cardiovascular adverse effects.

Introduction

During cardiovascular surgical procedures, a number of drugs are administered depending on the patients' condition and the surgical procedure. Among these, protamine sulfate (PS) is commonly used as an antidote for heparin. Being a polycationic peptide, it reverses the anticoagulant effects of the highly negatively charged heparin by forming a complex with heparin (Viaro et al., 2002).

Although studies on healthy volunteers who received PS intravenously showed rapid decrease in protamine blood concentration, no significant changes were found in heart rate, mean arterial blood pressure, or cardiac output (Butterworth et al., 2002). However, increasing evidence from clinical and experimental data indicated a direct influence of PS on the myocardial tissue. Negative cardiovascular side effects of PS have been described in human patients (Conahan et al., 1981; Shapira et al., 1982) as well as in animals (Goldman et al.,1969). The drug administration resulted in decrease of cardiac oxygen consumption (Sethna et al., 1982; Wakefield et al., 1990).

Further clinical studies have provided information on adverse conditions associated with PS, e.g. systemic hypotension, bradycardia, pulmonary artery hypertension or hypotension, thrombocytopenia, and leucopenia (Wakefield et al., 1992). It has been established that PS mediates dose- and time-dependent detrimental effects on cardiac function independent of the presence of heparin (Hendry et al., 1987; Wakefield et al., 1989; 1990).

However, the detailed molecular effects of PS on the cardiovascular system are not well understood. Fadali et al. found that hindering possible potential pathways leading to bradycardia and hypotension (e.g. ganglionic and adrenal medullary pathway blockade with hexamethonium chloride; postganglionic parasympathetic with atropine sulphate; alpha and beta adrenergic receptor by phenoxybenzamine and propranolol respectively; or depletion of endogenous histamine), were ineffective in abolishing the unwanted cardiovascular effects of PS. In the same approach, the group isolated the vascular tree from the heart during extracorporeal circulation. The findings of the study showed that hypotension and bradycardia were produced by a direct effect of protamine on the myocardium and peripheral

vascular system [Fadali et al., 1976). Earlier *in vitro* studies have also shown that PS application resulted in impaired mitochondrial respiration (Popinigis, 1974; Kossekova et al., 1975). Similar results were obtained with *in vivo* studies performed with dogs (Wakefield et al., 1989), as well as rabbit and rat hearts (Wakefield et al., 1990; 1992; Halpern et al., 1997). A rapid decline in ATP levels particularly in the presence of heparin occurred within minutes after application of PS to the cultured bovine pulmonary artery endothelial cells (Wakefield et al., 1989). Corresponding to these reports, a direct effect of PS on the contractile performance of isolated cardiomyocytes was also described previously (Hird et al., 1994).

The interaction of this drug to basic mitochondrial metabolic processes has an elementary relevance to the pathophsyiological understanding. A previous study exposed that the site of inhibition of mitochondrial respiration by PS might be localized between cytochromes c and aa_3 since sub-mitochondrial inside out particles were only slightly affected by protamine (Konstantinov, 1975). Therefore, we suppose an elementary influence of PS on the regulatory triangle between mitochondrial respiration, mitochondrial membrane potential and ROS production by its direct interaction with cytochrome c oxidase (CytOx) activity. Thus, this study addresses the effect of PS on the link between cytochrome c oxidase activity, mitochondrial membrane potential and ROS production representing a mitochondrial functional triangle that clarifies the known toxic effects of PS on the myocardium.

Material and Methods

All animal experiments had local approval (EX-8-2018) and were performed conforming to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Mitochondria were isolated by standard procedures of isolation as described previously (Ramzan et al., 2017). Briefly, whole heart tissue was excised rapidly from male Wistar rats (300-400 g) after decapitation and placed in ice-cold isolation medium (250 mM sucrose, 10 mM HEPES, 1 mM EDTA, and 0.2% fatty acid-free bovine serum albumin (BSA)

pH 7.4 at 4°C), washed and homogenized by a Teflon potter in 30 ml of ice-cold isolation medium. Homogenate was centrifuged at 2500 rpm for 10 min. Supernatant was carefully collected and centrifuged at 9000 rpm for 15 min. The mitochondrial pellet was resuspended in 5 ml of ice-cold isolation medium and centrifuged again at 9000 rpm for 10 min. Finally, the pellet was resuspended in 200 µl of isolation medium and stored on ice until further use.

Protein concentrations were estimated by the bicinchoninic acid assay (BCA assay) determining the total concentration of protein. The assay relies on two reactions. First, the peptide bonds in protein reduce Cu^{2+} ions from the copper (II) sulfate to Cu^{+}. The amount of Cu^{2+} reduced is proportional to the amount of protein present in the solution. Bicinchoninic acid chelate with each Cu^{+} ion forming purple-colored complexes that strongly absorb light at the wavelength of 562 nm. Bovine Serum Albumin was used as a standard. Stock concentrations of mitochondrial pellets were 29.4 mg/ml ± 6.0 (n = 5). Protamine sulfate from Herring source was purchased from Sigma-Aldrich.

Because tracking the changes in oxygen consumption is the easiest way to monitor drug alterations on mitochondrial function, we performed polarographic oxygen consumption measurements of mitochondrial samples using a Clark-type oxygen electrode (Hansatech Oxygraph System, Norfolk, UK) at 25°C and standard atmospheric pressure (101.32 kPa) in respiration buffer (130 mM KCl, 3 mM HEPES, 0.5 mM EDTA, 2 mM KH_2PO_4, 0.5% BSA, pH 7.4 at room temperature).

In the measuring chamber, 10 µl of stock mitochondrial sample corresponding to 29.4 mg/ml protein was added to 490 µl of constantly stirred respiration buffer, giving a final concentration of 0.589 mg/ ml ± 0.12 (n = 5 measurements). The oxygen consumption measurements were started and substrates (figure 1) were added as described in the legends to the figures 2A-2D. Rates of oxygen consumption were calculated. The measured respiratory control ratios (RCR) were found to be 4.38 ± 0.49 (n = 5).

Mitochondrial membrane potential and ROS production were measured by flow cytometry. Freshly isolated rat heart mitochondria were resuspended in respiration buffer at a concentration of 0.589 mg/ml ± 0.12. Substrates were added as mentioned above to a total

volume of 500µl respiratory buffer. Finally, samples were successively stained with the corresponding fluorescent dyes and measured immediately.

Mitochondrial reactive oxygen species were detected with 2.5µM MitoSOX^{TM} red indicator (Thermo Fisher Scientific, Darmstadt, Germany). Changes in mitochondrial membrane polarization were determined using 150nM tetramethylrhodamine ethyl ester (TMRE; Thermo Fisher Scientific, Darmstadt, Germany). Samples were measured with a total count of 50,000 events utilizing a Guava easyCyte 6-2L flow cytometer (Merck Millipore, Darmstadt, Germany) while data analysis and gating were performed with GuavaSoft 3.1.1 software. Data were plotted as dot plots using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA). Results were calculated as mean ± SD. Finally, statistical analysis including regression analysis, a standard one-way *ANOVA* and post hoc Kruskal-Wallis tests were performed by using IBM SPSS Statistics 21.

Results

Determining the quality of isolated mitochondrial preparations by Cytochrome c test

Immediately after isolation, the quality of fresh mitochondrial preparations was assessed by performing cytochrome c test as previously described (Ramzan et al., 2017). The study of this basic parameter confirmed the intactness and quality of mitochondrial preparations. The freshly isolated mitochondrial samples always showed minimal stimulation at 10µM cytochrome c indicating a high integrity of the outer mitochondrial membrane. The increase in oxygen consumption rate after 10µM cytochrome c addition in 4 different mitochondrial preparations was 9.0 ± 2.6 nmol O_2 min^{-1} ml^{-1}.

Determining the quality of isolated mitochondrial preparations by RCR measurements

Next, mitochondrial oxygen consumption was measured in order to assess the respiratory control ratio which is considered as a standard measure of coupling between mitochondrial respiration and oxidative phosphorylation. This is another widely established parameter for the measurement of mitochondrial intactness and is obtained by dividing the

oxidation rate calculated at state III (high steady state increase in oxygen consumption rate by ADP in the presence of respiratory substrates e.g. glutamate, malate) to the rate measured at state IV which occurs when all the added ADP is converted to ATP and respiration spontaneously and markedly decreases to a slower rate, which is also nearly constant. Our mitochondrial preparations retained a respiratory control ratio (RCR) of at least 4.38 ± 0.49 (see example of the original data in figure 2A, left side on top) indicating the desired intactness and quality. The Kruskal-Wallis test showed statistically significant differences in oxygen consumption between different groups, $X^2(4) = 21.313$, $p = 0.000$, with a mean rank score of 3.00 for RHM, 8.80 for Glu+Mal, 20.60 for ADP, 12.20 for StateIV and 20.40 for CCCP.

Inhibition of mitochondrial respiration by PS

In order to verify the effect of PS on mitochondrial respiration, 250µg/ml PS was added which strongly inhibited oxygen consumption rate at state III i.e. ADP-stimulated oxygen consumption (figure 2B) from 51.6 ± 6.9 nmol O_2 min^{-1} ml^{-1} to 12.4 ± 2.3 nmol O_2 min^{-1} ml^{-1} , corresponding to approximately 76% reduction ($p < 0.001$). Interestingly, the inhibition of state III respiration by PS was not released by the addition of 4µM CCCP (figure 2B). The Kruskal-Wallis test showed statistically significant differences in oxygen consumption between different groups, $X^2(4) = 19.176$, $p = 0.001$, with a mean rank score of 3.00 for RHM, 15.20 for Glu+Mal, 23 for ADP, 12.30 for PS and 11.50 for CCCP.

Dose- dependent inhibition of mitochondrial respiration by PS

Corresponding to clinically used concentrations of PS that induced adverse reactions on contractility in mammalian heart preparations or isolated myocardium (Hendry et al., 1987; Wakefield et al., 1990; 1992; Park et al., 1994), we performed titrations of PS at increasing concentrations beginning at 25µg/ml followed by 50, 100, 200, and up to the higher concentration of 250µg/ml (figure 2C) after inducing State III respiration.

The PS effect was found to be dose dependent. In fact, a direct correlation was found between the inhibition rate of mitochondrial oxygen consumption and the concentration of PS showing that mitochondrial respiration rate was reduced to half while doubling the concentration of the drug.

A simple linear regression analysis was performed to predict that an increase in PS decreases the consumption of oxygen. A significant regression equation was found, F (1,23) = 130.637, p < 0.000), with an R2 of .850. Furthermore, statistically significant differences were found by the Kruskal-Wallis test between different groups, $X^2(4) = 36.610$, p = 0.000, with a mean rank score of 5.90 for RHM, 24.70 for Glu+Mal, 38 for ADP, 33 for 25 µg/ml PS, 26.30 for 50 µg/ml PS, 17.60 for 100 µg/ml PS, 11.70 for 200 µg/ml PS and 6.80 for 200 µg/ml PS.

CytOx inhibition by PS

It has already been described that PS acts at a target structure between cytochrome c and cytochrome a and a_3 (Konstantinov, 1975) thereby inhibiting Cytochrome c oxidase (CytOx) activity. To assess this direct interaction of PS with CytOx alone and not to other members of the ETC, we measured the effect of the drug on mitochondrial respiration in the presence of antimycin A which binds to complex III, thus blocks the electron transfer between Complex III and IV (see figure 1). In order to specify PS action, we stimulated this antimycin A- inhibited respiration of mitochondria by adding the substrates for CytOx, i.e. ascorbate and TMPD which directly transfers electrons to cytochrome c (figure 2D). Electron transport to CytOx was stopped and mitochondrial oxygen consumption rate was clearly decreased when 250µg/ml PS was added (respiration after AA: 6.9 ± 1.0 nmol O_2 min^{-1} ml^{-1}, ascorbate and TMPD: 101.02 ± 8.9 nmol O_2 min^{-1} ml^{-1}, after PS: 38.0 ± 9.9 nmol O_2 min^{-1} ml^{-1}, respectively). Statistically significant differences were found by the Kruskal-Wallis test between different groups, $X^2(4) = 32.130$, p = 0.000, with a mean rank score of 3.00 for RHM, 13 for Glu+Mal, 27 for ADP, 8 for AA, 33 for Asc+TMPD, 21.60 for PS, and 20.40 for CCCP.

Mitochondrial hyperpolarization and stimulation of ROS production by PS

For establishing the link of PS-induced inhibition of mitochondrial respiration to another important mitochondrial basic process i.e. membrane potential and consequent ROS generation, we used two different concentrations of the drug i.e. 50µg/ml (minimum) and 250µg/ml (maximum) and correlated these with the control data (figure 3). Clearly, membrane potential ($\Delta\psi_m$) increased from 71.31 ± 2.14 % (Control) to 86.97 ± 1.78 % and 90.58 ± 1.89 % of relative TMRE fluorescence (figures 3A,B). Correspondingly, ROS production (measured as relative MitoSOX™ fluorescence) was also increased from 5.98 ± 1.80 % (control) to 39.91 ± 15.6 % in the presence of 50µg/ml PS and 52.41 ± 14.5 % in case of 250µg/ml PS (figures 3C,D). It is evident that PS- addition resulted in marked increase in membrane potential as well as ROS production ($p < 0.001$ and $p < 0.01$ compared to controls). In addition, we measured membrane potential and ROS in correlation to state II (induced by the addition of substrates for complex I: glutamate + malate) and state III (generated by the addition of ADP in the presence of complex I substrates) conditions of mitochondria (figure 4). Initially, addition of ADP in the presence of substrates initiated oxidative phosphorylation and resulted in an increased mitochondrial respiration as described earlier (figure 2C); followed by partial depolarization of the membrane potential (figures 4A,B) and low ROS (figures 4C,D). When PS added at state III of the mitochondria, again an increase in membrane potential (glutamate+malate = 69.66 ± 6.54 % and glutamate+malate+ADP = 64.21 ± 4.72 % vs. glutamate+malate+ADP+50µg/ml PS = 83.03 ± 6.72 % and glutamate+malate+ADP+250µg/ml PS = 86.15 ± 4.31 % relative TMRE fluorescence) as well as an increase in ROS production was measured (glutamate+malate = 3.93 ± 2.71 % and glutamate+malate+ADP = 3.83 ± 1.93 % vs. glutamate+malate+ADP+50µg/ml PS = 19.73 ± 5.33 % and glutamate+malate+ADP+250µg/ml PS = 29.64 ± 6.08 % relative MitoSOX™ fluorescence). This increased production of ROS after PS exposure appeared to be dose dependent ($p < 0.001$).

A simple linear regression analysis was performed to predict that addition of PS increases the mitochondrial membrane potential as well as ROS production. Significant regression equations were found in both cases: $F(1,13) = 72.378$, $p < 0.000$), with an R^2 of .848 for mitochondrial hyperpolarization and $F(1,13) = 31.746$, $p < 0.000$), with an R^2 of .709 for ROS production.

Additionally simple linear regression analysis was performed to predict that an increase in PS increases the mitochondrial membrane potential as well as ROS production. Significant regression equations were found in these cases as well: $F(1,13) = 28.435$, $p < 0.000$), with an R^2 of .686 for membrane potential and $F(1,13) = 70.586$, $p < 0.000$), with an R^2 of .844 for ROS production. The Kruskal-Wallis test showed statistically significant differences among different groups at $p < 0.000$ level.

Mitochondrial hyperpolarization and stimulation of ROS production by PS after CytOx inhibition

In order to evaluate the direct effect of PS on CytOx under substrate stimulation, mitochondrial respiratory chain was blocked at complex III (bc1-complex) by 2.5µM antimycin A which distinctly decreased the membrane potential (glutamate+malate+ADP = 64.21 ± 4.73 % vs. glutamate+malate+ADP+AA = 39.62 ± 9.89 % relative TMRE fluorescence). Under these conditions of inhibited respiratory chain, CytOx (Complex IV) was stimulated by the addition of its substrates i.e. ascorbate and TMPD. Corresponding to previous results, when PS was added to the mitochondria in the presence of glutamate+malate+ADP+AA+ascorbate+TMPD, a significantly increase in the membrane potential (from 49.96 ± 11.22 % to 77.32 ± 3.47 % of relative TMRE fluorescence) as well as in the ROS production (from 6.19 ± 4.22 to 23.60 ± 8.79 % relative MitoSOX™ fluorescence) was found (figures 5A-D). Statistically significant differences were found among different groups at $p < 0.000$ level by the Kruskal-Wallis test.

Discussion

Protamine sulfate is a small polycationic protein, originally isolated from the sperm of salmon (Bolan and Klein, 2013). Usually the recommended dose is 1 mg of protamine to antagonize 100 Units of heparin. Beside some difficulties in calculations, protamine dosage is usually based on body weight (3 to 4 mg/kg) or in a ratio to the heparin dosage (Lindblad, 1989; DiNardo et al., 2011) and depends on the time elapsed since the heparin infusion was discontinued. We adjusted our PS concentrations according to the previous studies (Hendry, 1987; Wakefield et al., 1990; 1992; Park et al., 1994), so finally our experimental conditions were relevant to the clinical scenario. Although it is routinely used, the optimal rate of drug administration is also a matter of consideration (Kumar et al., 2013].

Concerning the potential cytotoxic effects of PS, the heart and certain blood cells (white blood cells and platelets) appear to be a relevant target for this widely used pharmacological compound. Notably, the heart appears to be the primary target for protamine since it contains a high number of mitochondria. Heart energy supply relies mainly on fat metabolism (Lemieux and Hoppel, 2009) and mitochondrial dysfunction is one of the major causes for heart failure (Rosca and Hoppel, 2013). Excessive production of reactive oxygen species (ROS) plays an important role in cellular injury (Knowlton et al., 2014) e.g. in cardiac arrhythmias (Jeong et al., 2012). Mitochondria are the main source of ROS generation in the cell (Suski et al., 2012) which participate in the process of ischemia and reperfusion, hence contributes to the development of myocardial infarction and stroke (Kalogeris et al., 2014). Amongst various forms of ROS, the superoxide anion is considered to be the most frequent of mitochondrial ROS (Murphy, 2009).

The central parameter that controls the generation of reactive oxygen species in mitochondria remains to be the membrane potential (Turrens, 2003; Nicholls, 2004). An exponential increase in the production of ROS has been measured in mitochondria with a membrane potential of >140 mV (Korshunov et al., 1997). An increase in membrane potential could be a result of ATP synthase inhibition or closure of the mitochondrial permeability transition pore (Wojtczak et al., 1999). It has been reported that mitochondria show

significant ROS production when they are not synthesizing ATP and consequently have a high membrane potential and an increased pool of reduced CoQ (coenzyme Q) (Murphy, 2009).

Data from previous studies indicate that protamine affects mitochondria at the membrane level (Popinigis et al., 1971). Although a biphasic effect of protamine on intact mitochondria has been reported previously stating stimulation of respiration at lower concentrations while inhibition of respiration at higher concentrations, but this biphasic effect was found to be mainly dependent on the media used to study mitochondrial respiration (Popinigis, 1974; Kossekova et al., 1975). Since we used nearly physiological KCl buffer which is routinely used for oxidative phosphorylation studies, so in our measurements we have found a dose dependent inhibitory effect of the drug on mitochondrial respiration (figure 2C) conforming to previous studies (Person and Fine, 1961) performed with fresh rat heart homogenate. Here, we found that the respiration was inhibited even at low concentrations of the drug and the extent of this inhibition was increased at increasing concentrations of PS. This could indeed explain the known side effects of the drug on energy metabolism and cardiovascular functions (Horrow, 1985). Figure 2D shows that protamine sulfate inhibits mitochondrial respiration by inhibiting the activity of cytochrome c oxidase as reported previously (Person and Fine, 1961).

Additionally, structural changes in mitochondria may be associated with the effects of PS since contraction of intermembrane space together with swollen cristae and extreme vacuolization were observed after incubation of mitochondria with the drug (Popinnigis et al., 1971; Wakefield et al., 1990). These structural changes might result in desorption of cytochrome c from the cristae of the inner mitochondria membrane in the presence of protamine and thus blocking oxidative phosphorylation (Kossekova et al., 1975). Moreover, the measured membrane hyperpolarization in the presence of PS independent of different experimental conditions (figures 3B, 4B, 5B), and concomitant inhibition of mitochondrial respiration was not released by CCCP (figures 2B, 2D) which may be due to the fact that protamine blocks proton leakage and inhibits other exchange diffusion barriers, in turn

building up a high pH gradient between intramembrane space and matrix with an apparent pronounced increase in pH within the mitochondrial matrix (Popinigis et al., 1970). Consequently, all the complexes of the ETC except CytOx are reduced. Furthermore, cytochrome c, which is not involved in electron transport because of PS action could serve as an amplifier of ROS production (Akopova et al., 2012).

Deterioration of metabolism results in the reduction of blood supply (Nicholls, 2004) which cause slower heart beat (bradycardia) and low blood pressure (systemic or pulmonary artery hypotension). Contrarily an increased blood pressure within the arteries of the lungs results in pulmonary artery hypertension where mitochondrial dysfunction plays an essential role in the pathogenesis of the disease (Freund-Michel V et al., 2014). Overall, all these factors are the major hallmarks of PS unwanted side effects on the cardiovascular system (figure 6). The combination of reduced ATP synthesis together with excessive formation of ROS eventually results in heart failure (Sabbah, 2016). Correspondingly, protamine-induced thrombocytopenia (decreased number of blood platelets) and leucopenia (decreased number of white blood cells) have already been reported (Al-Mondhiry et al., 1985). In case of blood platelets, it has been described that an increase in ROS production leads to thrombus formation (Zharikov and Shiva, 2013) which in turn may lead to thrombocytopenia.

Finally, the inhibitory effect of PS on mitochondrial respiration not only affects the mitochondrial membrane potential but also the redox state of mitochondria to such an extent that both are strongly increased while the mitochondrial respiration remains inhibited between the cytochrome c and cytochrome a binding site. Extensive increase in ROS levels results in oxidative stress. Correspondingly, monitoring the redox status of patients before and after cardiovascular surgical procedures by determining redox parameters i.e. total antioxidant status(TAOS), total glutathione concentrations, glutathione peroxidase activity, and both nitrite and nitrate concentrations in the peripheral blood of patients, can be of great impact (Schuh et al., 2018) for optimal treatment in the postoperative time period. In future clinical studies, when PS is used, combination of mitochondria- specific antioxidants or ROS scavengers, to reverse the side effect of PS, should be evaluated. Nevertheless, in clinical

routine, measures should be taken to anticipate and attenuate the potential risks of PS-induced oxidative stress before its use, in relation to mitochondrial bioenergetics profile.

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Authorship Contributions

Participated in conception and research design: Ramzan and Vogt

Conducted experiments: Ramzan, Michels, Rhiel, Weber

Contributed to the analysis and interpretation of data: Ramzan, Rastan and Vogt

Contributed to the writing of manuscript: Ramzan, Michels, Irqsusi, Rastan, Culmsee, Vogt

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Legends for Figures

Figure 1

Simplified and modified scheme of the mitochondrial respiratory chain or electron transport chain with some inhibitors. ETC means a series of complexes (I to IV) that transfer electrons from electron donors to electron acceptors via redox reactions. Electron transfer is coupled with the transfer of protons (H^+) across the mitochondrial inner membrane by complex I, III and IV, only. This creates an electrochemical proton gradient that drives the synthesis of ATP through the ATP synthase (complex V). The ETC also includes peptides and enzymes, which are proteins or protein complexes essential for electron transport i.e. Cyt b, c and c1; coenzyme Q or ubiquinone, Fe–S proteins; FMN -flavin mononucleotide. Heme a is a coordination complex consisting of a macrocyclic ligand a porphyrin, chelating an iron atom. It is a part of the CytOx- holoenzyme complex. Heme a differs from heme a3 in a methyl side chain at ring position 8. It is oxidized to a formyl group and a hydroxyethylfarnesyl group. An isoprenoid chain has been attached to the vinyl side chain at ring position 2 of the iron tetrapyrrole heme. The complex IV contains two hemes, cytochrome a and cytochrome a3, and two copper centers, the CuA and CuB centers. The final acceptor of electrons in the ETC during aerobic respiration is molecular oxygen (dioxygen). Four electrons are transferred from the binding site at the CytOx to oxygen for water production. Antimycin A (in a frame) is a potent inhibitor of complex III, so that cytochrome c cannot shuttle electrons to the enzyme. External reductants like ascorbate and TMPD transfer electrons directly to complex IV and thus are used to test activity under “isolated” conditions. Protamine Sulfate binds as a polycationic compound directly to the cytochrome c binding site and blocks electron transfer.

In polarographic measurements glutamate and malate are added to test mitochondrial respiratory function. The glutamate-aspartate antiporter imports glutamate from the cytosol into the matrix and exports aspartate from the matrix to the cytosol. Aspartate is converted by cytosolic aspartate aminotransferase to oxaloacetate. In the cytosol, malate dehydrogenase

catalyzes the reaction of oxaloacetate and NADH to produce malate and NAD+. Herein, two electrons generated from NADH, and an accompanying H^+, are attached to oxaloacetate to form malate. The malate-aspartate shuttle is a biochemical system for translocating electrons produced during glycolysis across the semi-permeable inner membrane of the mitochondrion for oxidative phosphorylation in eukaryotes. These electrons enter the electron transport chain of the mitochondria via reduction equivalents to generate ATP. The shuttle system is required because the mitochondrial inner membrane is impermeable to NADH, the primary reducing equivalent of the electron transport chain. Therefore, malate transports reducing equivalents across the membrane. Finally, the malate-aspartate shuttle is sustaining the redox reactions, because NADH in the cytosol is oxidized to NAD^+, and NAD^+ in the matrix is reduced to NADH. The NAD^+ in the cytosol can be reduced again by further glycolysis, and the NADH in the matrix can be used to pass electrons to the electron transport chain for ATP synthesis. The mitochondrial membrane potential ($\Delta\psi_m$) generated by proton pumps (Complexes I, III and IV of the ETC) is an essential component in the process of energy storage during oxidative phosphorylation in the mitochondria. The sum of the membrane potential and the pH gradient are together known as the proton motive force (PMF). This indicates the total potential energy stored in the transmembrane gradients, which is available to drive protons back into the matrix space, and provide the power for biologically useful processes. PMF finally drives the ATP synthase to make ATP. Note, all ETC complexes (except complex II) participate in proton pumping to form $\Delta\psi_m$.

ETC = Electron transport chain; ATP = Adenosine triphosphate; (Cyt b = Cytochrome b; Cyt c = Cytochrome c; Cyt c1 = Cytochrome c1; Q = Coenzyme Q or ubiquinone; Fe–S = Iron–sulfur proteins; FMN = Flavin mononucleotide; NAD = Nicotinamide adenine dinucleotide; NADH = Nicotinamide adenine dinucleotide (reduced); FAD = flavin adenine dinucleotide; $FADH_2$ = flavin adenine dinucleotide (reduced); CytOx = Cytochrome c oxidase; TMPD = Tetramethyl phenylene diamine; H_2S = Hydrogen sulphide; CO = Carbon monoxide; CN^- = Cyanide; Cu_A = Copper A; Cu_B = Copper A; TCA cycle = Tricarboxylic acid cycle

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Figure 2A-D

Measurements of mitochondrial oxygen consumption by polarography. Original protocols of oxygen measurements are shown on the left side while combined data of the same time course with statistical analysis (gray arrow at x- axis) are on the right. Freshly isolated rat heart mitochondria (0.589 mg/ml ± 0.12) were added to the total volume of 500µl respiratory buffer in the oxygraph chamber and measurements were started after closing the chamber. Substrates were added during each measurement as indicated.

A) Oxygen consumption measurements for calculating respiratory control ratio (n = 5). Mitochondrial preparation with a high respiratory control ratio (RCR) of at least 4.38 ± 0.49, thus indicating the higher intactness and good quality of mitochondria after isolation.

B) Inhibition of ADP stimulated respiration (state III) by protamine sulfate (n = 5).

Addition of 250µg/ml PS to the isolated mitochondria at state III i.e. ADP-stimulated oxygen consumption state strongly inhibit the rate from 51.6 ± 6.9 nmol O_2 min^{-1} ml^{-1} to 12.4 ± 2.3 nmol O_2 min^{-1} ml^{-1}, corresponding to approximately 76% reduction in oxygen consumption ($p < 0.001$ calculated through standard one-way *ANOVA* analysis). Furthermore, the PS inhibition of state III respiration was not released by the addition of 4µM CCCP.

C) Dose dependent effect of PS on ADP stimulated (state III) respiration (n = 5).

The PS effect was found to be dose dependent. A direct correlation was found between the inhibition rate of mitochondrial oxygen consumption and the concentration of PS showing that mitochondrial respiration rate was reduced to half when doubling the concentration of the drug.

D) Effect of protamine sulfate on oxygen consumption activity of CytOx in isolated intact rat heart mitochondria (n = 5).

The specific action of PS is related to the inhibition of Cytochrome c oxidase (CytOx) activity. The antimycin A-inhibited mitochondrial respiration was stimulated by adding the substrates for CytOx, i.e. ascorbate and TMPD. Here, the electron transport to CytOx was stopped by adding 250µg/ml PS which clearly decreased the mitochondrial oxygen consumption rate in the presence of ascorbate and TMPD: 101.02 ± 8.9 nmol O_2 min^{-1} ml^{-1} to 38.0 ± 9.9 nmol O_2 min^{-1} ml^{-1} after PS addition.

RHM = Rat heart mitochondria; Glu = Glutamate; Mal = Malate; ADP = Adenosine diphosphate; CCCP = Carbonyl cyanide m-chlorophenyl hydrazone; PS = Protamine sulfate; AA = Antimycin A; Asc = Ascorbate; TMPD = Tetramethyl phenylene diamine; State II = Substrate induced mitochondrial respiration; State III = ADP stimulated mitochondrial respiration; State IV = ADP completely phosphorlyted to form ATP

(***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ calculated through standard one-way *ANOVA* analysis)

Figure 3A-D

Measurements of mitochondrial membrane potential (A,B) and ROS production (C,D) by flow cytometry at two different concentrations of PS in comparison to the control.: A and C show the respective histograms with gating of one representative measurement. B and D display the percentage of mitochondria within the gate of n=5 independent experiments. Freshly isolated rat heart mitochondria were resuspended in 500µl of respiration buffer at a concentration of 0.589 mg/ml ± 0.12. Substrates were added as indicated. Finally, fluorescence was measured immediately after adding 150 nM TMRE to the samples. In case of mitochondrial reactive oxygen species (ROS) measurements, 2.5 µM MitoSOX™ was added to the samples. Measurements were performed for n= 5 experiments. The data are shown as mean ± SD.

Distinctly, addition of PS even at 50µg/ml concentration has increased mitochondrial membrane potential ($\Delta\psi_m$) from 71.65 ± 1.33 % (Control) to 86.72 ± 2.31 % which stimulated further to 91.10 ± 2.1 % of relative TMRE fluorescence at 250µg/ml PS. Correspondingly, ROS production was also increased from 6.55 ± 0.34 % (Control) to 48.31 ± 14.7 % and

62.17 ± 8.19 % relative MitoSOX™ fluorescence at the same 2 PS concentrations, respectively.

Ctr = Control; PS = Protamine sulfate. (***p < 0.001, **p < 0.01, *p < 0.05 calculated through standard one-way *ANOVA* analysis)

Figure 4A-D

Measurements of mitochondrial membrane potential (A,B) and ROS production (C,D) by flow cytometry: A and C show the respective histograms with gating of one representative measurement. B and D display the percentage of mitochondria within the gate of n=5 independent experiments. Mitochondrial membrane potential and ROS measurements at state II, state III and at two different concentrations of PS, added at state III.

Addition of ADP in the presence of glutamate and malate initiated oxidative phosphorylation and resulted in an increased mitochondrial respiration followed by partial depolarization of the membrane potential and consequently low ROS. Addition of PS at mitochondrial state III again resulted in an increased membrane potential and increase in ROS ($\Delta\psi_m$: 65.6 ± 4.0 % and 62.0 ± 3.8 % vs. 82.5 ± 9.1 % and 86.5 ± 6.0 % of relative TMRE fluorescence; ROS: 2.3 ± 2.2 % and 3.2 ± 2.4 % vs. 16.7 ± 4.4 % and 26.5 ± 6.0 % relative MitoSOX™ fluorescence).

Glu = Glutamate; Mal = Malate; ADP = Adenosine diphosphate; PS = Protamine sulfate

(***p < 0.001, **p < 0.01, *p < 0.05 calculated through standard one-way *ANOVA* analysis)

Figure 5A-D

Measurements of mitochondrial membrane potential (A,B) and ROS production (C,D) by flow cytometry: A and C show the respective histograms with gating of one representative measurement. B and D display the percentage of mitochondria within the gate of n=5 independent experiments. Comparison of mitochondrial membrane potential and ROS

measurements after inhibiting the respiratory chain by antimycin A, followed by the addition of substrates (ascorbate +TMPD) for CytOx in the presence and absence of 250µg/ml PS. Once again, the addition of PS under these conditions as well, significantly increased the membrane potential and ROS production, both.

Glu = Glutamate; Mal = Malate; ADP = Adenosine diphosphate ; AA = Antimycin A; Asc = Ascorbate; TMPD (tetramethyl phenylene diamine); PS = Protamine sulfate; ROS = Reactive oxygen species;

(***p < 0.001, calculated through standard one-way *ANOVA* analysis)

Figure 6

Proposed mechanism of protamine sulfate- induced cardiovascular adverse reactions.

After administration into the blood circulation, PS forms a complex with heparin depending on the dosage ratio. Since the mechanism of elimination or metabolic pathway of PS is not known yet, it is suggested that the rest of the drug which is not bound to heparin easily enters into mitochondria because of its chemical properties, i.e. being highly basic small molecular size protein (approximately 4500 Da). Once accumulating inside the mitochondrial intermembrane space, PS binds between the binding site of cytochrome c and cytochrome a_3 at the outer surface of the inner mitochondrial membrane thereby inhibiting mitochondrial respiration. At the same time ETC remains highly reduced. Consequently, an increased membrane potential is maintained through reduced complexes I and III, while simultaneously electrons may leak from the ETC and react with dioxygen contributing to the generation of ROS [30]. Excessive ROS production at high membrane potential (Turrens, 2003) is followed by slowed or inhibited oxidative phosphorylation in mitochondria. These excessive ROS act as signaling molecules to start various pathways in the heart as well as in white blood cells and platelets (the only blood cells containing/harboring mitochondria). Concomitantly, although the membrane potential is maintained high but cannot be used by ATP synthase to produce ATP because of its limited capacity since the respiration is inhibited. Finally, this results in proton and electron leakage, membrane disruption and energy deficiency.

In conclusion, inhibition of mitochondrial respiration by protamine sulfate at the site of cytochrome oxidase results in the inhibition of oxidative phosphorylation on one side and an increase in the reduced components of prior electron transport chain by electron transmission on the other side, followed by an increase in membrane potential and consequent increase in ROS production. The decrease in ATP levels and chronic increase in ROS production contribute to the development of drug related adverse conditions which ultimately may lead to the reduction of myocardial contractility.

ETC = Electron transport chain; ROS = Reactive oxygen species; OxPhos = Oxidative phosphorylation; ATP = Adenosine triphosphate

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Figure 1

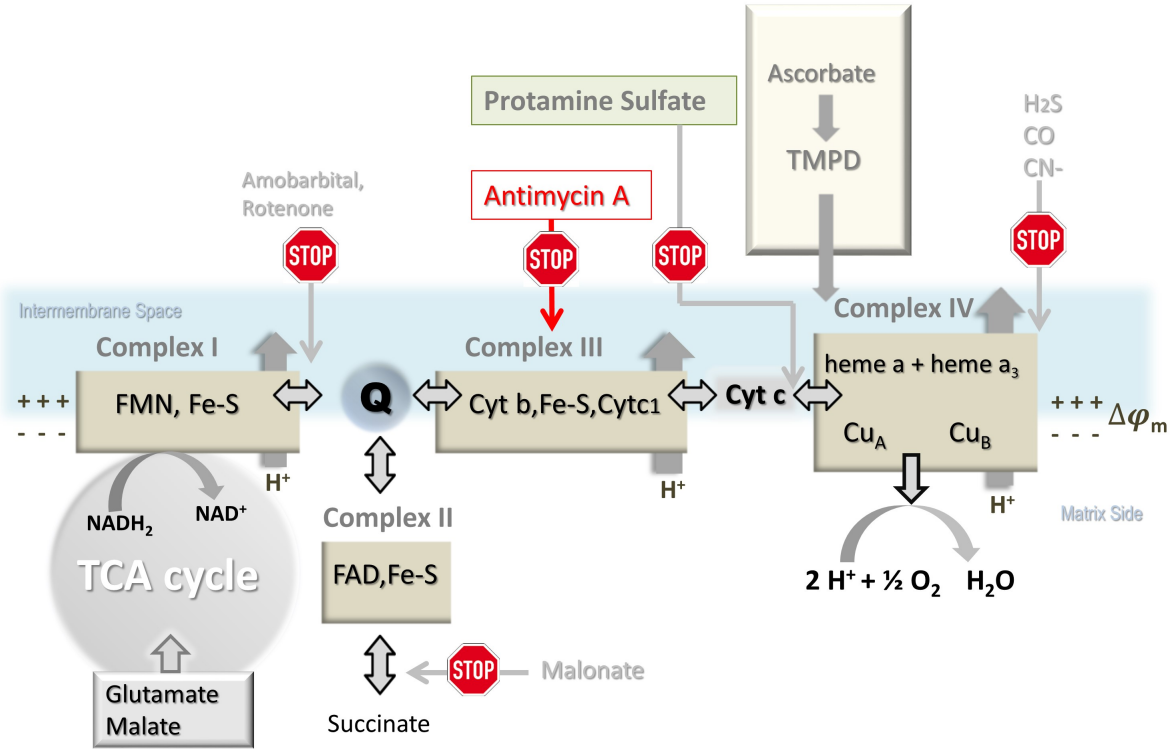

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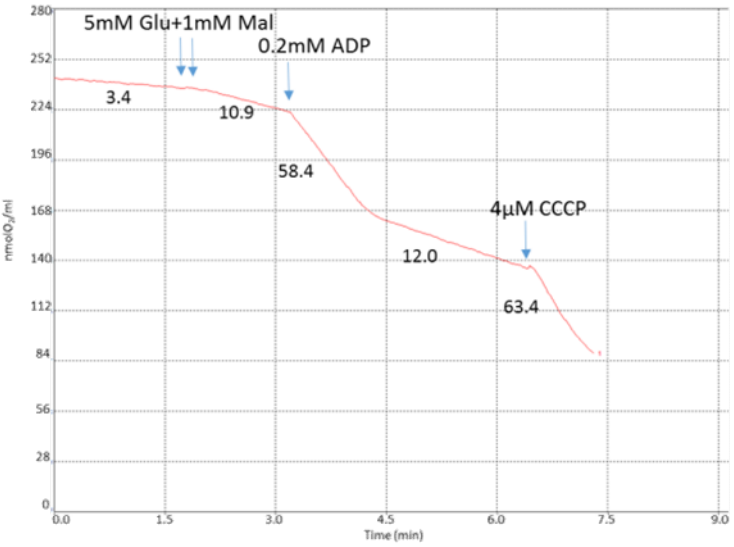
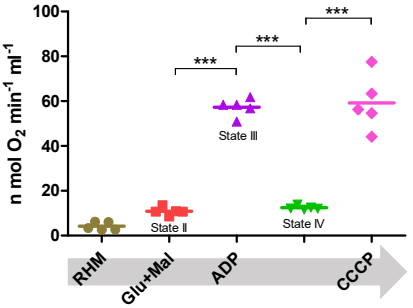
Figure 2 A - D

A





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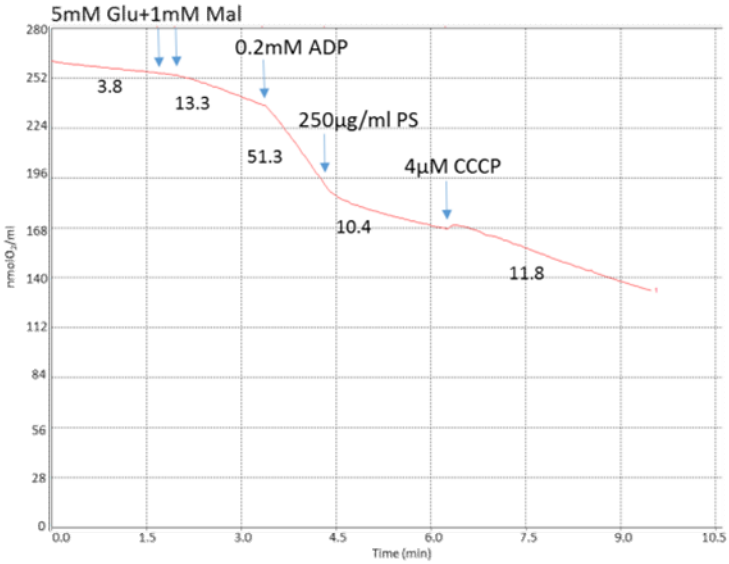

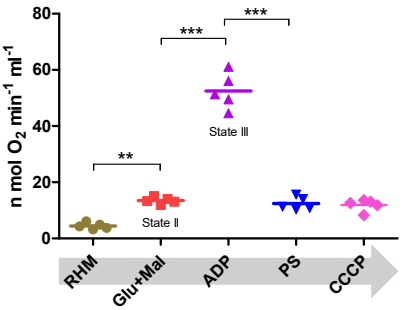

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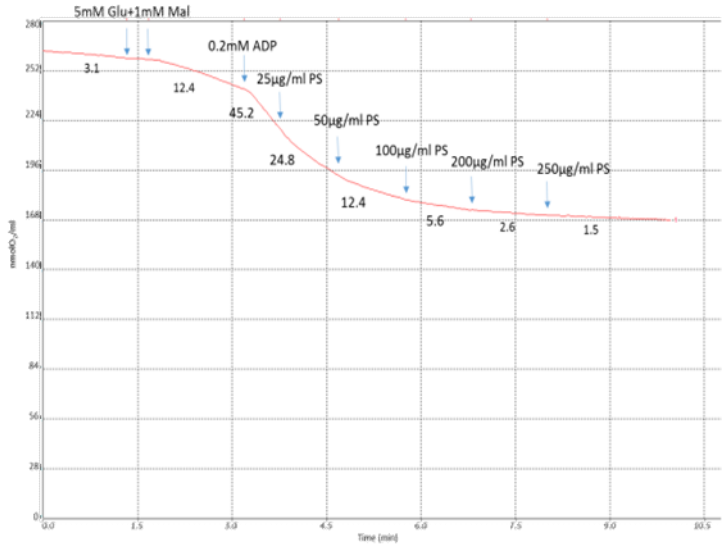
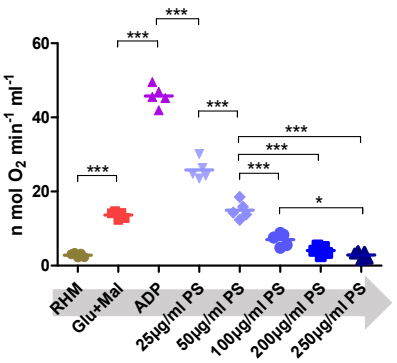
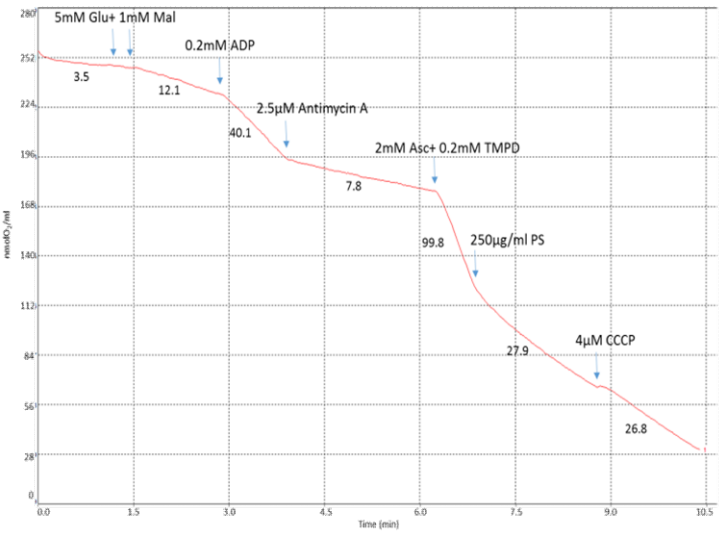
C





D



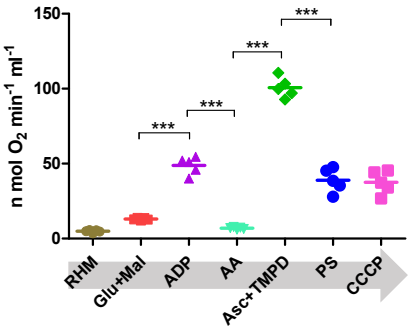


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Figure 3 A -D

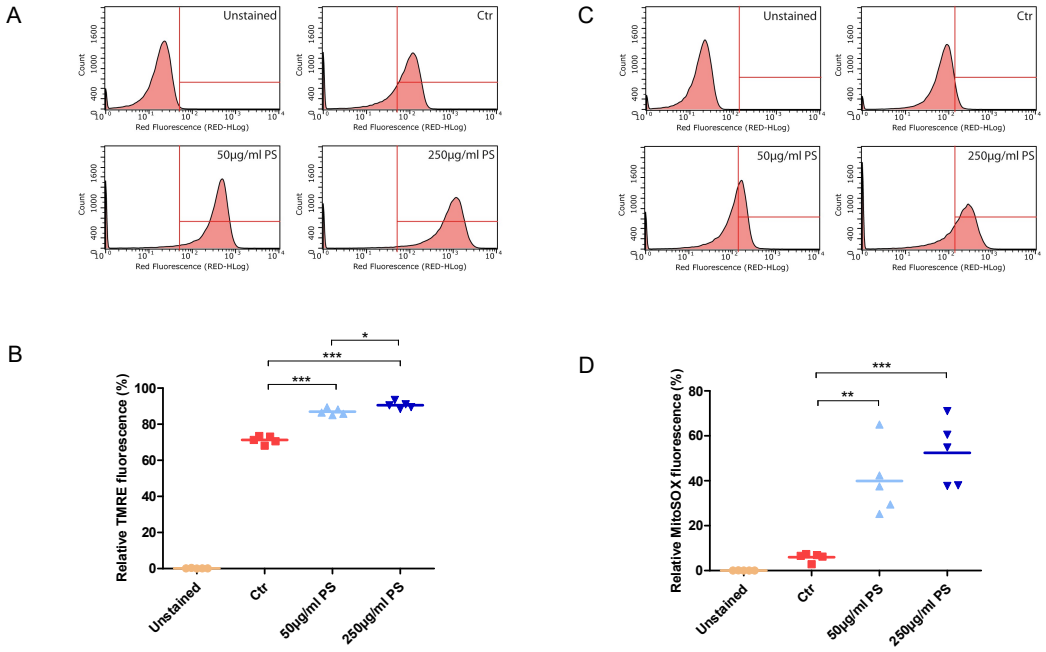

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Figure 4 A-D

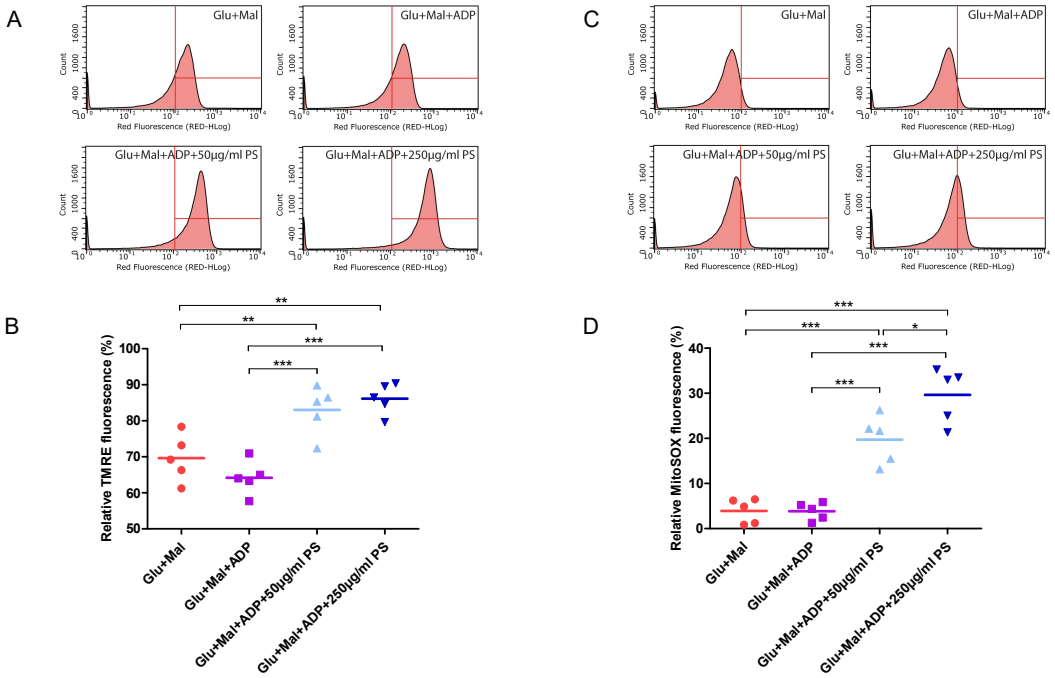

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Figure 5 A-D

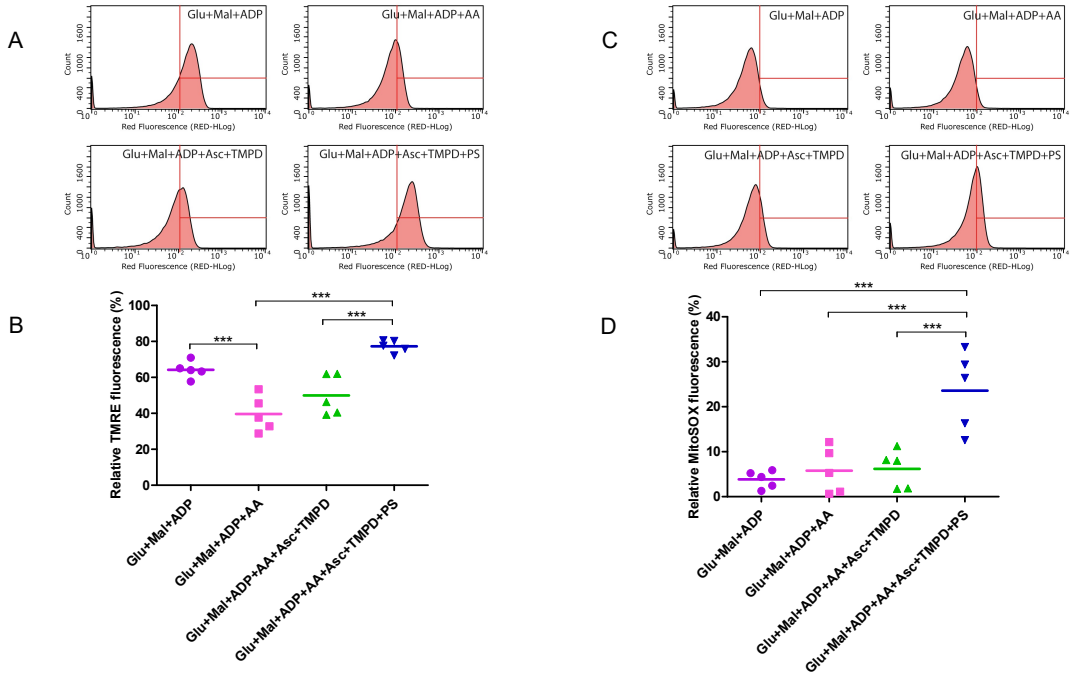

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Figure 6