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Existence of a distinct concentration window governing daunorubicin-induced mammalian liver mitotoxicity—implication for determining therapeutic window

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ABSTRACT

Daunorubicin (DNR) is a well known anticancer drug believed to act mainly by topoisomerase II inhibition and mitochondria-mediated free radical generation. Though several studies were dedicated to elucidate the mechanism of action of DNR, however the mechanism still remains illusive. DNR is reported to affect mitochondrial respiration. However, there are contradictory reports regarding DNR effect on oxygen consumption. Interestingly, DNR at low concentration (<10 μM) dose-dependently augments respiration but at higher concentration inhibits respiration. To investigate, if a concentration window exists in which the effect of DNR on mitochondria is optimum, dose-dependent effect of DNR on mitochondria was studied. DNR inhibited electron transfer and generates reactive oxygen species (ROS) at complex I and III but not at complex II. DNR-induced ROS generation was found instrumental in mitochondrial membrane potential collapse and mitochondrial permeability transition (MPT) opening. MPT closure reduced the observed respiratory burst. Thus, at lower DNR concentration, MPT opening leads to a sudden burst of respiration while at higher concentration electron transfer gets inhibited, therefore respiration gets repressed. We for the first time, provide a possible explanation for the reports regarding the differential regulation of respiration by DNR. Thus, further establishing the concept of concentration window and justifying the need for dose optimization for maximal therapeutic effect.

1. Introduction

The anthracycline antibiotic, DNR is one of the major antitumor agents widely used in the treatment of a variety of malignancies, soft-tissue sarcomas, non-Hodgkin's lymphoma and primarily in the treatment of acute myeloid leukemia and acute lymphocytic leukemia [1–3]. DNR is a high-affinity; sequence selective DNA intercalating agent [4,5], targeting topoisomerase II and causing mitochondrial redox reaction mediated ROS-induced macromolecular damage like lipid peroxidation [6–9]. The mechanism of action of DNR is still not fully understood. Among the several proposed mechanism the free radical hypothesis [6] for DNR toxicity has received a great attention and is believed to generate toxic oxygen radicals by ways of oxidation–reduction cycling. Inside the cell, the quinone moieties of DNR are reduced to

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semiquinone radical species which participate in electron accepting and donating reactions, generating superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl ion (OH^-) [9]. Inhibition of mitochondrial respiration is an important parameter governing cell survival and apoptosis. The effect of DNR on cellular respiration provides a controversial overview. Suslova et al. reported DNR-induced lipid peroxidation mediated increase in cellular respiration of rat liver mitochondria [10]. Bachur et al. has also reported similar increase in respiration in rat liver treated with DNR [11]. While Gorskaia et al. reported a DNR-induced decrease in ADP/O ratio and respiratory control ratios and stimulated state 4 respiration [12]. Shinozawa et al. reported a decrease in oxygen uptake of state 3 respiration and level of respiration control index [13]. Souid et al. reported that the rate of respiration increased by 32–60% when rat liver sub-mitochondrial particles were incubated with DNR, on the other hand they illustrate that 50–100 μM DNR produced a 50% inhibition of cellular respiration [14]. These observations are particularly interesting keeping in mind that mitochondrial functions like electron transfer, ROS production, mitochondrial membrane potential ($\Delta\Psi_m$), MPT opening are all correlated with respiration. Hence, we attempted to study the underlying cause and establish a proper correlation among the different mitochondrial functions that are affected by DNR.

A lot of effort has been channelized towards DNR mediated cardiotoxicity but to the best of our knowledge there are very few reports on the effect of DNR on mammalian liver mitochondria. Considering the functional importance of liver and the use of high drug concentration during cancer therapy, we studied the effect of DNR on rat liver mitochondria. We have investigated the immediate effects of increasing DNR doses on the liver mitochondrial functions *in vitro*, in order to exemplify the probable existence of a concentration window for maximal mitotoxicity. We have analyzed the concentration-dependent effect of DNR on the status of electron transfer, ROS formation, oxygen consumption, $\Delta\Psi_m$ collapse, MPT opening, lipid peroxidation and their interrelationship.

2. Materials and methods

2.1. Materials

DNR, rotenone, antimycin A, malonate, menadione, bovine serum albumin (BSA), 3′-dithiothreitol (DTT), reduced nicotinamide adenine dinucleotide (NADH), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), succinic acid, dichlorophenolindo-phenol (DCPIP), 4-morpholinepropanesulfonic acid (MOPS) buffer, bradford reagent, phenylmethylsulfonyl-fluoride (PMSF), safranine O, superoxide dismutase (SOD), valinomycin, oligomycin and cyclosporin A were purchased from Sigma (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) buffer and sucrose were purchased from Hi media Laboratories Pvt. Ltd. (Mumbai, India). Unless otherwise mentioned all other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Animals

Male Sprague-Dawley rats (200–250 g) obtained from Central Animal Facility of National Institute of Pharmaceutical Education and Research (NIPER) were used for all the studies. Animals were housed in Institute's temperature controlled (22 ± 1 °C) room with 12 h light/12 h dark cycles and allowed free access to food (standard laboratory rat-chow) and water *ad libitum*. All studies were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutes Ethical Committee, NIPER. Rats were killed by decapitation and liver was quickly excised in ice-cold buffer.

2.3. Isolation of rat liver mitochondria

Liver mitochondria were prepared by differential centrifugation. Excised liver was quickly chopped in ice-cold homogenization medium (250 mM sucrose, 1 mM EGTA and 10 mM HEPES, pH 7.4) and 0.1% defatted BSA. The minced tissue was suspended in 40 ml of isolation medium and homogenized with a glass homogenizer and Teflon pestle (Polytron PX OS 2000). The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C [15]. Mitochondria were isolated by subjecting the supernatant to centrifugation at 17,000 × *g* for 10 min at 4 °C. The pellet was resuspended in the isolation medium (1 g tissue/10 ml medium) and then centrifuged at 17,000 × *g* for 10 min at 4 °C [16] and isolated mitochondria were resuspended in a minimal volume of respiration buffer (125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, pH 7.2, 5 mM $MgCl_2$, 2.5 mM potassium phosphate, 0.5% BSA, 0.5 mM EGTA, pH 7.4) supplemented with 0.3% (w/v) BSA and freshly isolated mitochondria was used for every experiment. Protein concentration was determined by using the Bradford method using BSA as standard [17]. The mitochondria isolated were characterized by respiratory ratio, succinate dehydrogenase activity and transmission electron microscopy (supplementary Fig. 1).

2.4. Oxygen consumption

Oxygen uptake was measured polarographically using a Clark type oxygen electrode (Rank Brothers Ltd., UK) with a Graphic 1000, Seatallan Ltd. oxygraph, in a 1.5 ml water jacketed closed chamber with magnetic stirring at 37 °C. The reaction mixture contained mitochondria (500 μg/ml protein) in respiratory buffer, pH 7.2 and substrate concentrations used were 500 μM NADH and 20 mM succinate for complex I and complex II mediated oxygen consumption, respectively [16].

2.5. Complex I and II mediated electron transfer assay

Complex I (NADH dehydrogenase) activity was determined spectrophotometrically (Perkin-Elmer, Lambda-25 spectrophotometer) by monitoring the oxidation of NADH at 340 nm using 50 μg mitochondria protein in a final volume of 1 ml (0.1 M phosphate buffer, pH 8.0) at 30 °C. The reaction was initiated by adding 200 μM menadione and 150 μM NADH and the change in the optical density (OD) was analyzed for 8 min. [18]. The amount of NADH oxidized was calculated using molar absorptivity of NADH (ε = 6.22 mM^{-1} cm^{-1}).

Complex II (succinate dehydrogenase) activity was determined spectrophotometrically by monitoring DCPIP reduction at 600 nm using 100 μg protein in a final volume of 1 ml (0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA). The reaction was initiated with 50 μM DCPIP and 20 mM succinate and the change in the OD was analyzed for 8 min [19].

2.6. Estimation of ROS production

H_2O_2 production was detected using DCFH-DA dye (non-fluorescent), which when oxidized gets converted into fluorescent 2′,7′-dichlorofluorescein (DCF) (ex: 488 nm, em: 540 nm). Mitochondria (500 μg) were incubated in presence and absence of DNR for 6 min in 1 ml respiration buffer, pH 7.2. To this 20 μM $DCFDAH_2$, 400 μM NADH (for complex I) or 20 mM succinate (for complex II) was added, mixed and fluorescence was detected using a ALS50B Perkin-Elmer fluorimeter [20]. To determine which side of the mitochondrial inner membrane $O_2^{\bullet -}$ was generated and to further confirm the existence of concentration window, the rate of H_2O_2 production was measured in the presence and absence of exogenous SOD (50 U/ml) [21].

2.7. Determination of membrane potential using safranine O

Mitochondrial $\Delta\psi_m$ was evaluated with the lipophilic cationic dye safranine O. The standard reaction medium, containing 250 mM sucrose, 20 mM MOPS buffer, pH 7.2, 20 mM succinate, 2 mM NaH_2PO_4, and 1 μM rotenone, was supplemented with mitochondria (500 μg/ml protein) and safranine O (10 μM). Fluorescence was measured with a Perkin-Elmer fluorimeter (ex: 495 nm and em: 586 nm; slit width: 5.0) [22]. The change in fluorescence due to generation of $\Delta\psi_m$ upon addition of succinate was observed in the presence of DNR and uncoupler CCCP (50 μM). For the calibration of the dependence of safranine fluorescence on $\Delta\Psi_m$, K^+ diffusion potential were induced by 20 nM valinomycin in the presence of 1 μM rotenone and 2.5 μM oligomycin [23].

2.8. Mitochondrial swelling

Mitochondrial swelling was used as an indicator for mitochondrial pore transition pore opening. Mitochondrial swelling was measured as the decrease in light scattering by following optical density at 540 nm (A_{540}) over time. Mitochondrial volume changes were measured from the absorbance changes at 540 nm using a dual wavelength Perkin-Elmer spectrophotometer equipped with a magnetic stirrer assembly and thermostatic control [24]. With this method, a decrease in absorbance is indicative of an increase in mitochondrial volume. Mitochondria (1 mg ml^{-1}) were incubated at 25 °C in respiration buffer and were energized with 20 mM succinate to support swelling using varying concentrations of DNR and Ca^{++} ions and swelling was inhibited using CsA [25]. Succinate was added after calcium addition. Swelling amplitude was calculated as the difference between initial (before succinate addition) and final optical density.

2.9. Lipid peroxidation

Lipid peroxidation by DNR was studied following the protocol of Placer et al. [26]. Briefly, 500 μg of freshly isolated mitochondrial sample in 1.3 ml of Tris-maleate (pH 5.9) buffer in stoppered glass tubes was treated with different concentration of DNR then incubated for 30 min at 37 °C. To the reaction mix, 1.5 ml of thiobarbituric acid was added and then boiled for 10 min at 80 °C. To the cooled tube, 3 ml of pyridine–butanol and 1 ml of 1N NaOH were added. Samples were shaken thoroughly and absorbance measured at 548 nm using a Perkin-Elmer spectrophotometer. Amount of malonyldialdehyde formed was calculated using its standard curve prepared under the same conditions.

2.10. Statistical analyses

The data are expressed as mean ± SEM. For comparison of two groups, *P*-values were calculated by two-tailed unpaired student's *t*-test. In all the cases $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. DNR-induced complex I mediated oxygen consumption, electron transfer and ROS generation

The immediate effect of DNR (2–40 μM) on mitochondrial respiration (incubated for 6 min) in the absence of external ADP was studied. Standard complex I inhibitor rotenone (10 μM) inhibited cell respiration about 80% (data not shown). Interestingly DNR showed a dose-dependent activation (~10–30%) of the mitochondrial respiration up to a concentration of 10 μM. With increasing DNR concentration (20–40 μM) inhibition of respiration as compared to control was noted with complex I substrate (Fig. 1A). With still higher concentration (100 μM) a 65% inhibition of respiration was observed (data not shown).

Respiration is governed by substrate channeling; hence, effect of DNR on electron transfer was studied. DNR at a dose range of 2–10 μM exhibited insignificant electron transport inhibition as evident from the kinetic profile and percentage activity (Fig. 1B) in functionally active mitochondria oxidizing NADH. Complex I mediated electron transfer was dose-dependently inhibited by DNR at higher concentration. 20–40 μM concentration of DNR was required to obtain ~16–20% inhibition of complex I mediated electron transfer. DNR-induced complex I inhibition was also observed with other complex I substrates like glutamate and α-ketoglutarate (data not shown). Hence, DNR caused a dose-dependent inhibition of complex I mediated electron transfer (insignificant inhibition at 2–10 μM concentration).

As DNR is reported to produce ROS from complex I [5]; hence, complex I mediated ROS generation was studied. Incubation (6 min) of the liver mitochondria with DNR caused a time and dose-dependent generation of H_2O_2 using NADH as substrate (Fig. 1C). It is interesting to note that DNR being a redox agent increased ROS production even at very low concentration (2–10 μM). However, at high concentration

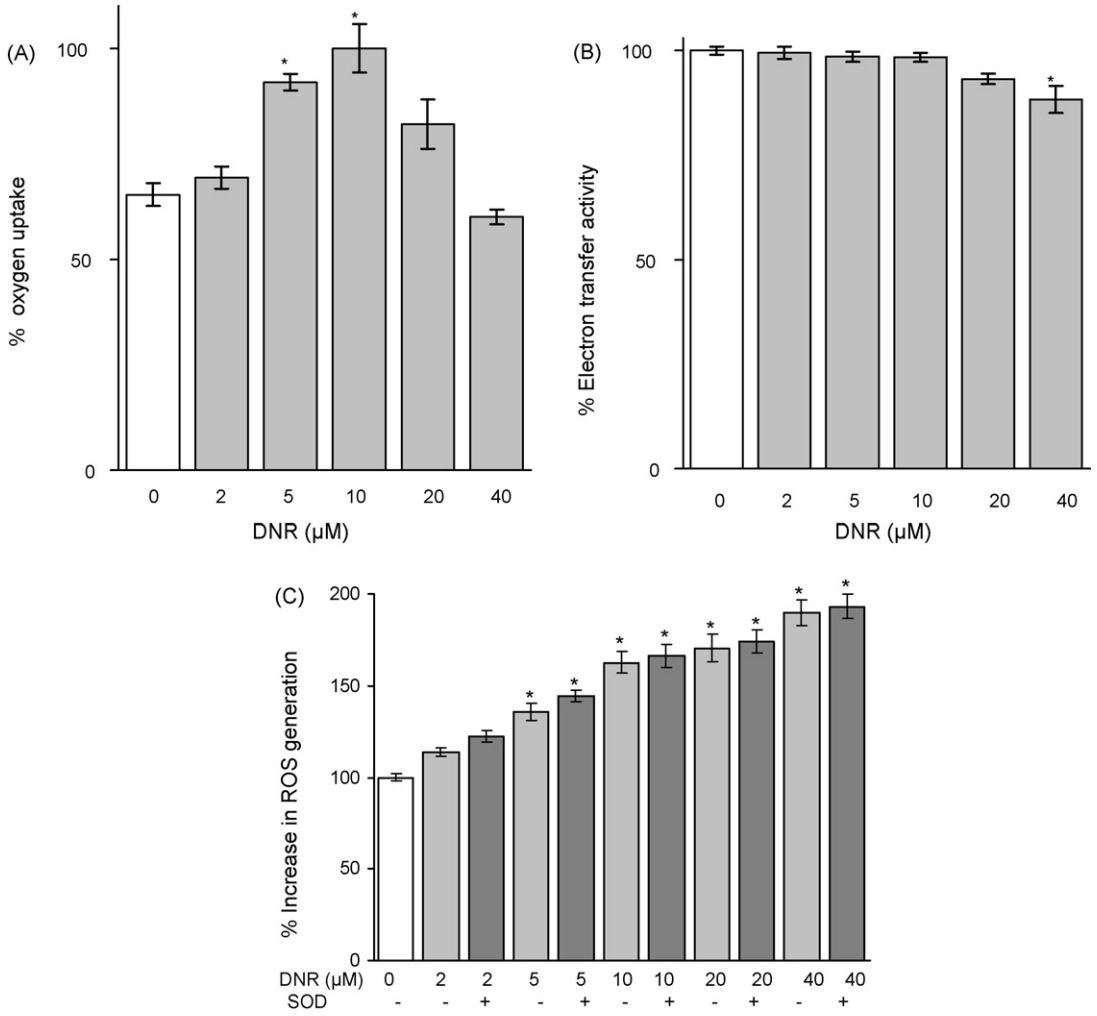

Fig. 1 – Effect of DNR on complex I mediated oxygen consumption, electron transfer and ROS generation. (A) Oxygen consumption rates of DNR treated (for 6 min) mitochondria was measured as an indicator of mitochondrial respiration, using the Rank Brothers system as described in Section 2. Percent oxygen uptake corresponds to oxygen consumed per min per mg of protein (nmol/mg protein/min) for mitochondria energized with NADH. State IV, mitochondrial oxygen uptake in the presence of DNR (2–40 μM) and 0.4 mM NADH as substrate was measured. Bar diagram showing the % oxygen uptake after 8 min in the presence or absence of DNR. (B) Mitochondria (50 μg of protein) were suspended in 0.1 M potassium phosphate buffer pH 8.0 to a final volume of 1.0 ml. DNR (2–40 μM) was incubated for 6 min with mitochondria and the reaction then initiated by addition of 200 μM menadione and 50 μM NADH. DNR concentrations are as indicated. Bar diagram showing the % complex I mediated electron transfer activity of control and DNR treated mitochondria. 100% represents the electron transfer activity for mitochondria energized with 50 μM NADH. (C) Mitochondria (0.5 mg of protein) were suspended in a medium containing 135 mM KCl, 5 mM HEPES-NaOH, pH 7.2, 0.1 mM EGTA, and 5 mM $MgCl_2$ to a final volume of 1.0 ml. DNR was incubated for 6 min with the mitochondria and the reaction then initiated by addition of 0.4 mM NADH and 20 μM DCFDA. Bar graph representing the effect of DNR on the complex I mediated ROS production of liver mitochondria after 8 min of reaction in presence and absence of exogenous SOD. DNR concentrations are as indicated. The ROS production in absence of DNR was considered as 100% and accordingly the increase in % ROS production was calculated for other concentrations. Values represents mean ± SEM; $n = 4$. $^{*}P < 0.05$, for difference from control value.

(>10 μM) DNR exhibited saturation kinetics for ROS production (Fig. 1C). To further confirm the finding, exogenous SOD was used. SOD in the presence of low concentration of DNR (2–10 μM) increased the rate of H_2O_2 production compared to control but because of the small increase in response compared to DNR treated we consider them to be unreliable. While at higher DNR concentration (>10–40 μM) H_2O_2 production was insensitive towards SOD addition (Fig. 1C).

3.2. *DNR-induced complex II mediated oxygen consumption, electron transfer and ROS generation*

Mitochondria incubated with complex II substrate (succinate) exhibited very similar results regarding the effect of DNR on mitochondrial respiration. With 2–10 μM DNR, activation (~10–25%) of respiration as compared to control was obtained. With an increase in DNR concentration

(20–40 μM) decrease in respiration was observed (Fig. 2A). Standard complex II inhibitor malonate (20 μM) significantly inhibited respiration by ~85% (data not shown). The result indicates the existence of a complex concentration window of DNR which invokes further study to explain this phenomenon.

DNR even at 40 μM concentration did not exhibit a statistically significant inhibition of complex II mediated

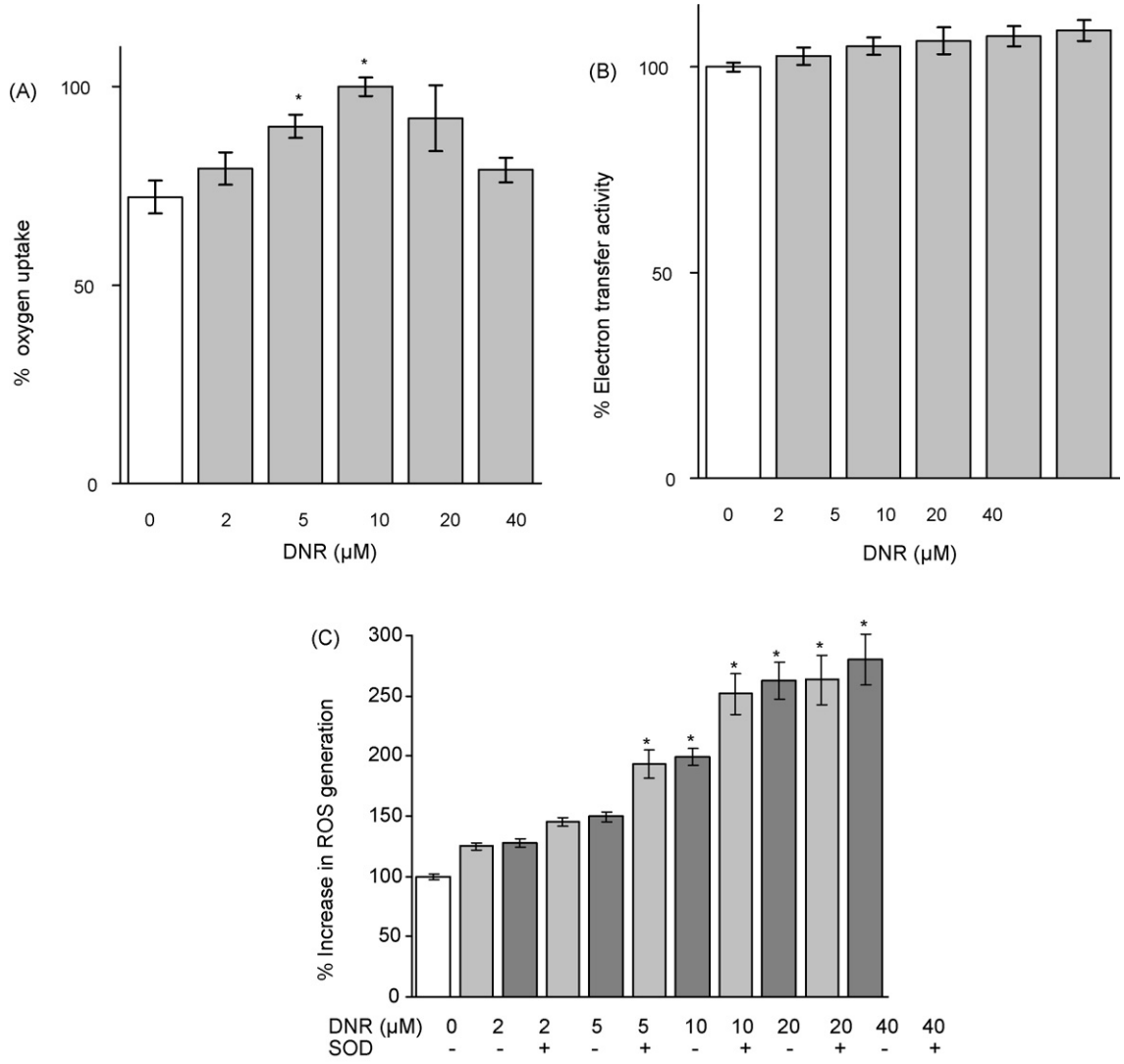

Fig. 2 – Effect of DNR on complex II mediated oxygen consumption, electron transfer and ROS generation. (A) Bar diagram represents oxygen consumption rates of DNR (2–40 μM) treated (for 6 min) mitochondria measured up to 8 min as an indicator of mitochondrial respiration. Percent oxygen uptake corresponds to nmol oxygen consumed per min per mg of protein for mitochondria energized with succinate. State IV, mitochondrial oxygen uptake in the presence of DNR (2–40 μM) and 20 mM succinate as substrate. Values represent mean±SEM; $n = 3$. $^{*}P < 0.05$, for difference from control values. (B) Mitochondria (0.1 mg of protein) were suspended in a medium as earlier described to a final volume of 1.0 ml with phosphate buffer, pH 7.4. DNR was incubated for 6 min with the mitochondria and the reaction was then initiated by addition of 50 μM DCPIP and 20 mM succinate. DNR concentrations are as indicated. Bar diagram showing the % complex II mediated electron transfer activity of control and DNR treated mitochondria. The ΔOD_{600} was calculated and specific activity of succinate dehydrogenase was calculated by using molar absorptivity of DCPIP ($\varepsilon = 21\ mM^{-1}\ cm^{-1}$). 100% represents the electron transfer activity for mitochondria energized with 20 mM succinate. Values represent mean ± SEM; $n = 4$. (C) Mitochondria (0.5 mg of protein) were suspended in a medium containing 135 mM KCl, 5 mM HEPES-NaOH, pH 7.2, 0.1 mM EGTA, and 5 mM $MgCl_2$ to a final volume of 1.0 ml. DNR was incubated for 6 min with the mitochondria and the reaction was then initiated by addition of 20 mM succinate and 20 μM DCFDA. DNR concentrations are as indicated. Bar graph representing the effect of DNR on the % increase of complex II substrate-mediated ROS production of liver mitochondria in presence and absence of exogenous SOD after 8 min of reaction. The ROS production in absence of DNR was considered as 100% and accordingly the increase in % ROS production was calculated for other concentrations. Values represent mean ± SEM; $n = 4$. $^{*}P < 0.05$.

succinate utilization (Fig. 2B) and at even higher concentration (50–100 μM) a insignificant but dose-dependent increase in electron transfer was observed (data not shown).

We further investigated DNR-induced complex II mediated ROS (using succinate) generation (Fig. 2C). In the absence of electron transfer inhibition, significant amount of ROS production was observed while using complex II substrate. As it is reported that, complex I and III are the major site of ROS production [21] and DNR has an insignificant effect on complex II mediated electron transfer inhibition, we can very well assume that complex II substrate may induce ROS generation from complex III in the presence of DNR. To further elucidate the existence of a concentration window exogenous SOD was used. Addition of exogenous SOD to succinate supplemented mitochondria increased DNR-induced H_2O_2 generation compared to control but the signal was minimal compared to DNR treated. Addition of SOD showed increasing trend with increasing DNR concentration (Fig. 2C).

Hence, we find an interesting correlation between the concentrations needed for electron transfer inhibition, ROS production and respiration. We further extended our work to check whether the correlation exists among other interrelated parameters like $\Delta\psi_m$, MPT and lipid peroxidation.

3.3. *Effect of DNR on mitochondrial membrane potential ($\Delta\psi_m$)*

DNR at a lower concentration range (2–5 μM) induces a partial mitochondrial depolarization, while at a concentration of 10 μM and above there is complete depolarization of liver mitochondria. CCCP was added (arrows) to check the extent of $\Delta\psi_m$ depolarization (Fig. 3A).

In order to study whether DNR (10 μM) mediated membrane potential dissipation is a consequence of DNR-induced ROS generation, we used the common antioxidant ascorbic acid (0.5 mM) to scavenge the ROS generated by DNR (Fig. 3B). Ascorbic acid ameliorates DNR-induced mitochondrial depolarization suggesting a clear role of DNR-induced ROS in DNR-induced $\Delta\psi_m$ collapse.

3.4. *Effect of DNR on MPT pore induction*

Functional MPT was checked using different concentrations of standard activator (calcium) and inhibitor (cyclosporin A) of MPT (data not shown). MPT pore opening was obtained using succinate as an electron donor, as succinate exhibits better MPT response as compared to NADH in our experiment. We observed that DNR in a dose-dependent manner induced mitochondrial swelling similar to calcium in a concentration range of 2–20 μM (Fig. 4A), higher concentration does not led to any further swelling (data not shown). The results suggest that the MPT pore opening operates at lower concentrations like ROS generation.

To further evaluate the role of DNR induced ROS formation in MPT opening, the effect of the antioxidant ascorbic acid was studied. DNR mediated MPT induction was almost completely inhibited by ascorbic acid. Ascorbic acid at a concentration range of 1–5 mM protected the MPT opening in presence of DNR (Fig. 4B). Thus, strengthening the role of DNR-induced

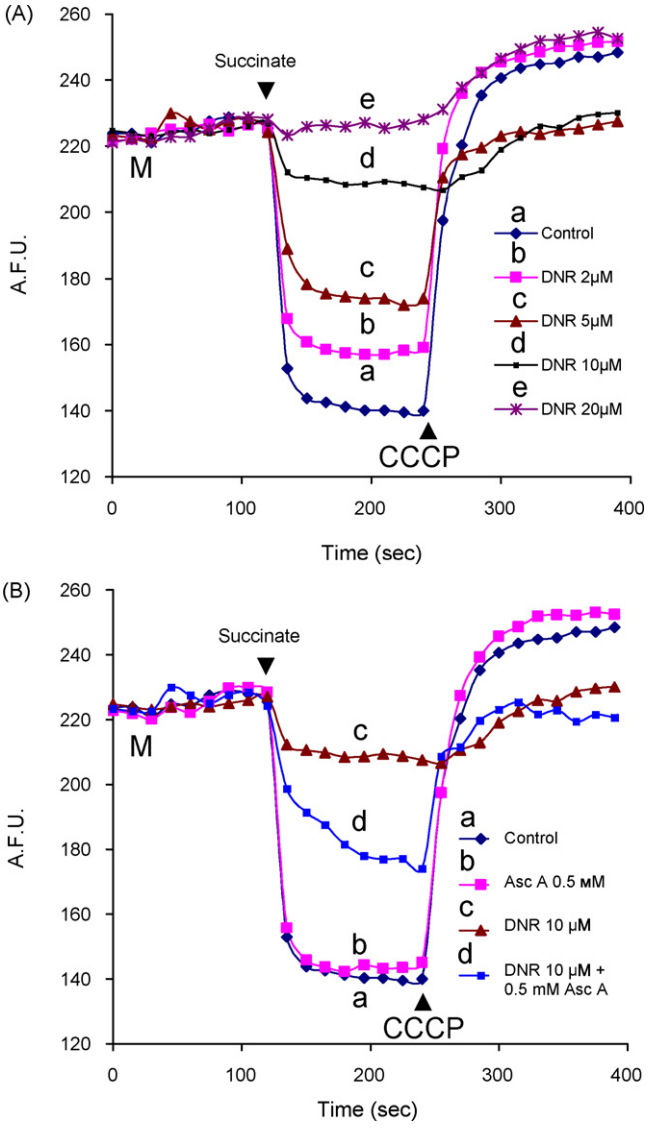

Fig. 3 – (A) Effect of DNR on mitochondrial membrane potential. The membrane potential ($\Delta\psi_m$) of mitochondrial preparations (50 μg) was estimated by fluorescence changes of safranine O (10 μM) in a standard medium (1 ml), as described in Section 2.7 and energized with 20 mM succinate. Mitochondria were incubated with DNR for 6 min before adding succinate to the mitochondria (indicated by bold arrow). Traces for the last 2 min of incubation have been shown before the addition of succinate. M (bold) represents that mitochondria is already present in the system. CCCP was added as indicated (bold arrow) to collapse $\Delta\psi_m$. The traces are the representative of three independent experiments. (B) Representative traces showing the effect of ascorbic acid (0.5 mM) on DNR (10 μM)-induced $\Delta\psi_m$ depolarization.

ROS in the induction of MPT pore and subsequent $\Delta\psi_m$ depolarization. It was observed that the phenomenon of MPT occurred much earlier (within 2 min) than the initiation of $\Delta\Psi_m$ collapse as investigated by a temporal study involving $\Delta\psi_m$ and MPT together (data not shown).

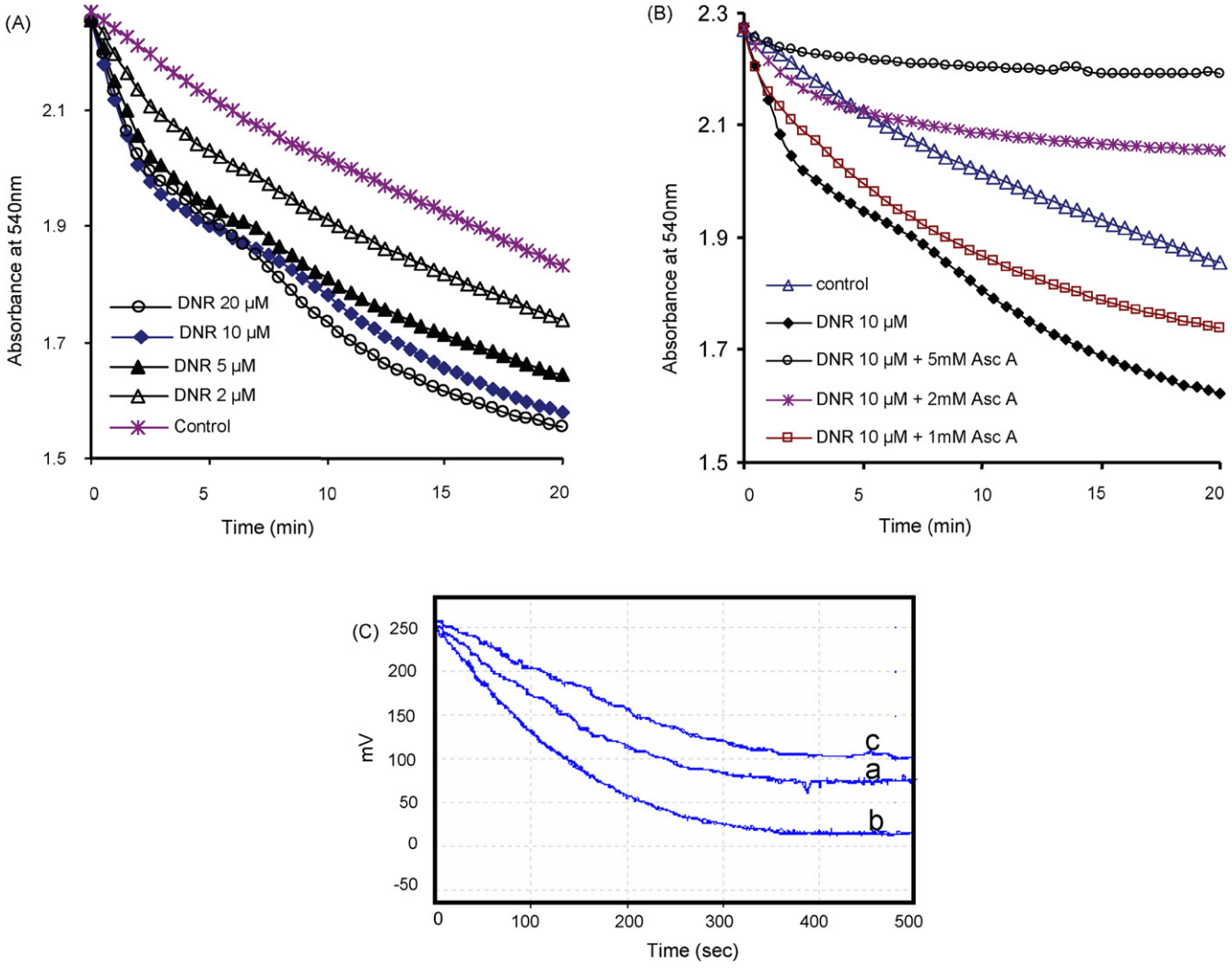

Fig. 4 – (A) Effect of DNR on the amplitude and rate of swelling of liver mitochondria energized with succinate (20 mM). Mitochondria (1 mg) were incubated at 25 °C in respiration buffer. DNR was incubated for 1 min with the mitochondria before additions of the substrate. The traces are representative of recordings of four experiments. Swelling is indicated by a downward and shrinkage by an upward deflection of the spectrophotometric trace. (B) Effect of ascorbic acid on DNR-dependent mitochondrial swelling. The experiments were carried out in the presence of 20 mM succinate to induce energy dependent swelling; $n = 3$. DNR (10 μM) was found to induce MPT which in turn was inhibited in presence of antioxidant ascorbic acid, dose dependently (1–5 mM). (C) Traces show the effect of CsA on DNR-induced respiration. Trace (a) represents oxygen uptake by normal succinate energized mitochondria. Trace (b) represents oxygen consumption mediated by DNR only. Trace (c) represents oxygen consumption mediated by DNR in the presence of CsA (2 μM). Direct tracings are shown as a representative of three individual experiments; y axis denotes values in mV.

We further checked whether the increase in respiration is a consequence of MPT opening by studying DNR-induced respiration in the presence of cyclosporin A (CsA). It was observed that DNR-induced respiration increase was inhibited partially by CsA (Fig. 4C). Thus, MPT opening may be considered to play a role in the observed respiratory burst.

3.5. *Effect of DNR on lipid peroxidation*

Since lipid peroxidation is an expression of oxidative stress in cells, we examined the level of MDA formation in presence of different concentrations of DNR after 2 h of incubation. A DNR-induced dose-dependent increase in lipid peroxidation was noted. The amount of lipid peroxidation at lower concentration (5–10 μM) of DNR was significantly less (~2–5% as compared to control) in comparison to higher concentration (20–80 μM) mediated peroxidation (~30–45%) (Fig. 5). Indicating that at low concentration there is very small amount of lipid peroxidation that may not be sufficient to produce an uncoupled state.

4. Discussion

Despite the fact that DNR has been extensively studied in mitochondrial energetics, controversial reports exist regard-

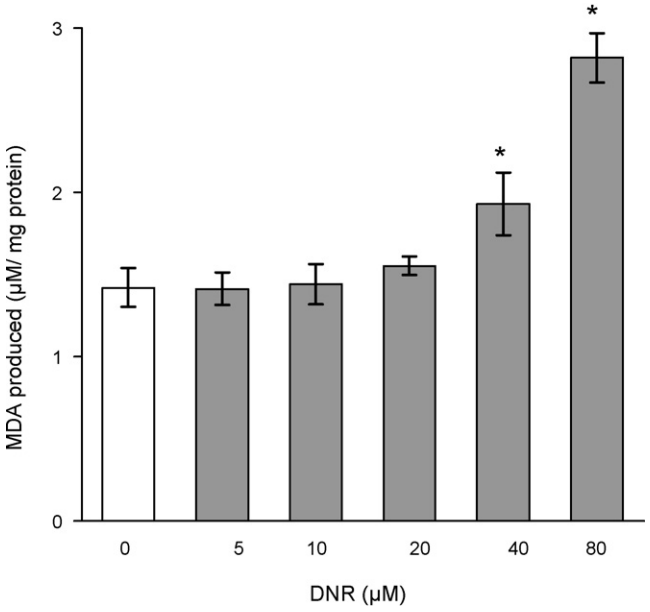

Fig. 5 – Bar graph representing DNR-induced lipid peroxidation as indicated by MDA formation in the normal and DNR treated mitochondria. DNR concentrations are as indicated. $\Delta_{548\ nm}$ was used to study the amount of MDA formed and data expressed as MDA produced (μM/mg mitochondrial protein). Values represent mean ± SEM; $n = 3$. $^{*}P < 0.05$ for difference from control value.

ing DNR-induced oxygen uptake [10,12,13] and no clear picture has emerged. This study examined the immediate effect of DNR concentrations on mitochondrial functions. Results suggests that DNR at low concentration (2–10 μM) augments respiration while at high concentration (>10 μM) inhibits respiration supported by complex I and complex II substrates; thus, indicating the existence of a plausible concentration window for DNR and was further scrutinized. Complex I mediated electron transfer was significantly inhibited by DNR, while complex II mediated electron transfer remained unaffected. DNR produced both complex I and III mediated ROS generation. At complex I the NAD^+/NADH couple has a redox potential of −320 mV. Electrons travel downhill from this −320 mV to ubiquinone through several intermediate redox state molecules. DNR is reported to have a similar redox potential (~320 mV) [27]. Understanding of ROS production at complex I is still limited. Both FMN and FeS centers are implicated in ROS generation [28]. Of the two, FMN has the redox potential close to that of NADH and DNR, whereas FeS has a redox potential further downhill (~250 mV). Hence, FMN holds redox compatibility for electron leakage to form DNR semiquinone (Supplementary Scheme 1). DNR as reported earlier, after being reduced at complex I into DNR-semiquinone forms superoxide [29] and hydrogen peroxide as a consequence of redox cycling of anthracyclines [30]. DNR being lipophilic accumulates in the inner mitochondrial membrane and enhances electron deviation from the regular respiratory chain pathway to the formation of ROS, leading to amplification of mitochondrial dysfunction [9]. Thus, providing further evidence that DNR-induced mitochondrial dysfunction is caused by inhibition of complex I and probably complex III of the electron transport chain (ETC). At low concentration (2–10 μM) where ROS is produced maximally, electron transfer is only partially inhibited (10–25%). It is quite interesting to note the stoichiometry of concentrations required to inhibit electron transfer and ROS production. During forward electron transfer DNR acts as an electron cycler, getting reduced to the semiquinone radical species and back. DNR, unlike routinely used ETC inhibitors, acts as an electron withdrawer, rather than a blocker. The redox state of iron can be converted between the iron (II) and iron (III) states by interaction with anthracyclines, generating toxic ROS [1,27]. As complex I and III both has 'FMN' center thus, are involved in ROS generation (Supplementary Scheme 1 and 2). The minor extent of electron transfer inhibition as compared to very high ROS formation by DNR may be due the high efficiency of electron slippage through semiquinone formation and inhibition of any cofactor at complex I and complex III of the ETC. To further confirm the result H_2O_2 generation was studied in the presence of exogenous SOD. If mitochondria produce $O_2^{\bullet-}$ on the cytosolic face of the inner membrane, $O_2^{\bullet-}$ will be dismutated to produce more H_2O_2 which in turn will be detected by the DCFDHA probe. Exogenous SOD will not be in a position to act on $O_2^{\bullet-}$ generated by mitochondria on the matrix side of the inner membrane [21]. At lower concentration NADH-induced DNR-mediated ROS generation increased in the presence of SOD, while at higher concentrations of DNR no increase was observed. At low concentration electron transfer at complex I is almost uninhibited by DNR and ROS generation occurs. As electron transfer partially inhibited hence electron travels downstream to complex III, where DNR acts to produce complex III mediated ROS at the cytoplasmic side. The ROS generated at complex III may be scavenged by SOD to produce a minimal increase in H_2O_2 signal as observed. At high concentration, the electron transfer is mostly blocked through complex I to complex III. Hence, complex III mediated ROS generation ceases and thus the SOD-mediated insensitivity is noticed. Exogenous SOD addition augmented succinate induced DNR-mediated ROS generation in a dose-dependent manner but gets saturated at a DNR concentration >20 μM. Thus, further indicating the existence of a distinct concentration window.

Biochemical and physiological data favor the hypothesis that DNR causes free radical-mediated lipid peroxidation and alters cellular membrane integrity and may induce MPT pore opening [31,32]. We found that MPT pore opening is an early event than membrane potential depolarisation, operates at lower DNR concentration (2–10 μM) and is sensitive to DNR-induced ROS as evident from the antioxidant experiment. On the contrary, $\Delta\psi_m$ depolarization is a delayed event, operates at a higher DNR concentration and is also ROS sensitive. DNR-induced ROS-mediated lipid peroxidation was reported at higher DNR concentrations (>100 μM) in mouse heart and liver [13]. Thus, our work correlates well with the reports of Gewirtz that limited DNR toxicity in H-35 rat hepatoma due to the apparent absence of free radical-mediated cellular damage thus substantiating the role of DNR-induced free radicals in cell death [33]. In this work, for the first time we are demonstrating that the DNR-mediated

opening of MPT is responsible for the transient increase of respiration. Though reports suggest that induction of the MPT is at least in part responsible for decreased mitochondrial respiration [34]. Our results suggests that the immediate effect of DNR-induced ROS mediated MPT opening leads to respiratory burst as specific MPT inhibitor CsA was found to inhibit such increase in respiration. Apart from MPT inhibition, CsA is also reported to inhibit calcineurin, a protein phosphatase [35]. The calcium-activated phosphatase, calcineurin plays important roles in the transduction of Ca^{2+} signals to regulate various cellular processes [36]. CsA also inhibits calcineurin-dependent signaling pathways, thereby attenuates the expression of nitric oxide synthase 2. Nitric oxide is reported to induce the MPT and to inhibit mitochondrial electron transport [37]. Interestingly, Nicolli et al. has clearly demonstrated that inhibition of calcineurin activity is not required for the inhibitory effect of CsA on MPT of rat liver mitochondria [38]. Therefore, considering the *in vitro* experimental set up, it is less likely that CsA will inhibit calcineurin and produce any effect on mitochondrial functions.

ROS is an upstream event than $\Delta\Psi_m$ collapse in DNR-induced liver toxicity and our studies correlate well with that of Chibowska et al. [39]. At lower DNR concentration (2–10 μM) stimulation of respiration occurs. This phenomenon may be attributed to maximal ROS production leading to MPT pore opening and partial membrane potential collapse leading to an uncoupled state. MPT opening may also be associated with massive ATP hydrolysis, which may induce state III respiration and a slight increase in complex II mediated electron channeling. At higher concentrations, there were significant inhibitions of electron transfer, decrease in respiration (due to reduced flow of electrons to molecular oxygen), saturation of ROS production and maximal $\Delta\Psi_m$ collapse (supplementary Scheme 3). Our data correlate well with the report of Masqueliera et al., suggesting the existence of DNR concentration window, where at low drug levels apoptosis was induced but at higher levels necrosis is triggered in leukemia cells [40].

Some studies suggest high DNR dose improves chemotherapy results [36]. However, other groups suggest that dose intensification does not significantly improve outcome [32,39] due to toxicity problem. Thus, we describe the existence of a DNR concentration window, which has serious implication in drug dosage and determination of the therapeutic window.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.06.009.

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