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NAD^+ supplementation reverses the oxidative stress induced PARP1 signalling in *D. discoideum*

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Increased oxidative stress leads to cell death by inducing DNA damage, PARP activation and energy depletion in age related disorders which are a growing concern due to increased life expectancy. Indeed, cellular NAD^+ levels, depletion of which is one of the consequences of overactive PARP, also decline with age. We previously showed rescue in oxidative stress induced paraptotic and necrotic cell death by PARP1 inhibition in *D. discoideum*. Inhibition of PARP1 activity prevented cellular depletion of its substrate NAD^+. To understand the significance of NAD^+ depletion in PARP1 mediated oxidative stress induced cell death, exogenous addition of NAD^+ was done. Addition of NAD^+ prevented PARP1 mediated oxidative stress induced cell death at low doses upto 10 mM NAD^+, nevertheless led to an anticipated increase in PARP1 activity. NAD^+ significantly prevented oxidative stress induced cell death in *D. discoideum*. Exogenous NAD^+ averted depletion of cellular NAD^+ and mitochondrial membrane potential changes that were triggered by oxidative stress, without getting affected by the elevated ROS levels. Altogether, this study ascertains that NAD^+ replenishment overcomes cadmium or H_2O_2 induced cell death by preventing cellular energy collapse incited by PARP1 activation. Thus, our results explicitly demonstrate that PARP1 overactivation led NAD^+ depletion but not PARP1 activity *per se* is of consequential significance in causing oxidative stress induced *D. discoideum* cell death. Moreover, NAD^+ supplementation could be a beneficial approach in aging and age-related disorders mediated by PARP1.

Keywords: Metabolite, NAD^+ supplementation, Necrotic cell death, Paraptotic cell death, PARP1, Pharmacological inhibition

Oxidative stress is a perpetrator in age-related conditions while NAD^+ deficit is concurrent with aging, age-related pathologies and several diseases[1-3]. Overactivity of Poly (ADP-ribose) polymerases (PARP1) could be a connecting link between oxidative stress and NAD^+ depletion. As the ADP-ribose donor for PARP1 catalyzed PARylation reactions, NAD^+ becomes significant for PARP1 activity and in determining its consequences. That is, hyperactivation of PARP depletes cellular NAD^+ and ATP and may result in paraptotic and necrotic cell death[4-9]. This observation has led to the 'suicide hypothesis' which suggests that rapid catabolism of NAD^+ due to PARP1 activation affects cellular energy metabolism and ultimately leads to cell death[10]. Put simply, during aging, progressive generation of oxidative stress would augment DNA damage mounting PARP activation which would translate into significant and consistent utilization of NAD^+ and ultimately precipitate as age-linked pathologies. In support of this theory, NAD^+ supplementation exhibits a positive impact on aging[11], neurological and mitochondrial diseases[12] through DNA repair[13]; however, data from PARP activity perspective is missing.

In this study, we examined the effect of exogenously supplemented NAD^+ on oxidative stress induced PARP mediated cell death. We used *D. discoideum* as the model organism wherein we have established the role of PARP1 in paraptotic and necrotic cell death, development, and mitochondrial regulation[14-22] and showed the kinetics of cell death events[4-6,23]. The results obtained in this study highlight the significance and advantage of NAD^+ supplementation on PARP1 mediated oxidative stress induced paraptotic and necrotic cell death.

Materials & Methods

D. discoideum strains and growth conditions

D. discoideum AX-2 amoebae were cultured in HL5 medium pH 6.5 on a rotary shaker at 22°C and 150 rpm. Log phase cells at a density of ~2.5×10^6 cells/mL were used for experiments[6].

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Cell death analysis

Cell viability and integrity was monitored by trypan blue dye exclusion assay. Aliquots were removed and mixed with trypan blue in 1:1 ratio and cells were counted using hemocytometer.

ROS measurement

2×10^6 cells were harvested and washed with 1X SB (Sorenson's buffer) twice. 50 nM 2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA) was added to cells and incubated for 15 min at 22°C with shaking, followed by two washes with 1X SB. Fluorescence unit was measured by fluorimeter (F7000, Hitachi, Japan) using 200 µL sample diluted 5 times in 1X SB buffer. Excitation (λex) and emission (λem) wavelengths used for fluorometric studies were 480 and 525 nm, respectively.

PARP activity assay

PARP activity was assayed by indirect immunofluorescence as described previously[6] using anti-PAR mouse mAb (10H) (Calbiochem, Germany) at a concentration of 0.5 µg/mL and anti-mouse IgG (whole molecule) FITC conjugate (Sigma) at a dilution of 1:200.

NAD measurement

Intracellular levels of NAD^+ were determined using previously described method[4]. In brief, cells were exposed to cumene H_2O_2 or cadmium for 1 h and cultures were washed twice with ice cold PBS and NAD^+ was extracted with 1 mL of 0.5M perchloric acid and then neutralized with 1N KOH. NAD^+ levels were estimated by taking the absorbance at 570 nm following protein estimation.

Mitochondrial membrane potential change

The dye DiOC6 (3,3'-dihexyloxacarbocyanine iodide) (Sigma) was used to evaluate changes in mitochondrial membrane potential (MMP). Dissipation of the mitochondrial membrane potential reduces the affinity of binding of the dye. Briefly, ~2.0×10^6 cells were pelleted and washed twice with 1X SB. Cells were stained with DiOC6 (400 nM) for 15 min in dark and then washed once with 1X SB and monitored for the fluorescence[4].

Results

Toxicity effect of NAD^+ supplementation on *D. discoideum*

To standardize the NAD^+ concentration for replenishment studies, *D. discoideum* cells were treated with different concentrations of NAD^+ (1, 10 and 20 mM) and a trypan blue exclusion assay was done to assess cell death. 1 to 10 mM NAD^+ did not induce cell death while, 20 mM NAD^+ led to ~40% cell death (Fig. 1A). Based on these results 10 mM NAD^+ was selected for further studies.

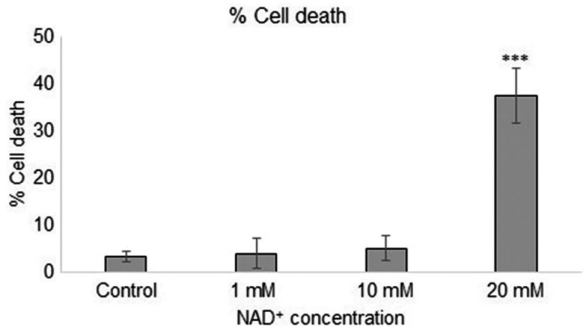

Fig. 1 — Dose dependent effect of exogenous addition of NAD^+ on *D. discoideum* cell death. Results are the mean ± SE of three independent experiments. ***P <0.001 compared to control

NAD^+ supplementation reduces oxidative stress induced cell death

Our previous studies have established paraptotic (LD_{25}) and necrotic (LD_{50}) doses of H_2O_2 and Cd[24]. In the present study, we used the paraptotic and necrotic doses of the oxidants to study the protective effect of NAD^+ supplementation on *D. discoideum* paraptotic and necrotic cell death, respectively. *D. discoideum* cells were supplemented with 10 mM NAD^+ for 2 h prior to oxidant exposure (H_2O_2 or Cd) and cell death was monitored by trypan blue assay after 24 h of treatment.

As can be seen in Figure 2A, NAD^+ supplementation partially intercepted Cd-induced cell death in *D. discoideum*. Paraptotic cell death with Cd was found to be reduced from 25% to 12% in cells pre-treated with 10 mM NAD^+, whereas, necrotic cell death was reduced from 50% to 30% upon 10 mM NAD^+ treatment.

Similarly, *D. discoideum* cells were supplemented with 10 mM NAD^+ for 2 h before H_2O_2 treatment and cell death was monitored by trypan blue assay. As can be seen in Figure 2B paraptotic cell death was reduced from 25% to 8% with 10 mM NAD^+, while necrotic cell death was reduced from 50% to 30% with 10 mM NAD^+ supplementation. Thus, NAD^+ supplementation could rescue paraptotic and necrotic cell death in *D. discoideum.*

Effect of NAD^+ supplementation on oxidative stress induced ROS production

ROS levels were monitored after 10 min of Cd and H_2O_2 treatment to find out whether NAD^+

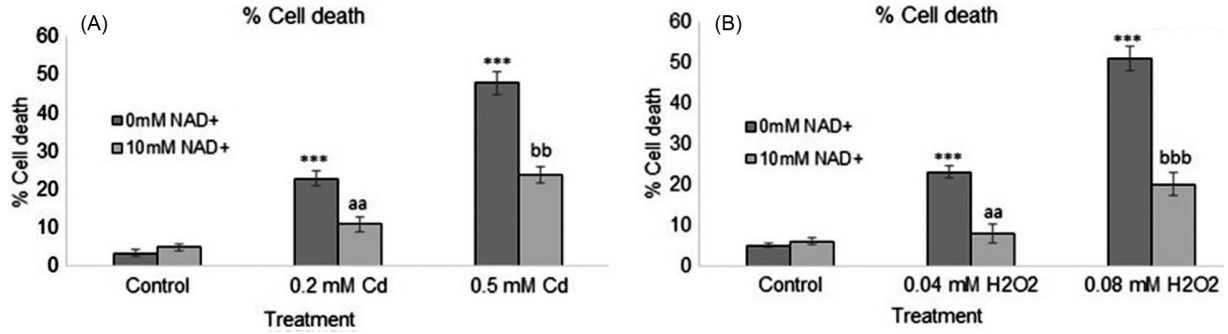

Fig. 2 — Effect of exogenous addition of NAD$^+$ on oxidative stress induced cell death (A) NAD$^+$ supplementation effect was observed on Cd induced cell death. 10 mM NAD$^+$ partially restored the Cd induced cell death. ***P <0.001 compared to control; aa & bb P <0.01 compared to 0.2 mM and 0.5 mM Cd, respectively; and (B) NAD$^+$ supplementation effect was observed on H_2O_2 induced cell death. 10mM NAD$^+$ partially restored the H_2O_2 induced cell death. ***P <0.001 compared to control; aa P <0.01compared to 0.04 mM H_2O_2; bbb P <0.001 compared to 0.08 mM H_2O_2

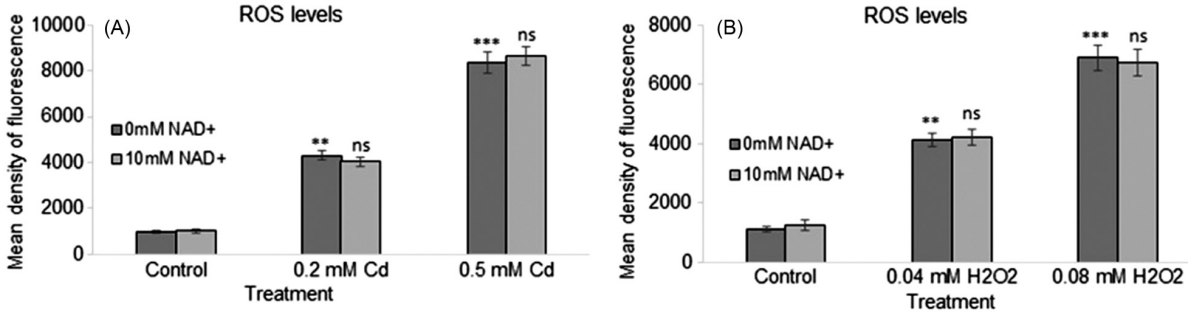

Fig. 3 — Effect of exogenous addition of NAD$^+$ on Cd and H_2O_2 induced ROS production. (A) No change was observed in ROS levels with NAD$^+$ supplementation in Cd treated *D. discoideum*. ***P <0.001; **P <0.01 compared to control; ns - non significant as compared to respective Cd doses; and (B) ROS levels were unchanged upon exogenous addition of NAD$^+$ after H_2O_2 treatment. ***P <0.001; **P <0.01 compared to control; ns- non significant as compared to respective treatment

supplementation reduces the ROS that was produced by the oxidants.

As can be seen from the Figures 3A & 3B, NAD$^+$ (10 mM) supplementation, had no effect on the ROS production 10 min post treatment of Cd and H_2O_2.

NAD$^+$ supplementation augments oxidative stress induced PARP1 activation

PARP1 gets activated in response to DNA damage. PARP1 activity in *D. discoideum* cells was assayed 5 min post oxidant treatment. PARP1 activity increased initially and peaked at 5 min post exposure to paraptotic and necrotic doses of Cd and H_2O_2. It was observed that NAD$^+$ supplementation further increased the PARP1 activity significantly 5 min post treatment of Cd and H_2O_2 (Fig. 4A & B).

NAD$^+$ supplementation rescues oxidative stress induced NAD$^+$ depletion

As per our hypothesis, NAD$^+$ may act as a currency coin or signalling molecule during oxidative stress induced PARP1 mediated cell death. NAD$^+$ supplementation could significantly rescue Cd and H_2O_2 induced NAD$^+$ depletion.

40% NAD$^+$ depletion was seen in paraptotic dose of Cd, while NAD$^+$ levels could be restored to 87% when *D. discoideum* cells were pre-treated with 10mM NAD$^+$ prior to Cd stress induction. However, 70% NAD$^+$ depletion was seen in a necrotic dose of Cd which could be restored to 65% in 10mM NAD$^+$ pre-treated cells (Fig. 5A). Similarly, 10mM NAD$^+$ also partially restored NAD$^+$ levels when H_2O_2 was used as the oxidant (Fig. 5B).

NAD$^+$ supplementation rescues oxidative stress induced MMP changes

Mitochondria play a crucial role in life and death processes. As mitochondrial membrane potential (MMP) change is the first event to be sensed NAD$^+$ depletion, if NAD$^+$ depletion is averted, mitochondria may function normally. We studied the change in MMP by using the mitochondrial membrane potential ($\Delta\psi_m$)-sensitive fluorescent dye $DiOC_6$ and monitored the changes in fluorescence quantitatively by fluorimeter and visually by fluorescence microscope.

As can be seen in Figures 6A-6D paraptotic and necrotic doses of Cd and H_2O_2 induced reduction

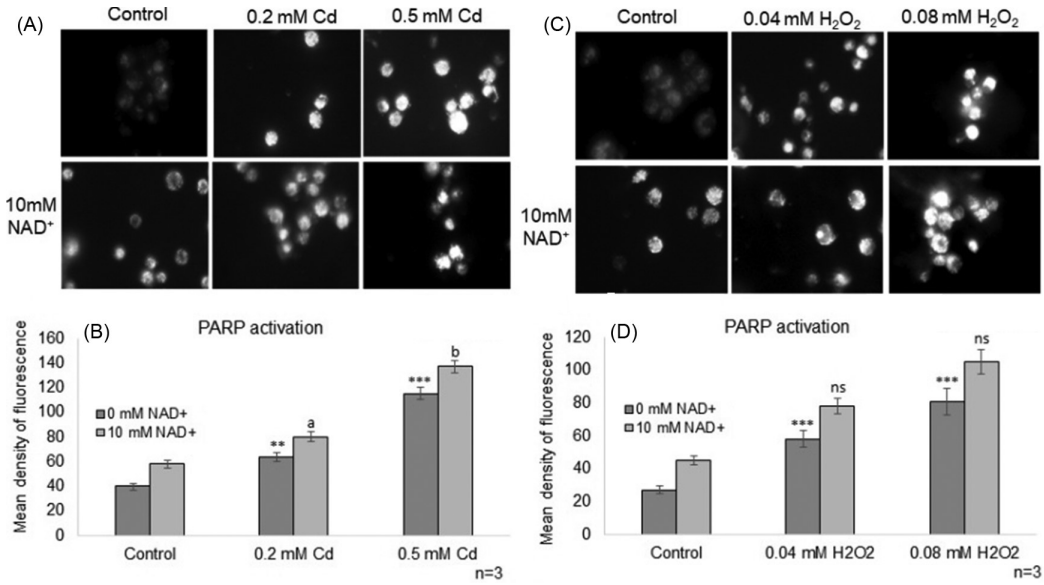

Fig. 4 — PARP activation after exogenous addition of NAD^+ in oxidative stress treated *D. discoideum*. (A) Effect of exogenous addition of NAD^+ on Cd induced PARP1 activation; (B) Densitometric analysis of effect of exogenous addition of NAD^+ on Cd induced PARP1 activation. ***P <0.001; **P <0.01 as compared to control; a P <0.05 as compared to 0.2 mM Cd; b P <0.05 as compared to 0.5 mM Cd; (C) Effect of exogenous addition of NAD^+ on H_2O_2 induced PARP1 activation; and (D) Densitometric analysis of effect of exogenous addition of NAD^+ on H_2O_2 induced PARP1 activation. ***P <0.001; ns- non significant as compared to respective control

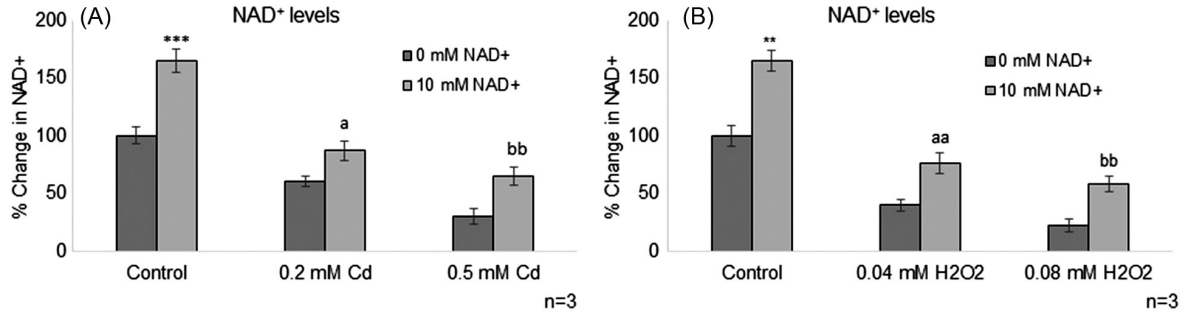

Fig. 5 — Effect of exogenous addition of NAD^+ on oxidative stress induced NAD^+ depletion. (A) NAD^+ levels were restored in *D. discoideum* cells pre-treated with 10 mM NAD^+ prior to Cd stress induction. ***P <0.001 compared to control; a P <0.05 compared to 0.2 mM Cd; bb P <0.01 compared to 0.5 mM Cd; and (B) Partial restoration was also observed upon exogenous addition of NAD^+ on H_2O_2 induced NAD^+ depletion. **P <0.01 compared to control; aa P <0.01 compared to 0.04 mM H_2O_2; bb P <0.01 compared to 0.08 mM H_2O_2

of MMP could be prevented significantly by NAD^+supplementation. NAD^+ supplementation partially restored MMP changes induced by the paraptotic dose of Cd and significantly restored MMP at both paraptotic and necrotic doses of H_2O_2.

Discussion

Nicotinamide adenine dinucleotide (NAD^+) is known to regulate major processes in a cell, including cell metabolism and DNA repair. NAD^+ controls the bioenergetics of a cell. It serves as a coenzyme for several proteins, including SIRTs and PARPs.

Changes in NAD^+ levels are associated with aging and age-related pathologies. Several pathological conditions can be improved by elevating the overall content of NAD^+. With aging there is increased oxidative stress and decreased DNA repair attributed to low NAD^+ levels[25]. Recent years have seen an increasing interest in the scientific community for NAD^+ and NAD^+ metabolism, and its role in mitochondrial dysfunction and aging. Tremendous efforts are being put into studying the therapeutic role of NAD^+ in aging. Several studies on NAD^+ supplementation have shown profound effects on aging and age-related disorders. Pioneering work has been done using *in vivo* models such as *C. elegans* and rodents[2,26]. Maintaining the overall NAD^+ levels has proved to be an effective strategy to combat

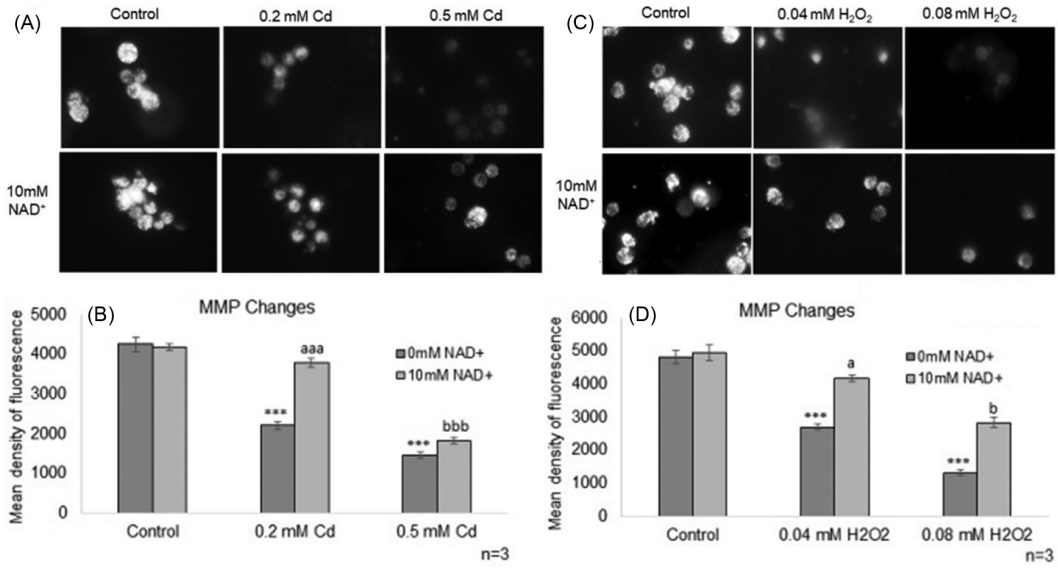

Fig. 6 — Effect of exogenous addition of NAD$^+$ on oxidative stress induced mitochondrial membrane potential changes (A) Cd induced reduction of MMP was significantly prevented by NAD$^+$ supplementation; (B) Exogenous addition of NAD$^+$ prevented MMP changes induced by Cd as monitored by fluorimeter. ***P <0.001 compared to control; aaa & bbb P <0.001 compared to 0.2 mM Cd and 0.5 mM Cd, respectively; (C) H_2O_2 induced reduction of MMP was significantly prevented by NAD$^+$ supplementation; and (D) Exogenous addition of NAD$^+$ prevented MMP changes induced by H_2O_2. ***P <0.001 compared to control; a P <0.05 compared to 0.04 mM H_2O_2; b P <0.05 compared to 0.08 mM H_2O_2

numerous diseases including cancer[27]. Administration of NAD$^+$ precursors such as nicotinamide riboside (NR) has shown to prevent oxidative stress, organ injury and improved the survival in sepsis[28]. Another NAD$^+$ precursor dihydronicotinamide riboside (NRH) induces cell specific cytotoxicity in HepG3 cancer cells[29], suggesting altered fate of NRH in cell specific manner.

PARP1 consumption of NAD$^+$ has led to either parthanatos[10] or survival of damaged cells by switching to oxphos-mediated metabolism[30]. Previous studies from our lab showed the importance of PARP inhibition in stress induced cell death. Oxidative stress induced cell death is caused by ROS production leading to, PARP1 activation followed by NAD$^+$ depletion and ultimately cell death[24]. In the present study, we observed the effect of NAD$^+$ supplementation on oxidative stress induced PARP1 mediated cell death of *D. discoideum*.

Under basal conditions (no oxidative stress) 20 mM NAD$^+$ led to ~20% cell death; supplementation with lower concentrations induced no toxicity. Firstly, these data suggest cellular uptake of NAD$^+$ by *D. discoideum* cells. Secondly, the data corroborates with the fact that these unicellular organisms have higher basal ROS levels[31] and therefore have higher tolerance for NAD$^+$, whereas for human retinal pigment epithelium (RPE) cells, supplementation even with as low as 1 mM NAD$^+$ was toxic[32]. Nonetheless, supplementation with non-toxic doses of NAD$^+$ reduced oxidative stress induced cell death in *D. discoideum*. These results suggest NAD$^+$ could rescue oxidative stress induced cell death in a dose dependent manner.

One possibility for this protective effect of NAD$^+$ supplementation could be by ROS/oxidative homeostasis. ROS estimations were done with various pre-treatments to find out if NAD$^+$ supplementation rescued oxidative stress induced paraptotic and necrotic cell death in *D. discoideum* by scavenging the ROS. No change was observed in ROS levels induced by Cd or H_2O_2 with NAD$^+$ supplementation (Figs. 3A & B).

Another mechanism for the protective effect of exogenous NAD$^+$ could be by maintaining physiological NAD$^+$ homeostasis. The exogenous addition of NAD$^+$ restores intracellular NAD$^+$ levels and counteracts cell death induced by the novel drug FK866[33]. We have previously shown activation of PARP1 after oxidative stress depletes cellular NAD$^+$ and ultimately cell death. Therefore, exogenous addition of NAD$^+$ prevents cellular NAD$^+$ depletion subsequent to PARP1 activation, preventing oxidative stress induced cell death. Interestingly, however, NAD$^+$ being a substrate for PARP, its supplementation showed increased PARP activation

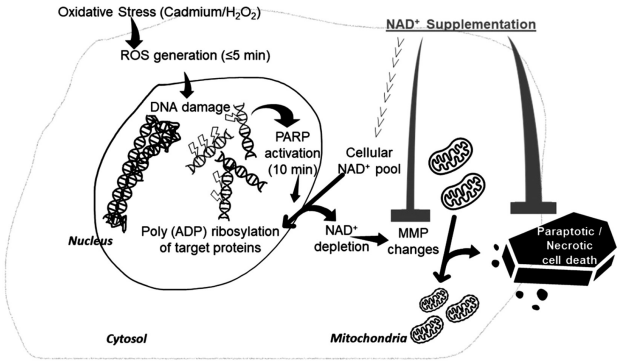

Fig. 7 — Summarization of the effect of NAD^+ supplementation on oxidative stress induced cell death in *D. discoideum*: Oxidative stress cause DNA damage that leads to PARP activation. PARP utilizes NAD^+ to PARylate its substrates. Therefore, over activation of PARP depletes cellular content of NAD^+ that later reflects in mitochondrial damage and cellular death. Supplementation with exogenous NAD^+ under such conditions replenishes cellular NAD^+ pools and prevents the ensuing mitochondrial impairment and death without affecting the upstream events in *D. discoideum* cells

in a dose dependent manner (Figs. 4A and 4B). Significant rescue in cell death with exogenous NAD^+ despite increasing PARP1 activity further projects PARP1 as an "Innocent killer protein" (as its actual role is to recruit DNA repair machinery upon DNA damage). These results are in par with the recent evidence where the exogenous addition of NAD^+ enhances the DNA repair capacity mediated by PARP1[34]. This further suggests ROS production to be an event upstream to PARP1 activation as we proposed in our earlier studies[4,7,24].

To find out the signal which is involved during nuclear-mitochondrial cross-talk we monitored the NAD^+ levels. Our results showed a 62% and 78% reduction in NAD^+ levels in paraptotic and necrotic doses of Cd and H_2O_2[24]. Additionally, NAD^+ supplementation showed maximum rescue in cell death. NAD^+ supplementation studies in mice have demonstrated beneficial effects in muscle regeneration[26,35], cardiovascular function[36], improved glucose metabolism[37], mitochondrial rejuvenation[12] and also alters body composition[38].

Irrespective of the morphological features of end-stage cell death (that may be apoptotic, necrotic, autophagic, or mitotic), mitochondrial membrane permeabilization (MMP) is frequently the decisive event that commits the cell to death[39]. The inhibition of MMP changes constitutes an important strategy for the pharmacological prevention of unwarranted cell death. Reduction in NAD^+ content in a cell induces redox imbalance followed by mitochondrial damage[40]. Diet supplementation with NAD^+ precursors partially recover mitochondrial defects[41] and reverts metabolic impairment[42]. Reportedly, NAD^+ boosting therapies also improve mitochondrial functioning[43]. Our results showed a dose-dependent effect of oxidative stress on MMP changes, where NAD^+ depletion caused by PARP1 overactivation triggers changes in MMP. With Cd and H_2O_2, a reduction in MMP was observed which was partially restored with NAD^+ supplementation (Fig. 6A & B). NAD^+ supplementation showed better rescue in MMP changes as compared to our previous data with other pharmacological agents like benzamide. This suggests NAD^+ supplementation as one of the effective approaches to reverse oxidative stress induced cell death. This strategy would be effective in diseases like neurodegenerative disease[44], and diabetes also where NAD, purine and pyrimidine levels were reduced drastically[45]. As can be seen, NAD^+ pre-treated *D. discoideum* cells could prevent NAD^+ depletion followed by MMP changes induced by oxidative stress. In other words, NAD^+ depletion is a turning point during the entire cell death cascade, suggesting NAD^+ to be the "Currency coin" during nuclear-mitochondrial cross-talk (Fig. 7). Thus, preventing NAD^+ depletion could block the downstream events leading to cell death. Extracellular NAD^+ is reported to be effective not only in age related pathologies but is found to have beneficial effects in immune cell modulation and osteoporosis[46-47].

Conclusion

Our results strongly support that replenishment of NAD^+, despite it being the central metabolite in several biological pathways, would serve the best as a pharmacological agent against diseases related to oxidative stress induced PARP1-mediated cell death. Altogether, these results imply that NAD^+ supplementation rescues PARP1 mediated oxidative stress induced cell death (Fig. 7) and could be a beneficial approach in aging and age-related disorders mediated by PARP1.

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Conflict of interests

All authors declare no conflict of interest.

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