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ORIGINAL ARTICLE

Low-dose curcumin reduced TNBS-associated mucin depleted foci in mice by scavenging superoxide anion and lipid peroxides, rebalancing matrix NO synthase and aconitase activities, and recoupling mitochondria

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Abstract

Background The role of mitochondrial dysfunction in the pathogenesis of inflammatory bowel diseases (IBD) is still being investigated. This study evaluated the therapeutic effect of curcumin (Cur), a polyphenolic electrophile in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced chronic colitis and mitochondrial dysfunction, in mice.

Methods Colitis was induced by rectal instillation to mice of 30 mg kg^{-1} TNBS, alone or followed by daily intraperitoneal injections of Cur 25 mg kg^{-1}. Animals were euthanized at days 3, 7, and 14, post TNBS challenge. Colon mitochondria of control mice were treated with 5 µM Cur, and TNBS (50, 100 µM)-toxicity was evaluated by measuring swelling, respiration, and aconitase and fumarase activities. Redox status was evaluated in colon mucosa and in mitochondria.

Results In vitro, a short-term Cur treatment controlled the dose and time dependent mitochondrial toxicity induced by TNBS, by collapsing the generation of superoxide anion and hydroperoxy lipids, rebalancing nitric oxide synthase and aconitase activities, and recoupling mitochondria. In vivo, a daily low-dose Cur abolished mice mortality which reached 27% in model group. Cur improved in a time dependent manner mucosal redox homeostasis, cell apoptosis, mucin depleted crypts and crypt abscesses by controlling prooxidant activity of myeloperoxidase and NO synthase associated to phagocytes influx, quenching hydroperoxy lipids, and reboosting GSH levels.

Conclusion Cur, by quenching intra and extra mitochondrial ROS generation, rebalancing aconitase/fumarase and MDA/GSH ratios, and recoupling mitochondria, may support mithormesis priming and remitting in IBD.

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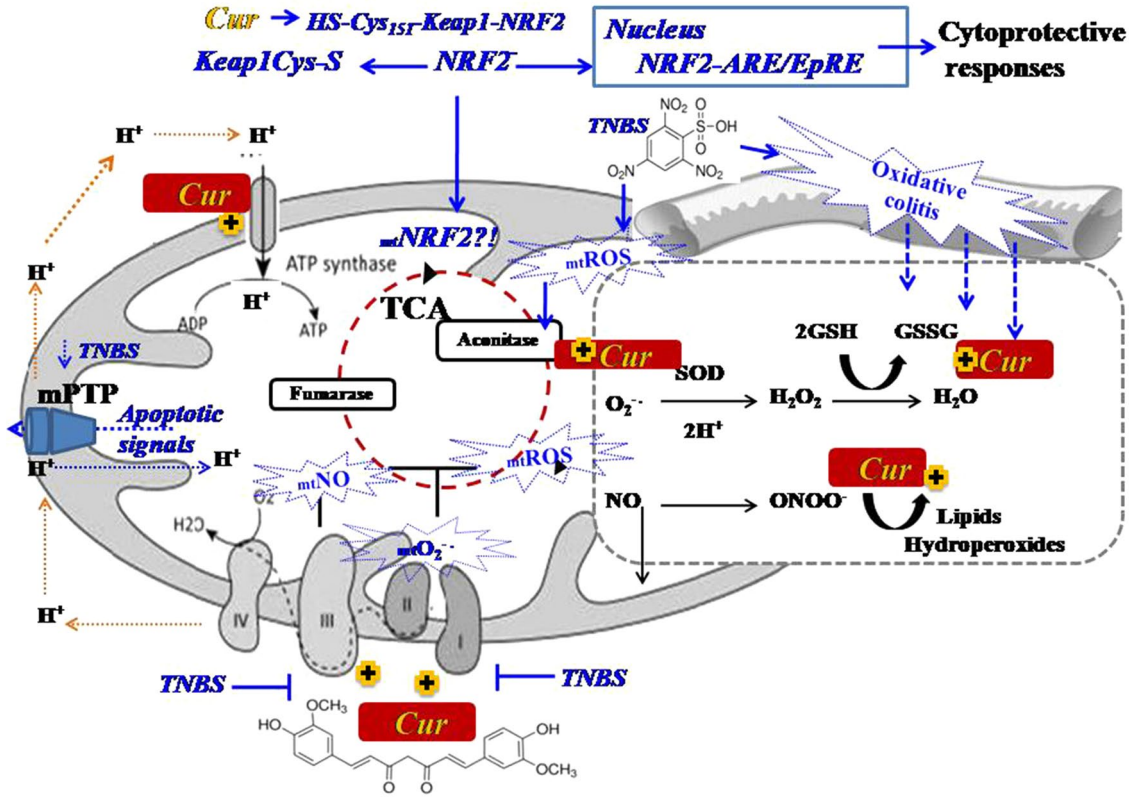
Graphic abstract



Keywords Aconitase · Curcumin · Electrophile · MDA/GSH · Mucin depleted crypts · TNBS

Abbreviations

CD	Crohn's disease
Cul3	Cullin3-based Cullin-RING E3 ubiquitin ligase
Cur	Curcumin
ARE/EpRE	Antioxidant response element/electrophile response element
ETC	Electron transfer chain; GSH, reduced glutathione
IBD	Inflammatory bowel diseases
I(O)MM	Inner (outer) mitochondrial membrane
iNOS	Inducible NO synthase
Keap1	Kelch-like ECH associated protein 1
MDA	Malondialdehyde
MPO	Myeloperoxidase
mPTP	Mitochondrial permeability transition pore
NO	Nitric oxide
NOX2	NADPH Oxidase2
$O_2^{\cdot -}$	Superoxide anion
NRF2	Nuclear factor (erythroid-derived 2)-like 2
OxPhos	Oxidative phosphorylation
mt	Mitochondria
PN	Neutrophils
ROS	Reactive oxygen species
TNBS	2,4,6-Trinitrobenzene sulfonic acid

Introduction

Crohn's disease (CD) is a typical inflammatory bowel disease (IBD) affecting sensitive subjects developing chronic intestinal oxidative inflammation associated to imbalanced immune tolerance (Circu and Aw 2011; Novak and Mollen 2015). Mitochondrial stress has been suggested as a predisposing factor initiating intestinal inflammation through increase of epithelial permeability to gut microbiota (Nazli et al. 2004; Lewis and McKay 2009; Wang et al. 2014).

Mitochondria are dynamic autonomous self-replicating organelles, centrally involved in cell redox homeostasis (Murphy 2009; Handy and Loscalzo 2012; Brand 2016; Spinelli and Haigis 2018). One characteristic feature of mitochondria is to provide cells in energy by oxidative phosphorylation (OxPhos), a bioenergetic process coupling electrons transfer through the respirasomes (respiratory

complexes I–IV, ETC), with the phosphorylation of ADP to ATP by ATP synthase. Physiologically, few electrons leaked out from respirasomes I and III to reduce oxygen into superoxide anion ($O_2^{\cdot-}$) (Wong et al. 2017). This OxPhos by product may react with nitric oxide (NO) released by matrix NO synthase, to generate various reactive oxygen and nitrogen species (mtROS) (Murphy 2009).

The biphasic discharge of low-dose mtROS involved in eustress conditions and redox signaling pathways and high-dose mtROS causing nitro-oxidative stress, are characteristic features of mithormesis (Brand 2016; Wong et al. 2017).

Abrupt mtROS levels can cause major changes to mitochondria architecture and function (Zorov et al. 2014).

The histones free mtDNA is critically sensitive to nitro-oxidative stress, bringing cell into adaptative response, or apoptosis (Chen et al. 2005; Circu and AW 2011; Izem-Meziane et al. 2012). Also, ROS attack of polyunsaturated fatty acids of phospholipid bilayer generates hydroperoxy lipids, primarily hydroxynonenal (HNE) and malondialdehyde (MDA). These aldehydic adductors exacerbate mitochondrial permeability transition pore (mPTP) opening, leading to mitochondria uncoupling, and the collapse of mitochondrial membrane potential in the expense of ATP synthesis (Chen and Yu 1994; Bernardi et al. 2015). In addition, cardiolipin, a complex phospholipid exclusive to the inner mitochondrial membrane (IMM) leaflet, is intimately associated with respirasomes. Its ROS-dependent oxidation strongly alters the fluidity of lipid bilayer and impairs electrons transfer chain (ETC) activity (Pfeiffer et al. 2003; Yin and Zhu 2012). More, the nitrosation of iron-sulfur (Fe-S)-containing proteins such as aconitase, a redox sensitive enzyme of tri carboxylic acid (TCA) cycle and respirasomes I and III, blocks TCA cycle and respiration, resulting in bioenergetic stress (Chen et al. 2005).

Because of the multifactorial etiology of IBD, and the dose dependent side effects of classical therapies, plant-derived secondary metabolites have been successfully assayed as alternative adjuvant treatments for IBD. They include numerous chemically related compounds polyphenols, flavonoids, terpens, alkaloids, etc.

Inflammation and pain are prominent symptoms associated-disability in Crohn's disease patients that highly impacts their quality of life (Grossi et al. 2019). Thus, in addition to their controlled use as adjuvant analgesics in chronic pain, the terpenopolyphenolic group of cannabinoids has been shown to be effective on mucosal oxidative

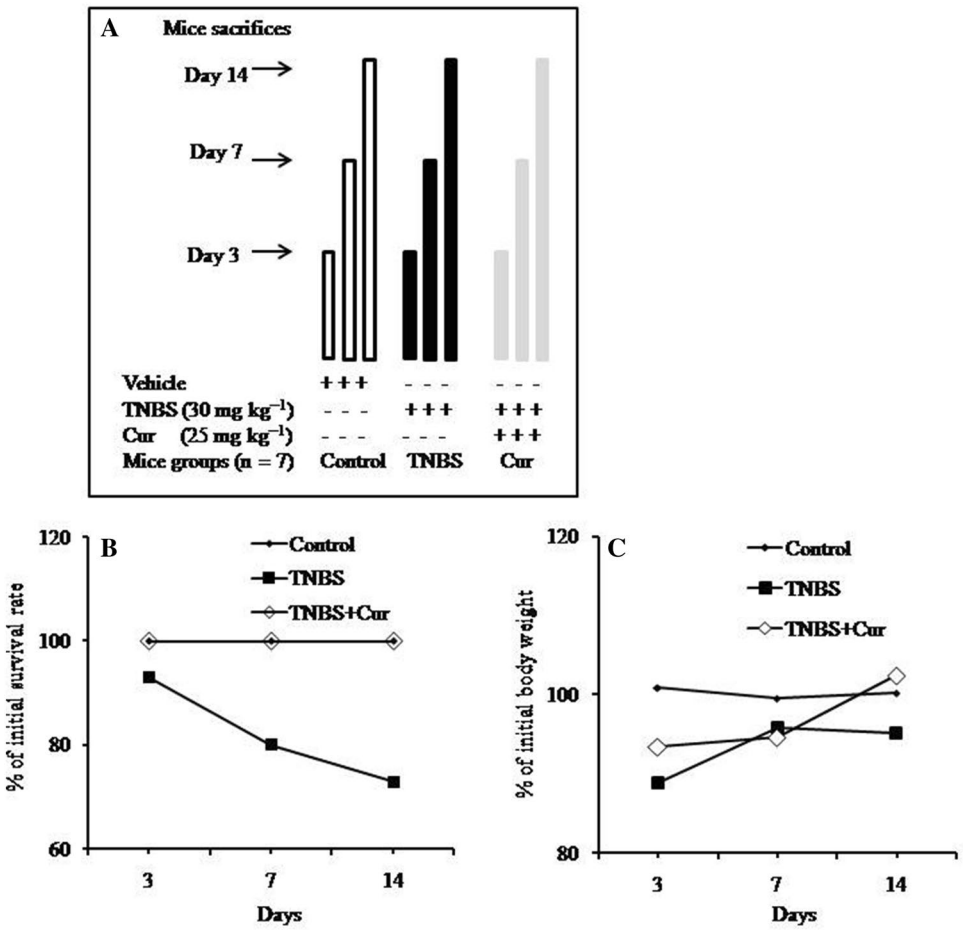


Fig. 1 Curcumin prevented TNBS-induced mice mortality and body weight loss. In vivo protocol for colitis induction in mice ($n=7$) using the haptenic weak acid 2,4,6 trinitrobenzene sulfonic acid (TNBS) (**a**). Mice were given 30% ethanol (control), TNBS (30 mg kg^{-1} in 30% ethanol, intrarectal instillation, ir. (TNBS group), or TNBS + Cur (25 mg kg^{-1}, intraperitoneal, ip; Cur group). Mice were euthanized at days 3, 7 and 14 days post-colitis induction. Evaluation of survival rate (**b**) and body weight variation (**c**)

inflammation (Millar et al. 2019; Pagano et al. 2016, 2019). They exert their pharmacological effects through the cross talk of various receptors namely, the G protein-coupled cannabinoid (CB1, CB2) receptors, and the transient receptors potential cation channel subfamily V (TRPV) (Zádor and Wollemann 2015). Likewise, curcuminoids was associated with significant reduction in the disease activity index (Szebeni et al. 2019).

Curcumin (Cur) [(1E,6E)-1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the major curcuminoid antioxidant of *Curcuma longa L.* This low biovailable lipophilic phytochemical displays multiple molecular pharmacological effects ranging from antioxidant, anti- inflammatory and anti-infectious to sensitizing addicted cancer cells to anticancer therapy (Saha et al. 2012; Heger et al. 2013; Kunnumakkara et al. 2017). The methoxy phenols and α,β-unsaturated carbonyl (enone) electrophile backbone of Cur support its ROS scavenging capacity for superoxide anion, peroxinitrite, hydroperoxy lipids and hydroxyl radical generated by ferrous oxidation in Fenton reaction (Aggarwal et al. 2013, 2015; Kunnumakkara et al. 2017).

Cur can maintain eustress conditions through the reactivation of endogenous antioxidant system, while inhibiting ROS metabolizing enzymes primarily NADPH oxidases, NO synthases, cytochromes P450 and cyclooxygenases (Aggarwal et al. 2013, 2015; Peng et al. 2017).

This polyphenolic electrophile can modulate immune response through inhibition of redox sensitive kinases, and proinflammatory pathways involving the NLRP3 inflammasome and the transcription factorNF-kB (Aggarwal et al. 2013; Heger et al. 2013; Edwards et al. 2017; Luis et al. 2017; Hennig et al. 2018). Cur can restore adaptive cell response to nitro-oxidative stress by thiols modulation of Kelch-like ECH associated protein 1 (Keap1), an endogenous inhibitor of the nuclear factor (erythroid-derived 2)-like 2(Nrf-2). The latter is the master transcription factor of genes for NADPH and GSH biosynthesis and for antioxidant and detoxifying enzymes (Balogun et al. 2003; Li and Kong 2009; Edwards et al. 2017; Luis et al. 2017).

This study investigated the potential of Cur in a mice model of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-associated colitis and mitochondrial toxicity.

Materials and methods

Ethics statement

The experiments were performed in accordance with the Ethical Committee for animals' welfare of University of Science and Technology Houari Boumediene [ATRSS 2011 (ATRSS/PNR 08/N_305)], and with Algerian Legislation for the protection of animal experiments used in scientific purposes [Law No. 12-235/2012; Executive Decrees No. 10-80/10-90].

Adult male NMRI mice from Institut Pasteur (Algiers, Algeria), were kept under controlled conditions, and experienced with chemicals obtained from Sigma Aldrich (St Louis, MO), except otherwise indicated.

Evaluation of low-dose curcumin treatment in TNBS-induced colitis in mice

In vivo *protocol*

Colitis was induced in 3 model groups of mice ($n = 7$) instilled rectally with 100 µl TNBS (30 mg kg^{-1} in 30% ethanol) (Morris et al. 1989). Three additional groups of mice received daily intraperitoneal injections of curcumin (Cur, 25 mg kg^{-1}), according to previous reports (Ukil et al. 2003; Mouzaoui et al. 2012), immediately after TNBS challenge. Mice were observed for mortality and body weight loss, and were serially euthanized by cervical translocation at days 3, 7, and 14 post colitis induction. Mice instilled with 30% ethanol (control group) were sacrificed at day 14 (Fig. 1). Colons were cut opened longitudinally, washed with ice-cold saline and scored for macroscopic damages (Fedorak et al. 1992).

Histological analysis of colonic lesions

Distal and proximal colons samples were fixed overnight in 10% phosphate buffered formalin and processed for histological analysis. Thin sections stained with hematoxylin–eosin (H&E) or periodic-acid-Schiff (PAS), were analyzed for colon architecture and mucus content by light microscopy (Carl Zeiss, Germany), and scored for microscopic damages (Neurath et al. 1995).

Hoechst 33258-stained colon sections were evaluated for apoptosis (Yamaoka et al. 2008). Apoptotic cells displaying high fluorescence intensity due to chromatin condensation and nuclear fragmentation, were counted in 10 fields under fluorescence microscopy (Carl Zeiss inc., Thornwood, NY).

Preparation of colon homogenates

Colonic tissues were homogenized in ice-cold 50 mM phosphate buffer (pH 7.4) containing 0.5%Triton X-100 (Ultraturrax $T_{25,}$ England). After freeze-thawing three times, homogenates were centrifuged at 10,000*g*, 15 min at 4 °C. The resulting supernatants were stored at − 40 °C.

Evaluation of short-term curcumin treatment in TNBS-induced mitochondrial toxicity

Isolation of colon mitochondria

Colon mitochondria were isolated according to the method of Towers et al. (1972), with slight modifications. Colons recovered from control mice were homogenized in ice cold 10% (*w:v*) MOPS (10 mM, pH 7.4) containing 125 mM sucrose and 0.05% BSA (Ultra-turrax $T_{25,}$ England). Unbroken cells and nuclei were pelleted at 800 g, 5 min at 4 °C. Supernatants were further centrifuged at 12,000*g*, 20 min at 4 °C. Crude mitochondria were washed twice and suspended in ice cold respiratory buffer (pH 7.4), and used within 2 h.

Mitochondrial swelling assay

Swelling was measured as an index of mitochondrial permeability transition pore (mPTP) opening (Masubuchi et al. 2002). Colon mitochondria (0.5 mg prot ml^{-1}) were incubated in 10 mM MOPS buffer (pH 7.4) containing 125 mM sucrose, 5 mM succinate, 20 µM $CaCl_2$ with or without 5 µM Cur (Izem-Meziane et al. 2012), 10 min at 25 °C. The change in matrix volume following 50 and 100 µM TNBS treatment was monitored at 540 nm, 10 min at 25 °C. Results were expressed as ΔOD_{540nm} mg^{-1} prot.

Mitochondrial complex I assay

The rate of NADH oxidation was used as a measure of complex I activity (Kuznetsov and Gnaiger 2003). Colon mitochondria were disrupted by sonication and complex I activity was measured in 50 mM phosphate buffer (pH 7.4) containing 0.1 mM ubiquinone-1, 1 mM KCN and 2 mM NaN_3, with or without 5 µM Cur for 5 min. Complex I activity was monitored in the presence of 50 or 100 µM TNBS at 340 nm for 2 min. Results were expressed as µmol of NADH mg^{-1} prot.

Determination of respiration and superoxide anion production

Mitochondria (0.5 mg prot ml^{-1}) were incubated in 15 mM MOPS buffer (pH 7.4) containing 200 mM mannitol, 50 mM sucrose, 150 mM KCl, 0.5 mM EDTA, 50 µM KCN, 1 mM potassium ferricyanide [$K_3Fe(CN)_6$], with or without 5 µM Cur for 5 min. The respiration was initiated by 5 mM succinate in the presence of 50 or 100 µM TNBS, and followed at 420 nm, 5 min at 25 °C (Forman and Wilson 1982).

Table 1 Curcumin improved macroscopic and microscopic scores damage in TNBS-induced colitis

Treatments (days)	Macroscopic scores	Microscopic scores	
		Distal colon	Proximal colon
Control	0.6 ± 0.2	0.4 ± 0.2	0.4 ± 0.2
TNBS (3)	2.8 ± 0.4**	2.2 ± 0.4**	1.2 ± 0.6*
TNBS (7)	3.2 ± 0.4***	2.8 ± 0.4**	1.6 ± 0.6**
TNBS (14)	3.6 ± 0.2***	1.8 ± 0.4*	2.2 ± 0.4***
TNBS + Cur (3)	1.2 ± 0.4$^{\#\#}$	1.6 ± 0.5$^{\#}$	0.8 ± 0.4$^{\#}$
TNBS + Cur (7)	1.6 ± 0.2$^{\#\#}$	0.8 ± 0.4$^{\#\#}$	1.0 ± 3 $^{\#}$
TNBS + Cur (14)	1.2 ± 0.5$^{\#\#}$	0.6 ± 0.4$^{\#}$	0.6 ± 0.4$^{\#\#}$

Mice were given 30% ethanol (control), TNBS (30 mg kg^{-1} in 30% ethanol, TNBS group), or TNBS + Cur (25 mg kg^{-1}; Cur group) and euthanized at days 3, 7 and 14 days post-colitis induction. Colons were evaluated for macroscopic and microscopic damages (Fedorak et al. 1992; Neurath et al. 1995). Results from mice treated with TNBS (30 mg kg^{-1}) were matched to control mice with Mann Whitney's *U* test. Data are means ± SEM of 7 mice for each group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. TNBS treated group

Results were expressed as µmol of reduced potassium ferricyanide mg^{-1} prot.

The potential of Cur to scavenge superoxide anion ($O_2^{\cdot-}$) was determined using mitochondria (0.5 mg prot ml^{-1}) incubated in 10 mM Tris buffer (pH 7.4) containing 250 mM sucrose, 100 µM cytochrome c, with or without 5 µM Cur for 5 min. After addition of 50 or 100 µM TNBS, the generation of $O_2^{\cdot-}$ was monitored by the reduction of cytochrome c at 550 nm, 2 min at 25 °C. Results were expressed as µM $O_2^{\cdot-}mg^{-1}$ prot.

Determination of mitochondrial aconitase and fumarase activity ratio

Aconitase activity was measured by monitoring the formation of *cis*-aconitate from isocitrate at 240 nm in 50 mM Tris–HCl buffer (pH 7.4) containing 0.6 mM $MnCl_2$ and 2 mM isocitrate (Hausladen and Firdovich 1996). One unit (U) was defined as the amount of enzyme necessary to produce 1 µmol of cis-aconitate min^{-1}. Fumarase activity was determined by measuring the increase in absorbance at 240 nm in 30 mM potassium phosphate buffer (pH 7.4) and 5 mM succinate (Racker 1950). One unit (U) was defined as the amount of enzyme that produces 1 µmol of fumarate min^{-1}.

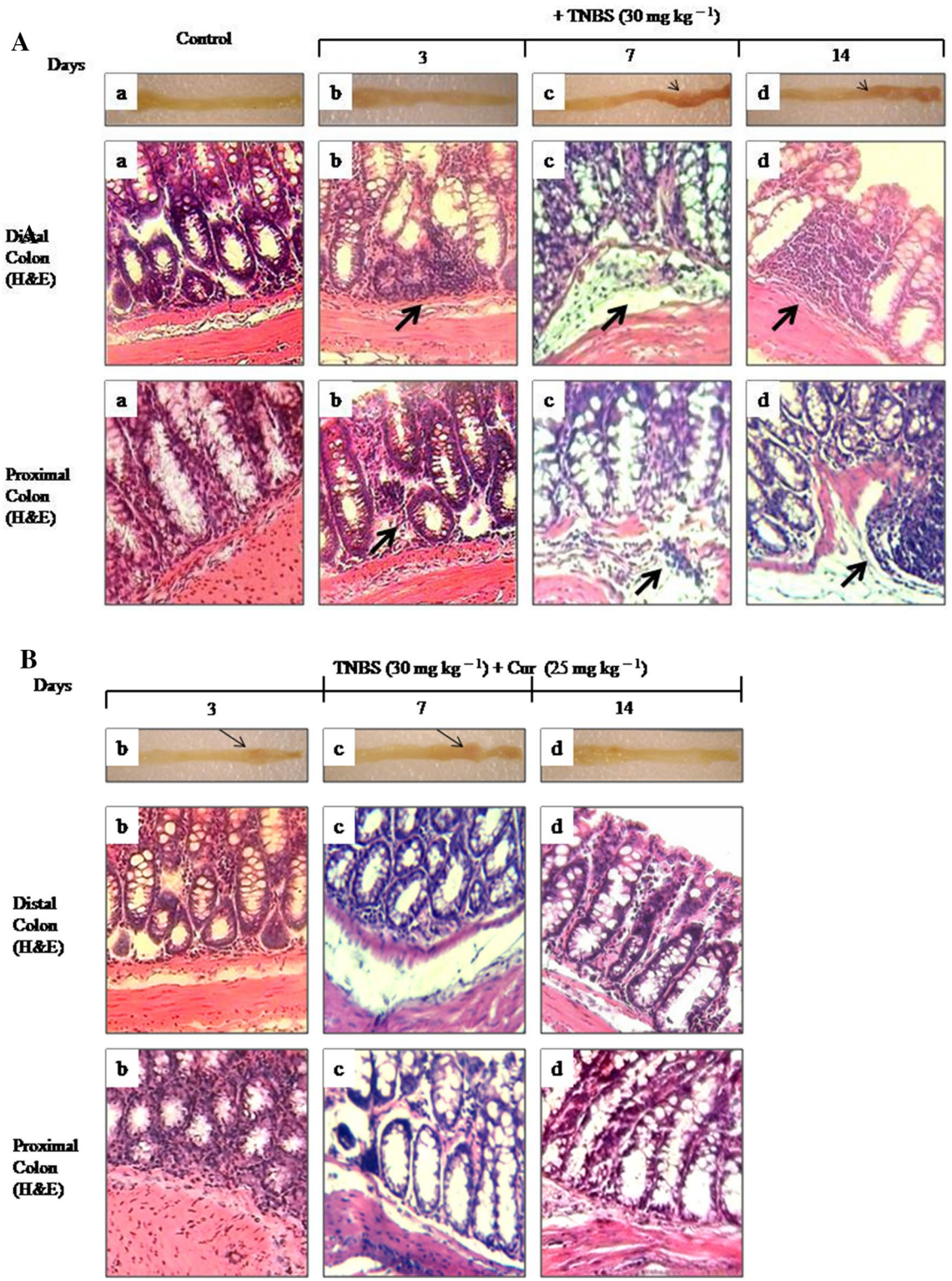


Fig. 2 Curcumin improved TNBS-induced macroscopic and microscopic colon lesions. Mice receiving vehicle (control group), TNBS (30 mg kg^{-1}, ir; TNBS group) alone (**A**) or followed by daily treatment of Cur (25 mg kg^{-1}, ip; Cur group) (**B**) were sacrificed at days 3, 7 and 14 post colitis induction. Specimens of distal and proximal colons were submitted to histological analysis as described in materials and methods section. Hematoxylin and eosin (H&E)-stained colon sections of control (**a**) and TNBS-treated mice for 3 (**b**), 7 (**c**), 14 (**d**) days in distal and proximal colon were analyzed by light microscopy (400 ×). Representative H&E-stained colon sections of luminal wall of colon mucosa showing foci of hemorrhages (arrowhead), inflamed areas (arrow). Microscopic damage were assessed according to Neurath et al. (1995)

Biochemical evaluation of colon mucosa and mitochondria redox status

The level of reduced glutathione (GSH) was measured as a marker of antioxidant potential in colon homogenates and mitochondria (nmol of GSH mg^{-1} prot) (Ellman 1959). Nitric oxide (NO) synthase activity was measured by nitrites content, the stable nitric oxide metabolite (Green et al. 1982), and expressed in µM nitrites mg^{-1} prot, using a sodium nitrite curve as standard. The level of malondialdehyde (MDA), a marker of lipid peroxides (nmol of MDA mg^{-1} prot) was measured as described (Ohkawa et al. 1979). Neutrophils recruitment into inflamed colons was determined by myeloperoxidase (MPO) activity (µM H_2O_2 consumed min^{-1} mg^{-1} prot) according to Krawisz et al. (1984). Biochemical values were normalized to samples protein concentration (Bradford 1976).

Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was performed using the Student's *t*-test, $p < 0.001$, 0.05 and 0.01 differences between groups were considered significant. Histological damage scores were analyzed by Mann–Whitney's *U* test.

Results

Low-dose Cur treatment erased TNBS-induced chronic nitro-oxidative colitis in mice

The protective effect of low dose Cur in TNBS-induced drastic mice mortality and chronic colitis scores, was checked at three end points (3, 7 and 14 days), post colitis induction.

Cur suppressed mice mortality by reducing colitis and histological scores

TNBS-colitis is an hapten sensitizer-based model mimicking the colonic immune dependent symptoms of Crohn's disease (Morris et al. 1989; Hölttä et al. 2008; Tu et al. 2017).

Our results show that TNBS induced 20–30% mice mortality, at the 2nd week post colitis induction. The surviving mice showed a time dependent increase in colitis scores (Table 1, Fig. 1).

TNBS-treated colons revealed hemorrhagic edemas in mucosa and submucosa infiltrated by mixte inflammatory cells. The early disruption of colon architecture, was associated with mucin depleted foci (MDF) as attested by the low number of alcian blue (AB) positive goblet cells. Crypt abscesses highly invaded by neutrophils occurred at the 2nd week, primarily in distal colon (Fig. 2A, B).

Daily low-dose Cur suppressed mortality and improved the hallmarks of chronic colitis, including crypt architecture, goblet cells number and mucin content more efficiently in proximal colon, compared with the damaged distal colon (Table 1; Figs. 2, 3).

Cur prevented phagocytes influx and redox imbalance in distal colon

One hallmark of IBD is the transmural infiltration of primed neutrophils and macrophages where they accumulate substantial ROS amounts generating persistent oxidative stress (Circu and Aw 2011; Mouzaoui et al. 2012; Aviello and Knaus 2016; Peng et al. 2017).

Compared with control, nitrites level enhanced by three- to fourfold, while MPO activity rapidly increased by sevenfold ($p < 0.001$) to stabilise at 3.5-fold of control at day 14, in inflamed distal colon ($p < 0.001$), indicating phagocytes dependent oxidative activity. MDA levels increased by three- to fivefold ($p < 0.01$), while GSH levels decreased by 62%, 83% and 52% ($p < 0.001$), respectively, compared to control.

Cell apoptosis increased by 3- ($p < 0.01$), 5- ($p < 0.001$) and 2.5-fold ($p < 0.01$) at days 3, 7 and 14, respectively, post colitis induction.

Low-dose Cur reduced progressively nitrite level by 55% ($p < 0.01$) and 70% ($p < 0.001$), and MPO activity by 70% and 85% ($p < 0.01$), compared to model group. It blocked cell apoptosis by 60% ($p < 0.01$), 58% ($p < 0.001$), 40% ($p < 0.05$), and MDA levels by 67%, 66% and 62% ($p < 0.001$), while restoring GSH content by 34% and 25% ($p < 0.01$) in inflamed distal colon, compared to TNBS-treated group (Tables 2, 3; Fig. 3A, B).

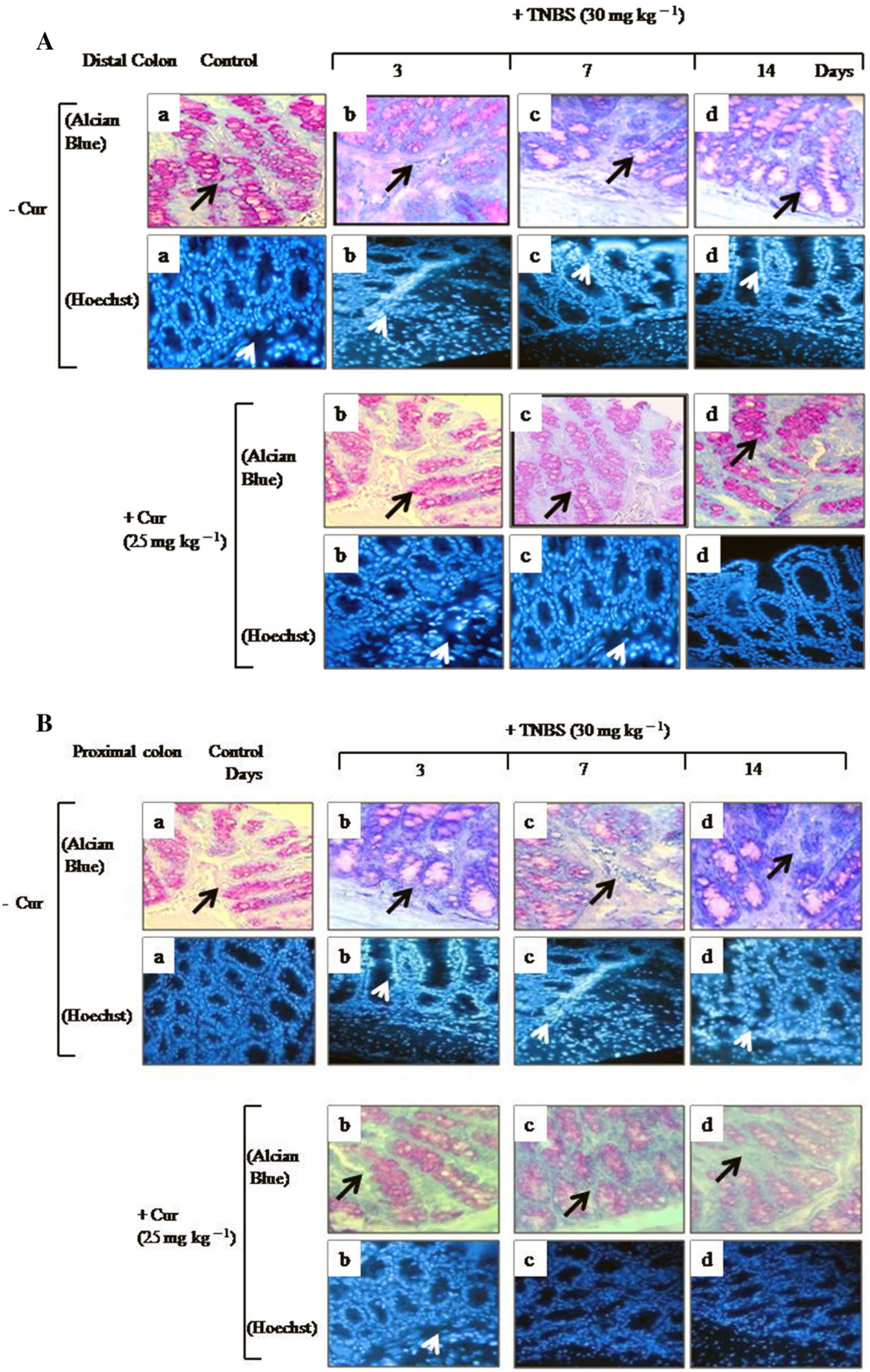
Protective effect of Cur in inflamed proximal colon

As predicted by the differences in genetic profile, metabolism and interactions with gut microbiota, the proximal and distal colon display specific sensitivity to oxidative inflammation and cancer (Glebov et al. 2003; Sun et al. 2020).

We show that TNBS-associated inflammation and nitro-oxidative stress extended progressively to proximal colon (Table 1). Nitrites level doubled ($p < 0.01$) after 3 days to reach fivefold increase ($p < 0.001$) at week 2. MPO activity rapidly doubled ($p < 0.01$) to stabilize at fourfold increase ($p < 0.001$) at day 14 (Table 2).

TNBS enhanced MDA level by three- ($p < 0.01$), five- and eightfold ($p < 0.001$), and cell apoptosis by four-, five- ($p < 0.001$) and threefold ($p < 0.01$), respectively, 3, 7 and 14 days post TNBS challenge (Tables 3, 4).

Cur lowered time dependently nitrites levels by 80% ($p < 0.001$) and 70% ($p < 0.001$) and MPO activity by 25%


A
Distal Colon
Control
+ TNBS (30 mg kg −1)
3
7
14
Days
- Cur
(Alcian Blue)
(Hoechst)
+ Cur (25 mg kg −1)
a
b
c
d
B
Proximal colon
Control
Days
+ TNBS (30 mg kg −1)
3
7
14
- Cur
(Alcian Blue)
(Hoechst)
+ Cur (25 mg kg −1)

◂**Fig. 3** Curcumin attenuated cell apoptosis and goblet cells loss related to oxidative stress in TNBS-colitis. Sections of distal (**A**) and proximal (**B**) colons were obtained as described in Fig. 2. Representative Alcian blue (AB) and Hoechst 33258-stained distal (**A**) and proximal (**B**) colon sections, of TNBS-treated for 3 (**b**), 7 (**c**), 14 (**d**) days followed (+Cur) or not (− Cur) by Cur treatment were analyzed by light microscopy (400x), and assessed for microscopic damages (Neurath et al. 1995)

($p < 0.05$), 57% ($p < 0.01$) and 75% ($p < 0.001$), respectively, at days 7 and 14. Cur improved cell apoptosis by 30% ($p < 0.05$), 35% ($p < 0.01$) and 10% ($p > 0.05$), and MDA levels by 11%, 65% and 73% ($p < 0.001$), compared with model group.

TNBS depressed GSH level by about tenfold ($p < 0.001$) which was fully restored by Cur supplementation.

Short-term Cur treatment improved TNBS-induced mitochondrial toxicity in vitro

Recently, mitochondrial dysfunction in IBD has been associated to nitro-oxidative stress (Wang et al. 2014; Zorov et al. 2014; Packiriswamy et al. 2017; Wong et al. 2017).

To furher explore the role of mitochodrial stress in the severity of colitis, the protective effect of short term Cur treatment was analyzed on TNBS-inflamed mitochondria isolated from control mice and from in vivo protocol.

Cur prevented TNBS-induced mitochondrial swelling

Compared with control, 50 and 100 µM TNBS enhanced mitochondrial swelling by 3.5- and 3.8-fold ($p < 0.001$), and by 3.7- and 4.2-fold ($p < 0.001$), respectively after 5 and 10 min, suggesting that this haptenic sensitizer induced a dose and time dependent uncoupling of colon mitochondria. Short-term Cur attenuated mitochondrial swelling by 77% ($p < 0.001$) and 68% ($p < 0.01$), and by 67% and 68% ($p < 0.01$), respectively, compared to the respective controls (Fig. 4).

Cur prevented inhibition of mitochondrial respiration by TNBS

Compared with control, 50 and 100 µM TNBS (5 min) decreased respiration by 50% and 65% ($p < 0.001$), and electron transfer through complex I by 38% ($p < 0.05$) and 65% ($p < 0.01$), respectively. Cur restored mitochondrial respiration by 30% ($p < 0.05$) and 26% ($p < 0.05$), and complex I activity by 33% and 9% ($p < 0.05$), respectively, in TNBS-treated mitochondria (Fig. 5a, b).

Cur quenched matrix superoxide anion and restored aconitase activity

To further explore the mechanism of mitochondria dysfunction attested by uncoupling, inhibition of respiration, complex I and aconitase activity, we evaluated the impact of Cur in TNBS-induced mtROS generation.

Superoxide generation was amplified by two- ($p < 0.05$) and fourfold ($p < 0.001$), whereas aconitase activity was depressed by 50% ($p < 0.05$) and 77% ($p < 0.01$) in colon mitochondria exposed to 50 µM and 100 µM TNBS, respectively, compared to control.

Cur quenched TNBS-generated superoxide anion by 55% ($p < 0.05$) and 60% ($p < 0.001$), (Fig. 5c), and restored aconitase activity by 50% ($p < 0.05$), and 70% ($p < 0.01$), respectively. In contrast, TNBS and Cur had no effect on fumarase activity (Fig. 6a, b).

Cur prevented TNBS-induced mitochondrial redox imbalance

Cur can restore mitochondrial redox balance through its ability to scavenge mtnitrites, to inhibit lipids peroxidation and to restore mtGSH stores.

The level of mtnitrites and mtMDA increased by 1.2- ($p > 0.05$) and 1.5-fold ($p < 0.05$), and by 1.3- ($p < 0.05$) and 1.8-fold ($p < 0.01$), in response to 50 and 100 µM TNBS (5 min), respectively. Whereas the level of mtGSH was lowered by 10% ($p > 0.05$) and 30% ($p < 0.05$), indicating that TNBS-toxicity is related to the dose dependent mitochondrial redox imbalance.

Cur fully restored mtGSH level and dropped mtMDA by 36% ($p < 0.05$) and 45% ($p < 0.01$) in colon mitochondria exposed to 50 and 100 µM TNBS, thus rebalancing mtMDA/mtGSH ratio, while mtnitrites level were slightly restored (19% and 1%; $p > 0.05$), in 50 and 100 µM TNBS-treated mitochondria (Figs. 5d, 7).

Discussion

This study reports the pharmacological effect of Cur in TNBS-mediated mitochondria toxicity and colitis.

As a weak acid, TNBS accumulated within matrix can collapse pH gradient (mtΔpH) and disrupt inner membrane potential (mtΔΨ), which uncouples ETC activity from ATP synthesis (Fig. 4). In addition to sulphite-peroxyl and sulphate radicals generated by oxidative metabolism of TNBS, intestinal nitro-reductases accumulate nitro and superoxide radicals, in vivo. More, mucosal oxidative damages are amplified by TNBS redox cycling and its toxicogenic

Table 2 Curcumin reduced phagocytes cells influx and oxidants-releasing enzymes

Treatments (days)	Distal colon		Proximal colon	
	MPO (μM H_2O_2 mg^{-1} prot)	Nitrites (μM mg^{-1} prot)	MPO (μM H_2O_2 mg^{-1} prot)	Nitrites (μM mg^{-1} prot)
Control	04.42 ± 1.11	10.47 ± 1.44	05.71 ± 0.90	04.35 ± 1.70
TNBS (3)	25.61 ± 1.34***	31.78 ± 5.23***	11.787 ± 1.36**	09.36 ± 0.51**
TNBS (7)	26.15 ± 2.92***	30.84 ± 2.63***	11.416 ± 2.66**	23.14 ± 3.51***
TNBS (14)	13.0 ± 3.09	37.5 ± 3.96***	23.27 ± 3.05***	22.33 ± 1.36***
TNBS + Cur (3)	27.15 ± 3.98	13.69 ± 2.9##	08.50 ± 0.58#	15.29 ± 3.08
TNBS + Cur (7)	08.67 ± 1.95##	0 8.74 ± 0.94###	04.92 ± 0.29##	04.66 ± 1.07###
TNBS + Cur (14)	04.51 ± 0.85##	11.39 ± 1.44###	05.80 ± 1.3###	06.83 ± 0.53###

Mice were given 30% ethanol (control), TNBS (30 mg kg^{-1} in 30% ethanol, TNBS group), or TNBS + Cur (25 mg kg^{-1}; Cur group), and euthanized at days 3, 7 and 14 days post-colitis induction. Distal and proximal colon homogenates were prepared and assayed for MPO activity (a) and NO level (b), as described in materials and methods section. Data are means ± SEM ($n = 7$). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. control group; #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ vs. TNBS treated group

Table 3 Curcumin attenuated cell apoptosis in TNBS-colitis

Treatments (days)	Apoptosis (mean/10 fields)	
	Distal colon	Proximal colon
Control	2.33 ± 0.42	1.8 ± 0.41
TNBS (3)	6.83 ± 0.65**	7.3 ± 0.64***
TNBS (7)	11.5 ± 0.84***	10.1 ± 0.58***
TNBS (14)	5.83 ± 0.75**	15.4 ± 0.65**
TNBS + Cur (3)	2.67 ± 0.75##	5.2 ± 0.47#
TNBS + Cur (7)	4.83 ± 0.6###	6.6 ± 0.73##
TNBS + Cur (14)	3.5 ± 0.62#	4.9 ± 0.62

Sections of distal (3A) and proximal (3B) colons were obtained as described in Fig. 2. Hoechst 33258-stained distal (A) and proximal (B) colon sections of TNBS-treated for 3 (b), 7 (c), 14 (d) days followed (+Cur) or not (− Cur) by Cur treatment were assessed for cell apoptosis by fluoresence microscopy (400 ×). Results Data are means ± SEM ($n = 7$). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. control group; #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ vs. TNBS treated group

potential on mitochondria enriched colon epithelial cells (Grisham et al. 1991; Chamulitrat 1999).

TNBS-stressed mitochondria show hallmark features of a ROS dependent dysfunction. The half and two third decrease in respiration and respirasome I activity may be related to the drop in aconitase activity, the redox check point of TCA cycle (Titov et al. 2016). A mitochondrial NO synthase (mtNOS) has been convincingly identified as a variant of constitutive neuronal NOS (Kanai et al. 2001). Low-dose NO is protective to respirasome I by neutralizing superoxide to nitrate (Figs. 5, 6) (Sharpley et al. 2006). At higher doses, NO cross reaction with superoxide releases the highly reactive peroxynitrite (ONOO-), which covalently binds to Fe-S-containing respirasomes I, III, triggering a reverse electron transfer flow and mitochondria uncoupling (Brown and Borutaite 2004; Ohtani et al. 2012). Mitochondria uncoupling is a physiological adaptive response to mild oxidative stress (Fig. 4). mtROS and lipid peroxides can induce antioxidant activity of uncoupling protein

Table 4 Curcumin reduced MDA/GSH ratio in inflamed colon

Treatments (days)	Distal colon			Proximal colon		
	MDA (nmol mg^{-1} prot)	GSH	MDA/GSH	MDA (nmol mg^{-1} prot)	GSH	MDA/GSH
Control	27.56 ± 0.96	62.06 ± 11.32	0.44	10.58 ± 1.33	38.46 ± 5.51	0.27
TNBS (3)	69.23 ± 7.31**	23 ± 4.04***	3.01 (6.84[a])	31.41 ± 12.37**	3.24 ± 0.37***	9.69 (35.88[a])
TNBS (7)	135.25 ± 12.37***	10. 48 ± 2.13***	12.90 (29.31[a])	48.59 ± 16.66***	3.90 ± 0.37***	12.45 (46.11[a])
TNBS (14)	133.33 ± 20.96***	29. 56 ± 0.65***	4.51 (10.25[a])	85.71 ± 12.82***	3.90 ± 0.66***	21.97 (81.37[a])
TNBS + Cur (3)	23.07 ± 2.24###	22.42 ± 4.04	1.03 (2.34[a] 0.33[b])	28.21 ± 6.28	7.5 ± 1.1##	3.76 (13.93[a] 0.39[b])
TNBS + Cur (7)	46.15 ± 9.36###	31.91 ± 2.96##	1.46 (3.31[a] 0.11[b])	17.12 ± 1.80###	8.31 ± 0.88##	2.06 (7.63[a] 0.165[b])
TNBS + Cur (14)	50 ± 5.13###	45. 07 ± 4.09###	1.1(2.5[a] 0.24[b])	23.53 ± 6.15###	35.07 ± 2.5##	0.67 (2.48[a] 0.17[b])

Mice were given 30% ethanol (control), TNBS (30 mg kg^{-1} in 30% ethanol, TNBS group), or TNBS + Cur (25 mg kg^{-1}; Cur group), and euthanized at days 3, 7 and 14 days post-colitis induction. Distal and proximal colon homogenates were prepared and assayed for MDA (a) and GSH level (b), as described in materials and methods section. MDA/GSH ratio was calculated as an index of mucosal toxicity. Data are means ± SEM ($n = 7$). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. control group; #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ vs. TNBS treated group

[a]Fold of control

[b]Fold of TNBS

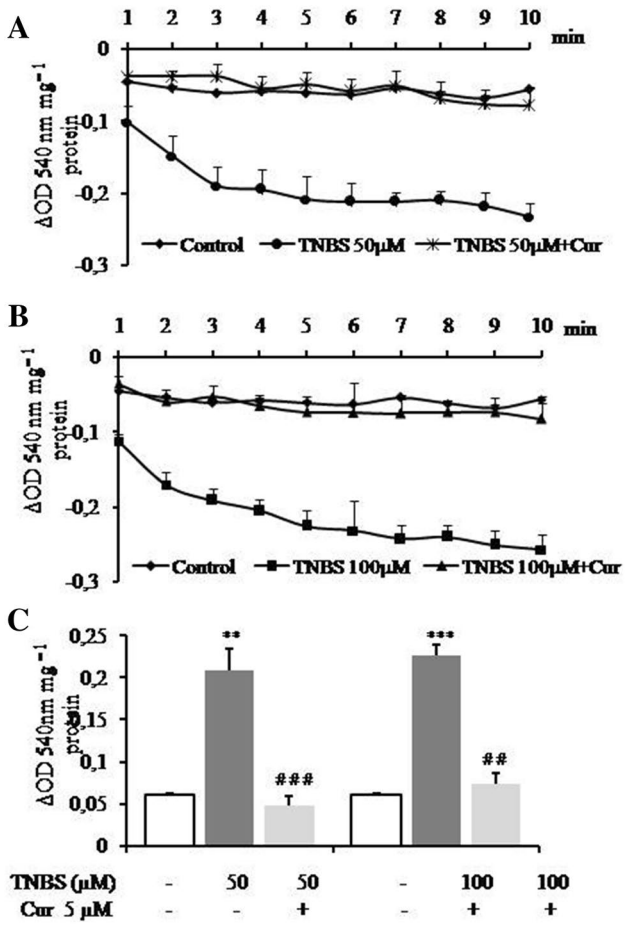


Fig. 4 Curcumin improved the opening of mitochondrial permeability transition pore (mPTP) induced by TNBS. Control colon mitochondria were incubated in swelling buffer as described in materials and methods section. Mitochondrial swelling was initiated by the addition of TNBS 50 (**a**) or 100 µM (**b**, **c**) in the presence or absence of 5 µM Cur for 10 min at 25 °C. Data are means ± SEM. $n = 5$ for controls; $n = 5$ for TNBS. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. TNBS treated mitochondria

2 (UCP2), an ubiquitous mitochondrial carrier associated to inner mitochondrial membrane, which catalyzes protons re entry into matrix and energy dissipation in the expense of ATP synthesis (Houten and Wanders 2010; Chen et al. 2018; Huang et al. 2019). Also, UCP2 blocks fatty acids entry, while exporting various TCA cycle metabolites to the cytoplasm. This limits NADH and FADH2 generation, the electron donors for mtROS-releasing respiration (Vozza et al. 2014).

The drastic consumption of GSH and sustained accumulation of MDA, a marker of electrophilic hydroperoxy lipids, exacerbates MDA/GSH ratios in stressed mitochondria, and in inflamed distal and proximal colon, suggesting MDA/GSH ratio as a potential index of nitro-oxidative stress at tissular and subcellular level. The nitro-oxidative process in proximal colon reached threefold the level of distal colon, and may account for colon epithelial cell apoptosis (Tables 3, 4; Fig. 7).

Superoxide dismutases (SOD) block propagating oxidative chain reactions through dismutation of superoxide into hydrogen peroxide (H_2O_2) in matrix (MnSOD) and cytosol (Cu/Zn SOD) (Murphy 2009). Then, glutathione reductase (GSH/GS-SG)/glutathione peroxidase shuttle takes up H_2O_2 and oxidizes NADPH (H^+) through the thiol redox chain thioredoxin (TrxR)/TRX/Peroxiredoxin/catalase, facilitating the reduction of hydroperoxy lipids and H_2O_2 (Awasthi et al. 2000; Cox et al. 2009; Handy and Loscalzo 2012).

By restoring GSH stores, Cur can restart this ROS-detoxifying machinery (Table 4; Fig. 7).

By quenching TNBS-generated mtROS and trapping hydroperoxy lipids around respirasomes (Fig. 8), Cur can support mitochondria recoupling through the fine modulation of mPTP opening, inner mitochondrial membrane fluidity and physiological electrons transfer by respirasomes. The powerful antioxidant and metal chelating capacity are supported by the redox activity of methoxyphenols and α,β-unsaturated carbonyl (enone) electrophile backbone of Cur (Priyadarsini et al. 2003; Jiao et al. 2006; Wei et al. 2006; Aggarwal et al. 2015).

Cur may prime mithormesis, the adaptive mitochondrial response to nitro-oxidative stress, by up-regulating genes involved in GSH and NADPH neosynthsesis through NRF2-EpRE pathway (Aggarwal et al. 2013; Forman et al. 2014).

Mitochondrial stress has been evidenced in intestinal epithelium of IBD patients, and ultrastructural changes of colon mitochondria have been earlier attributed to deficient fatty acid β-oxidation (Houten and Wanders 2010; Sifroni et al. 2010; Packiriswamy et al. 2017).

Besides, the uncontrolled immune response of CD mimicked by TNBS is sustained by Th1 and Th7 dependent pro inflammatory cytokines IFN-γ, IL-1β, TNF-α resulting from the massive influx of inflammatory Th1/Th17 cells and neutrophils (Hölttä et al. 2008; Beltrán et al. 2010).

Macrophages accumulated in luminal epithelium, while neutrophils invaded crypt pits, forming abscesses in distal colon (Figs. 2, 3). The respiratory burst and NO synthase activity of phagocytes infiltrating colon mucosa are significant sources of extracellular ROS. The coupling of phagocytes NADPH oxidase (NOX2)-derived superoxide anion (ROS precursor), NO and MPO activity concentrate mixed reactive nitrogen and oxygen species in distal and proximal colon at days 3, 7 and 14 days of TNBS-colitis (Tables 2, 4). Active inflammatory foci are maintained in intestinal mucosa by the persistent flow of mitochondria and NOXs-derived ROS, fuelled by inducible colonic NOX1, uncoupled endothelial NOS activity and various oxidative metabolic activities (Beltrán et al. 2010; Mouzaoui et al. 2012; Aviello and Knaus 2016). NO-related colonic damages have been attributed to macrophage 1 (M1) subset. The alternative

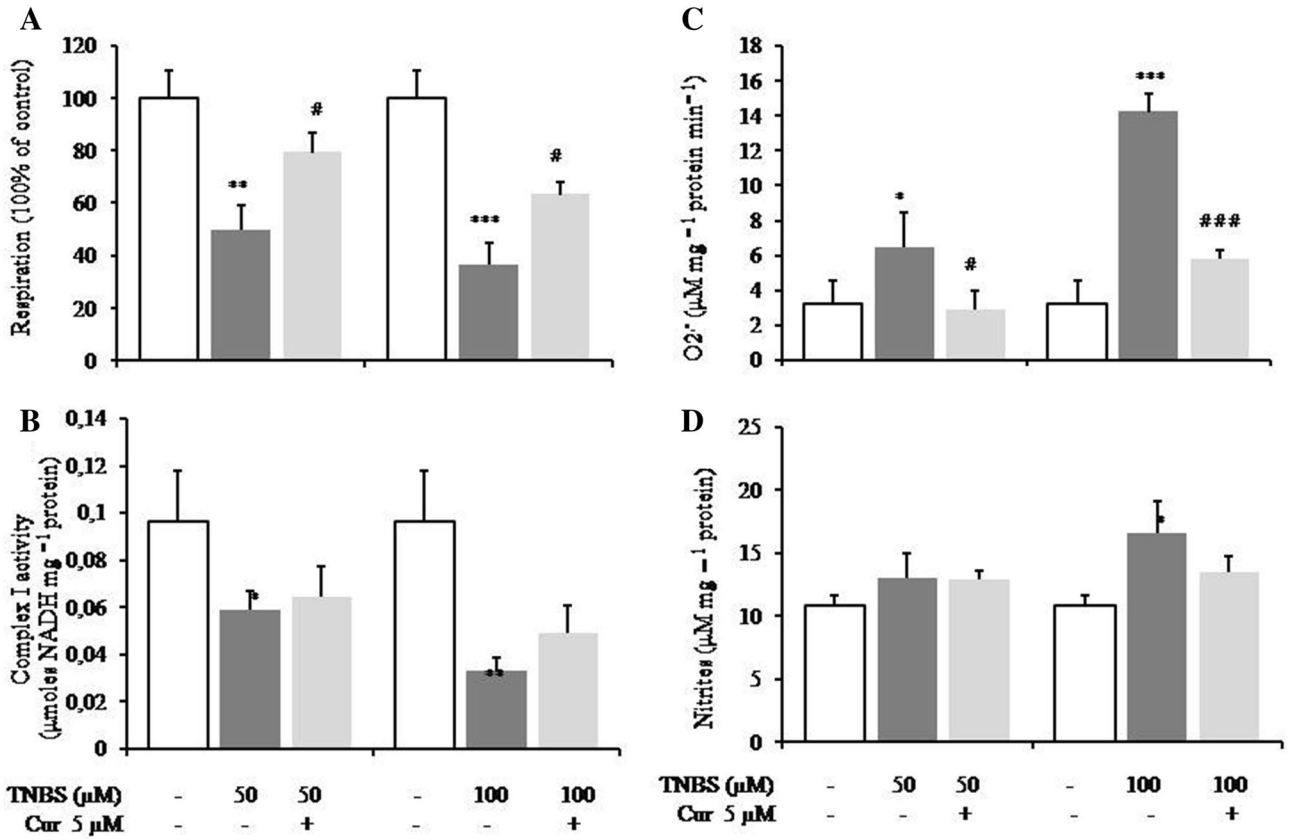


Fig. 5 Curcumin ameliorated TNBS-induced colon mitochondria toxicity in vitro. Colon mitochondria (0.5 mg prot^{-1}) were incubated with 100 µM TNBS in the presence or absence of 5 µM Cur as described in materials and methods section and assayed for respiration (**a**), complex I activity (**b**) and superoxide (**c**) and nitric oxide (**d**) production. Data are means ± SEM. $n = 5$ for controls; $n = 5$ for TNBS. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. TNBS treated mitochondria

activation of macrophages 2 (M2) with up regulated arginase activity is involved in epithelia cells proliferation and mucosa healing (Tu et al. 2017).

Importantly, low-dose Cur abolished the drastic mortality in mice, and improved in a time dependent manner macroscopic and histological scores including crypts disruption, intestinal fibrosis and hemorrhagic edema, while restoring mucosal function by progressive recovery of mucin-producing crypts (Table 1; Figs. 2, 3), intestinal permeability and gut microbiota (Nazli et al. 2004; Kaulmann and Bohn 2016). Mucin depleted foci (MDF) with β catenin mutations are preneoplastic formations. They are identified in animal models of colon carcinogenesis and in humans with CRC. They are emerging as biomarkers of colon carcinogenesis (Femia et al. 2005; Sakai et al. 2012).

Low-dose Cur attenuated TNBS-oxidative bowel inflammation by inhibiting the prooxidant activity of myeloperoxidase and NO synthase associated to phagocytes influx, primarily through the blockade of adhesion to vascular endothelium (Table 2; Fig. 5) (Kunnumakkara et al. 2017; Luis et al. 2017). Cur reboosted catalase activity in colon mucosa (data not shown), indicating that oxidative lesions and mitochondrial damages also results from inefficient antioxidant system.

Phytochemicals encomprise a wide set of electrophilic adductors of the cytoplasmic Keap1/Cul3-NRF2 redox sensor. Keap1 is a zinc metalloprotein wearing critical 25–27 Cys residues (Dinkova-Kostova et al. 2005). Among them, Cys 151,257, 273, 288, and 297 of BTB/ERV domains can finely appreciate electrophile and oxidative stress. Various electrophiles and oxidants including TNBS, lipid peroxides, and reactive oxygen and nitrogen species can interact with Keap1 to weaken interactions with DLG and ETGE binding domains of NRF2.

The electrophilic capacity of Cur is associated to α,β-unsaturated carbonyl (enone) moiety. Its cytoprotective effect is potentiated by auto-oxidation and oxidative metabolism which generate more efficient derived electrophiles with enhanced nucleophile binding sites (Priyadarsini et al. 2003; Edwards et al. 2017; Luis et al. 2017).

Cur and its oxidized derivatives can adduct Keap1 through oxidation of its most reactive Cys151 and

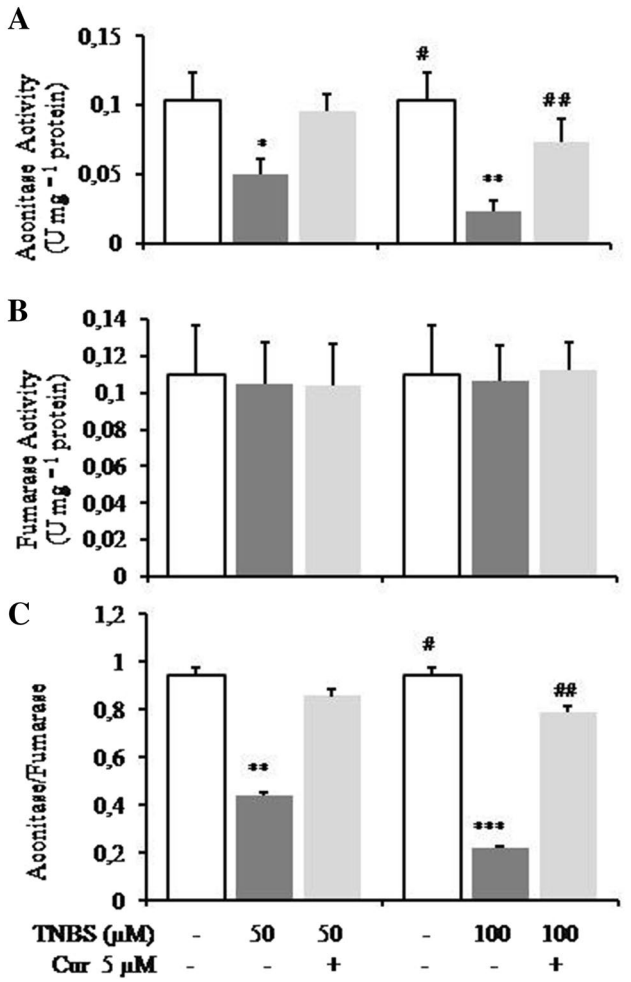


Fig. 6 Curcumin prevented TNBS-collapsing redox sensor activities. (0.5 mg prot^{-1}) were incubated with 100 µM TNBS in the presence or absence of 5 µM Cur as described in materials and methods section and assayed for aconitase activity (**a**), fumarase activity (**b**). Evaluation of aconitase/fumarase ratio (**c**). Data are means ± SEM. $n = 5$ for controls; $n = 5$ for TNBS. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. TNBS treated mitochondria

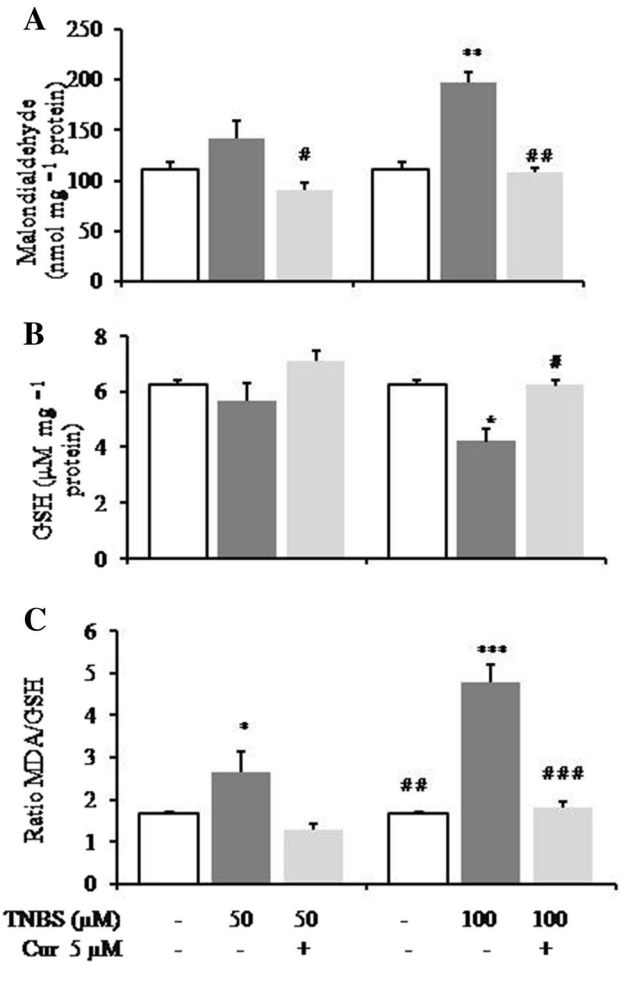


Fig. 7 Curcumin prevented TNBS-induced mitochondria redox imbalance. Colon mitochondria (0.5 mg prot^{-1}) were incubated with 100 µM TNBS in the presence or absence of 5 µM Cur as described in materials and methods section and assayed for MDA (**a**) and GSH (**b**) levels. Evaluation of colon mitochondria MDA/GSH ratio (**c**). Data are means ± SEM. $n = 5$ for controls; $n = 5$ for TNBS. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. TNBS treated mitochondria

polyphenols sensitive Cys of BTB/ERV domain, to induce conformational change. This releases a stabilized form of NRF2 which translocates to the nucleus where it adapts on Maf protein to lock the antioxidant/electrophile response element (ARE/EpRE) on DNA, and triggers the transcription of a network of cytoprotective genes, such as heme-oxygenase-1, NADP(H) quinine oxidorectase 1, thioredoxin…, which restores redox homeostasis at systemic and cellular level (Wang et al. 1997; Edwards et al. 2017).

Complex interplay between NLRP3 inflammasome, NFKB and NRF2, modulating pro- and anti inflammatory pathways is potentially mastered by the regulatory protein p62 (Hennig et al. 2018).

Taken together, our results indicate that short term Cur, restore primary mitochondrial architecture and function through the modulation of mPTP opening (swelling), ROS leakage by the redox loops of the electron transfer chain (ROS generation). This critical property is likely related to its lipophilic nature, allowing spontaneous "vectorization" to the mitochondria. Cur can collapse the mtROS gradient, by quenching superoxide, nitrites and hydroxyl radical. This ROS-detoxifiyng capacity sustains mitochondria recoupling and colon epithelium healing.

Herbal medicine is growing, worldwide. Despite its low bioavailability, Cur, a phytochemical electrophile with high

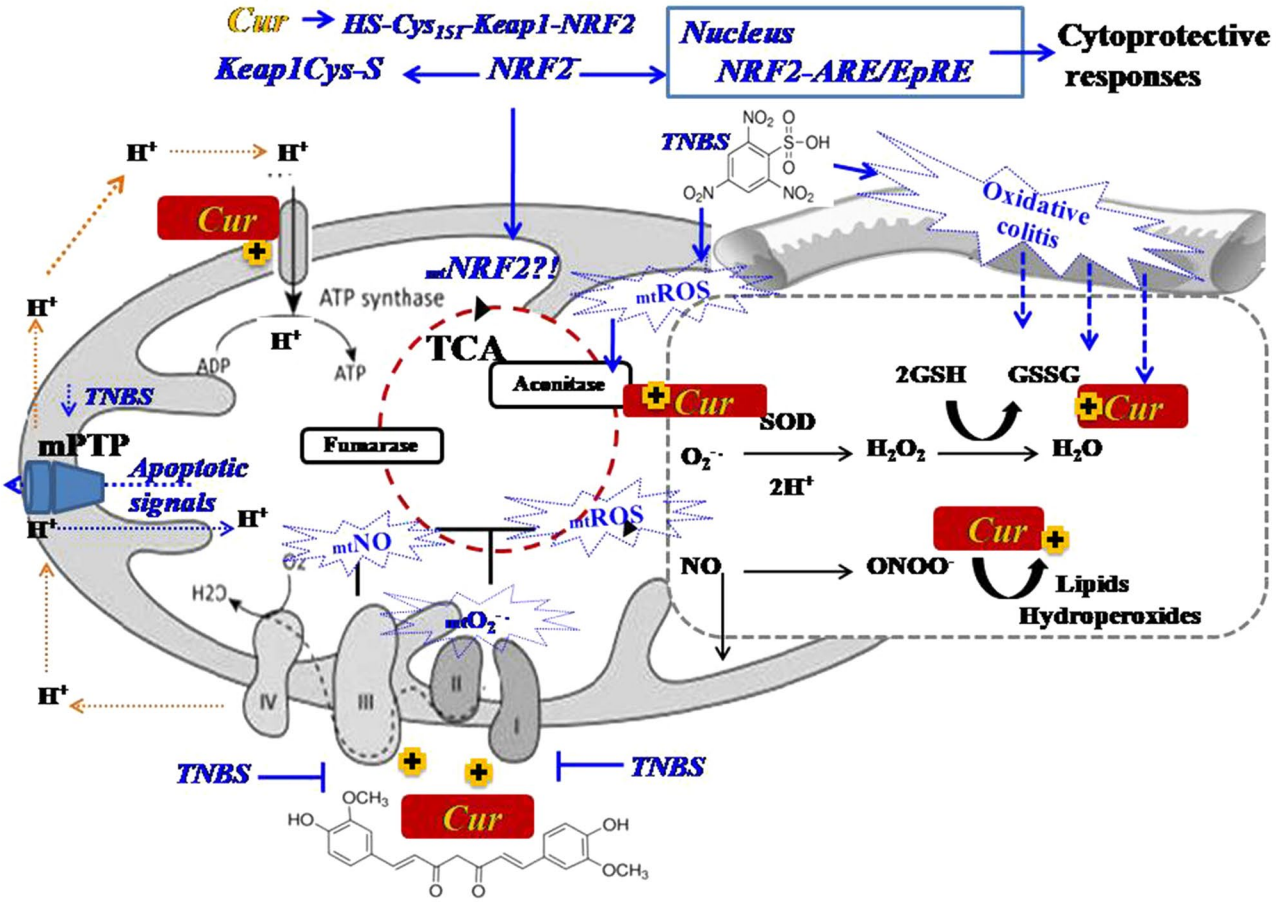


Fig. 8 Recoupling of TNBS-stressed mitochondria by curcumin. TNBS induced mitochondrial stress, and sustained reactive oxygen and nitrogen species (mtROS) levels. The mtROS dependent oxidation of polyunsaturated fatty acids of phospholipid bilayer generates hydroperoxy lipids adductors which leads to mitochondria uncoupling, and the collapse of mitochondrial membrane potential. In addition, the nitrosation of iron-sulfur (Fe-S)-clusters of respirasomes I and III, and the redox sensitive aconitase, blocks tri carboxylic acid (TCA) cycle and respiration, resulting in bioenergetic stress. Curcumin (Cur) as a lipophilic phytochemical can insert into lipid bilayer which ii interfers with lipids and proteins oxidation by scavenging superoxide anion, peroxinitrite, hydroperoxy lipids and hydroxyl radical. Cur can also reactivate endogenous antioxidant system and restore adaptive cell response to nitro-oxidative stress by thiols modulation of (Keap1), an endogenous inhibitor of the transcription nuclear factor (erythroid-derived 2)-like 2(Nrf-2) of genes for NADPH and GSH biosynthesis and for antioxidant and detoxifying enzymes. In addition, matrix superoxide dismutase (MnSOD) block propagating oxidative chain reactions through dismutation of superoxide into hydrogen peroxide (H_2O_2). Then, glutathione reductase (GSH/GS-SG)/glutathione peroxidase shuttle takes up H_2O_2 and oxidizes NADPH (H^+) through the thiol redox chain thioredoxin (TrxR)/TRX/Peroxiredoxin/catalase, then facilitating the reduction of hydroperoxy lipids and H_2O_2

safety and tolerability is promising as mitochondrial targeted antioxidant, for IBD.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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