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ORIGINAL PAPER

N-acetyl-L-cysteine modulates multiple signaling pathways to rescue male germ cells from apoptosis induced by chronic hCG administration to rats

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Abstract The present study was aimed to investigate the beneficial effects of *N*-acetyl-L-cysteine (NAC, 150 mg/kg bw twice/week) against testicular germ cell apoptosis in rats induced by chronic hCG administration (100 IU/rat/day for 30 days). NAC co-treatment improved serum testosterone, prevented rise in lipid peroxidation, intracellular H_2O_2 and the activities of antioxidant enzymes in germ cells. Replenishment of intracellular GSH and total antioxidant capacity was seen. There was a marked reduction in TUNEL positive germ cells and expression of caspase-3 ($p < 0.01$) and PARP cleavage. Pro-apoptotic markers Fas, FasL, caspase-8 were also significantly downregulated. While Bcl-2 was fully restored, rise in Bax, caspase-9, phospho-JNK/JNK and phospho-c-Jun/c-Jun expression was significantly arrested. Anti-apoptotic phospho-Akt/Akt and NF-κB were otherwise found upregulated. Taken together, the above findings demonstrate that NAC intervention rescued the testicular germ cells from demise following chronic hCG treatment through regulation of multiple signaling mechanisms of metazoan apoptosis.

Keywords Chronic hCG treatment · Testis · Oxidative stress · Apoptosis · NAC co-treatment · Germ cell survival

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Introduction

Hormonal therapy in the form of human chorionic gonadotropin (hCG) treatment has been a standard mode of treatment in various conditions of cryptorchidism [1–3] and male hypogonadotropic hypogonadism [4, 5]. However, such interventions are both beneficial and detrimental to the seminiferous epithelium depending on the dose, duration and the spermatogenic status at the time of treatment. The positive effects include testicular descent and enlargement of testis in conditions of cryptorchidism [6] to improvement in sperm quality and return of fertility in men with hypogonadotropic hypogonadism [7]. On the other hand, there are associated detrimental effects like inflammation-like morphological changes due to increase in intratesticular pressure. However, most critical is the apoptotic induction of germ cells which rises significantly after hCG treatment [8]. Cryptorchid boys who were followed-up into adulthood show that hCG-treated testes were 50% smaller and that there was an inverse relationship between the degree of apoptosis at the end of hCG treatment and testicular volume in adulthood [3, 9].

hCG administration in adults also induces focal disruption of spermatogenesis, deteriorates seminiferous tubule histology and induces germ cell apoptosis in rats identical to what has been reported with hCG use in clinical conditions [10–12]. The rise in germ cell apoptosis was found to be associated with an identical rise in testicular oxidative stress [13]. Recently it has been reported from our laboratories that chronic hCG treatment to rats induces Leydig cell apoptosis [14]. *N*-Acetyl-L-cysteine (NAC) is a well established thiol antioxidant and has been effectively utilized to alleviate oxidative stress following experimentally induced conditions like testicular torsion [15], varicocele [16] and ischemia/reperfusion injury in rat testis [17]. NAC

was also shown to prevent testicular germ cell apoptosis induced through H_2O_2 exposure in vitro [18]. However, there have been very few attempts or interventions in vivo that counteracted hCG induced detrimental effects leading to germ cell apoptosis in the seminiferous epithelium. Besides, a transient rise in intracellular H_2O_2 concentration beyond optimal levels could initiate apoptotic induction in germ cells [19] and whether or not such an event actually occurs following chronic hCG treatment needs to be confirmed beyond doubt. The present study was therefore initiated to investigate the beneficial effects of NAC intervention promoting germ cell survival in the testis during chronic hCG treatment and the underlying molecular mechanisms of regulation associated with it.

Materials and methods

Chemicals and reagents

All chemicals were procured from Sigma-Aldrich (St. Louis, MO, USA) unless and otherwise specified.

Animals and treatment

Forty-eight adult male albino rats (Holtzman strain) weighing 200 ± 20 g were used and divided in six groups of eight animals each. The animals were maintained under control temperature (25 ± 2°C) and constant photoperiodic conditions (12 h light: 12 h dark) with food and water ad libitum. Treatments were carried out under strict compliance with Institutional Guidelines for Animal Care as prescribed by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India. All animal experiments were approved by Institutional animal ethics committee. Both hCG and NAC were dissolved in PBS and administered intraperitoneally for 30 days. The dose of hCG was selected on the basis of the findings and its usefulness from our previous study [13]. The dose of NAC, on the other hand, was extrapolated from the data reported elsewhere [15, 20]. To determine the efficacy of intervention, the frequency of NAC administration was varied without altering the dose. The details of the treatment are described as follows:

Gr 1: PBS (100 μL, vehicle control)/day
Gr 2: hCG (100 IU in 100 μL)/day
Gr 3: NAC (150 mg/kg b.w in 100 μL) once/week
Gr 4: hCG (100 IU in 100 μL)/day + NAC (150 mg/kg b.w in 100 μL) once/week
Gr 5: NAC (150 mg/kg b.w in 100 μL) twice/week
Gr 6: hCG (100 IU in 100 μL)/day + NAC (150 mg/kg b.w in 100 μL) twice/week.

Following the completion of 30 days of treatment, animals were sacrificed and testes were weighed. One testis from each animal was immediately fixed for histological analysis. The other testis was decapsulated and germ cells were isolated as described [18] and stored at −20°C till further use.

Blood was collected from the tail vein of rats at different time intervals (0th, 7th, 15th and 30th day) under mild ether anesthesia. Serum was separated by centrifugation at 5,000×*g* for 15 min and stored at −20°C till assayed for testosterone.

Histology of testes and quantitation of spermatogenesis

Testes fixed with buffered formalin for 4 h were cut at the two poles in both sides and allowed to remain in the fixative at 4°C for another 24 h. The fixative was then removed, tissues washed, dehydrated in upgraded series of alcohol, cleared in xylene and finally embedded in paraffin at 60°C. Using the paraffin embedded blocks; sections (4 μm) were cut with the help of a semiautomatic microtome (Leica Microsystems Inc., Bannockburn, IL, USA) and layered on poly-L-lysine coated glass slides. Sections were deparaffinized, cleared in xylene, rehydrated, stained with haematoxylin and eosin, examined and photographed using the microscope fitted with an image analyzer (Nikon, Eclipse E600). Quantitation of the spermatogenesis [21] in the testes of control and treated rats ($n = 8$) was carried out from 20 randomly selected seminiferous tubules in each testicular section (one section each from five different regions of testis). Number and types of germ cells present in a tubule were recorded.

ELISA of testosterone

Testosterone was measured from serum using commercially available ELISA kit, according to the manufacturer's (DRG testosterone ELISA, EIA-1559, DRG Instruments, GmbH, Germany) instructions. Briefly, 25 μL of standards, control and test samples were dispensed in separate wells to which 200 μL of enzyme conjugate was added and the mixture was incubated for 1 h at room temperature (RT). Contents were briskly shaken, wells washed three times with diluted wash solution following which 200 μL of substrate solution was added and incubated for 15 min at RT. The reaction was stopped by adding stop solution (100 μL). The absorbance was read at 450 nm. The sensitivity of the kit was 0.083 ng/mL. Concentrations of testosterone in samples were calculated using the standard graph.

Intracellular H_2O_2 measurement

Chloromethyl-2′, 7′-dichlorofluorescein diacetate (CM-H_2DCFDA) was used to measure H_2O_2. Chemically reduced and acetylated forms of 2′,7′-dichlorofluorescein (DCF) is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. Esterase cleavage of the lipophilic blocking groups yields a charged form of the dye that is much better retained by cells than the parent compound. Oxidation of these probed is assessed using a fluorometer as previously described methods with modifications [22].

Briefly, 5×10^6 cells (germs and interstitial cells of treated and control group) were resuspended in 500 µL of PBS and 2.5 µL of 1mM CM-H_2DCFDA (Molecular Probes, Eugene, OR, USA) dye solution (final 5 µM) was added to each tube. Content was mixed by gentle tapping and tubes were incubated at 37°C for 15 min in dark. Cells were pelleted and washed twice with PBS to remove excess unbound dye and resuspended in 500 µL of PBS. 100 µL of the suspension were plated in triplicate using an ELISA plate. Fluorescence was measured by a multiplate reader (Bio Tek Inc., Winooski, VT, USA) at excitation wavelength of 485 nm and emission wavelength of 530 nm. H_2O_2 (100–1,000 nM) was used preparing a standard plot against which H_2O_2 concentrations of test samples were determined.

Lipid peroxidation and antioxidant enzymes activity

Isolated testicular germ cells from treated or untreated rats were sonicated for 30 s and divided into two equal parts. One part was assayed for lipid peroxidation through the formation of thiobarbituric acid reactive substances (TBARS) in the reaction mixture [23]. The second part was centrifuged at 10,000×*g* for 5 min and the supernatant was assayed for antioxidant enzyme activity. SOD was measured as described earlier [24]. Catalase was estimated [25] by the degradation of hydrogen peroxide (6 mM). Glutathione-s-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate [26].

Total antioxidant capacity (TAC)

TAC in isolated germ cells was assayed as per manufacturer's instructions (Cayman Chemical Company, Ann Arbor, USA). The assay measures the ability of combined antioxidants (vitamin, protein, lipids, glutathione, uric acid etc.) present in the cell lysate to inhibit the oxidation of 2,2-Azino-di-(3-ethylbenzthiazoline sulphonate (ABTS) by metmyoglobin. The amount of oxidized ABTS produced was measured at 750 nm. Total antioxidant capacity (mM) was calculated from the Trolox standard curve.

Intracellular glutathione levels

ApoGSH Glutathione Detection Kit (Biovision, Mountain view, CA, USA) was used to measure the total glutathione levels from the isolated testicular germ cells according to manufacturer's instructions. Briefly 5×10^6 germ cells were allowed to lyse for 10 min at 4°C in 200 µL of lysis buffer provided in the kit, centrifuged at 12,000×*g* and supernatant was collected. Samples were deproteinized using 10 kDa filters. 40 µL of samples were taken in fluorometric plate and diluted with lysis buffer (final 100 µL). 2 µL of GST reagent (50 U/ml) and 2 µL of MCB (25 mM Monochlorobimane) dye was added to each standard and test samples in the plate, shaken well and incubated at 37°C for 30 min. Fluorescence was measured using micro-titre plate reader (Bio Tek Inc., Winooski, VT, USA) at Excitation/Emission ratio of 380/460 nm. Total glutathione was calculated for each sample using the standard curve.

TdT-mediated deoxyuridine-triphosphate dUTP nick end labeling (TUNEL) assay

TUNEL kit (R&D system Inc., Minneapolis, USA), as per manufacturer's instructions was utilized to detect the DNA strand breaks in the whole testis section or among the isolated testicular germ cells. Testis sections were rehydrated in a downgraded series of alcohol, washed subsequently with H_2O (3 min) and PBS (10 min) and permeabilized with Proteinase-K solution (50 µL) for 10 min at RT. Following two washes with PBS, the sections were incubated with freshly prepared quenching solution (3% H_2O_2 in methanol) for 5 min to remove the endogenous peroxidase. Biotinylated nucleotides were incorporated by incubation with TdT reaction mixture (1 µL TdT-dNTP, 1 µL Mn^{2+} stock, 1 µL TdT enzyme, 50 µL 1× TdT labeling buffer) and detected by using streptavidin-HRP. Methyl green was used as a counter stain. Stained tissue sections were washed using 1-butanol and mounted in DPX. Ten random sites were examined from each section for the presence of TUNEL positive cells/tubule and photographed using Nikon Image analyzer (Nikon E600).

Isolated testicular germ cells were smeared on poly-L-Lysine coated slides and fixed in 4% formaldehyde. Cells were treated with cytonin for 10 min followed by quenching with H_2O_2. Biotinylated nucleotides were similarly incorporated into the 3′-OH ends of the DNA fragments by TdT, and detected by using streptavidin-HRP. The colour was developed by diaminobenzidine (DAB) solution and counter stained with methyl green later. The slides were examined using a Nikon microscope. TUNEL positive cells were scored from 100 stained cells and from five randomly selected sites on each slide.

Caspase -3, -8, -9 activities

Caspase colorimetric assay kits (Biovision, San Diego, CA, USA) were used to measure the activity of caspase-3/8/9 as per the manufacturer's instructions. Germ cells were resuspended in cold lysis buffer and incubated for 10 min. Cell lysates were centrifuged for 2 min at 10,000×*g* at 4°C. An aliquot of supernatant (100 μg protein/50 μl) was added to 50 μL of reaction buffer containing 200 μM of chromogen (Ac-DEVD-pNA/Ac-IETD-pNA/Ac-LEHD-pNA for caspase-3, -8, -9 respectively), kept at 37°C for 2 h and terminated by adding stop buffer. The change in the absorbance due to the release of p-nitroanilide (p-NA) was measured at 405 nm using micro-titre plate reader (BioTek Inc., Winooski, VT, USA).

Immunofluorescence for Bax

Localization of Bax antigen through immunocytochemical staining was carried out using specific antibody (Santa Cruz Biotechnology, CA, USA). Isolated testicular germ cells were washed with PBS and smeared on poly-L-Lysine coated slides and fixed in 4% formaldehyde for 10 min. Cells were washed three times in PBS and incubated in 0.05% Triton X-100 for 10 min for permeabilization. Following two washes in PBS, the cells were incubated with 50 μL mouse monoclonal primary antibody (1:100, in PBS containing 0.01% BSA) for 1 h and subsequently with anti-mouse secondary antibody tagged with TRITC (1:1,000, in PBS containing 0.1% BSA) for 30 min. Washed twice with ice cold PBS, slides were mounted in 50% glycerol, examined under microscope and photographed with the help of an image analyzer (Nikon Eclipse 80i) using suitable filter for rhodamine.

Western blot analysis

Whole cell lysates from isolated testicular germ cells were prepared in 200 μl lysis buffer containing 20 mM HEPES (pH 7.4), 2 mM EDTA, 50 mM *β*-glycerophosphate, 1% Triton X-100, 150 mM NaCl, 10% Glycerol and protease inhibitor cocktail (Roche, Basel, Switzerland). Western blotting was carried out as previously described [19]. Primary antibodies (rabbit polyclonal), anti- PARP, anti-Caspase-3, anti-Caspase 9, anti-Fas, anti-FasL, anti-p53, anti-Bax, anti-JNK, anti-c-Jun, and (mouse monoclonal) anti-Caspase 8, anti-p-JNK, anti-Bcl-2, anti-Akt, anti-NF-*κ*B, anti-I*κ*B anti-*β*-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit monoclonal anti-p–c-Jun and anti-p-Akt (Cell Signaling Technology Inc, Danvers, MA, USA) and goat anti rabbit/mouse-HRP conjugate secondary antibody (Santa Cruz Biotechnology, CA, USA) were utilized. *β*-actin was used to monitor equal loading of protein. Densitometric analysis (Table S1) was performed with the help of Image analysis software (Lab Works 4.0, UVP, Upland, CA, USA).

RNA isolation and RT-PCR analysis

Total RNA was extracted from the isolated testicular germ cells using TRI-Reagent (Ambion, TX, USA). Primers for specific genes were from Eurofins MWG Operon (Whitefield, Bangalore, India) and R&D system Inc., Minneapolis, USA. cDNA was synthesized using 2 μg of total RNA by omniscript RT kit (Qiagen, Hilden, Germany). 2 μL of the RT reaction was then used for PCR using the HotStar HiFidelity DNA polymerase (Qiagen). The PCR reactions were carried out under the temperature conditions: (1) denaturation at 95°C for 15 min; (2) 28–30 cycles of 95°C for 30 s, 55–67°C for 1 min, 72°C for 1 min; (3) a final extension for 10 min at 72°C. The sequence, source, annealing temperature, Mg^{2+} concentration, PCR cycles and product size of primers are shown in Table 1. *β*-actin was used as an internal control. Prior to expression analysis of each gene, the house keeping gene was investigated for its product size after 28, 30, 32 and 34 cycles, the product size analysed and the number of cycles for each gene expression was selected accordingly. The products were separated on 1.5% agarose and documented with the help of gel documentation system (UVP, upland, CA, USA). Densitometric analysis was performed as previously described (Table S2).

Statistical analysis

Each Figure (for western blotting and RT-PCR) is a representative of three independent experiments with similar results. The error bars represent standard deviations (SD) of three experiments. Statistical analysis was performed using Student's *t* test and one way ANOVA followed by Tukey's test. Results were considered statistically significant at $p < 0.05$.

Results

NAC co-administration restores the hCG induced decrease in testis weight and spermatogenic arrest

NAC co-treatment (twice/week) arrested the decline in testes weight (Fig. 1) leading to a significant ($p < 0.01$) improvement in spermatogenesis (Fig. 2f) impaired due to chronic hCG administration (Fig. 2b). In contrast, NAC co-treatment (once/week) failed to show any favorable improvement (Fig. 2d). NAC given alone, once or twice/week (Fig. 2c, e) demonstrated no deleterious effects on

Table 1 Primer-specific conditions used for PCR amplification of candidate genes

Name	Primer sequence	Mg^{2+} conc. (mM)	Annealing temp. (°C)	PCR cycles	Product size (bp)
β-Actin[a]	F CTGTGCCCATCTATGAGGGTTAC	2.5	60	28	539
	R AATCCACACAGAGTACTTGCGCT				
Bax[b]	F CCAGGATCGAGCAGAGAGG	2.5	60	30	342
	R CGGAGGAAGTCCAGTGTCC				
Bcl-2[c]	F CTTTGTGGAACTGTACGGCCCCAGCATGCG	2.5	67	30	232
	R ACAGCCTGCAGCTTTGTTTCATGGTACATC				
p53[d]	F GGCCATCTACAAGAAGTCAC	2.5	55	30	317
	R CCAGAAGATTCCCACTGGAG				
Caspase-8[a]	F CTGGGAAGGATCGACGATTA	2.5	62	30	123
	R CATGTCCTGCATTTTGATGG				
Caspase-9[a]	F AGCCAGATGCTGTCCCATAC	2.5	65	30	132
	R CAGGAGACAAAACCTGGGAA				
Fas[a]	F GCAATGCTTCTCTCTGTGACCACT	3.5	65	28	351
	R GCTGTTGTGCTCGATCTCATCG				
FasL[a]	F GGAATGGGAAGACACATATGGAACTGC	1.5	65	28	238
	R CATATCTGGCCAGTAGTGCAGTAATTC				

[a] Maheshwari et al. [18]

[b] Ruwanpura et al. [27]

[c] Bozec et al. [28]

[d] Fogarty et al. [29]

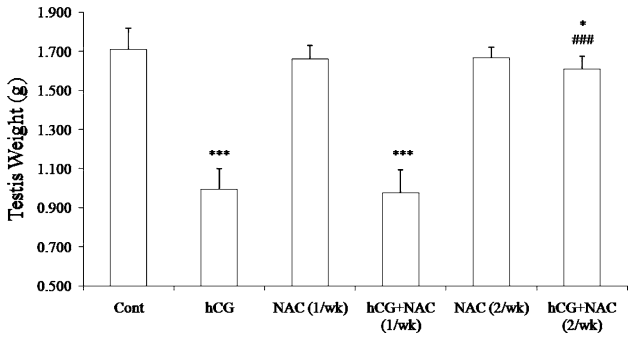

Fig. 1 Testis weight in rats after 30 days of various treatments. $^{*}p < 0.05$, $^{***}p < 0.001$, compared to vehicle treated control and $^{\#}p < 0.001$ compared to hCG treated ($n = 8$/gr)

testicular histology which was comparable to the vehicle treated controls (Fig. 2a). Reduction in testicular weight in hCG treated rats coincided well with hypo-spermatogenesis (Fig. 2b) which was associated with a significant decline in the various populations of developing germ cells (Fig. 2g). The round spermatids declined significantly ($p < 0.001$) more than other cell types (Fig. 2g). NAC co-treatment (twice/week) simultaneously helped to restore the germ cell numbers and spermatogenesis (Fig. 2f, g).

Effect of NAC on Leydig cell function

Leydig cell function as demonstrated by serum testosterone levels continued to decline from 15th day and was extremely low after 30th day of hCG administration (Fig. 3). NAC co-treatment twice/week but not once/week was able to arrest the falling testosterone levels improving the Leydig cell function leading to restoration of serum testosterone at par with vehicle treated control levels (Fig. 3).

Evaluation of intracellular H_2O_2 and oxidative stress in germ cells

H_2O_2 is an important ROS molecule and a signaling agent of apoptosis. In order to find out alterations, if any, on H_2O_2 levels in germ cells, intracellular levels of H_2O_2 were measured using a fluorescent dye CM-H_2DCFDA. A significant rise ($p < 0.001$) in the levels of H_2O_2 was observed both in germ and interstitial cells after 30 days of chronic hCG treatment. The rise was more pronounced in interstitial cells as compared to the tubular cells (Fig. 4a). NAC (twice/week) completely inhibited the rise in H_2O_2 levels in the tubular cells. The arrest of H_2O_2 rise through NAC mediation was significant too in interstitial cells. NAC

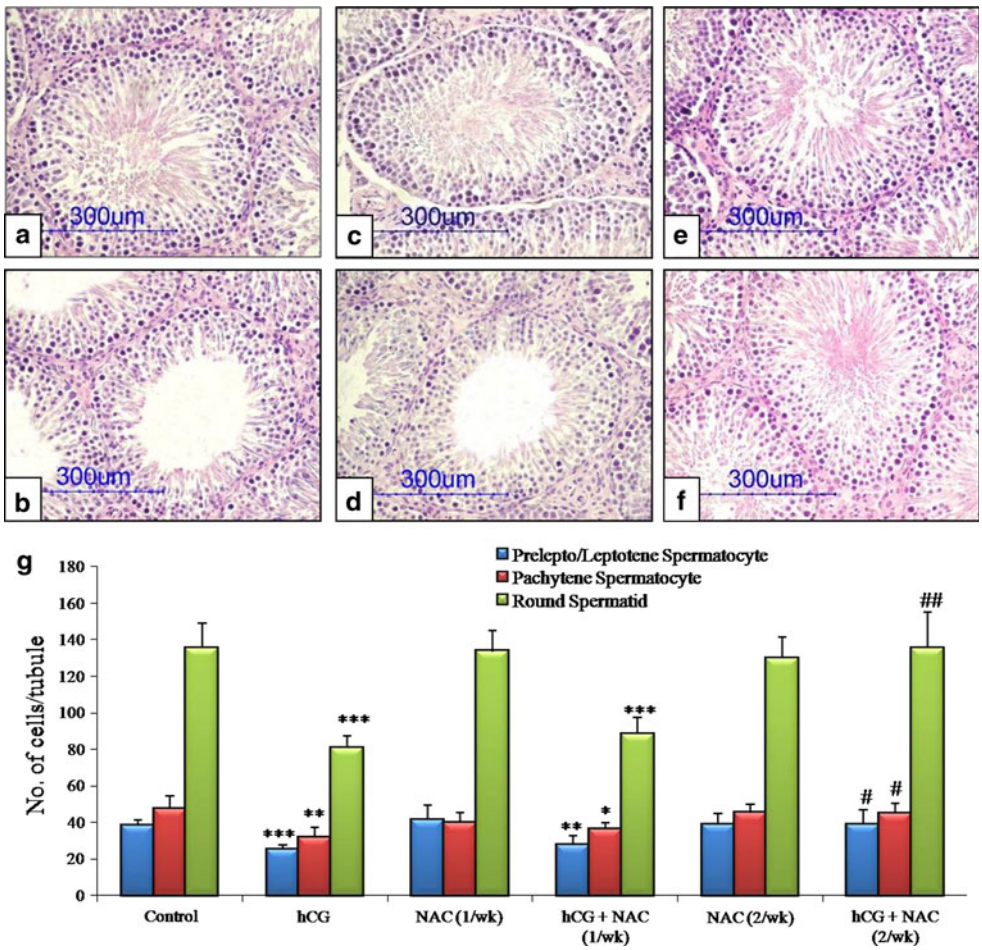

Fig. 2 Histological sections of rat testis after 30 days of **a** vehicle treated control, **b** hCG only, **c** NAC once/week, **d** hCG + NAC once/week, **e** NAC twice/week, **f** hCG + NAC twice/week ($n = 8$/gr) treatment. **g** Histogram showing quantitation of germ cells/seminiferous tubule in the treated rats. ×400 $^{*}p < 0.01$, $^{**}p < 0.001$ compared to vehicle treated and $^{\#}p < 0.01$, $^{\#\#}p < 0.001$ compared to hCG treated

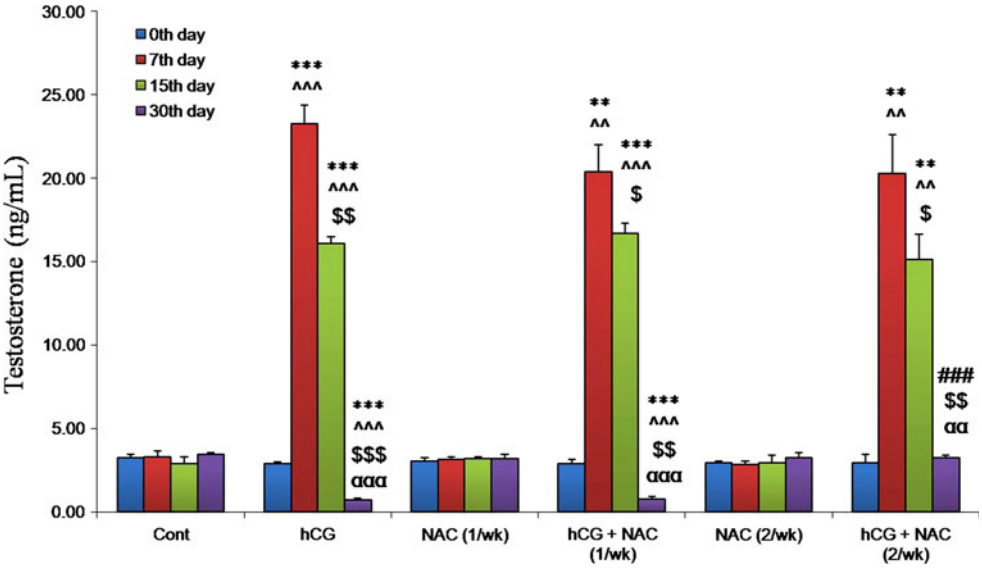

Fig. 3 Serum testosterone in rats following treatment ($n = 8$/gr) for different durations. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to 0 day in vehicle treated control; $^{\#\#\#}p < 0.01$ compared to 0 day hCG treated group; $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ compared to respective treatment group of 0 day; $^{\$}p < 0.01$, $^{\$\$}p < 0.001$ compared to respective treatment groups of 7th day; $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha}p < 0.001$ compared to respective treatment groups of 15th day

co-treatment for once/week, however, demonstrated little protective effect (Fig. 4a).

Identically, the increase in lipid peroxidation in germ cells following hCG treatment was effectively checked by NAC co-intervention (twice/week). NAC on its own did not induce any excess TBARS formation but also failed to curb the same when given once/week in combination with hCG (Fig. 4b).

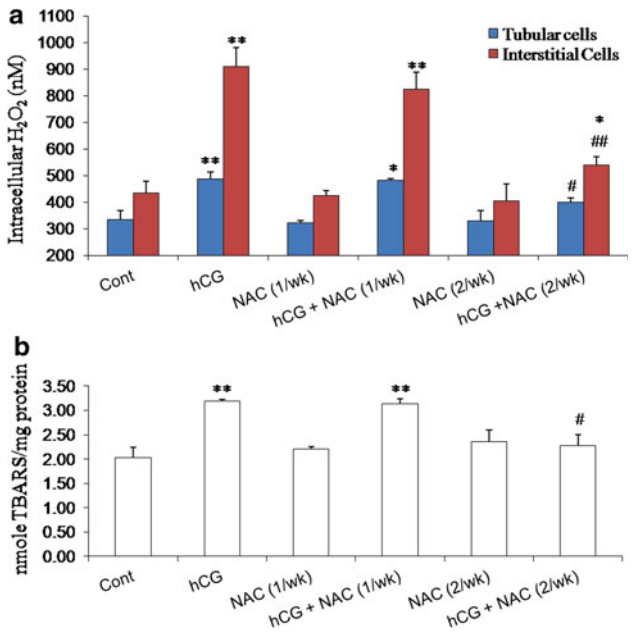

Fig. 4 Assessment of NAC counteraction of rise in intracellular H_2O_2 and oxidative stress ($n = 8$/gr). **a** Depicts intracellular H_2O_2 levels in tubular and interstitial cells and **b** lipid peroxidation in germ cells isolated from testis of various treated groups. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to vehicle treated; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to hCG treated

Improvement in antioxidant status following NAC + hCG co-treatment

Significant improvement in the activities of antioxidant enzymes, SOD (Fig. 5a), catalase (Fig. 5b) and GST (Fig. 5c) in germ cells was observed following NAC + hCG co-treatment (twice/week) compared to those from the hCG only treated group. Total glutathione (Fig. 6a) and antioxidant capacity (Fig. 6b) of these cells attenuated after hCG administration were also replenished back to control levels following NAC (twice/week) intervention. In contrast, NAC given once/week was ineffective.

NAC promoted cell survival by preventing hCG induced apoptosis of germ cells

The NAC mediated germ cell survival was studied through TUNEL assay both in testis sections and isolated germ cell populations from rats receiving chronic hCG treatment. TUNEL positive germ cells were much more prevalent in the seminiferous epithelium of hCG only treated rats (Fig. 7c) compared to vehicle treated control rats (Fig. 7b). NAC only administration (once/week, Fig. 7d or twice/week, Fig. 7f) demonstrated no adverse effects on the seminiferous epithelium. On the other hand, NAC intervention (once/week) to chronically hCG treated rats did not incite any favorable response in bringing down the number of apoptotic germ cells (Fig. 7e). However, with NAC intervention (twice/week), a significant reduction in the germ cell apoptosis was resolved (Fig. 7g). Figure 7a represented the negative control testis section. The distribution of TUNEL positive germ cells/tubule in different groups is shown in Fig. 7h.

Identical rise in TUNEL positivity was observed in testicular germ cells isolated in vitro from the hCG treated group. Compared to vehicle treated controls (Fig. 8a), approximately 17% of the germ cells were found TUNEL positive (Fig. 8b, g) which declined to control levels

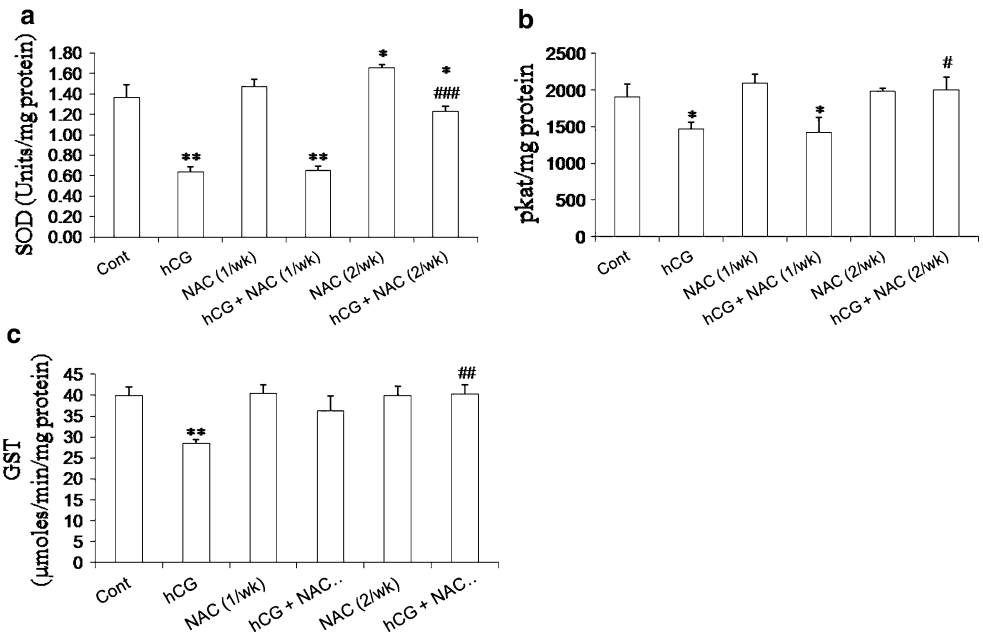

Fig. 5 Activities of antioxidant enzyme activity in isolated germ cells from the rat testis, vehicle control versus other treatments ($n = 8$/gr). **a** SOD, **b** Catalase, **c** GST. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to vehicle control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to hCG treated. Strong augmentation in antioxidant enzyme activity was seen with NAC intervention

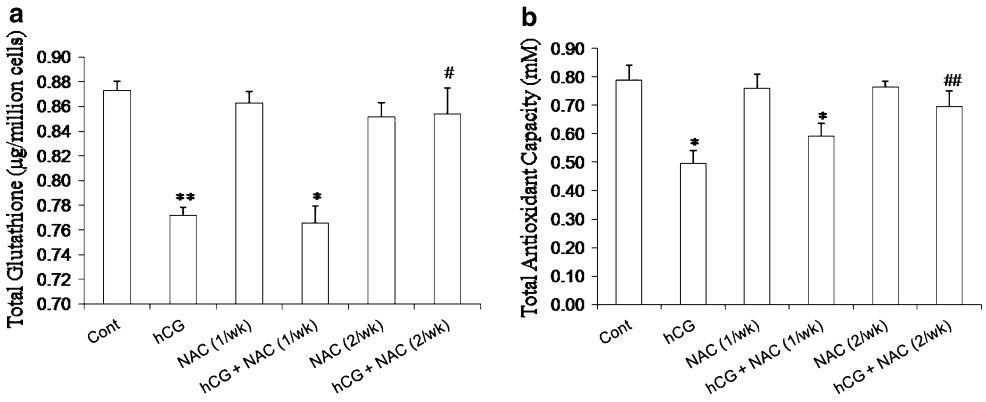

Fig. 6 Improvement in total glutathione and antioxidant capacity of germ germ cells after NAC intervention (twice/week) in hCG treated rats ($n = 8$/gr). **a** Represents total glutathione and **b** total antioxidant capacity. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to vehicle treated control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to only hCG treated

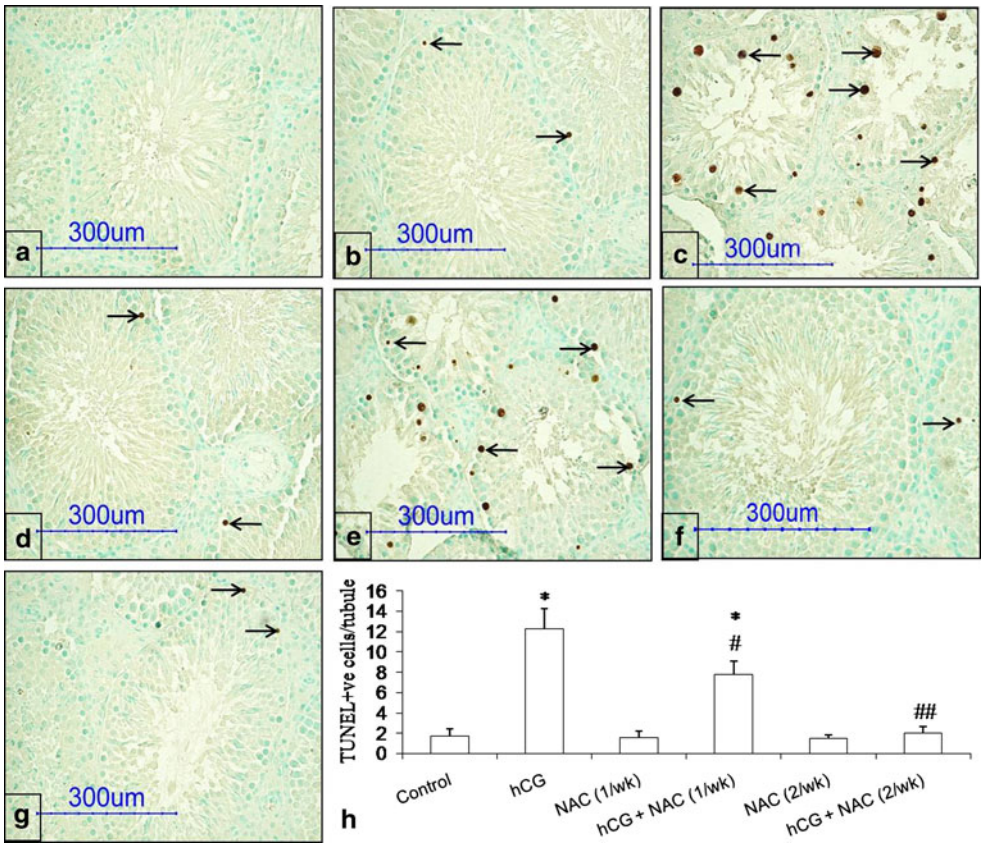

Fig. 7 TUNEL positive (→) germ cells in testis sections of rats ($n = 8$/gr) after 30 days of treatment (×400). **a** –ve control, **b** vehicle treated control, **c** hCG only, **d** NAC once/week, **e** hCG + NAC once/week, **f** NAC twice/week, **g** hCG + NAC twice/week, **h** Graph showing TUNEL +ve cells/tubule. $^{*}p < 0.001$ compared to vehicle treated; $^{\#}p < 0.01$, $^{\#\#}p < 0.001$ compared to hCG treated

following NAC intervention (twice/week. Fig. 8f, g). NAC given alone (once/week, Fig. 8c or twice/week, Fig. 8e) had little effect on apoptotic induction among germ cells.

Regulation of caspase-3 activity & PARP cleavage

Germ cells isolated from the hCG administered rats revealed a significant rise ($p < 0.01$) in caspase-3 activity (Fig. 9a) and expression of its active (p17) subunit (Fig. 9b). There was also a demonstrable increase in the expression of PARP cleavage (Fig. 9b) protein. NAC co-administration (twice/week) to hCG treated rats completely prevented the rise in caspase-3 activity and expression of caspase-3 and PARP cleavage (Fig. 9b).

Modulation of extrinsic & intrinsic pathways of germ cell apoptosis

In order to determine the role of NAC in the modulation of extrinsic and intrinsic pathways of apoptosis, activities and

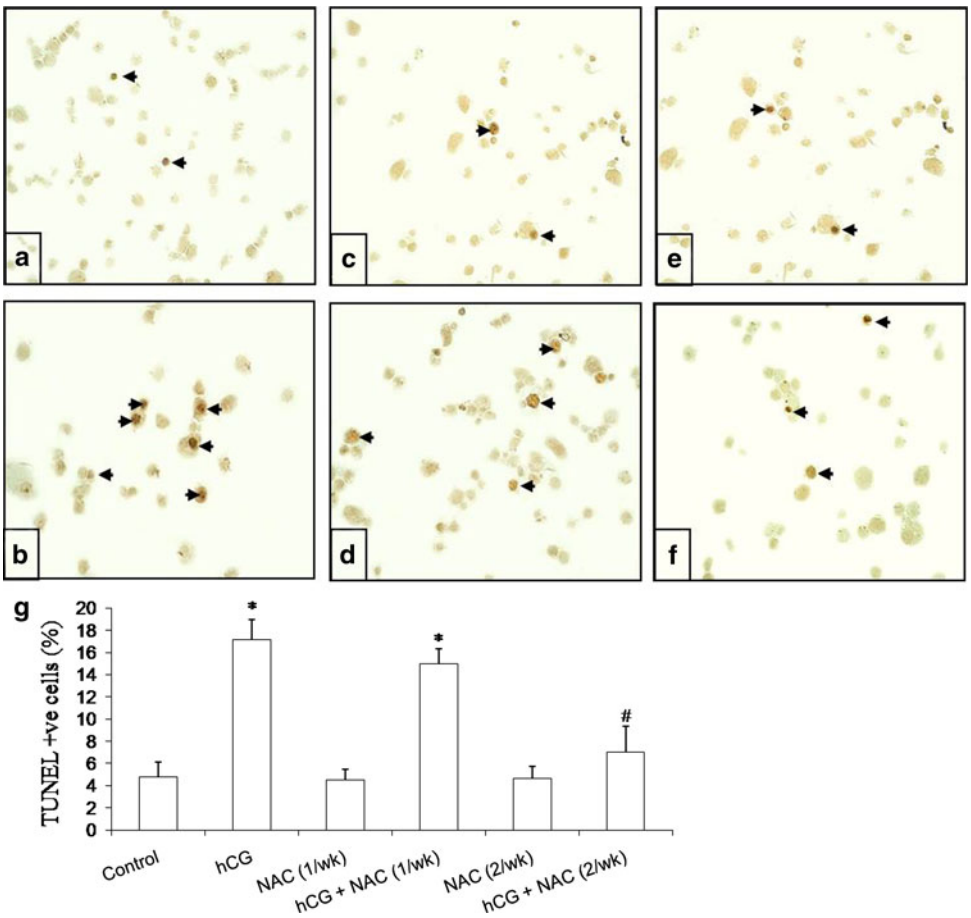

Fig. 8 TUNEL (→) germ cells isolated from testis after 30 days of treatment ($n = 8$/gr). **a** vehicle treated control, **b** hCG only, **c** NAC once/week, **d** hCG + NAC once/week, **e** NAC twice/week, **f** hCG + NAC twice/week, **g** Graph showing % TUNEL +ve germ cells in different groups. $^{*}p < 0.001$ compared to vehicle treated; $^{\#}p < 0.001$ compared to hCG treated

expressions of the various pro-/anti-apoptotic markers were examined. Chronic hCG treatment induced a significant increase in activities of caspase-8 (Fig. 10a) and caspase-9 (Fig. 11a) in the isolated germ cells. Simultaneous upregulation in the protein and mRNA expressions for caspase 8 (Fig. 10b, c) and caspase 9 (Fig. 11b, c) were also observed. Over expression of upstream extrinsic, Fas and FasL (Fig. 10b, c) and intrinsic Bax (Fig. 11b, c) markers was seen. The expression of Bcl-2, being anti-apoptotic, declined following hCG treatment (Fig. 11b, c). Compared to vehicle treated controls (Fig. 12a), rise in Bax expression in isolated germ cells from hCG treated rats was further confirmed by immune-fluorescence (Fig. 12b). NAC intervention (twice/week) favorably modulated the expression of these apoptotic proteins (Figs. 10b, c, 11b, c, 12d).

Regulation of p53 expression

p53, which acts as sensor to DNA damage was found over-expressed both in protein and transcript levels following hCG administration. However, NAC co-administration (twice/week) successfully prevented the rise in its expression (Fig. 13a, b).

Regulation of NF-κB, JNK and Akt expression

Role of NAC in modulating other pathways of germ cell apoptosis was also examined. The degradation of IκB, an inhibitory protein of anti-apoptotic NF-κB that led to attenuation of NF-κB (Fig. 13c) following chronic hCG treatment was restored back to control levels by NAC intervention. Similarly, NAC also prevented the increase in phosphorylation of pro-apoptotic JNK and its downstream transcription factor, c-Jun. On the other hand, it stimulated the phosphorylation of anti-apoptotic Akt (Fig. 13c).

Discussion

The present findings establish that NAC co-administration with hCG rescued the germ cells from apoptotic induction which was mainly achieved through modulation of multiple signaling pathways of metazoan apoptosis. It augmented the intracellular antioxidant defense in germ cells leading to a reduction in oxidative stress. Simultaneous improvement in testosterone availability too promoted better germ cell survival and helped to restore normal spermatogenesis.

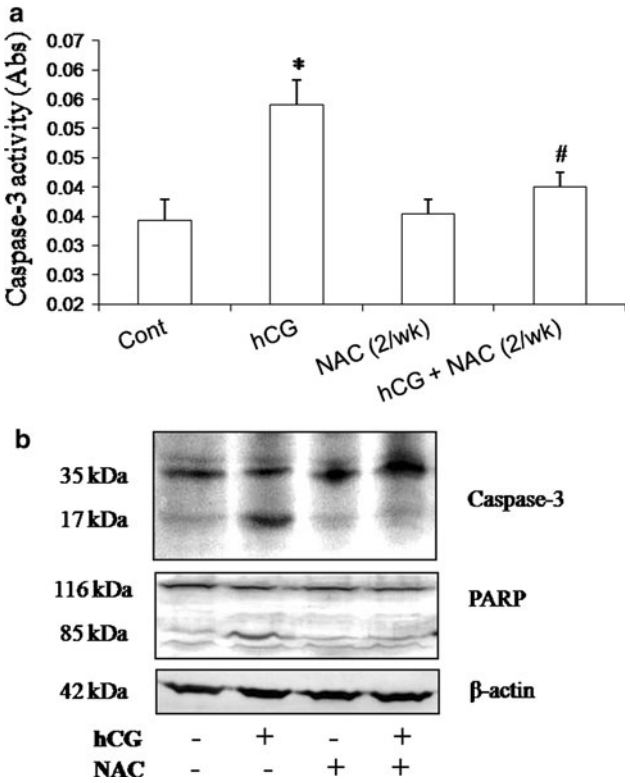

Fig. 9 **a** Caspase 3 activity in testicular germ cells of rats ($n = 8$/gr). NAC inhibited the rise in caspase-3 activity after chronic hCG treatment. **b** Identical inhibition of caspase-3 and PARP cleavage protein expression by NAC is seen in western blots. $^{*}p < 0.01$ compared to vehicle treated; $^{\#}p < 0.05$ compared to hCG treated

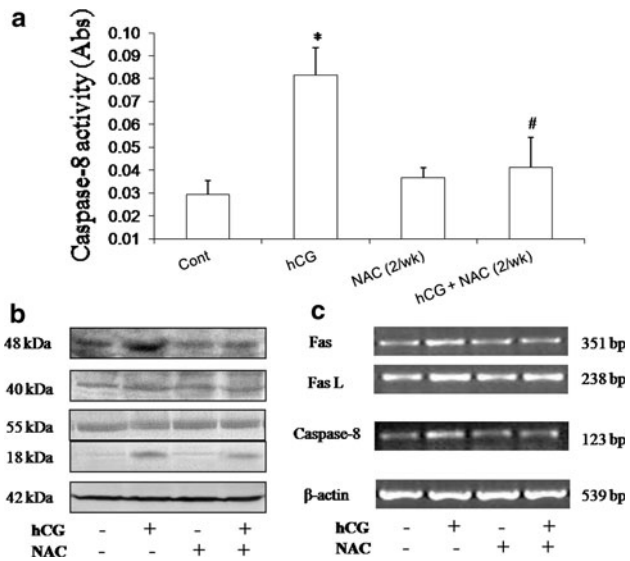

Fig. 10 Role of NAC in modulating the extrinsic pathway of apoptosis in isolated germ cells ($n = 8$/gr). **a** Caspase-8 activity along with **b** expression and Fas and FasL expression, **b** protein and **c** transcript levels were found significantly downregulated with NAC intervention. $^{*}p < 0.01$ compared to vehicle treated; $^{\#}p < 0.05$ compared to hCG treated ($n = 8$/group)

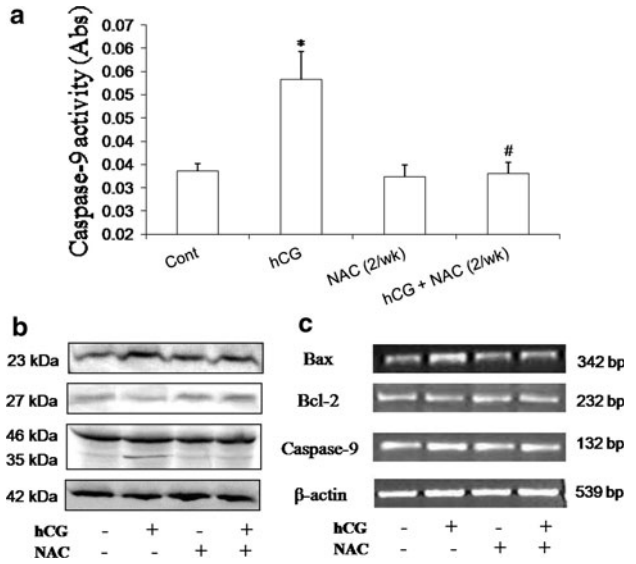

Fig. 11 Role of NAC in modulating the intrinsic pathway of apoptosis in isolated germ cells of rats ($n = 8$/gr). **a** Caspase-9 activity along with **b** expression and Bax expression, **b** protein and **c** transcript levels were found significantly downregulated with NAC intervention. Bcl2 on the other hand was upregulated. $^{*}p < 0.05$ compared to vehicle treated; $^{\#}p < 0.05$ compared to hCG treated ($n = 8$/group)

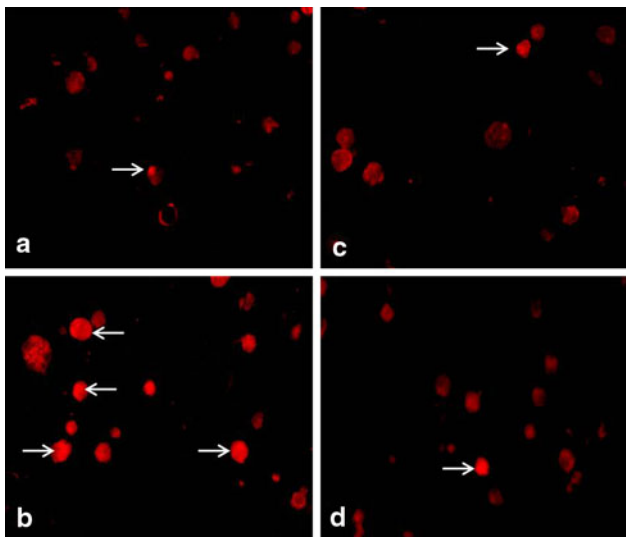

Fig. 12 Bax expression in isolated germ cells of rats through immunofluorescence. **a** Vehicle treated control, **b** hCG alone, **c** NAC twice/week, **d** hCG + NAC twice/week. The rise in Bax expression in germ cells after hCG treatment is curtailed hCG + NAC co-administration

One of the therapeutic options in the treatment of cryptorchidism is the hormonal therapy in the form of hCG treatment for several weeks which stimulates spermatogenesis leading to an increase in the diameter of seminiferous tubules [3]. In patients with hypogonadotropic

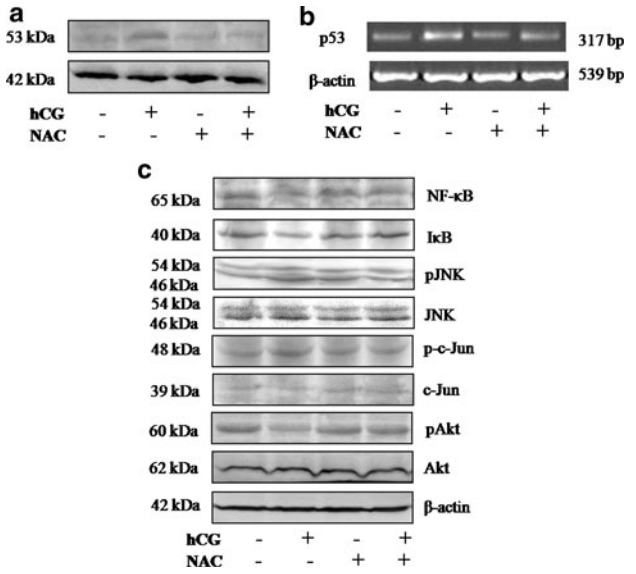

Fig. 13 NAC modulation of other pathways of germ cell apoptosis. NAC + hCG co-treatment significantly contained the rise in the **a** protein, **b** transcript levels of p53, **b** protein expression of pJNK, JNK, p-cJun, and stimulated expression of NF-κB, IκB and phosphorylation of Akt

hypogonadism, spermatogenesis can also be initiated and maintained by identical treatment with hCG, though the treatment options have now been modified to include human menopausal gonadotropin or recombinant follicle stimulating hormone as well [4, 30]. Besides all the above beneficial effects, hormone therapy with hCG also induces adverse affects in the form of germ cell apoptosis [13], the remedial interventions of which have not been tested clinically as yet. Since hCG treatment in adult animal models induces identical deterioration in the histology of testes [12], the same principle was utilized in the present work to maximize the apoptotic cell death in the seminiferous epithelium before simultaneous interventions with NAC was explored to counteract the adverse effect. NAC given twice/week during hCG stimulation promoted improved germ cell survival the molecular mechanisms of modulation in apoptotic pathways were subsequently investigated in the present work.

It is reported that pre-pubertal hCG injections affect post-pubertal germ cell maturation and androgen production in rat testis [31]. Testosterone availability is critical for germ cell survival. Serum levels of testosterone demonstrated significant attenuation (Fig. 3) as Leydig cell function deteriorated following chronic hCG treatment to animals. It is not only the refractoriness of cells to further stimulation that affected the Leydig cell function but also affected the very survival of cells under such conditions of repeated stimulation. Co-treatment with NAC (twice/week), however, salvaged the situation as serum testosterone was restored to control levels (Fig. 3) indicating improved Leydig cell function [14]. Such alteration in the hormonal milieu induces hypospermatogenesis leading to reduced testicular weight in rodents. Chronic estrogen treatment results similar alterations in reproductive hormone levels that impairs spermatogenesis [32]. In the present study too, spermatogenesis is not completely arrested but impaired with significant reduction in the number of immature germ cells/tubule (Fig. 2). As a result, testis weight decreases (Fig. 1) only to be regained following NAC co-administration (twice/week) that subsequently restores spermatogenesis (Fig. 2).

NAC is a thiol and precursor of L-cysteine and reduced glutathione. It is a source of sulfhydryl groups in cells and scavenger of free radicals as it interacts with reactive oxygen substances (ROS) such as hydroxyl radical and H_2O_2 [33]. It is permeable through plasma membrane and has been used as an alternate source to restore the GSH levels in cells [34]. The scavenging effect of NAC against ROS has been very effectively used clinically in different psychiatric disorders and also been explored as a therapeutic modality in male factor infertility [35, 36]. However, its therapeutic use in conditions of repeated or bolus hCG administration against the subsequent adverse effects on germ cells has never been reported before. This is primarily due to absence of any in vivo supportive data linking NAC administration with better germ cell survival under adverse conditions as examined in the present study. NAC is relatively a safe drug as it is neither teratogenic nor mutagenic [37]. However, the effective dose and duration have to be worked out in each case in order to maximize the benefit. A dose of 600/mg/day for a period of 3 months has been utilized previously to improve the oxidative status in idiopathic infertility. In the present study, while NAC (150 mg/kg b.w) given once/week has no positive impact, germ cell survival was significantly augmented when the frequency of NAC administration was increased twice/week keeping the dose of NAC unchanged.

Alteration in the serum levels of reproductive hormones also leads to increase in testicular oxidative stress [32, 38]. When chronic hCG treatment was utilized as a mode to alter the reproductive hormonal levels, an identical effect was obtained [13]. The later work further revealed that the rise in testicular oxidative stress was also associated with simultaneous increase in testicular H_2O_2 levels, the exact source of its production, intra- or inter tubular, remained unexplored. In this work, we attempted to seek an answer for the same by estimating the concentration of H_2O_2 in cells isolated from both these specific areas. The findings reveal that H_2O_2 rise was simultaneous both in intra- and inter tubular cells following the hCG administration, though the amplitude was much higher in interstitial cells compared to the tubular cells (Fig. 4a). Despite the

difference in the rise in levels of H_2O_2 in the cells of the two different compartments, the elevated levels of H_2O_2 among the tubular cells might constitute one of the important factors leading to germ cell apoptosis as presently observed (Fig. 7c). Since, H_2O_2 as a molecule has the ability to leach out from the source of its production and affect the neighbouring cells, the rise in its levels in the interstitial space may very well contribute to the germ cell demise which can only be confirmed further in future studies.

H_2O_2 has earlier been implicated as an endogenous messenger stimulating platelet derived growth factor (PDGF) in vascular smooth muscle cells. PDGF receptor binding caused peroxide formation which was inhibited by intracellular expression of catalases. Antioxidants, particularly thiol-reducing agents such as NAC could mimic the inhibitory effects of catalase and prevent redox activation of ligand-coupled protein kinase cascades [39]. We have reported recently that NAC is able to inhibit H_2O_2 induced germ cell apoptosis by regulating oxidative stress and modulating intrinsic as well JNK/c-Jun pathways of apoptosis [18]. Therefore, identical intervention with NAC (twice/week) was tried in the present in vivo study, which demonstrated successful inhibition of intracellular rise in H_2O_2 levels, lipid peroxidation (Fig. 4b), improved antioxidant enzyme activity (Fig. 5) and total cellular anti-oxidant capacity (Fig. 6) subsequently leading to a decline in germ cell apoptosis (Fig. 7f) in the seminiferous epithelium.

Signal transduction for apoptosis can be triggered by alterations in the redox status inside the cell [40]. The physiological relevance of apoptosis is very well documented which is common during normal spermatogenesis but gets accentuated in many pathological or experimentally induced conditions [41, 42]. The molecular mechanisms of hCG induced germ cell apoptosis and their subsequent modulation by NAC were further investigated in the present study. While hCG + NAC (once/week) did not show any positive impact on apoptosis inhibition, increasing the frequency of NAC administration (twice/week) significantly attenuated the number of TUNEL positive germ cells in the seminiferous epithelium (Fig. 7f). Caspase-3 activity/expression and PARP cleavage in the target cells were significantly downregulated. The present findings are similar to earlier reports of NAC mediated attenuation of apoptosis in which many different cell types including testicular germ cells were used [20, 43–45].

Fas and Fas ligand are expressed in the testis [46] and considerable attention is given in the past understanding the role of these proteins in the control of germ cell apoptosis in the testis. It is reported that upregulation of Fas receptor is associated with spermatocyte apoptosis during the first round of spermatogenesis in the rat [47, 48]. With estradiol induced germ cell apoptosis, FasL upregulation independent of any alteration in Fas expression is found sufficient to initiate the extrinsic pathway [49]. In the human testis, Fas/FasL expression is developmentally regulated and under gonadotropin control. Patients with post-meiotic germ cell arrest show increased Fas expression in germ cells suggesting that the Fas/FasL system is somewhat similar to a mechanism of controlling quality of cells that eliminates defective germ cells [50]. In the current work, there is an increase in Fas transcript and protein expression following chronic hCG administration that is brought down to control levels by NAC co-administration. FasL expression, on the contrary, remained unchanged (Fig. 10b, c). Further, the rise in caspase-8 activity, its transcript levels, and cleaved form were effectively countered by NAC (Fig. 10a–c). Identical findings are reported in Chinese hamster ovarian cells in which NAC was shown to inhibit acrolein induced apoptosis by inhibiting activity and processing of caspase-8,-9 and PARP [51]. The present observation on the rise in caspase transcript levels associated with similar rise in cleavage of the protein is well supported by findings from other studies as well [52, 53].

Germ cells isolated from hCG administered rats showed a marked increase in caspase-9 expression, processing to its active form, p35 and simultaneous upregulation in caspase-9 activity. However, NAC intervention significantly moderated the rise (Fig. 11a–c). This is in agreement with similar findings in CHO cells [51]. On the other hand, anti-apoptotic protein Bcl-2 demonstrated a completely reversed trend (Fig. 11b, c). The reported decrease in Bcl-2 mRNA expression in isolated immature Leydig cells [54] following 6 h of hCG (50 ng/mL) follows a similar trend as observed in the present study. NAC intervention also promoted neuroprotection of cutaneous sensory neurons through considerable upregulation of Bcl-2 and downregulation of Bax [44] which is identical to what presently observed through either expression analysis (Fig. 11b, c) or immuno-fluorescence (Fig. 12d).

While investigating the ability of NAC influencing other pathway of apoptosis, it is seen that following repeated hCG stimulation germ cells undergoing apoptosis accumulate p53 (Fig. 13a, b). p53 is a central mediator of the cellular response to DNA damage. It senses DNA damage and its activation provides the cell an opportunity to repair the damage before the induction of apoptosis. Cellular redox alterations lead to p53-induced cell apoptosis as absence of p53 expression correlated well with the attenuation of germ cell apoptosis after mono-(2-ethylhexyl)-phthalate exposure [55]. In our work, NAC + hCG co-administration successfully brought down the rise in expression of p53 (Fig. 13a, b) in the germ cells similar to cisplatin exposed LX-1 SCLC cells [43].

The preventive role of antioxidants on cell death is primarily through activation or inhibition of various factors associated with the signal transduction pathway of

metazoan apoptosis. These include the apoptosis-inducing factor, NF-κB, caspases, Fas, TNF, p53 and the products of the Bcl-2 gene family [56]. NAC reportedly inhibits activation of JNK, p38 MAP kinase, and NF-κB positively modulating the expression of numerous associated genes [57, 58] which ultimately favors cell growth and differentiation. In a similar manner, NAC intervention in the present study prevented I-κB degradation and promoted NF-κB accumulation (Fig. 13c) in the germ cells leading to their improved survival.

Extracellular signal regulated kinases (ERKs) are activated by mitogenic and proliferative stimuli and therefore, mitogen activated protein kinases (MAPKs) play a crucial role in regulating cell proliferation and apoptosis [59]. The JNK and p38 MAPKs also respond to environmental stress and chemotherapeutic drugs. The MAPK pathway activation represents a form of regulatory signal by which cells respond to stress in a stimuli specific manner. MAPK are among the protein kinases that are responsible for phosphorylation of the immediate early transcription factor for their respective genes like c-Jun and c-Fos. On the other hand, the anti-apoptotic phospho-kinase-B or Akt is an important protein that phosphorylates its downstream target Bad and the phosphorylated Bad bound by 14-3-3 proteins becomes unavailable for triggering apoptosis. Activated Akt can thus rescue cells from apoptosis induced by various stress signals. Akt is also shown to phosphorylate and inactivate ASK1, a kinase that transduces stress signals to the JNK and p38 pathways [60]. Systemic NAC administration was earlier seen to ameliorate ischemia associated activation of JNK [61]. In our studies, NAC co-administration efficiently downregulated JNK and induced Akt phosphorylation, thus promoted better survival of testicular germ cells (Fig. 13c).

In summary, the above findings indicate that germ cell apoptosis in the testis is one of the associated adverse effects of in vivo hCG treatment which may be effectively counteracted with NAC intervention. The benefit is not only limited to rescue the germ cells from apoptosis by positively modulating the multiple signaling pathways of metazoan apoptosis but also to improve testosterone availability as well leading to restoration of spermatogenesis. The present data on animals strongly favor NAC intervention in those clinical conditions requiring repeated hCG stimulation. However, the dose and duration of such intervention needs to be properly evaluated and extrapolated separately for maximizing the therapeutic benefit and curtailing the hCG induced adverse effects on testis.

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Conflict of interest No potential conflicts of interest to disclose.

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