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Differential modulation of apoptotic gene expression by *N*-acetyl-L-cysteine in Leydig cells stimulated persistently with hCG in vivo

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ABSTRACT

The present study was designed to investigate the molecular mechanisms of NAC (150 mg/kg bw twice/week) action in vivo under repeated hCG (100 IU/rat/day) stimulation to adult rats. Leydig cell refractoriness led to a significant decline in serum testosterone and intracellular cAMP by day 30 of chronic hCG intervention which improved significantly following NAC co-administration. It inhibited the rise in lipid peroxidation, improved the activity of antioxidant enzymes along with intracellular glutathione and total antioxidant capacity in the target cells. Leydig cell apoptosis declined significantly ($P < 0.001$) with down-regulation of upstream, Fas, FasL, caspase-8, Bax and caspase-9, JNK/pJNK and downstream caspase-3 and PARP. On the other hand, anti-apoptotic Bcl2, NF-kβ, and Akt were up-regulated. Taken together, the above findings indicate that the specificity of NAC action was not restricted to regulating marker proteins in the extrinsic and JNK pathways as seen in vitro but extended to include intrinsic pathway of metazoan apoptosis as well.

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1. Introduction

Leydig cell dysfunction in clinical cryptorchidism can be overcome by hormonal treatment with hCG (Dunkel et al., 1997; Aycan et al., 2006). However, the success rates with hCG administration vary widely from 6% to 80% (Kolon and Miller, 2001). This is described primarily due to the detrimental effects of hCG on both interstitial and tubular cells. It is reported that unsuccessful hCG treatment followed by orchidopexy even results in higher prevalence of azoospermia than orchidopexia alone (Chilvers et al., 1986). In animal studies, the dose dependence and the reversibility of effects of hCG on the histology of bilateral descended testes revealed a dose dependent significant decrease in mean germinal membrane thickness in the treated rats. However, the effect was found reversed at 3 months after treatment (Karaman et al., 2006). hCG administration has also been reported to induce focal disruption of spermatogenesis and apoptosis of testicular germ cells (Kerr and Sharpe, 1989; Gautam et al., 2007). Since modalities of hCG use in clinical conditions vary widely with dose and durations of treatment, the side effects of such treatments have not been extensively evaluated. There have been also limited attempts using animal models with appropriate interventions in vivo which can mitigate such effects.

The detrimental effects of persistent stimulation of Leydig cells were earlier reported from our laboratories (Aggarwal et al., 2009). It was found associated with significant rise in oxidative stress leading to Leydig cell apoptosis when analysed in vitro. However, when *N*-acetyl-L-cysteine (NAC) was simultaneously added along with hCG, it prevented oxidative stress and significantly stimulated the activities of antioxidant enzymes in the target cells resulting improved cell survival. The mechanism of inhibition of apoptosis as a sequel to NAC intervention was found mainly regulated through extrinsic and JNK pathways of metazoan apoptosis (Aggarwal et al., 2010). NAC is a well established thiol precursor that after its uptake deacetylates and converts to glutathione functioning both as a redox buffer and a reactive oxygen intermediate scavenger (Roederer et al., 1992; Mayer and Noble, 1994; Erkkila et al., 1998). NAC has also been effectively utilized in many different experimental animal models like oxidative injury following testicular torsion (Payabvash et al., 2007), varicocele (Duarte et al., 2010) and ischemia/reperfusion injury in the testis (Aktas et al., 2010) of rats. While ameliorative effect of NAC against oxidative stress is very well recognized, the mechanism by which it prevents apoptosis is cell and system specific and reported to be mediated through multiple modulations of gene expressions and signal transduction pathways (De Flora et al., 2004) The present study was therefore initiated to find out whether or not the ameliorative effect of NAC on persistently stimulated Leydig cells in vitro is similar when carried out in identical situations in vivo. In addition, the molecular

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mechanisms associated with such an effect were also investigated for comparison.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.2. Animals and treatment

Forty-eight adult male albino rats (Holtzman strain), weighing 200 ± 20 g were 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*. Animal experiments were carried out under strict compliance with the Guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India guidelines and Institutional guidelines for animal care. Both hCG and NAC were dissolved in PBS before being administered intra-peritoneally for 30 days. The details of the treatment plan with hCG alone or in combination with NAC were carried out as described below.

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

The dose of hCG was selected on the basis of its effective use in our earlier study (Gautam et al., 2007). The dose of NAC, on the other hand, was extrapolated from the data reported elsewhere (Payabvash et al., 2007). Without altering the dose, NAC administration was tried at two different time intervals to determine the efficacy of intervention. At the end of 30 days of treatment, animals were sacrificed and testes were weighed. One testis from each animal was immediately fixed in buffered formalin for histological analysis. The other testis was decapsulated and Leydig cells were isolated as described (Anakwe and Moger, 1986). Fraction of freshly isolated cells from all the groups were washed with PBS and smeared on poly-L-lysine coated slides and fixed in 4% formaldehyde for TUNEL assay and the rest were stored at −20 °C for biochemical and molecular analysis.

Blood was collected from the tail vein of rats at different time intervals (0th, 1st, 3rd, 7th, 15th and 30th day) of intervention under mild ether anesthesia and allowed to clot. Serum was separated by centrifugation at 5000*g* for 15 min and stored at −20 °C till assayed for hormones.

2.3. Testes histology and quantitation of spermatogenesis

Following 4 h in buffered formalin, the two poles of testis were cut with a fine blade and left for 24 h more at 4 °C. Tissues were washed off the fixative, dehydrated in upgraded series of alcohol, cleared in xylene and finally embedded in paraffin at 60 °C. Sections (4 μm) were cut from paraffin embedded blocks precooled in ice using a semiautomatic microtome (Leica Microsystems Inc., Bannockburn, IL, USA) and layered on poly-L-lysine coated glass slides. The tissue sections were deparaffinized, cleared with xylene and rehydrated before stained with haematoxylin and eosin and mounted in DPX. Testis sections were examined under the microscope (Nikon, Eclipse E600) and photographed with the help of image analyzer. Quantitation of the spermatogenesis in the testes of control and treated rats ($n = 6$) was carried out as described (Russell and Clermont, 1977). Briefly, 20 tubules in each testicular section (one section each from five different regions of testis) were randomly picked up and included in the analysis. Numbers of different types of germ cells present in a tubule were recorded through microscopic examination.

2.4. ELISA for Testosterone measurement

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

2.5. ELISA for cAMP assay

Intracellular cAMP in the Leydig cell ($\sim 5 \times 10^6$ cells) lysates were measured using the cAMP assay kit (R&D system Inc., USA). Briefly, reagents, samples and standards were prepared as per manufacturer's instructions. Primary antibody was added to all the wells (excluding NSB) followed by the cAMP conjugate. After 3 h of incubation on a shaker at RT, each well was aspirated, washed four times with washing buffer and added with substrate solution. The color development was carried out in dark at RT. Reaction was terminated after 30 min by adding stop solution and absorbance was read at 450 nm. Concentrations of cAMP in samples were calculated using the standard graph.

2.6. Lipid peroxidation and antioxidant enzymes activity

Leydig cells were sonicated for 30 s and divided in two parts. One part was assayed for lipid peroxidation as described (Ohkawa et al., 1979). The second part was centrifuged at 10,000*g* for 5 min and the supernatant was assayed for antioxidant enzyme activities. Superoxide dismutase (SOD) was measured (Das et al., 2000) and catalase was estimated (Aebi, 1984) by the degradation of hydrogen peroxide (6 mM). Glutathione-*S*-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig et al., 1974). The enzyme activities were normalized against protein present in the sample (Bradford, 1976).

2.7. Total Antioxidant Capacity (TAC)

TAC of Leydig cells from control and treated rats was assessed as per manufacturer's (Cayman Chemical Company, Ann Arbor, USA) instructions. The assay relies on 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 and the total antioxidant capacity (mM) was calculated from the trolox standard curve.

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