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siRNA as a tool to delineate pathway channelization in H_2O_2 induced apoptosis of primary Leydig cells in vitro

Himani Anand · M. M. Misro · S. B. Sharma · Sant Prakash

Abstract Using siRNA as a tool, the channelization of pathway in H_2O_2 induced apoptosis of primary Leydig cells was investigated in vitro. Exposure (4 h) to H_2O_2 (250 µM) induced maximum apoptosis but affected Leydig cell viability significantly. Therefore, expression of apoptotic marker genes, caspase-8, -9, -3 and polyadenosine ribose polymerase was subsequently investigated using the same concentration post 1 h exposure. Incubation with siRNA (20 nM) either for caspase-8 or -9, inhibited their individual expressions by 55–60 % and activity, 50–55 %. The inhibition efficiency using siRNA was comparable with post- or pre-H_2O_2 treatment of cells. Like siRNA, *Eugenia jambolana* (100 µg/ml) plant extract too, effectively countered over-expression of all apoptotic marker proteins. Silencing expressions of caspase 8 but not 9 through siRNA leads to a profound inhibition of caspase 3 implying that H_2O_2 induced Leydig cell apoptosis is preferably channeled through extrinsic and later extending to other pathways.

H. Anand · M. M. Misro (✉)
Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, Baba Gang Nath Marg, Munirka, New Delhi 110067, India
e-mail: mm_misro@yahoo.com

H. Anand · S. Prakash
Department of Zoology, Dayalbagh Educational Institute, Dayalbagh, Agra 282005, India

S. B. Sharma
Department of Biochemistry, University College of Medical Sciences, University of Delhi, Dilshad Garden, New Delhi 110095, India

Introduction

Impaired Leydig cell function during prenatal period has been associated with a number of human male developmental disorders such as, cryptorchidism, hypospadiasis and testicular cancers as well as decline in semen quality [1–3]. In adult life too, such a situation may lead to infertility or sub-fertility and needs to be corrected appropriately. However, various mechanisms of regulation of adult Leydig cell function are not very well understood. Leydig cell steroidogenesis though exclusively dependent on LH availability is also modulated by other intracrine and paracrine factors [4–6]. One such factor comes from the secretions of testicular macrophages located in close physical proximity with Leydig cells in the interstitium. Macrophage secretions are potentially rich in reactive oxygen species (ROS). Among others, H_2O_2, as a ROS, possesses the ability to diffuse in and out of cells and tissues easily [7] and has been recognized as one of the important biomolecules regulating physiological function in many cell and tissue systems [8–10]. The significance of this morphological association between testicular macrophages and Leydig cells has implications in regulating Leydig cell function locally since H_2O_2, at physiological concentrations, inhibited steroidogenesis and induced oxidative stress and apoptosis in isolated adult rat Leydig cells [11] in vitro. Though apoptosis in rat Leydig cells is reported to be channeled primarily through both extrinsic and intrinsic pathways [12, 13], the preference of one above the other following H_2O_2 induced apoptosis has not been worked out in detail.

Synthetic oligonucleotides are now used in functional genomics by targeting antisense knockdown of gene expression in cultured cells [14]. Such protocols use cationic lipids for transfecting DNA and oligonucleotides which are routinely carried out using common laboratory cancer cell lines [15]. However, such an approach is hardly applicable for primary cells which are non-dividing like adult Leydig cells which can be maintained in culture for few days only. To overcome the problems of transfection in such situations, we decided to make use of an N-TER^{TM}Nanoparticle siRNA (small interfering RNA) transfection system (Sigma-Aldrich, St. Louis, MO, USA) in the present work utilizing caspase 8 and caspase 9 siRNA to inhibit their individual expressions following H_2O_2 induced apoptosis of adult rat Leydig cells. The system has an N-TER^{TM} peptide that binds to siRNA non-covalently, forming a nanoparticle. The N-TER/siRNA nanoparticle possesses the ability to transfect into cells both in the presence and absence of serum allowing optimization of transfection conditions in a wide variety of cell types. This is for the first time that such a transfection is accomplished and reported here using primary mammalian testicular cells in vitro. Besides siRNA, the efficiency of the plant extract *Eugenia jambolana* (EJE) to inhibit the expression of apoptotic marker proteins was also examined in comparison since it was reported earlier to possess good antioxidant properties and rescued the Leydig cells from apoptosis under identical experimental conditions [16].

Materials and methods

Chemicals and reagents

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

Leydig cell isolation and treatment

Leydig cells were collected from adult male albino rats of Holtzman strain, 60–90 days of age, weighing about 200–220 g and maintained under controlled temperature (25 ± 2 °C) and constant photoperiodic conditions (12 h light: 12 h dark). The experimental protocols for the study were in accordance with the Institutional Guidelines for Animal Care issued by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India. The Leydig cell isolation was carried out as previously reported [17]. Briefly, the excised testes were rinsed in Medium 199 (M 199, pH 7.4) and the tunica albuginea, other connective tissues and blood vessels were carefully removed. The seminiferous tubule mass was transferred to 10 ml M 199 containing 0.25 mg/ml collagenase and kept for 15 min at 34 °C with constant shaking.

The dispersed tubules were allowed to sediment and the supernatant containing interstitial cells and blood cells was filtered through 100 and 43 μm mesh sequentially and centrifuged at 500 g for 10 min. The pellet was washed twice and resuspended in M 199 and plated as described [17]. In this way, contamination from macrophages, the source of endogenous H_2O_2 in the preparation was removed. The purity (~ 85 %) of the preparation (data not shown) was confirmed by 3β-hydroxy steroid dehydrogenase staining of the target cells [11]. Media containing cells were finally collated and approximately 5×10^6 cells were incubated with 100 and 250 μM or without (0 μM) H_2O_2 for 1, 2 and 4 h time durations. The dose and duration of H_2O_2 exposure (250 μM for 1 h) that induced maximum cell apoptosis without significantly reducing viability (>75 %) were subsequently utilized for further investigations.

Viable versus apoptotic Leydig cells

Following H_2O_2 exposure, viable and apoptotic Leydig cells (%) were detected using a Propidium iodide (PI)-AnnexinV-FITC kit system as per manufacturer's (Beckman Coulter, CA, USA) instructions. Briefly, cell pellets were resuspended in 100 μl ice cold AnnexinV-FITC binding buffer and incubated with 2.5 μg/ml AnnexinV-FITC and 0.3125 μg/ml PI on ice for 15 min in dark. Immediately after the incubation, 400 μl of binding buffer was added to each sample and the cells were analyzed on a Cell Lab Quanta SC Flow cytometer (Beckman Coulter, CA, USA). Red fluorescence signals emitted from PI stained cells were detected by a 488 nm (FL3 channel) argon-ion laser, while green fluorescence signals from AnnexinV-FITC stained cells were detected by 520 nm laser (FL1 channel). The cells (unstained), negative both for PI and AnnexinV-FITC are considered as viable.

Western blot analysis

Primary antibodies (rabbit polyclonal) anti-polyadenosine ribose polymerase (PARP), anti-caspase-3, anti-caspase-9 and (mouse monoclonal) anti-caspase-8 and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), were utilized to evaluate the upregulation of expression of apoptotic marker proteins following H_2O_2 exposure of Leydig cells. Goat anti-rabbit/mouse-HRP conjugate secondary antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Whole cell lysates were prepared in 200 μl lysis buffer containing 20 mM HEPES, 2 mM EDTA, 1 % Triton X-100, 10 % Glycerol, 150 mM NaCl, 50 mM β-glycerophosphate and protease inhibitor cocktail (Roche, Basel, Switzerland) and western blots were carried out as previously described [18]. Membranes were stained with 0.5 % ponceau-S to check loading and

transfer efficiency followed by blocking with 5 % non-fat milk for 2 h at room temperature. β-actin was used as an internal control and to ensure equal loading of protein. Densitometric analysis of the protein bands was carried out with the help of Image analysis software (Lab Works Image analysis software 4.0, UVP, Upland, CA, USA; Table S1, S2 and S3).

RT–PCR analysis

Total RNA was extracted using TRI-Reagent (Life Technologies Corp., Ambion®, TX, USA). Genomic DNA contamination, if any, was removed with DNase treatment (DNase I, 1 U/µg of RNA) at 37 °C for 30 min followed by EDTA (5 mM) at 65 °C for 10 min. Such a contamination was further assessed in samples by maintaining a 'nil' reverse transcriptase (RT) control during amplifications. cDNA was synthesized using 2 µg of total RNA by Omniscript RT kit (Qiagen, Hilden, Germany). PCR was carried out with 2 µl of RT reaction using the HotStar HiFidelity DNA polymerase (Qiagen, Hilden, Germany). Specific primers were obtained from Eurofins MWG Operon (Whitefield, Bangalore, India). For PCR reaction, the following temperature profiles were used; (1) denaturation at 95 °C for 15 min; (2) 30–32 cycles of 95 °C for 30 s, 55–67 °C for 1 min, 72 °C for 1 min; (3) 72 °C for 10 min for final extension. Table 1 shows the sequences, source, annealing temperatures, Mg^{2+} concentrations and product sizes of caspase-8, caspase-9 and β-actin primers presently used. β-actin was used as an internal as well as a non-template control. Prior to expression analysis of each gene, the house keeping gene was further investigated after 28, 30, 32 and 35 cycles, the product size analyzed and the number of cycles for caspase-8 and caspase-9 gene expression was selected accordingly. Post-amplification, the products were separated on a 1.5 % agarose gel and documented with the help of a gel documentation system (UVP). Densitometric analysis was performed as previously described (Table S4, S5 and S6).

siRNA transfection

Leydig cells were cultured in M 199 supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin at 37 °C with 5 % CO_2 and 95 % air. Leydig cells were plated at a density of 5×10^4 cells/well and treated with 250 µM H_2O_2 for 1 h. Post-exposure, cells were subjected to transfection with different concentrations of siRNA (5, 10 and 20 nM). Sense and antisense, rat specific caspase 8 and -9 siRNA (Accession Nos. NM_022277 and NM_031632, respectively) were obtained from Sigma-Aldrich, St. Louis, MO, USA (Table 2). Transfection was carried out using Sigma N-TER™ nanoparticle transfection system as per manufacturer's (Sigma-Aldrich, St Louis, MO, USA) instructions. Briefly, nano-particle formation solutions (NFS) were synthesized with siRNAs (caspase 8 or -9), N-TER peptide, N-TER buffer and nuclease free water, incubated at 37 °C for 45 min. The respective NFSs were then over layered on the plated cells and incubated at 37 °C overnight. The cells were harvested after 24 h, pelleted and stored at −20 °C until further use. The siRNA concentration (20 nM) that resulted maximum knockdown of expression (transcript and protein) was used in subsequent investigations. The efficiency of transfection was also assessed using siRNA in cells prior to H_2O_2 treatment. A scrambled universal negative siRNA (Sigma, St. Louis, MO, USA) was used as negative control. The interference of transfection reagent with siRNA duplexes was eliminated with un-transfected mock controls using cells treated with N-TER™ transfection reagent only.

Caspase-8 and -9 activities

Caspase 8 and -9 activities were assayed as per protocol supplied by the manufacturer (Biovision, San Diego, CA). Briefly, control and transfected cells ($\sim 5 \times 10^6$) were resuspended and incubated in cold lysis buffer for 10 min. The lysates were centrifuged at $10,000 \times g$ for 2 min at 4 °C and an aliquot of the supernatant (100 µg protein/50 µl) was added to 50 µl of the reaction buffer containing

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

Name	Primer sequence	Mg^{2+} concentrations (mM)	Annealing temperature (°C)	PCR cycles	Product size (bp)
Caspase 8[a]	F-CTGGGAAGGATCGACGATTA	2.5	62	32	123
	R-CATGTCCTGCATTTTGATGG				
Caspase 9[a]	F-AGCCAGATGCTGTCCCATAC	2.5	65	32	132
	R-CAGGAGACAAAACCTGGGAA				
β-actin[a]	FCTGTGCCCATCTATGAGGGTTAC	2.5	62	30	539
	RAATCCACACAGAGTACTTGCGCT				

[a] Maheshwari et al. [23]

Table 2 Characteristics and sequences of caspases 8 and 9 duplexes and their positions in the caspases-8 and -9 mRNAs

Target	Target sequence	siRNA sequence $5' \rightarrow 3'$	GC content
Caspase 8	AGACAACGTTTACCAAAT	S: CAGACAACGUUUACCAAAU dTdT	36.8 %
		AS: AUUUGGUAAACGUUGUCUG dTdT	
Caspase 9	GAGACATGCAGATATGGCA	S: GAGACAUGCAGAUAUGGCA dTdT	47.3 %
		AS: UGCCAUAUCUGCAUGUCUC dTdT	

200 μM of the chromogen [Ac-IETD-pNA (para nitroanilide) and Ac-LEHD-pNA for caspase-8 and -9, respectively]. The reaction mixture was incubated at 37 °C and terminated after 2 h by addition of stop-buffer. The release of pNA leads to a change in the absorbance which is measured at 405 nm using a microtitre plate reader (Bio-Tek, Inc., Winooski, VT, USA).

Analysis of caspase 8, -9 binding in transfected cells

Leydig cells showing positivity for caspase 8 or caspase 9 binding were analysed by flow cytometry on a Cell Lab Quanta SC flow cytometer (Beckman Coulter, CA, USA) using indirect immunofluorescence. Cells ($\sim 5 \times 10^4$/well) were transfected with 20 nM siRNA against caspase 8, -9 and universal negative siRNA as previously described. H_2O_2, treated or untreated and transfected or untransfected cells were washed with PBS and fixed with 4 % paraformaldehyde for 20 min at 4 °C. Cells were permeabilized in cell permeabilization buffer (CPB) containing 0.1 % cytonin and 1 % BSA in PBS (pH 7.2–7.4) for 10 min. Nonspecific binding was blocked by incubation in CPB containing 5 % BSA. Rabbit polyclonal anti-caspase 9 and mouse monoclonal anti-caspase 8 primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added (1:200 in CPB) and the cells were incubated in dark for 1 h at 4 °C. After washing with CPB, cells were again incubated in dark with FITC-conjugated goat anti-rabbit and goat anti-mouse secondary antibody, respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1,000 for 30 min at 4 °C. Following CPB wash, fluorescence signal from each cell was detected by 520 nm argon-ion laser. Data were collected for a minimum of 20,000 cells per sample. Fluorescence histograms were generated after gating the cell population using Cell Lab Quanta SC software (Beckman Coulter, CA, USA). Cells exposed to secondary antibody alone were used as negative control to assess the background staining which was subtracted from each acquisition to determine the percentage of actual positive binding.

Statistical analysis

All the figures (for Western Blotting and RT–PCR) are representative of three independent experiments with similar results. The results have been shown as the Mean ± SD of three experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's test. The significance of differences between percentage inhibition of caspase-8 and caspase-9 binding in pre-transfected and post-transfected H_2O_2 exposed cells was measured using student's t test. Statistical analysis was performed using Graph Pad Prism Software. $p < 0.05$ was considered statistically significant.

Results

H_2O_2 dose and duration of exposure on cell viability and apoptosis

In order to select the right concentration of H_2O_2 along with the period of exposure that would maximize apoptotic induction with minimal effect on cell vitality, Leydig cells were exposed to two different concentrations of H_2O_2, one lower (100 μM) and one higher (250 μM), with exposure time ranging from 1, 2 to 4 h. Post-exposure, apoptotic transformation in Leydig cells was highest (15.69 ± 1.2 %) with 250 μM of H_2O_2 which however was associated with a decline in cell viability to less than 70 % as determined by flow cytometry using the principle of PI-AnnexinV binding to cells (Fig. 1a–i). In contrast, using the same concentration and reducing the time of exposure to 1 h, cell viability (Fig. 1j) was maintained at a relatively much higher level (>75 %) with only a marginal decline in cell apoptosis to ~ 12 % (Fig. 1k). In subsequent experiments, therefore, the same dose and duration of H_2O_2 exposure was retained.

Over-expression of apoptotic marker proteins with H_2O_2

Irrespective of the cell system used, upregulation of gene expression in a shortest possible time has significant implications of studying the intricacies and molecular mechanisms associated with signal transduction pathways. In the present study, we have used this oxidized agent at a concentration that induced significant upregulation of apoptotic genes with a shortest possible time of 1 h. The selected apoptotic upstream marker proteins representing caspase 8 and -9 demonstrated over-expression in both protein and transcript levels after only
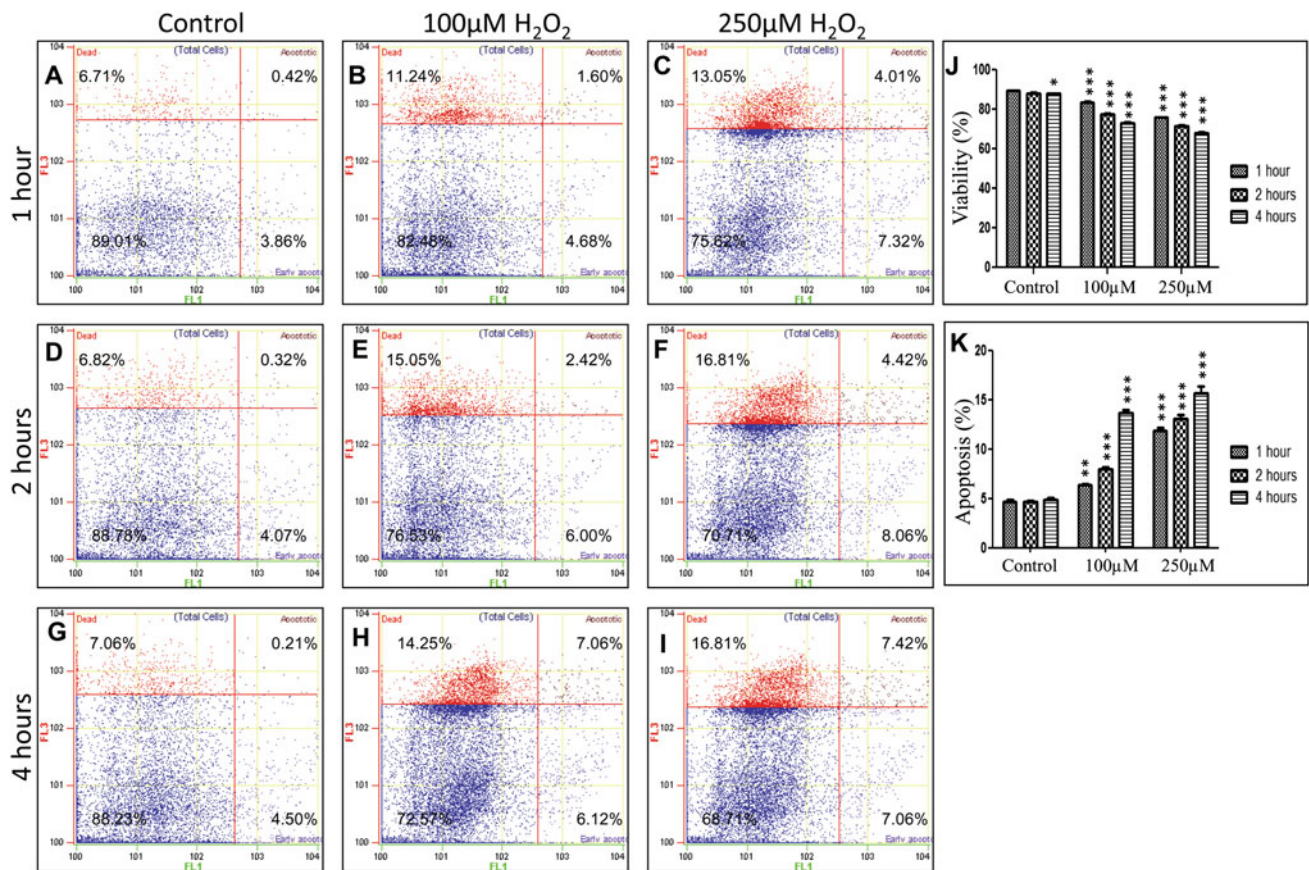
Fig. 1 Leydig cell viability and apoptosis by flow cytometry using FITC-labelled Annexin-V (*x*-axis) and PI (*y*-axis). Cell viability (%) and apoptosis (%) were assessed post H_2O_2 (100 and 250 μM) treatment for **a–c** 1, **d–f** 2 and (**g–i**) 4 h. **a–i** Viable cells in the *lower left* (AV−/PI−), apoptotic cells in the *lower* (AV+/PI−) and *upper right* (AV+/PI+), and dead cells in the *upper left* (AV−/PI+) quarter are shown. H_2O_2 (250 μM) exposure for 1 h maintained the cell (>75 %) viability (**j**) but induced ∼12 % cell (**k**) apoptosis. The *graphs* are representative of three independent experiments plotted as Mean ± SD. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to untreated control

1 h of exposure with H_2O_2 (Fig. 2a, b). It perfectly coincided with upregulated protein expression of downstream markers caspase 3 and PARP in western blots (Fig. 2a). Increasing the duration of exposure did not alter the protein but significantly raised the transcripts levels of caspase 8 and -9 (Fig. 2b).

siRNA use to knockdown the gene expression

siRNAs for two upstream apoptotic marker proteins namely caspase 8 (extrinsic) and caspase 9 (intrinsic), at different concentrations (5, 10, 20 nM) were initially tried to select the dose of siRNA that induced maximal knockdown of gene expression. By increasing the concentration of siRNA from 5 to 20 nM, the percentage of increase in the inhibition of protein expression for caspase 8 was 51 % while decline in transcripts was 15 %. For caspase 9, the corresponding define for protein and transcripts were 47 and 15 %, respectively (Fig. 3e). The cells, either treated or not treated with H_2O_2 but without siRNA, served as controls. Following selection of the dose (20 nM) of siRNA that induced

maximum downregulation of gene expression, the activity of the enzymes, both caspase 8 and -9 were subsequently analyzed. Significant ($p < 0.001$) attenuation in caspase 8 (50.1 %) and -9 (54.8 %) activity was observed with the use of corresponding siRNAs (Fig. 3f, g). Scrambled siRNA was used as a negative control which failed to inhibit the activity of either enzyme (Fig. 3f, g).

siRNA use in pre- versus post- H_2O_2 treatment leading to increase in cell survival

To determine whether or not preincubation of Leydig cells with siRNA followed by H_2O_2 treatment is a better option than post-H_2O_2 treatment, the cells from two different situations were analyzed for viability and apoptotic induction using PI-AnnexinV binding. In addition, the effect of the plant extract EJE which was recognized to have antioxidant properties and curtails apoptotic transformation in H_2O_2 treated cells, was investigated in comparison. Introducing siRNA (caspase 8, C-8 or -9, C-9) 24 h prior to H_2O_2
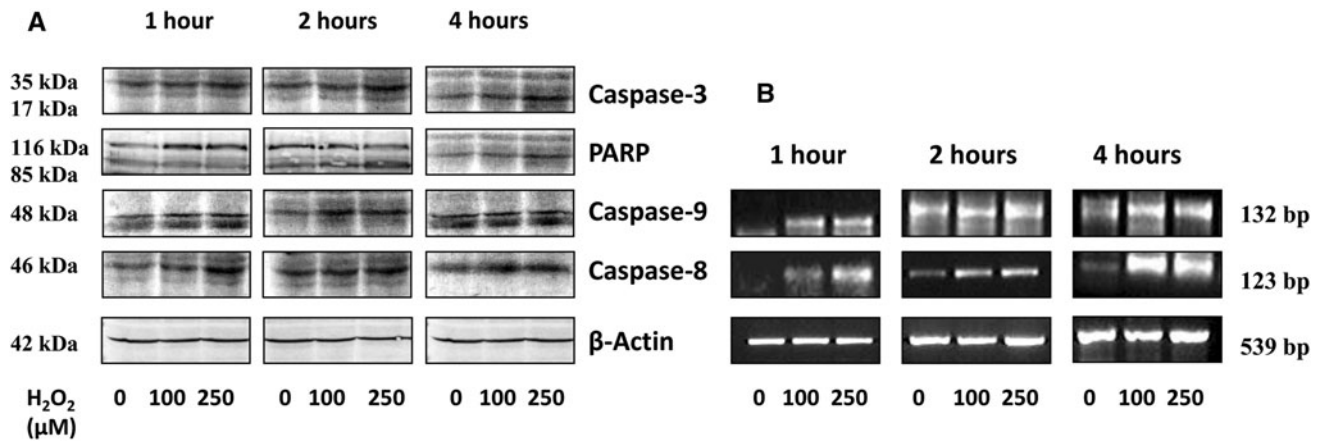
Fig. 2 H_2O_2 induced upregulation of apoptotic marker proteins. **a** Significant upregulation corresponding to duration of exposure in protein and **b** mRNA expression of *upstream* (caspase 8 and -9) and *downstream* (caspase 3 and PARP) apoptotic markers was seen
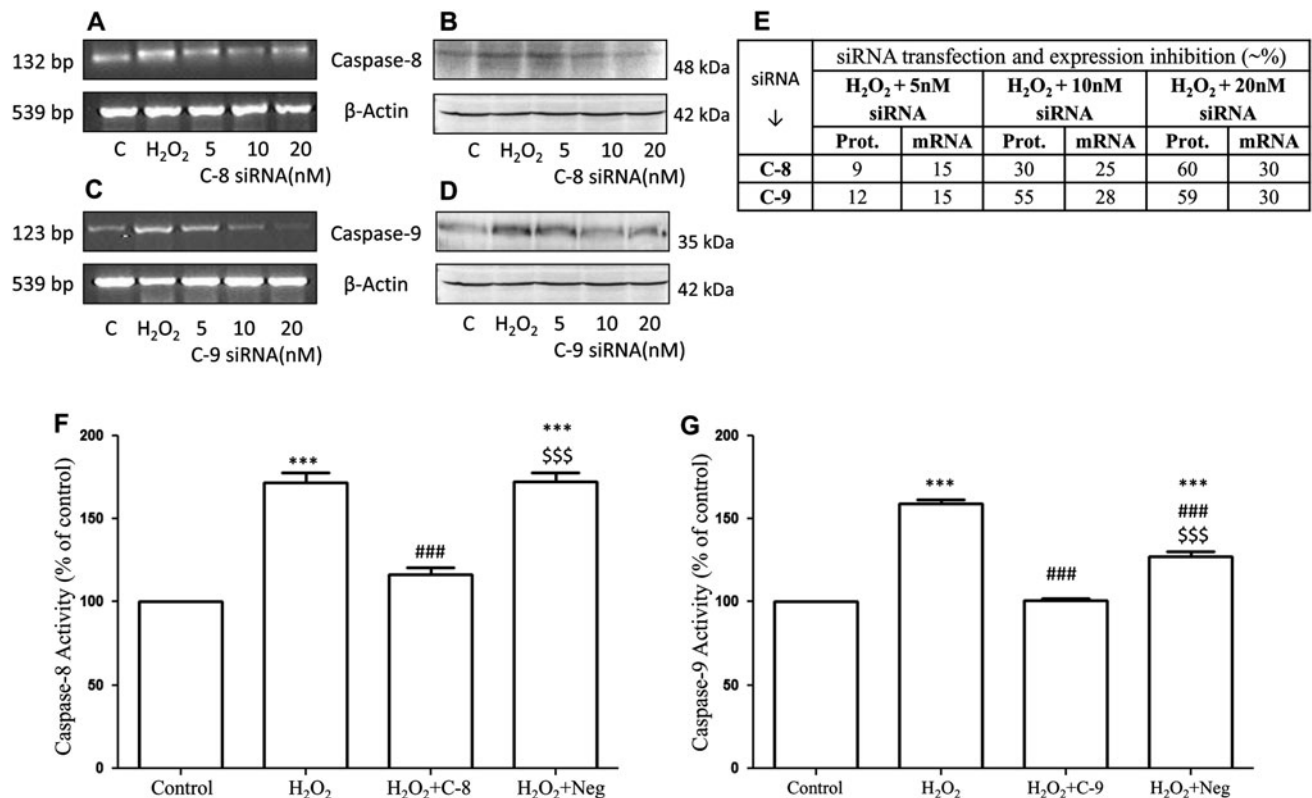

Fig. 3 Inhibition of gene expression with siRNA transfection. Out of different (5, 10 and 20 nM) concentrations of caspases 8 or -9 siRNA used, the inhibition efficiency [(**a**), transcripts and (**b**), protein] was maximum at the highest concentration. **c** Represents the percentage of

inhibition of expression. Corresponding decline in the activities of **f** caspase 8 and **g** caspase 9 was shown. ***$p < 0.001$ compared to untreated control, ###$p < 0.001$ compared to H_2O_2 treatment, \$\$\$$p < 0.001$ compared to H_2O_2 + siRNA

exposure, resulted a $\sim$6–7 % decrease in cell apoptosis (Fig. 4a–f, h). As a result, there was simultaneous improvement in cell survival compared to the group treated with H_2O_2 alone (Fig. 4a–f, g). On the other hand, introducing siRNA to cells post H_2O_2 exposure (1 h) yielded a comparatively better effect with a decline of 10–11 % in cell apoptosis (Fig. 5a–f, h) and restoring cell survival almost to control levels (Fig. 5a–g). EJE, either intervened pre or post

H_2O_2 treatment was equally effective suppressing ($\sim$7 %) Leydig cell apoptosis (Figs. 4h, 5h).

siRNA transfection efficiency in pre- versus post-H_2O_2 treatment by caspase 8,-9 binding

The efficiency of transfection with caspase 8, -9 siRNA in Leydig cells pre- versus post- H_2O_2 treatment was
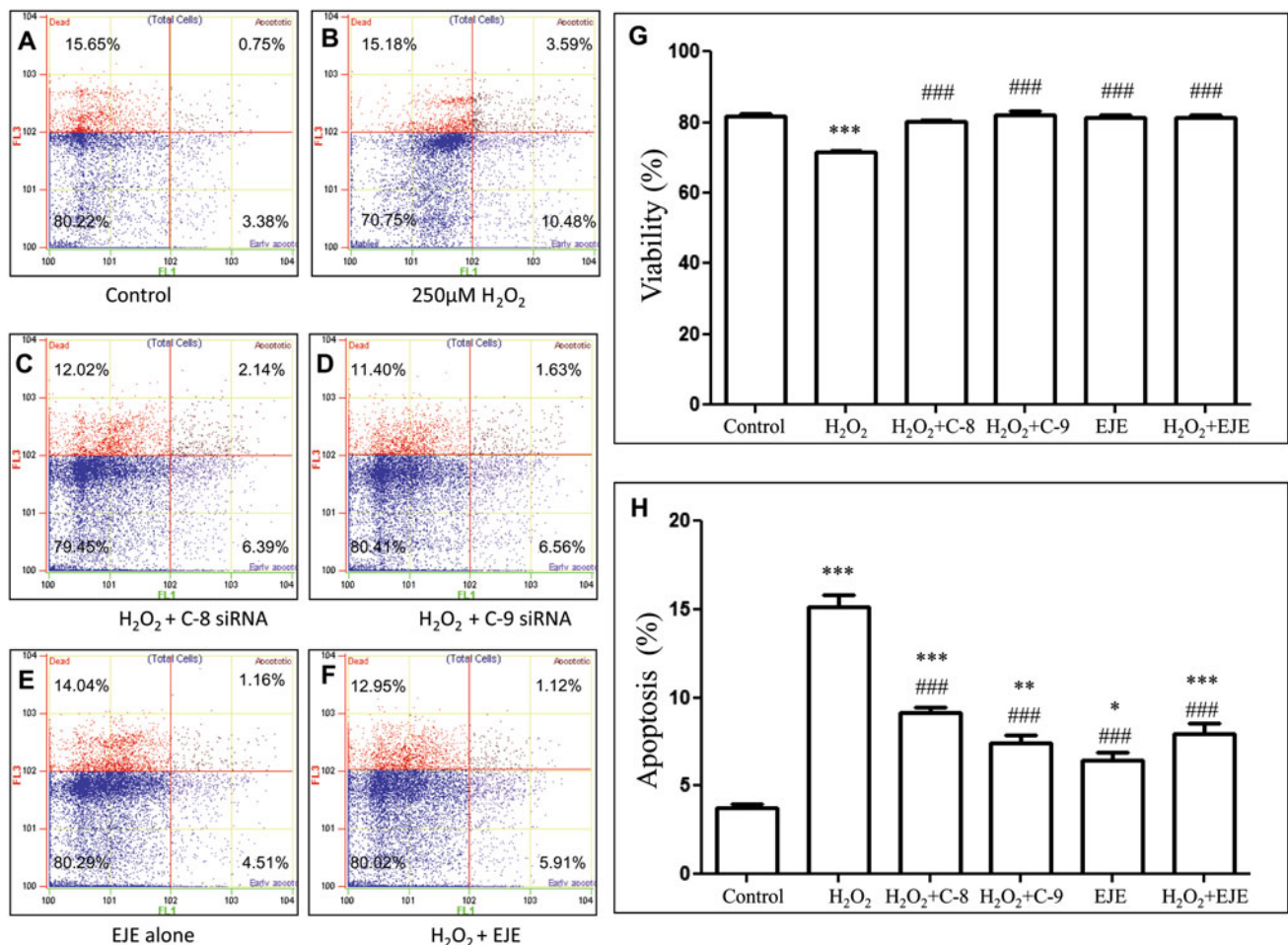
Fig. 4 siRNA transfection in Leydig cells prior to H_2O_2 treatment followed by flow cytometry to analyse cell viability and apoptosis (%) using FITC-labelled Annexin-V (x-axis) and PI (y-axis). **a** depicts untreated control, **b** H_2O_2 (250 μM) treatment, **c** H_2O_2 + caspase 8 (C-8) siRNA, **d**, H_2O_2 + caspase 9 (C-9) siRNA, **e** plant extract (EJE) alone and **f** EJE + H_2O_2. **g** With improved cell survival, siRNA transfection in cells 24 h prior to H_2O_2 exposure resulted in ~6–7 % decline in **h** apoptosis, siRNA caspase 9 showing a little better effect than caspase 8. The effect of plant extract (EJE) was comparable with that of expression inhibition using siRNAs. The *graphs* are representative of three independent experiments *plotted* as Mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to untreated control, ###$p < 0.001$ compared to H_2O_2 treatment

examined by indirect immunofluorescence using antibodies to caspase 8 and -9. Using caspase 8 (C-8) siRNA, caspase 8 positive cells were significantly ($p < 0.001$) brought down by 20 % of those treated with H_2O_2 alone (Fig. 6a–n). There was however, no significant difference in pre- versus post H_2O_2 incubation (Fig. 6a–l, o). EJE intervention, either pre- or post H_2O_2 treatment effectively reduced the number of caspase 8 positive cells by 22 % (Fig. 6f, l–n). With scrambled siRNA as negative control, no down trend in caspase 8 positivity was observed which was maintained at ~22–28 % compared to ~30 % with H_2O_2 alone.

Identically, caspase 9 positive cells showed a marked decline following siRNA transfection. However, preincubation with caspase 9 (C-9) siRNA induced a far more effective decline (22 %) in C-9 positivity than what was achieved (11 %) with post-H_2O_2 incubation (Fig. 7a–o).

EJE was equally effective in bringing down the caspase 9 positive cells, having a better efficacy when intervened during pre- than post-H_2O_2 treatment (Fig. 7f, l–n). Cells incubated with scrambled siRNA showed negligible effect in bringing down caspase 9 positivity (%) in the preparation.

Preferential initiation of extrinsic over intrinsic pathway of apoptosis

In order to identify whether or not there is a preferential channelization of extrinsic over intrinsic pathway during H_2O_2 induced Leydig cell apoptosis, protein expressions for caspase 8, -9, -3 and PARP were simultaneously examined with or without using siRNA to either caspase 8 (C-8) or -9 (C-9). Post-H_2O_2 exposure, inhibition of caspase 8 protein (↓60.6 %) induced identical inhibition (↓42 %) of caspase 3
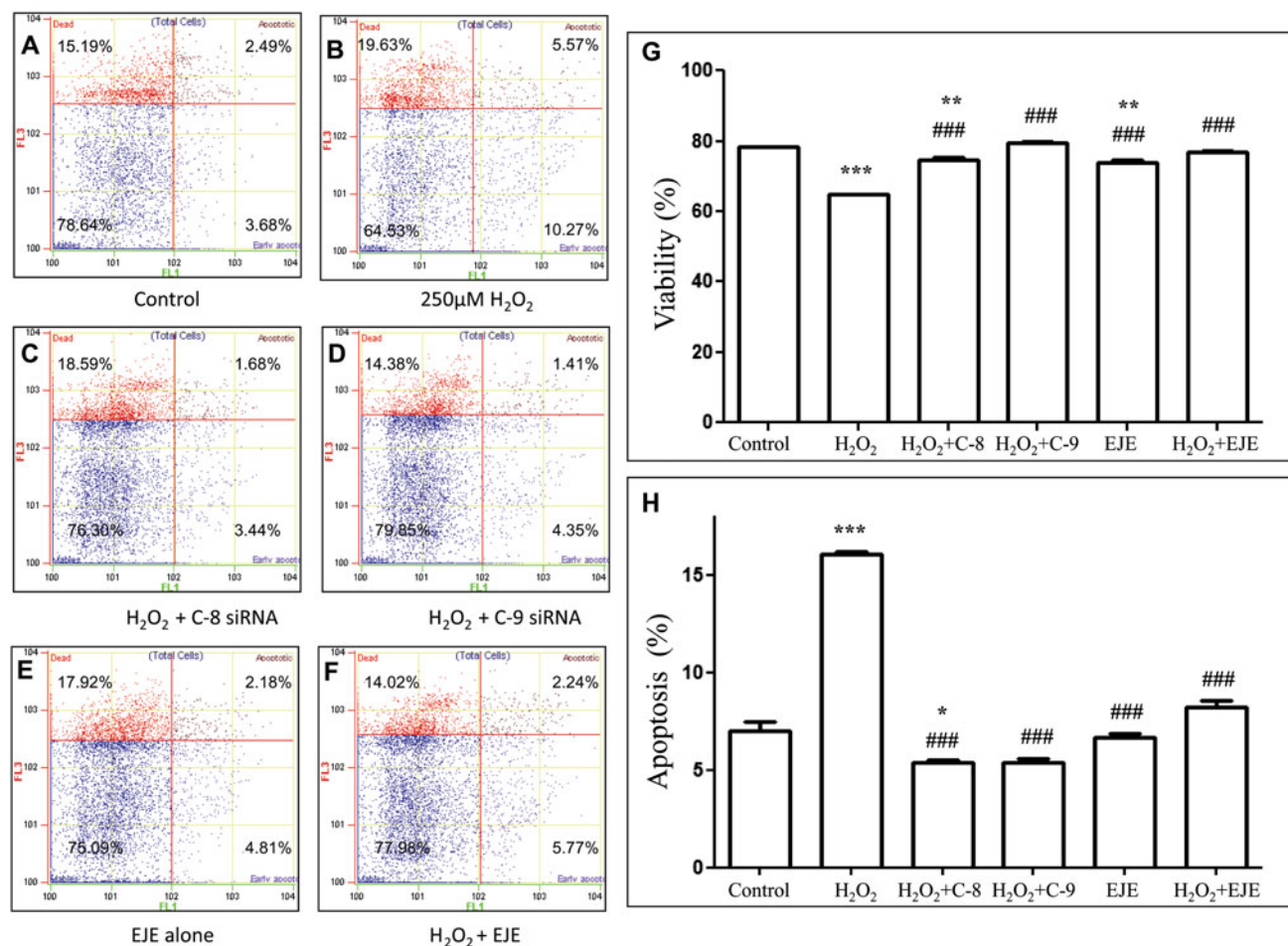
Fig. 5 siRNA transfection in Leydig cells post H_2O_2 treatment followed by flow cytometry to analyse cell viability and apoptosis (%)using FITC-labelled Annexin-V (*x*-axis) and PI (*y*-axis). **a** depicts untreated control, **b** H_2O_2 (250 μM) treatment, **c** H_2O_2 + caspase 8 (C-8) siRNA, **d** H_2O_2 + caspase 9 (C-9) siRNA, **e** plant extract (EJE) alone and **f** EJE + H_2O_2. **g** With improved cell survival, transfection in cells for 24 h post-H_2O_2 (1 h) exposure resulted in ∼10–11 % decline in (**h**) apoptosis with either caspases 8 or -9 siRNA. EJE was little less efficient than the siRNAs. The *graphs* are representative of three independent experiments *plotted* as Mean ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to untreated control, ###$p < 0.001$ compared to H_2O_2 treatment

protein. In contrast, caspase 9 inhibition (↓58.5 %) demonstrated a proportionate reduction of only 15.8 % in caspase 3 expression (Fig. 8a). PARP revealed no significant difference in expression using siRNA to either caspase 8 or -9. siRNA mediated inhibition of gene expression for caspase 8 (C-8) was further supported by corresponding attenuation in transcript levels as shown in Fig. 8b.

When siRNA was used in cells prior to H_2O_2 treatment, caspase 8 (C-8) downregulation (↓62 %) induced an identical decline (20 %) in caspase 3 expression. Subsequently, PARP cleavage was inhibited by 26 %. Using caspase 9 siRNA (C-9) the knockdown in expression was equally significant (↓56.5 %) but leading to only a marginal reduction (∼9 %) in caspase 3 expression. Reduction in PARP cleavage was only 5 % (Fig. 8c). Corresponding mRNA expressions for caspase 9 are shown in Fig. 8d.

Eugenia jambolana intervention in pre- or post-H_2O_2 treated cells demonstrated again a beneficial effect attenuating expressions of all apoptotic markers (Fig. 8a–d) with the lone exception of caspase 8 transcripts which displayed no significant difference (Fig. 8b, d).

Discussion

Upregulation as well as subsequent inhibition of specific gene expressions over a defined time not affecting viability in primary, nondividing mammalian cells has always been considered as a challenge. The findings from the present study indicate for the first time that it is possible to stimulate overexpression of apoptotic genes in a shortest possible time in adult Leydig cells with exposure to oxidant H_2O_2 for 1 h. The downregulation is followed by
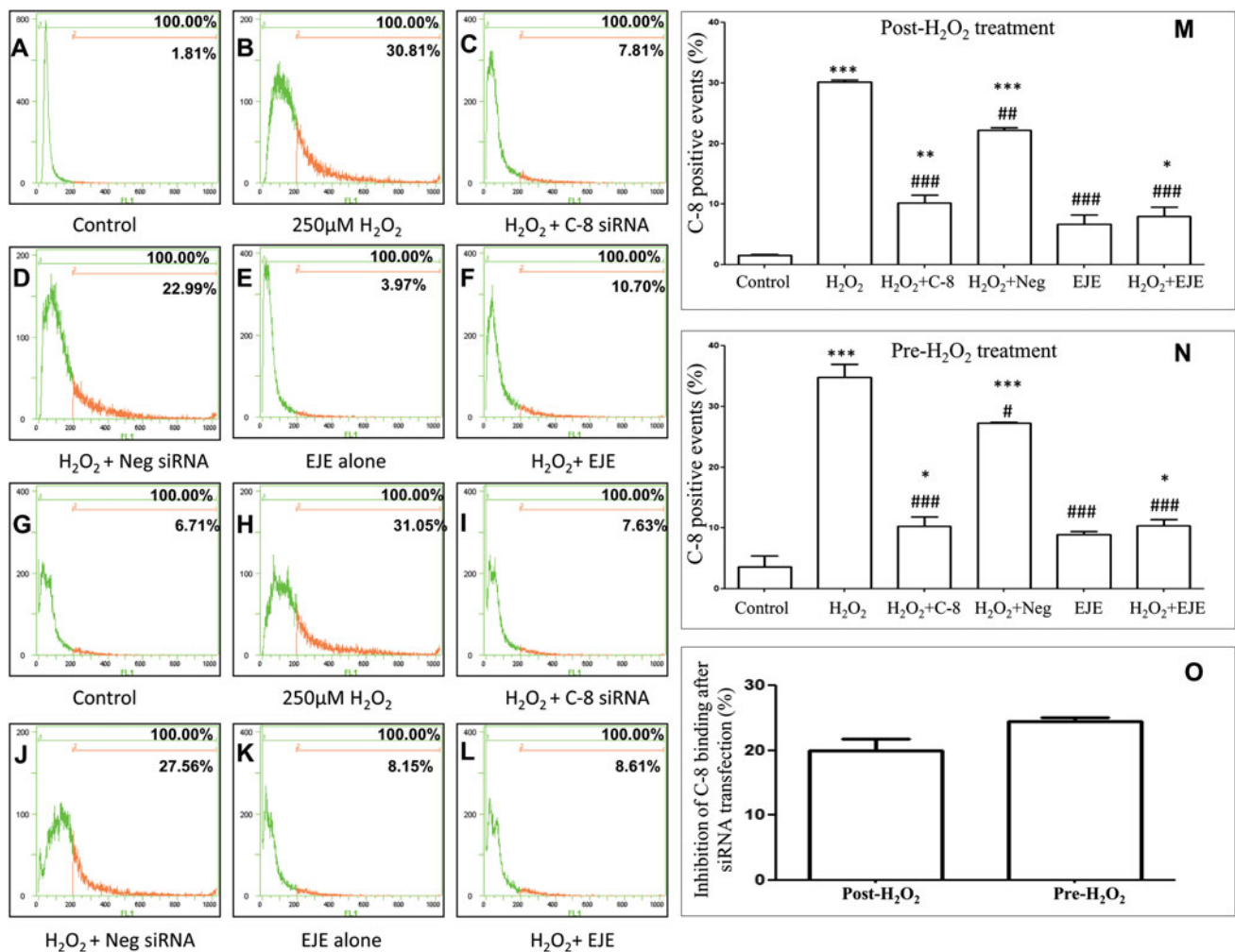
Fig. 6 Evaluation of transfection efficiency using caspase 8 (C-8) siRNA by flow cytometry through indirect immunofluorescence using caspase 8 antibody following **a–f** pre- and **g–l** post- H_2O_2 treatment. **a, g** Compared to untreated cells, the percentage of positive events for caspase 8 was significantly ($p < 0.001$) inhibited (~ 20 %) with the use of (**c, i**) C-8 siRNA. **d, j** Represents events using scrambled siRNA which were comparable to that of (**b, h**) H_2O_2 alone treatment which shows a significant rise (~ 30 %) in caspase 8 positivity ($p < 0.001$). Compared to EJE (**e, k**) alone, **f, l** EJE intervention against H_2O_2 exposure counters the rise in caspase 8 positive cells. **m, n** depicts % of viability and apoptosis in the different cell populations. **o** Inhibition of caspase 8 binding to cells following siRNA transfection was comparable with pre- or post-H_2O_2 exposure. The *graphs* are representative of three independent experiments *plotted* as Mean ± SD. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to untreated controls, ##$p < 0.05$ and ###$p < 0.001$ compared to H_2O_2 treatment

nanoparticle mediated knockdown of gene expression through caspase 8 or -9 siRNA which eventually indicated that H_2O_2 induced Leydig cell death is initially channeled through extrinsic but possibly extending later to intrinsic and other pathways of metazoan apoptosis.

Leydig cells are important somatic cells within the testicular interstitium. During testis development they originate and differentiate into two distinct populations, fetal and adult, that are regulated by a host of autocrine, paracrine, endocrine and other factors [19, 20]. There is a close association between adult Leydig cells and interstitial macrophages. Long microvillus-like Leydig cell processes are found inserted within coated pits of macrophages [21, 22].

Thus, more complex and less understood is the regulation, proliferation and differentiation in adult Leydig cells whose cellular function and survival is linked and affected by extraneous agents like H_2O_2 [11]. Cell death and replenishment would necessitate precursor cells to transform as fresh Leydig cells. Though the physiological significance of such a turnover in adult Leydig cell population is yet to be established, repeated hCG administration in rats was associated with a significant rise in H_2O_2 in the Leydig cells leading to their demise by apoptosis [23]. Since apoptosis requires upregulation of a number of marker genes and activation of proteins through signal transduction, in the present study, we investigated to find out the dose and duration of H_2O_2
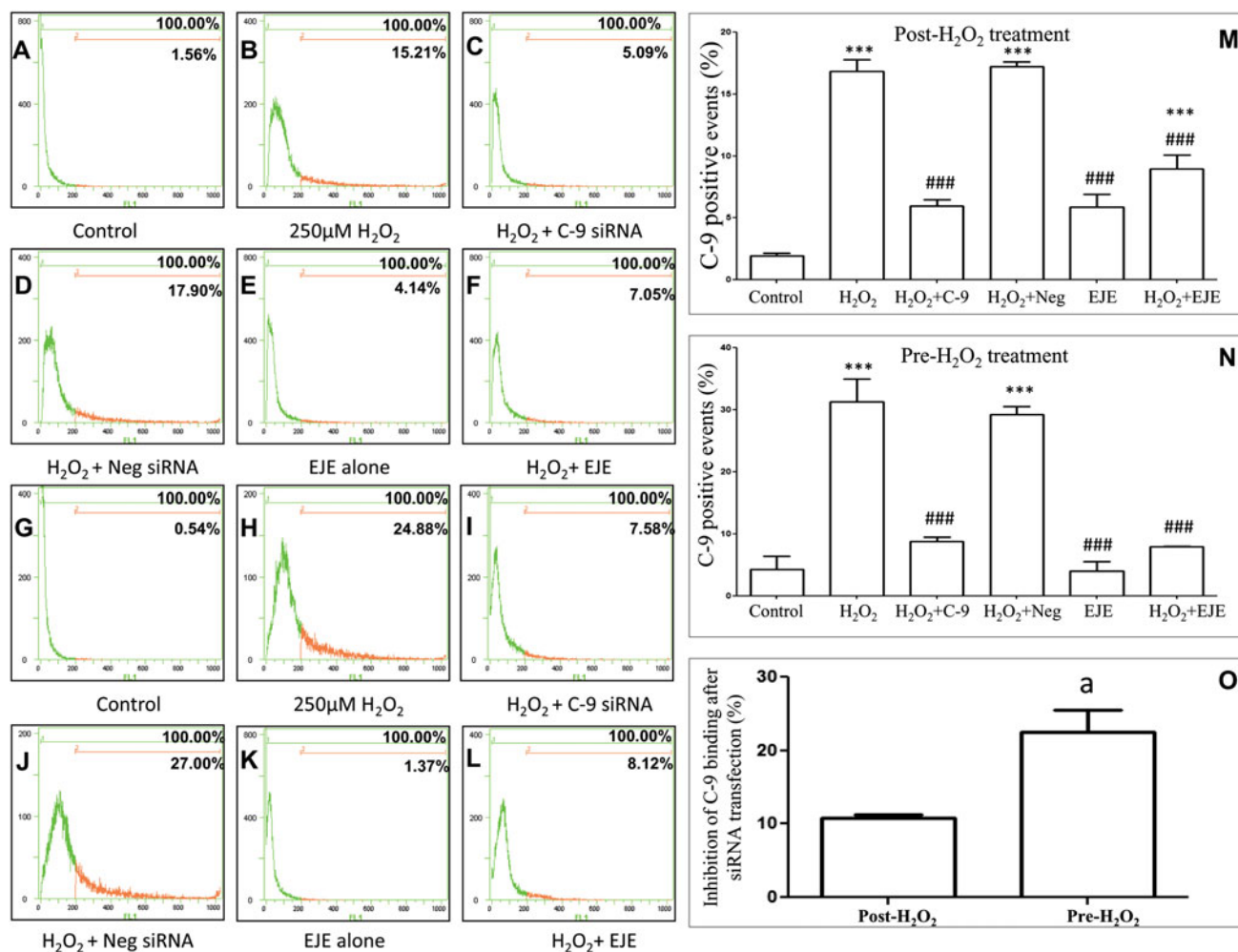
Fig. 7 Evaluation of transfection efficiency using caspase 9 (C-9) siRNA by flow cytometry through indirect immunofluorescence with caspase 9 antibody following **a–f** pre- and **g–l** post-H_2O_2 treatment. Compared to **a**, **g** untreated cells, the percentage of positive events for caspase 9 was significantly ($p < 0.001$) inhibited with the use of (**c–i**) C-9 siRNA. **d**, **j** Represent events using scrambled siRNA which were comparable to that of **b**, **h** H_2O_2 alone treatment which show more caspase-9 positivity in **b** pre- (25 %) compared to **h** post-(15 %) H_2O_2 exposure. Compared to EJE (**e**, **k**) alone, (**f**, **l**) shows the beneficial effects of EJE intervention against H_2O_2 exposure that counters the rise in caspase 9 positive cells. **m**, **n** Depicts % of viability and apoptosis in the different cell populations. **o** Inhibition of caspase 9 binding to cells following siRNA transfection was significantly ($p < 0.01$) higher with pre- than post-H_2O_2 exposure. The *graphs* are representative of three independent experiments *plotted* as Mean $\pm$ SD. ***$p < 0.001$ compared to untreated controls, ###$p < 0.001$ compared to H_2O_2 treatment, a$p < 0.05$ as compared to post-H_2O_2 exposed cells

challenge that could trigger an optimal gene overexpression without affecting cell survival.

There are only limited studies where H_2O_2 has been used to induce apoptosis both in primary cells and cancer cell lines. While inducing apoptosis in hepatocytes, concentrations of H_2O_2, 250–1,000 μM, were utilized [24]. However, in a recent study with HeLa cells in culture, 600 μM of H_2O_2 for a period of 8 h was used [25]. Though in our earlier work, 100 μM of H_2O_2 for 6 h was utilized to induce apoptosis in about 14 % of isolated adult Leydig cells [11], the same protocol was not followed in the present work. Instead, both the dose and the duration of exposure were altered so as to maximize H_2O_2 induced cell apoptosis within a considerably less period of time [Fig. 1].

250 μM of H_2O_2 for 1 h was found sufficient for the purpose as the event was synchronized with the upregulation of expressions for caspase 8, 9, 3 and PARP (Fig. 2). The short exposure further helped to maintain the cell viability but upregulated the expressions of marker proteins. It is quite possible that the same H_2O_2 dose and time may not work with all types of primary cells to upregulate the gene expressions, but need to be standardized with respect to each cell type. This is supported by the fact that using rat germ cells in culture identical gene upregulation is achieved with only 10 μM of H_2O_2 but without any alteration in exposure time [26].

The use of DNA/RNA oligonucleotides has been reported to be very beneficial for analyzing gene function

in cells/cell cultures [27]. For this purpose, DNA/RNA antisense nucleotides, containing a gene specific complimentary sequence to the translational initiation of the mRNA are used in various assays of gene silencing as a sequel to RNA mediated interference (RNAi). RNAi is characterized by specific mRNA degradation after the introduction of homologous double stranded RNA, known as siRNAs into cells [28, 29]. It is thus widely recognized that efficient siRNA transfection in different types of cells is crucial to down regulate the expression of a specific gene. However, considerable ambiguity exists selecting the right siRNA transfection protocol to inhibit the gene expressions using primary, nondividing cells like adult Leydig cells.

The delivery techniques of nucleic acids comprise various physical and chemical methods using viral and non-viral vector systems. There are advantages and disadvantages using conventional protocols like electroporation or calcium phosphate coprecipitation which are efficient but recognized as potentially harmful to the target cells [30]. Peptides as

shuttles for a controlled delivery of nucleic acids now represent a new and innovative concept [30, 31]. Considering the efficacy of such a method, an N-TER^{TM} Nanoparticle siRNA transfection system (Sigma-Aldrich, St Louis, MO, USA) was tried in the present study utilizing the adult Leydig cells with siRNAs to either caspase 8 or -9. With the increase in the doses of siRNA used, the inhibition of gene function in terms of protein expression was found upscaled by $\sim$60 % (Fig. 3a, e) and transcript levels by $\sim$30 % in either of the cases (Fig. 3b, e). Significant ($p < 0.001$) downregulation in the activity of both caspases was also seen (Fig. 3f, g). The findings demonstrate for the first time that the transfection system presently used has worked very efficiently involving rat testicular cells and retains a potential for similar use with any other type of primary and non-dividing cells in culture.

Peptide based delivery of siRNAs has been reported earlier with variable efficacies [32, 33]. The first study reportedly used siRNAs non-covalently complexed with the peptide leading to a strong downregulation of the target protein [34]. Covalent attachment of the cargo and the carrier
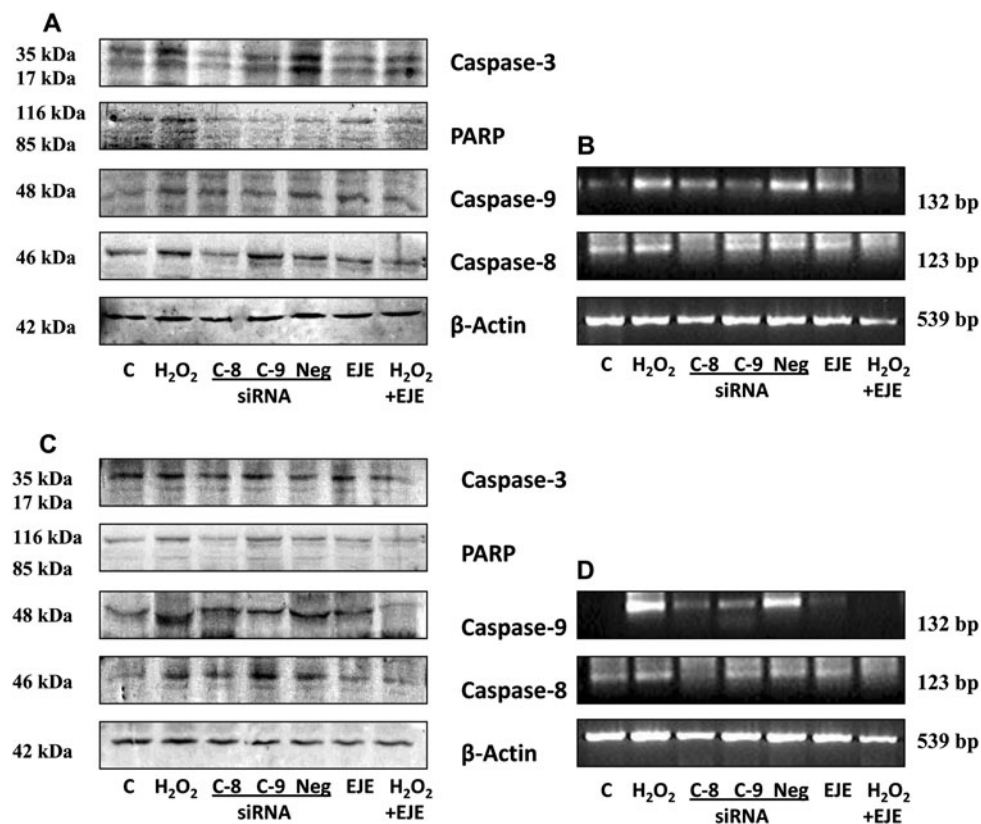

Fig. 8 Pathway initiation, extrinsic versus intrinsic, during H_2O_2 induced Leydig cell apoptosis. a Caspase 8 (C-8) siRNA inhibited its protein expression by $\sim$60 % simultaneously inhibiting caspase 3 by $\sim$42 %. In contrast, caspase 9 protein attenuation with caspase 9 (C-9) siRNA was $\sim$58.5 % leading to only a $\sim$15.8 % caspase 3 downregulation in cells post-H_2O_2 exposure. b Transcript levels corresponding to different treatments during post-H_2O_2 exposure are shown. c siRNA pre-treatment 24 h prior to H_2O_2 exposure knocked down caspase 8 expression by $\sim$62 % leading to only $\sim$20 % inhibition of caspase 3. Identically, $\sim$56 % of caspase 9 inhibitions led to only 9 % inhibition of caspase 3. d Transcript levels corresponding to different treatments during pre-H_2O_2 exposure to siRNA are shown. a–d The beneficial effects of EJE intervention show uniform suppression of apoptotic markers

was used later. In one such approach, anti-GFP or antiCDK9 siRNA were crosslinked to Tat peptide, but significant inhibition of the target protein could only be achieved with high concentrations of nucleic acids, ~ 300 nM or more [35]. In the system presently used, the efficacy of target protein downregulation was extremely good; approximately 60 % utilizing only 20 nm of siRNA (Fig. 3e). The efficiency of transfection leading to inhibition of target protein was further evaluated in pre- compared to post-H_2O_2 treated cell incubations with siRNA. The attenuation in expression was found much stronger in the latter approach than the former (Figs. 4, 5). This was supported from the subsequent studies of flow cytometry using indirect immunofluorescence through caspase 8 or -9 binding to the target Leydig cells in both pre- and post incubations with respective siRNAs of either caspase 8 or -9 (Figs. 6, 7).

Programmed cell death or apoptosis is a major control mechanism in cell proliferation by which cells die if DNA damage is not repaired [36, 37]. However, DNA damage in an apoptotic cell represents the final stages of the physiological programming controlled by a number of complex proteins triggered and activated by sequential signaling modes. Apoptosis occurs through two main pathways, extrinsic or cytoplasmic pathway through the Fas death receptor, a member of the tumor necrosis factor super family [38]. The other is the intrinsic or mitochondrial pathway, once stimulated releases cytochrome c from the mitochondria leading to the activation of the death signal [39]. The upstream events of both pathways are activated by initiator caspases, 8 or -9 representing extrinsic or intrinsic pathways respectively, but converge to a final common pathway activating effector caspases like caspase 3 that cleaves regulatory and structural molecules culminating target cell death. It is known that Fas mediated Leydig cell apoptosis occurs following ethane dimethyl sulfonate treatment to rats [40]. Identical apoptotic pathway is also followed during Leydig cell death in vitro following repeated hCG stimulation [13]. During H_2O_2-induced Leydig cell apoptosis, upregulation of both upstream caspases 8 and -9, and rise in FasL expression followed by caspase 3 activation are reported [11, 16]. Since, inhibition of caspase 8 but not -9 by siRNA directly inhibits significant ($p < 0.001$) caspase 3 expression (Fig. 8a), it supports the contention for the first time that H_2O_2-induced Leydig cell apoptosis is initially channeled through extrinsic and later extending to intrinsic and possibly to other pathways. There is a little dichotomy in PARP cleavage following caspase 8 or -9 mediated caspase 3 activation. Therefore, the extent of involvement of other apoptotic pathways and activation of effector caspases besides caspase 3 in the system presently used needs further elucidation in future studies. Transfection with siRNA in post-H_2O_2 treated cells seems to provide a far better attenuation of the target protein expression (Fig. 8a, b).

ROS including H_2O_2, have historically been considered mostly as damaging entities, mediating pathogenic processes through signal transduction. Under normal conditions, ROS are maintained within narrow boundaries by scavenging systems [41]. Redox balance, the ratio between oxidizing and reducing species within the cell plays a significant role modulating signaling pathways, including kinase, phosphatase activity and gene expression through regulation of transcription factor function [42, 43]. H_2O_2 exposure to Leydig cells raises oxidative stress and simultaneously decreases the activities of the intracellular antioxidant enzymes [11]. On the other hand, isolated Leydig cells co-treated with fruit pulp of EJE, an established antioxidant, and H_2O_2 demonstrated significant improvement in cell survival [16]. In the present study too, compared with siRNA treatment, the effect of EJE was beneficial as it uniformly counteracts the H_2O_2 induced rise in expression of PARP protein and all caspases with the lone exception of caspase 8 transcript levels (Figs. 4, 5, 6, 7, and 8).

In summary, the above findings clearly demonstrate that peptide based siRNA transfection is an excellent tool to inhibit target protein expression in non-dividing primary Leydig cells with the potential of similar use in many such cell systems. Efficient inhibition of caspase 3 simultaneous to caspase 8 downregulation which is relatively far less effective when caspase 9 is used indicates that H_2O_2-induced apoptosis in rat Leydig cells is preferentially channeled, at least initially through extrinsic pathway of metazoan apoptosis.

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