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Original Paper

Human Chorionic Gonadotropin Protects Vascular Endothelial Cells from Oxidative Stress by Apoptosis Inhibition, Cell Survival Signalling Activation and Mitochondrial Function Protection

Daniela Surico[a] Serena Farruggio[b] Patrizia Marotta[b] Giulia Raina[b] David Mary[b,c] Nicola Surico[a] Giovanni Vacca[b,c] Elena Grossini[b,c]

[a]Department of Obstetrics and Gynaecology, [b]Laboratory of Physiology, Department of Translational Medicine, University of East Piedmont "A. Avogadro", [c]Experimental Surgery, Azienda Ospedaliera Universitaria Maggiore della Carità, Novara, Italy

Key Words
Cell death • Mitochondria • Nitric oxide • Peroxidation

Abstract
Background/Aim: Previous reports have made it hypothetically possible that human chorionic gonadotropin (hCG) could protect against the onset of pregnancy-related pathological conditions by acting as an antioxidant. In the present study we planned to examine the effects of hCG against oxidative stress in human umbilical vein endothelial cells (HUVEC). ***Methods:*** HUVEC were subjected to peroxidation by hydrogen peroxide. The modulation of nitric oxide (NO) release by hCG and its effects on cell viability, glutathione (GSH) levels, mitochondrial membrane potential and mitochondrial transition pore opening (MPTP) were examined by specific dyes. Endothelial and inducible NO synthase (eNOS and iNOS), Akt and extracellular-signal-regulated kinases 1/2 (ERK1/2) activation and markers of apoptosis were analyzed by Western Blot. ***Results:*** In HUVEC, hCG reduced NO release by modulating eNOS and iNOS. Moreover, hCG protected HUVEC against oxidative stress by preventing GSH reduction and apoptosis, by maintaining Akt and ERK1/2 activation and by keeping mitochondrial function. ***Conclusion:*** The present results have for the first time shown protective effects exerted by hCG on vascular endothelial function, which would be achieved by modulation of NO release, antioxidant and antiapoptotic actions and activation of cell survival signalling. These findings could have clinical implications in the management of pregnancy-related disorders.

Dott. Elena Grossini

Laboratory of Physiology and Experimental Surgery, Department of. Translational Medicine, University East Piedmont "A. Avogadro", via Solaroli 17, I-28100 Novara, (Italy)
Tel. +390321660680, Fax +3903213733537, E-Mail grossini@med.unipmn.it

Introduction

The glycoprotein hormone, human chorionic gonadotropin (hCG), is one of the earliest embryonic proteins produced by the primate embryo and secreted before implantation [1]. In addition to its luteotropic function in pregnancy, previous reports have indicated that hCG could also exert cardiovascular effects. Hence, in anesthetized pigs hCG was recently reported to decrease arterial blood pressure, increase coronary blood flow and myocardial contractility in a dose-dependent way [2]. Similar vascular effects to those found in pigs were shown in uterine and mesenteric resistance arteries of rats [3].

Furthermore, the coronary effects of hCG were related to increased nitric oxide (NO) release. In addition, in porcine coronary artery endothelial cells, hCG was found to stimulate endothelial NO synthase (eNOS)-dependent NO production through the involvement of cAMP/protein kinase A (PKA), extracellular signal-regulated kinases (ERK)1/2, Akt, p38 mitogen-activated protein kinases (MAPK), which were activated as downstream effectors of β_2-adrenoceptor stimulation [2].

Those findings could have clinical implication regarding hemodynamic changes observed during assisted reproductive technologies or preeclampsia [4]. A combination of immunologic, environmental and genetic factors leading to the failure of normal trophoblastic invasion and remodelling of the uterine spiral arteries could be at basis of pre-eclampsia [5]. Pre-eclampsia is hypothesized to be a disorder secondary to decreased placental perfusion which would result in oxidative stress, endothelial activation, and a multi-system maternal disease [5]. Placental ischemia/hypoxia could lead to endothelial abnormalities [6, 7], which in turn would cause hypertension by impairing renal function and increasing total peripheral resistance. Changes in cytokines, reactive oxygen species (ROS) and NO release would be involved in the pathophysiology of pre-eclampsia [8]. Also the release of clustering of apoptotic nuclei into the maternal circulation could be at the basis of endothelial dysfunction observed in pre-eclampsia. Furthermore, widespread apoptosis of the syncytiotrophoblast may also impair trophoblast function leading to the reduction in nutrient transport and a worsening of trophoblast invasion [9].

Although the role of hCG in pre-eclampsia has not been clearly elucidated yet, reported data suggest that it might improve the uterine environment upon implantation by suppressing apoptotic responses in the maternal decidua under oxidative stress [10]. In addition, hCG could play an important role in the peri-implantation period by increasing uterine blood flow [11] and trophoblast invasion [12] as well as through modulation of peroxidation and endothelial function. All the above mechanisms could be at basis of protective effects elicited by hCG against pre-eclampsia.

For this reason in the present study we aimed to examine the effects of hCG against oxidative stress in human umbilical vein endothelial cells (HUVEC). In particular, we have focused on the modulation of NO release and the activation of the different NOS isoforms, the antioxidant system, apoptosis and mitochondria function.

Materials and Methods

Cell culture

HUVEC were isolated from voluntary umbilical cord donors who gave informed consent in accordance with the procedures approved by the local institutional ethics committee and according to the Declaration of Helsinki. Cells were cultured as previously described [13]. HUVEC were plated into 0.1% gelatin-coated flasks in a specific culture medium (EGM-2, endothelial growth media 2) until adhesion with the addition of human epidermal growth factor (hEGF), hydrocortisone, gentamicin-amphotericin B, 2% fetal bovine serum (FBS), vascular endothelial growth factor (VEGF), human fibroblastic growth factor (hFGF), recombinant analogue insulin-like growth factor 1 human, ascorbic acid, heparin, 2 mM glutamine and 1% penicillin-streptomycin and maintained at 37°C with 5% CO_2. The cells used for the experiments were obtained from passage 3 to passage 6.

For Griess study, $1x10^4$ cells were plated in gelatin-coated 24-well plates in EGM-2 complete medium in an incubator until adhesion and then incubated for 4-6 h in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 2 mM glutamine and 1% penicillin- streptomycin without FBS and red phenol (starvation medium). The same procedure was followed for mitochondrial membrane potential measurement and mitochondrial permeability transition pore (MPTP) opening, but using $5x10^4$ cells. For protein extract studies and glutathione (GSH) quantification, the cells were plated in 0.1% gelatin-coated flasks in complete medium and, at confluence they were incubated with starvation medium overnight.

NO production

The NO production was measured in HUVEC's culture supernatants using the Griess method (Promega, Milan, Italy). Cells were treated with H_2O_2 (200 μM) for 60 min. In addition, the effects of 60 min hCG (1 pM, 100 pM, 1 nM, 100 nM, 1 μM, 100 μM) on NO release in non-peroxidation condition (physiological condition) and during peroxidation were analyzed by giving hCG alone or before H_2O_2. Moreover, in some experiments HUVEC were pre-treated with 10 mM L-NG-nitroarginine methyl ester (L-NAME, Sigma) or with the mitochondrial ATP dependent potassium ($mitoK_{ATP}$) channels inhibitor, 5 hydroxydecanoate (5HD, 10 μM, Sigma) before hCG. Those agents and their vehicle were also tested alone. At the end of stimulations, NO production in the sample's supernatants was examined by adding an equal volume of Griess reagent following the manufacturer's instruction. The absorbance at 570 nm was measured by a spectrometer (BS1000 Spectra Count, San Jose, CA, USA) and the NO production was quantified in respect to nitrate standard curve [2, 14-16] and expressed as percentage. The values obtained corresponded to the NO (μmol) produced, after each stimulation, by samples containing 1.5 μg of proteins each.

Cell viability

The cells were treated with H_2O_2 (200 μM) for 60 min alone or in the presence of hCG (1 pM, 1 nM, 1 μM and 100 μM), which was given for 60 min before H_2O_2. In addition, in some samples, hCG (100 μM) was administrated after L-NAME (10 mM) or 5HD (10 μM). Control cells were treated with DMEM 0% FBS and phenol red only. To determine cell viability, the *In Vitro* Toxicology Assay Kit MTT Based (Life Technologies Italia, Monza; Italy) was used as previously described [16]. This kit is designed to determine cell number spectrophotometrically as a function of mitochondrial dehydrogenases activity in living cells. Briefly, after each treatment, the medium was removed and fresh culture medium without red phenol and FBS and with MTT dye was added in 96-well plates containing the cells and incubated for 2 h at 37°C in an incubator. Thereafter, the medium was removed and an MTT Solubilization Solution in equal volume to the original culture medium was added and mixed in a gyratory shaker until the complete dissolution of formazan crystals. The activity of living cells (cell survival) was determined by measuring the absorbance through a spectrometer (BS1000 Spectra Count, San Jose, CA) and cell viability calculated by comparing results with control cells (100% viable).

GSH quantification

For the GSH assay, $1X10^6$ cells were treated with 200 μM H_2O_2 for 60 min alone or in the presence of hCG (1 pM, 1 nM, 1 μM, 100 μM), which was given for 60 min before H_2O_2. Cells collected by trypsinization and centrifugation were deproteinized with 5% 5-sulfosalicylic acid solution (wt/vol) and centrifuged at 10,000g for 10 min to remove the precipitates. GSH determination was performed by following the manufacturer's instruction (BioVision Inc., Milpitas, CA) and as previously described [16, 17]. Briefly, HUVEC were homogenized on ice with 100 μl of ice cold Glutathione Assay Buffer. Thereafter, 60 μl of each homogenate was added to a pre-chilled tube containing perchloric acid (PCA) and vortexed for several seconds to achieve a uniform emulsion. After keeping on ice for 5 min, samples were spun for 2 min at 13000 G at 4°C and the supernatants were collected. Thereafter, 20 μl of ice cold 6N KOH was added to 40 μl of PCA preserved samples and after a further 2 min spinning at 13000 G at 4°C, 10 μl of the samples were transferred to a 96-well plate where GSH was detected following manufacturer's instructions. Glutathione Standard Curve was prepared using standard GSH following kit instructions. Samples and standards were read by a spectrometer (BS1000 Spectra Count) at an excitation and emission wavelength of 340 nm and 420 nm. GSH content was expressed as $nmol/10^6$ cells.

Cell lysates

HUVEC were stimulated with hCG for 60 min alone or before H_2O_2 (200 μM, 60 min). At the end of stimulation the cells were washed with PBS 1X supplemented with 1:200 sodium orthovanadate and 1:100 protease inhibitors cocktail (Sigma) and then lysed in an iced-Ripa-buffer (50 mM HEPES, 150 mM NaCl, 0,1% SDS, 1% Triton-X100, 1% sodium deoxycholate, 10% glycerol, 1.5 mM MgCl2, 1 mM EGTA, 1 mM sodium fluoride; Sigma) supplemented with 1:200 sodium orthovanadate and 1:100 protease inhibitors cocktail and phenylmethanesulfonyl fluoride (PMSF; 1:100; Sigma). The extract proteins were quantified through bicinchoninic acid protein (BCA; Pierce, Rockford, IL, USA BCA) and used for electrophoresis and immunoblotting studies.

Western blotting

Cell lysates (30 μg protein each sample) dissolved in Laemmli buffer 5X, boiled for 5 min, were resolved in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Bio-Rad Laboratories, Hercules, CA, USA), and after electrophoresis, transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories), which were incubated overnight at 4 °C with specific primary antibodies: anti phospho-ERK1/2 (p-ERK1/2, 1:1000; Thr202/Tyr204, Cell Signalling Technologies, Beverly, MA, USA), anti ERK1/2 (1:1000; Cell Signalling Technologies), anti phospho-Akt (p-Akt, 1:1000; Ser473, Cell Signalling Technologies), anti Akt (1:1000; Cell Signalling Technologies), anti phospho-eNOS (p-eNOS, 1:1000; Ser1177, Cell Signalling Technologies), anti eNOS (1:1000; Cell Signalling Technologies), anti iNOS (1:500; Santa Cruz Biotechnology, Inc, CA, USA), anti phospho-Bax (p-Bax, 1:500; Thr167; Assay Biotechnology Company, Sunnyvale, CA), anti Bax (1:500; Sigma), anti Cytochrome C (1:500, Sigma), anti cleaved Caspase-9 (1:500; Vinci-Biochem S.r.L., Vinci, Italy), Caspase-9 (1:500; Sigma), anti cleaved Caspase-3 p11 (1:500; Santa Cruz Biotechnology). The membranes were washed and then incubated with horseradish peroxidase-coupled goat anti rabbit IgG (Sigma), peroxidase-coupled rabbit anti goat IgG and horseradish peroxidase-coupled goat anti mouse IgG (Sigma) for 45 min, and were developed through a nonradioactive method using Western Lightning Chemiluminescence (PerkinElmer Life and Analytical Sciences, Waltham, MA). Phosphorylated protein expression was calculated as a ratio towards specific total protein expression or β-actin (1:5000; Sigma) detection.

Mitochondrial membrane potential measurement and mitochondrial permeability transition pore (MPTP) opening detection

HUVEC were treated as for cell viability and GSH determination. For mitochondrial membrane measurement the medium of the cells was removed and then incubated with 5,51,6,61-tetrachloro-1,11,3,31 tetraethylbenzimidazolyl carbocyanine iodide (JC-1) 1X diluted in Assay Buffer 1X for 15 min at 37°C in an incubator following the manufacturer's instruction (Invitrogen, Life Technologies Europe BV, Monza, Italy), as previously described [16]. The dyes were dissolved in dimethylsulfoxide (Sigma), and the percentage of the organic solvent in the samples never exceeded 1% vol/vol. After the incubation, the cells were washed twice with Assay Buffer 1X and then the suspensions were transferred in triplicates to a black 96-well plate. The red (excitation 550 nm/emission 600 nm) and green (excitation 485 nm/emission 535 nm) fluorescence was measured using a fluorescence spectrometer. To establish the cells undergoing apoptosis, the ratio of red to green fluorescence was determined and expressed as percentage.

For MPTP opening, the sarcolemmal membrane of HUVEC was permeabilized by using 10 mM of digitonin (Sigma) for 60 s and the cells were loaded with 5 μM acetomethoxy derivate of calcein (calcein/AM, Sigma) for 40 min at 37 °C. Thereafter, they were washed with Tyrode solution (Sigma) for 10 min to remove the excess dye. The calcein/AM fluorescence was measured by a fluorescence spectrometer with fluorescence excitation and emission of 488 and 510 nm, respectively.as previously described [16, 17].

Statistical analysis

All data were recorded using the Institution's database. Statistical analysis was performed by using STATVIEW version 5.0.1 for Microsoft Windows (SAS Institute Inc, Cary NC, USA). Data were checked for normality before statistical analysis. One-way ANOVA followed by Bonferroni *post hoc* tests were used to compare data obtained among various groups. Data represent means of at least five independent experiments for each experimental protocol and are expressed as mean ± SD. A value of $P < 0.05$ was considered statistically significant.

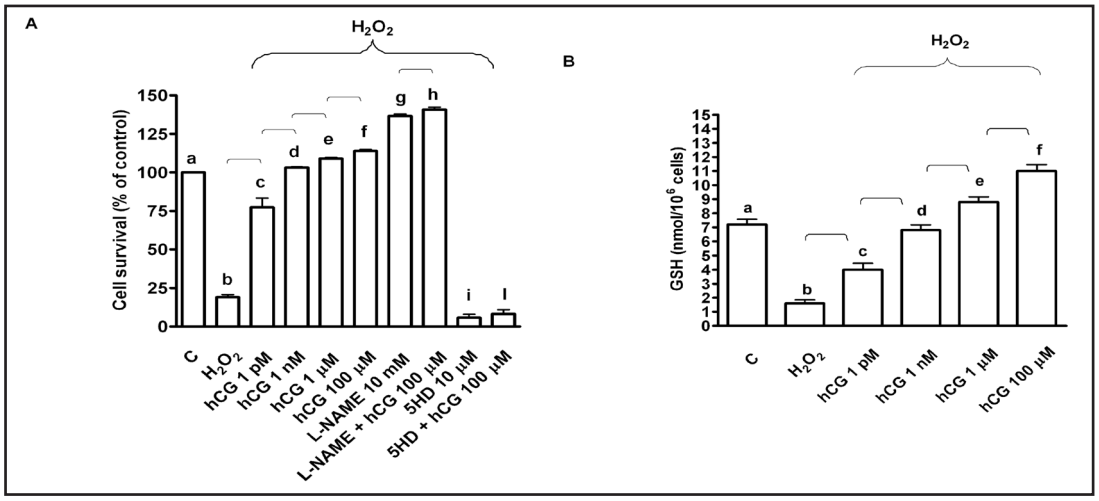
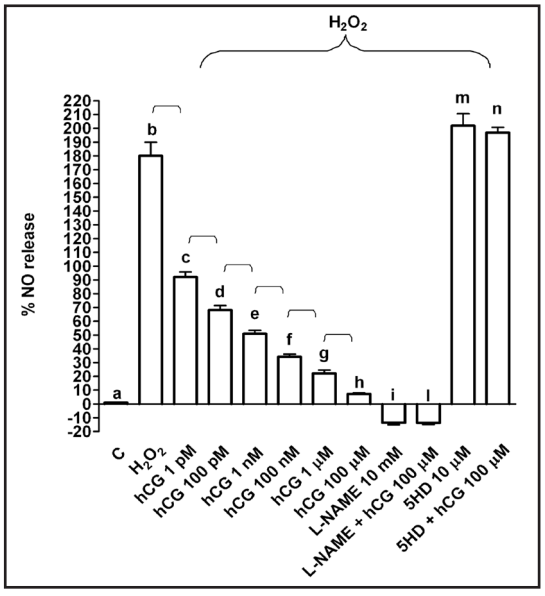


Fig. 1. Effects of hCG on cell survival (A) and glutathione (GSH; B) in HUVEC in peroxidative condition. Cell survival was examined by means of MTT assay, which is designed to determine cell number as a function of mitochondrial dehydrogenases activity in living cells. hCG was administrated at 1 pM, 1 nM, 1 µm and 100 µm for 60 min before 60 min H_2O_2 (200 µM). In some experiments HUVEC were pre-treated with L-NAME or 5HD before 100 µm hCG. C= control, L-NAME: L-NG-nitroarginine methyl ester; 5HD: 5 hydroxydecanoate. Reported data are mean ± SD of five independent experiments. In A, b, c, d, e, f, g, i $P<0.05$ vs a; d, e, f, g, i $P<0.05$ vs b; f $P<0.05$ vs c, d; h, l $P<0.05$ vs f. In B, b, c, e, f $P<0.05$ vs a; d, e, f $P<0.05$ vs b; f $P<0.05$ vs c, d. Square bracket indicates significance between the groups (P<0.05).

Fig. 2. Effects of hCG on NO release (%) caused by hydrogen peroxide in HUVEC. Griess assay was performed to examine NO release caused by hCG in peroxidative condition. hCG was administrated at 1 pM, 100 pM, 1 nM, 100 nM, 1 µm and 100 µm for 60 min before 60 min H_2O_2 (200 µM). In some experiments HUVEC were pre-treated with L-NAME or 5HD before 100 µm hCG. C= control, L-NAME: L-NG-nitroarginine methyl ester; 5HD: 5 hydroxydecanoate. b, c, d, e, f, g, h, i, m $P<0.05$ vs a; c, d, e, f, g, h, i, m $P<0.05$ vs b; e, f, g, h $P<0.05$ vs c; f, g, h $P<0.05$ vs d; g, h $P<0.05$ vs e; h $P<0.05$ vs f; l, n $P<0.05$ vs h. Square bracket indicates significance between the groups (P<0.05).



Results

hCG counteracts peroxidation in HUVEC by improving the antioxidant system, modulating NO release and mitochondria function

In HUVEC pre-treated with 200 µM H_2O_2, the activity of living cells was reduced to about 19% from control value of 100%, whereas GSH content decreased to 1.6 ± 0.8 nmol/10^6 cells from control value of 7.2 ± 0.8 nmol/10^6 cells (Fig. 1). Those effects were accompanied by increased NO release amounting to 180% of control values (Fig. 2) and by the collapse of mitochondrial membrane potential and activation of MPTP opening (Fig. 3).

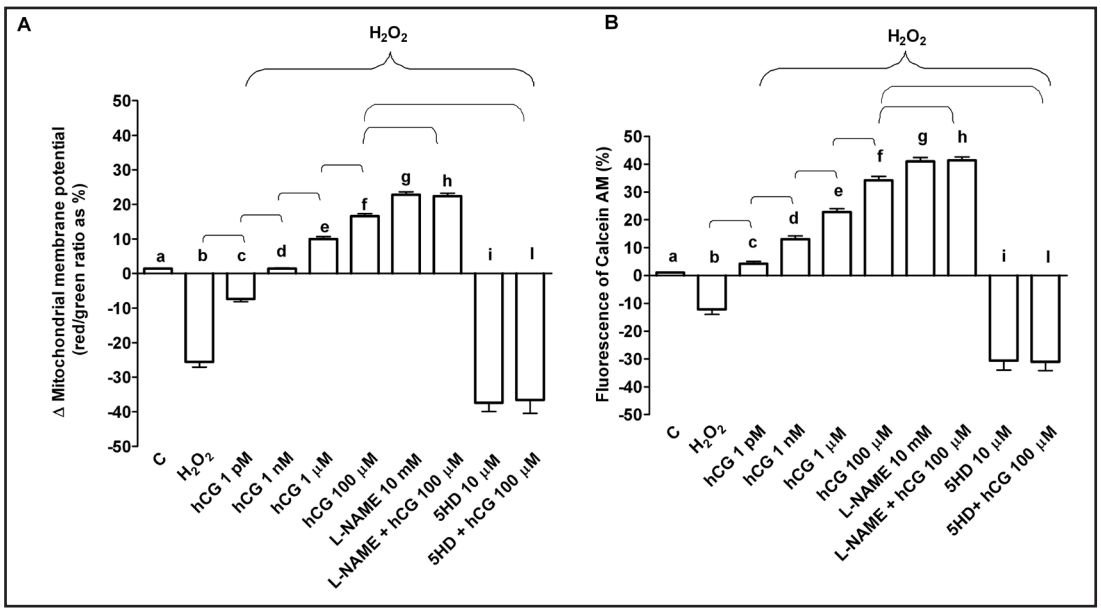


Fig. 3. Protective effects elicited by hCG on mitochondrial membrane potential (A) and transition pore (MPTP) opening (B) in HUVEC that underwent peroxidation. In A, JC-1 assay was performed to examine mitochondrial membrane potential caused by hCG in peroxidative condition. In B, changes of fluorescence of Calcein AM were quantified for examining the MPTP opening caused by hCG in peroxidative condition. hCG was administrated at 1 pM, 1 nM, 1 μm and 100 μm for 60 min before 60 min H_2O_2 (200 μM). In some experiments HUVEC were pre-treated with L-NAME or 5HD before 100 μm hCG. C= control, L-NAME: L-NG-nitroarginine methyl ester; 5HD: 5 hydroxydecanoate. In A, b, c, e, f, g, i $P<0.05$ vs a; d, e, f, g, i $P<0.05$ vs b; e, f $P<0.05$ vs c; f $P<0.05$ vs d; h, l $P<0.05$ vs f. In B, b, c, d, e, f, g, i $P<0.05$ vs a; d, e, f, g, i $P<0.05$ vs b; e, f $P<0.05$ vs c; f $P<0.05$ vs d; h, l $P<0.05$ vs f. Square bracket indicates significance between the groups (P<0.05).

In cells that were not treated with H_2O_2, hCG increased NO release and eNOS activation in HUVEC in a similar way as that observed in porcine aortic endothelial cells (PAE; Fig. 4A and B) [2]. No significant effects were observed on iNOS (Fig. 4C). Different results were obtained in peroxidative status, where the administration of 60 min hCG dose-dependently reduced NO release in HUVEC (Fig. 2). These effects were also accompanied by inhibition of eNOS and iNOS activation caused by hydrogen peroxide (Fig. 5).

Moreover, hCG prevented cell death and GSH reduction (Fig. 1) and inhibited the effects of hydrogen peroxide on mitochondrial membrane potential and MPTP opening (Fig. 3). As shown in Fig. 1-3, l-NAME, which abolished the increased NO release by hydrogen peroxide, was able to improve cell survival, mitochondrial membrane potential and MPTP opening. Opposite results were observed in HUVEC pre-treated with 5HD. In these cells, therefore, the survival and mitochondrial function were strongly reduced (Fig. 1 and 3). Moreover, in the presence of those agents, changes in the protective effects of hCG were observed in comparison with what was found in the absence of various inhibitors. The response to hCG of cell survival and mitochondrial function was increased in HUVEC pre-treated with L-NAME in comparison with HUVEC treated with hCG alone (Fig. 1 and 3), whereas after 5HD all protective effects were strongly inhibited and NO release caused by hydrogen peroxide was increased (Fig. 1-3).

hCG inhibits apoptosis and increases cell survival signalling in HUVEC that had undergone peroxidation

As reported in Fig. 6 and 7, in HUVEC pre-treated with hCG, p-Bax, Cytochrome C, p-Caspase 9 and Caspase 3 activation was reduced in comparison with that found in the

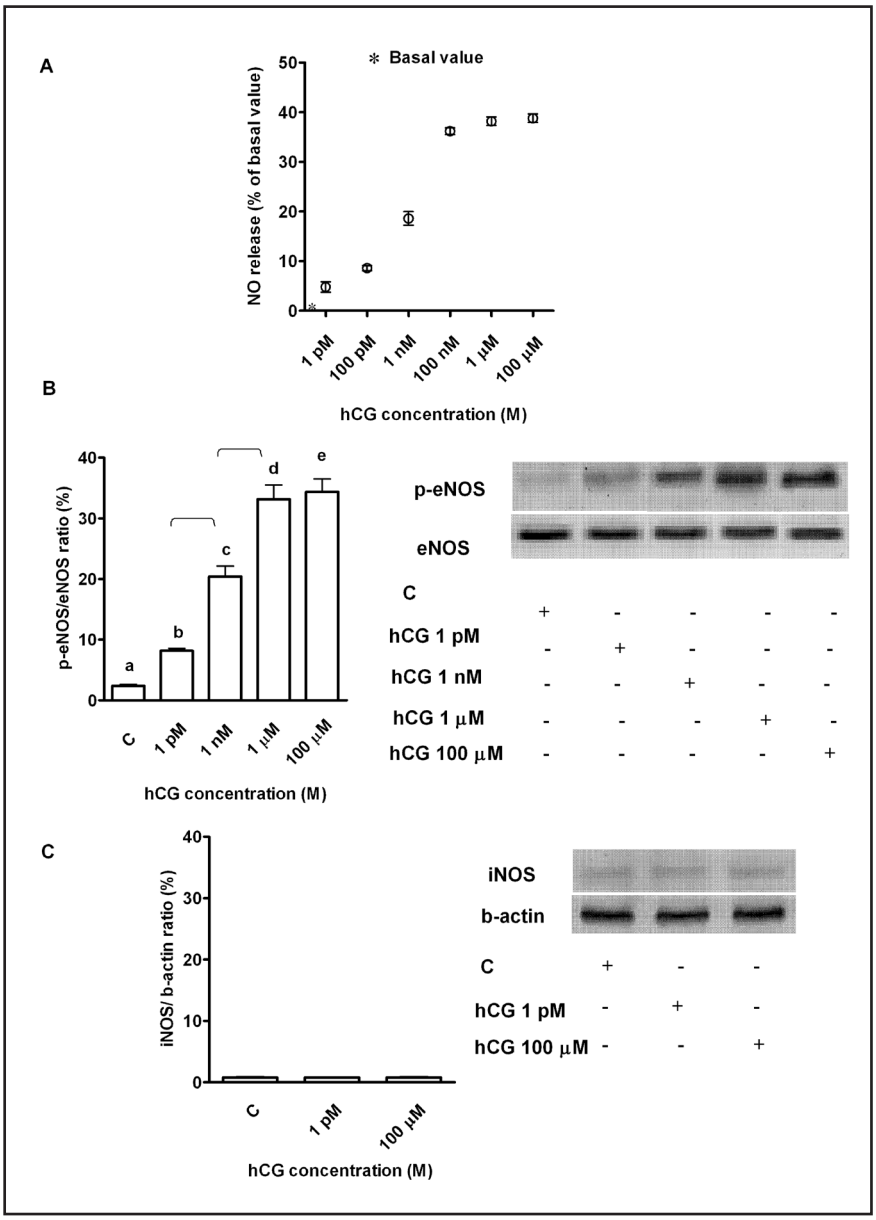


Fig. 4. Effects of hCG on NO release (A) and eNOS (B) and iNOS (C) activation in HUVEC. In A, a dose-response study of the effects of hCG (1 pM, 100 pM, 1 nM, 100 nM, 1 μm and 100 μm) on NO release was performed by Griess assay. In B and C, densitometric analysis and an example of Western Blot taken from 5 different experiments about eNOS and iNOS activation/expression are shown. For densitometric analysis p-eNOS was compared with eNOS, while iNOS was compared with b actin. b, c, d, e $P<0.05$ vs a; d, e $P<0.05$ vs b; e $P<0.05$ vs c. Square bracket indicates significance between the groups ($P<0.05$).

absence of hCG. Moreover, these effects were accompanied by increased activation of ERK1/2 and Akt (Fig. 8).

Discussion

The present results have shown that hCG can exert protection in human vascular vein endothelial cells against oxidative stress through the modulation of eNOS and iNOS, apoptosis inhibition and the keeping of mitochondrial function.

Pregnancy can be considered as a state of oxidative stress [18] arising from the increased metabolic activity in placental mitochondria and reduced scavenging power of antioxidants. However, in certain pathological pregnancies a heightened level of oxidative stress is encountered. This increased oxidative stress could affect placental function [19, 20] not only as a consequence of hypo-perfusion but also by enhanced apoptosis [21].

This is the first study showing that hCG can counteract peroxidative injuries in HUVEC by inhibition of apoptosis, the prevention of GSH reduction and the activation of

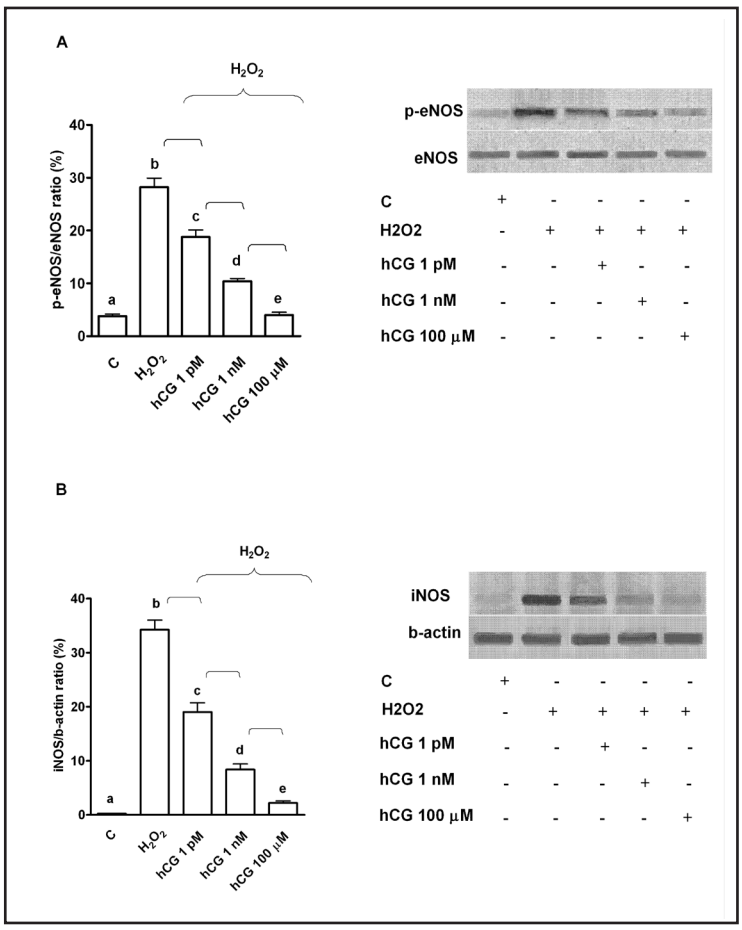


Fig. 5. Effects of hCG on eNOS (A) and iNOS (B) activation in HUVEC under peroxidative conditions. hCG was administrated at 1 pM, 1 nM, and 100 μm for 60 min before 60 min H_2O_2 (200 μM). Densitometric analysis and an example of Western Blot taken from 5 different experiments are shown. For densitometric analysis p-eNOS was compared with eNOS, while iNOS was compared with b actin. b, c, d, e $P<0.05$ vs a; d, e $P<0.05$ vs b; e $P<0.05$ vs c. Square bracket indicates significance between the groups ($P<0.05$).

cell survival signalling. Hence, pre-treatment of cells with hCG at concentrations similar to those previously used in PAE and corresponding to plasma levels which can be achieved during pregnancy [2, 3], dose-dependently reduced cell death caused by hydrogen peroxide and prevented antioxidant system consumption. In particular, the activity of living cells observed by using 1 pM hCG amounted to about 75% of control values of 100% and GSH content amounted to about 57% of control values. At 1 nM hCG concentration, the activity of living cells had nearly returned to control values, whereas GSH content was slightly lower. At concentration higher than 1 μM, which most frequently can be observed during pregnancy, both the activity of living cells and GSH content were higher than basal values. These responses were accompanied by inhibiting effects on apoptotic markers and increased activation of members of the so-called reperfusion injury salvage kinases pathway [16, 22]. It is of interest to note that findings about apoptosis inhibition are in agreement with those obtained in human endometrial stromal cells, where hCG was able to inhibit the expression of the proapoptotic Bax protein and to up-regulate antiapoptotic Bcl-2 [10].

Taken together those observations would suggest that hCG might improve the uterine environment upon implantation by suppressing apoptotic responses in the maternal decidua and vasculature under oxidative stress. In this way hCG could counteract peroxidative conditions associated with endothelial dysfunction, which has been considered as a central patho-physiological event in the development of pre-eclamptic state [19].

Previous studies have demonstrated that NO can affect cellular decisions of life and death by either turning on or shutting off apoptotic pathways, suggesting that NO can function differently depending on the dose and duration of exposure [23]. Regarding this issue it is notable that while NO produced in low concentration, as in the case of eNOS activation, would act as a messenger and cytoprotective factor *via* direct interactions with transition

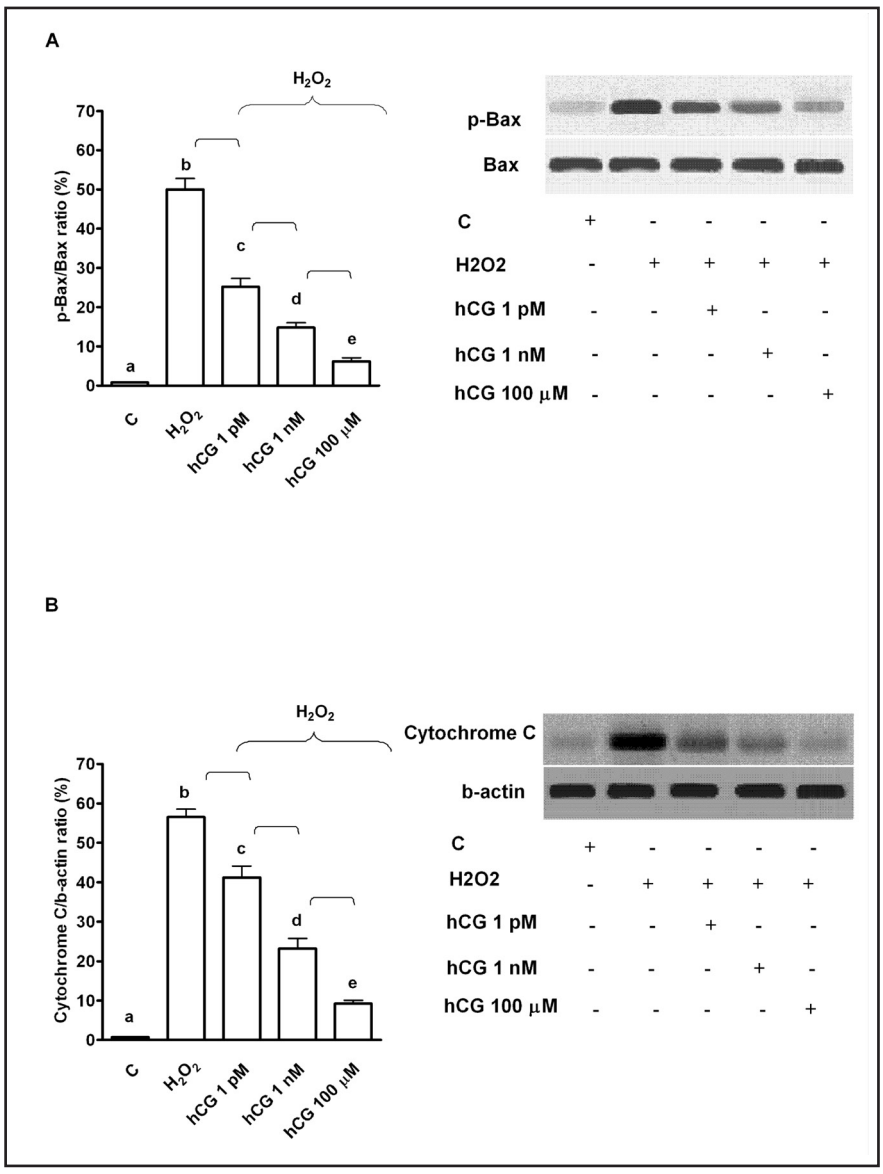


Fig. 6. Effects of hCG on p-Bax (A) and Cytochrome C (B) in HUVEC treated with hydrogen peroxide. hCG was administrated at 1 pM, 1 nM, and 100 μm for 60 min before 60 min H_2O_2 (200 μM). Densitometric analysis and an example of Western Blot taken from 5 different experiments are shown. For densitometric analysis p-Bax was compared with Bax, while Cytochrome C was compared with b actin. b, c, d, e $P<0.05$ vs a; d, e $P<0.05$ vs b; e $P<0.05$ vs c. Square bracket indicates significance between the groups (P<0.05).

metals and other free radicals [23] it could increase reactive nitrose species formation and cause cellular death when over-secreted [24].

It is notable that in HUVEC hCG increased NO release in physiological conditions in a dose-dependent way, as was observed in PAE [2]. Those effects were accompanied by the activation of the only endothelial NOS isoform, which could account for the small amount of NO release found in that condition.

Different results were observed in cells treated with hydrogen peroxide. As previously found [25, 26], in HUVEC that were subjected to peroxidation an increase of both eNOS and iNOS was observed. Both NOS isoforms could be involved in increased NO release found in HUVEC during peroxidation. Hence, it is widely accepted that NO production higher than 2 μMol/s, such as that found in HUVEC subjected to hydrogen peroxide, should be related to iNOS activation being NO release caused by eNOS in the range of nMol/s [17, 24]. It is notable in HUVEC that were subjected to peroxidation and pre-treatment with hCG, NO production was lower than 1 μMol/s. Secondly, although eNOS itself could take place in increased NO release caused by hydrogen peroxide, at high extent peroxidation it could change its function. In respect of this issue it is worth noting that eNOS has recently been reported to be a redox "hub", being regulated by and contributing to the regulation of intracellular

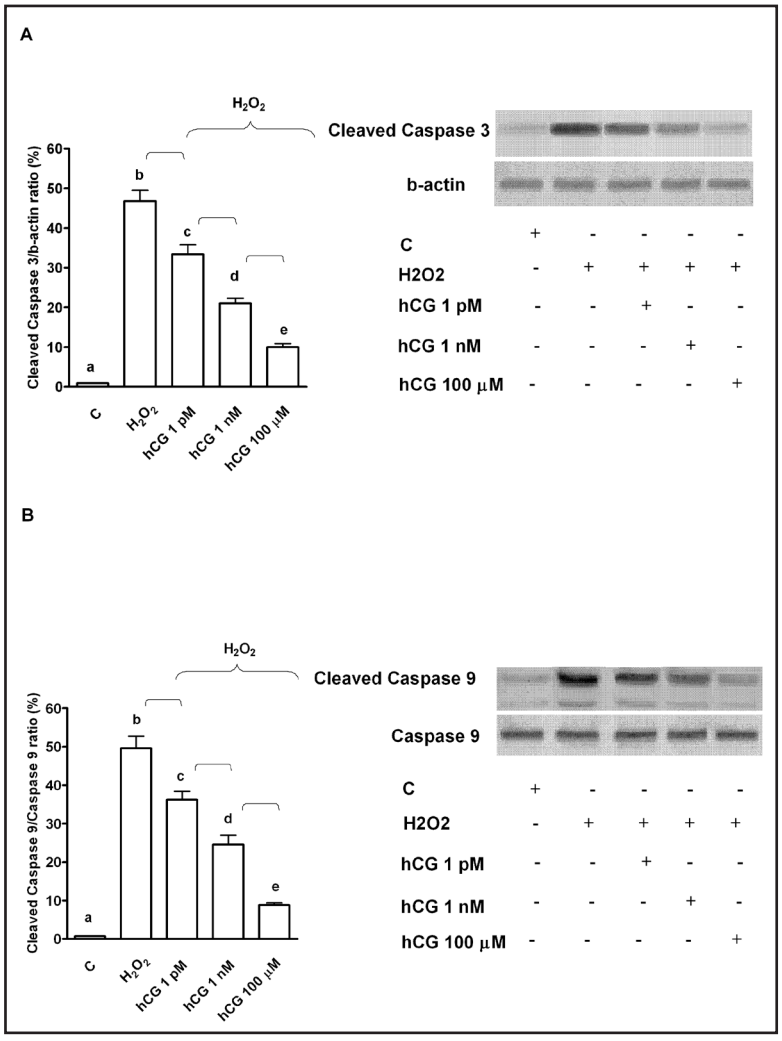


Fig. 7. Effects of hCG on cleaved Caspase 3 (A) and cleaved Caspase 9 (B) in HUVEC treated with hydrogen peroxide. hCG was administrated at 1 pM, 1 nM, and 100 μm for 60 min before 60 min H_2O_2 (200 μM). Densitometric analysis and an example of Western Blot taken from 5 different experiments are shown. For densitometric analysis cleaved Caspase 3 and Caspase 9 were compared with b-actin and Caspase 9. b, c, d, e $P<0.05$ vs a; d, e $P<0.05$ vs b; e $P<0.05$ vs c. Square bracket indicates significance between the groups ($P<0.05$).

redox homeostasis through mutually interacting tetrahydrobiopterin- and GSH-dependent pathways. Changes of GSH have been reported to cause eNOS uncoupling, which would cause the shift of the enzyme from NO to superoxide production [27]. This could, in turn, increase iNOS activation and strengthen NO release.

It could be hypothesized that in non-peroxidative condition hCG would activate eNOS, which in turn could increase NO release without exceeding physiological levels. Under peroxidation, eNOS activity, which would be increased by oxidative stress, could be impaired by concomitant uncoupling caused by GSH reduction. As a consequence, eNOS would augment the production of superoxide, which would potentiate the entire process by also increasing iNOS activity. Thus, hCG by restoring GSH levels could be useful for modulation of eNOS and iNOS function.

Anyway, although we could not precisely define the amount of NO caused by various NOS subtype, we can confirm the role of hCG in mediating protection against oxidative stress by the reduction of NO release, as previously reported [22]. This hypothesis has been confirmed by experiments performed with L-NAME, which also abolished NO release caused by hydrogen peroxide and caused an increase of the effects of hCG on cell survival and mitochondrial function.

Our findings are also of particular relevance when considering the different roles played by various NOS isoforms and related NO release in ovarian physiology and pregnancy [28, 29]. While NO produced in low concentration would act as cytoprotective factor [30], NO overproduction could interfere with mitochondrial respiration and result in significant

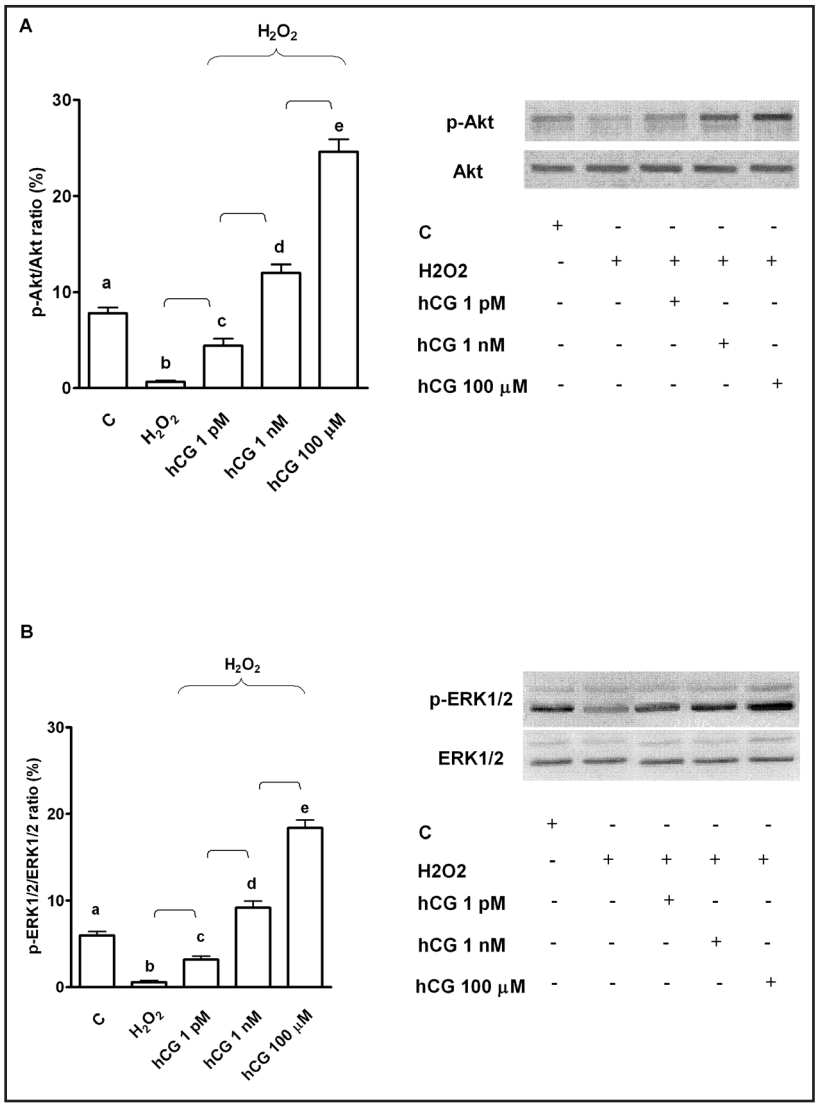


Fig. 8. Effects of hCG on p-Akt (A) and p-ERK1/2 (B) in HUVEC treated with hydrogen peroxide. hCG was administrated at 1 pM, 1 nM, and 100 μm for 60 min before 60 min H_2O_2 (200 μM). Densitometric analysis and an example of Western Blot taken from 5 different experiments are shown. For densitometric analysis p-Akt and p-ERK1/2 were compared with Akt and ERK1/2. b, c, d, e $P<0.05$ vs a; d, e $P<0.05$ vs b; e $P<0.05$ vs c. Square bracket indicates significance between the groups ($P<0.05$).

damage to cellular components and cellular death [31]. Also in ovaries, although follicular fluid NO derivatives are most likely to be necessary for oocyte activation at fertilization when produced within physiological limits, at higher doses they can have detrimental consequences on embryo quality, implantation and pregnancy rate [32].

The results obtained in this study about mitochondrial membrane potential and MPTP opening are of particular relevance and could be involved in the mechanism of protection exerted by hCG against peroxidation. Hence, the collapse of the mitochondrial membrane potential induced by MPTP opening could favour the release of Cytochrome c and downstream activation of effector caspases [33]. It is also notable that in endothelial cells from the umbilical artery of pre-eclamptic patients mitochondria have been found to be impaired [34, 35].

In the present study, pre-treatment of HUVEC with hCG prevented the collapse of mitochondrial membrane potential and MPTP opening caused by hydrogen peroxide. Furthermore, those effects were increased in cells pre-treated with L-NAME and counteracted by 5HD. These findings not only confirmed the above arguments about the role of NO in protective effects elicited by hCG but also highlighted, for the first time, the involvement of mitoK_{ATP} channels.

Taken together the results obtained in this study could have clinical implication in respect of the pathophysiology of pregnancy-related disorders and of the role of hCG. Hence,

women with pre-eclampsia have been found to exhibit higher serum hCG and hydrogen peroxide than normotensive pregnant women at term [36]. Both *in vivo* and *in vitro* findings have shown that H_2O_2 can trigger placental hormone synthesis. Thus, overexpression of hCG could balance the detrimental effects of peroxidation on endothelial function [37], by inhibition of apoptosis and helping cell survival and mitochondria function. In particular, the antioxidant effects of hCG could be attributed to the combination of GSH content modulation and inhibition of mitochondrial membrane potential collapse and MPTP opening.

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Disclosure Statement

None.

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