Original Article

Modulation of Antioxidant Enzyme Expression and Activity by Paraquat in Renal Epithelial NRK-52E Cells

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ABSTRACT

Renal toxicity produced by paraquat involves the generation of reactive oxygen species (ROS) which can overwhelm antioxidant defences, leading to oxidant injury. However, there are conflicting reports regarding the activity and/or expression of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) during oxidative stress injury. This study investigated the activity and expression of these enzymes in a renal epithelial cell line following exposure to paraquat. Confluent NRK-52E cells were incubated with increasing concentrations of paraquat (1-100mM) for up to 24 hours. Renal cell death was determined by measurement of lactate dehydrogenase release. Oxidant damage was determined via measurement of malondialdehyde formation and DNA strand breaks. The effects of paraquat on DNA and *de novo* protein synthesis were determined using radio-labelled thymidine and leucine respectively. ROS generation (superoxide anion and hydroxyl radical formation) was measured using nitro-bluetetrazolium and deoxyribose assays. Antioxidant enzyme activities and expression were measured using established biochemical assays and Western blot analysis. Exposure of confluent NRK-52E cells to paraquat resulted in significant cell death involving increased lipid peroxidation, DNA damage and inhibition of DNA and *de novo* protein synthesis. Renal cell injury and death were secondary to increased ROS generation. Incubation with paraquat reduced SOD and CAT activities; in contrast, GSH-Px activity increased significantly. Although SOD expression was significantly reduced, catalase expression was unaffected. These results indicate that paraquat mediates renal toxicity via oxidative stress involving both an increase in ROS generation and reductions in SOD and CAT activities with a concomitant reduction in SOD expression.

Keywords: Anti-oxidant enzymes, NRK-52E renal cell line, Oxidative stress, Paraquat

INTRODUCTION

Paraquat (1,1'-dimethyl-4,4'-bipyridium dichloride, also known as methyl viologen), is a widely used broad-spectrum and fast acting herbicide. However, it is extremely toxic, causing fatalities due to accidental or intentional poisoning; prevalently in developing countries (Gunnell and Eddleston, 2003; Eddleston and Phillips, 2004). Paraquat poisoning causes severe multiple organ failure, with the degree of poisoning dependent on the route of administration, the amount administered and

duration of exposure. It is rapidly distributed within the body with highest concentrations accumulating within the kidneys where it produces early and severe nephrotoxicity (Rose and Smith, 1977). Additionally, as it is primarily excreted unchanged via the kidneys, the consequent reduction in renal function increases plasma concentrations by up to 5-fold which contributes to paraquat toxicity in other organs; especially the lungs (Hawksworth *et al.*, 1981; Smith, 1987). Ultimately, respiratory failure, in the presence of nephrotoxic acute renal failure,

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is responsible for most deaths caused by paraquat (Smith, 1987; Nagata *et al.*, 1992). There is good evidence from laboratory-based research and clinical studies that oxidative stress plays a major role in the development of paraquat toxicity (Yumino *et al.*, 2002; Ranjbar *et al.*, 2002; Abdollahi *et al.*, 2004). This involves the generation of reactive oxygen species (ROS), such as superoxide anions (Tampo *et al.*, 1999), which contributes to the development of paraquat-induced toxicity (Togashi *et al.*, 1991; Liu *et al.*, 1995) especially nephrotoxicity (Molck and Friis, 1997; Samai *et al.*, 2007). Thus, during the past decade, research was therefore focussed on the therapeutic potential of antioxidants against paraquat-induced toxicity and particularly to agents which can degrade superoxide anions such as superoxide dismutase (SOD) mimetics (SODm) (Day *et al.*, 1995; Day and Crapo, 1996; Suntres, 2002; Mollace *et al.*, 2003; Peng *et al.*, 2005).

Recently, we demonstrated that tempol, a SODm with ROS-scavenging properties, and EUK-134, a SODm with catalase activity; can protect renal NRK-52E cells against renal toxicity mediated by paraquat (Samai *et al.*, 2007). The development of oxidative stress involves an imbalance between the generation of oxidants and their removal by antioxidants (Sies, 1991), with increasing production of ROS overwhelming antioxidant defences. However, oxidative stress also involves modulation of the expression or activity of antioxidant enzymes such as SOD, catalase (CAT) or glutathione peroxidase (GSH-Px). An increase in antioxidant enzyme activity may be a protective response, designed to remove increasing amounts of ROS such as superoxide anions or hydrogen peroxide. In contrast, down-regulation of antioxidant enzyme activity, which may occur due to oxidant damage to proteins, will contribute to oxidant injury by allowing ROS to exist for longer than normal.

The response of antioxidant enzymes to oxidative stress can be complicating. For example, under diabetic conditions in which oxidative stress plays an important role in the development of complications involving oxidant injury (Maiese *et al.*, 2007; Kaneto *et al.*, 2007), SOD activity has been shown to be reduced (Godin *et al.*, 1988) or unaffected (Kakkar *et al.*; 1995), CAT activity increased (Matkovics *et al.*, 1982; Yadav *et al.*,1997) or unaffected (Kakkar *et al.*,1995) and GSH-Px activity increased (Godin *et al.*, 1988;

Kakkar *et al.*, 1995; Duncan *et al.*, 1996) or decreased (Tagami *et al.*, 1992; Van den Branden *et al.*, 2000). Antioxidant enzyme activities are also altered in chronic renal failure (CRF) in which oxidative stress also plays an important part (Modlinger *et al.*, 2004), however, as with diabetic conditions, the true effects are still unclear. Specifically, CAT activity has been found to be down-regulated (Inal *et al.*, 1999; Mimc-Oka *et al.*, 1999; Sindhu *et al.*, 2005) or unaffected (Sommerburg *et al.*, 2002). GSH-Px activity has also been reported to be unaffected in CRF (Sindhu *et al.*, 2005). These differing results may be due to different responses to oxidative stress depending on cell type, tissue, organ, species or the experimental system used.

The effects of paraquat on antioxidant enzyme expression and activities, particularly within the kidney, are similarly unclear. In a recent study, administration of low doses (7.5 mg/kg) of paraquat to rats for up to 7 days increased the activities of SOD, CAT and GSH-Px in kidney tissues (Ray *et al.*, 2007). This may be a protective response to increased superoxide anion generation and is supported by other studies in which paraquat-resistant HeLa cells were found to possess increased levels of manganese (Mn)-SOD and copper/zinc (Cu/Zn)-SOD (Krall *et al.*, 1988) and in vero monkey fibroblasts in which incubation with 10 mM paraquat increased SOD activity and those of both glutathione reductase and glutathione-S-transferase (Garcia-Alfonso *et al.*, 1995).

However, there is conflicting evidence from investigations in which renal cell cultures have been exposed to increasing concentrations of paraquat. Specifically, short-term incubation of A74 rabbit kidney epithelial cells with 250 µM paraquat for 3 hours had no effect on the total SOD activity and those of Cu/Zn-SOD and GSH-Px (Senator *et al.*, 2004). In contrast, we have recently reported indirect evidence that SOD and CAT expression or activities may be reduced upon exposure of renal NRK-52E epithelial cells to paraquat, that is, EUK-134 – a SODm with CAT activity was able to provide significant protection against paraquat renocytotoxicity (Samai *et al.*, 2007). The aim of this study was to investigate the development of oxidative stress in the NRK-52E renal epithelial cell line exposed to clinically relevant concentrations of paraquat. Specifically, the role ROS generation and alteration in the activity and/or expression of the antioxidant enzymes such as SOD, CAT and GSH-

Px play in the development of oxidant injury caused by paraquat were studied.

MATERIALS AND METHODS
Chemical Compounds
Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK).

Culture of NRK-52E Cells
NRK-52E cells, which maintain characteristics of renal proximal tubular cells in culture (De Larco et al.; 1978), were obtained at passage 24 from the European Collection of Cell Cultures (ECACC, Salisbury, Wiltshire, UK). Cells were routinely cultured in 80 cm^2 Nunc flasks (Fisher Scientific, Loughborough, Leicestershire, UK) and grown in Dulbecco's Modified Eagle's medium (DMEM, Cambrex Bio Science, Wokingham, Berkshire, UK) containing 4.9g/L glucose and 0.584 g L-glutamine, supplemented with 10% (v/v) foetal bovine serum (FBS, Biosera, Ringmer, East Sussex, UK), 1% non-essential amino acid solution, 100 U/ml penicillin and 50 µg/ml streptomycin at 37°C in a humidified Heraeus incubator supplied with 5% carbon dioxide. Culture medium was changed every 48 hour. NRK-52E cells were subcultured at 85-100% confluence using a trypsin (0.1% w/v)/versene (0.02% w/v) mixture into 80 cm^2 Nunc culture flasks (Fisher Scientific) for subculture, or onto 6 or 24 well Nunc plates (Fisher Scientific) for in vitro experiments. Cells cultured on plates were grown in DMEM as described above except for the substitution of 5% (v/v) FBS. NRK-52E cells were used between passages 28 and 40.

Investigation of Paraquat Renocytotoxicity (LDH assay)
Confluent cultures of NRK-52E cells grown on 24 well plates were incubated for 4 or 24 hour with increasing concentrations of paraquat (0.003–100 mM) in incubation medium consisting of DMEM as described above but containing 1% (v/v) FBS. At the end of the incubation period, cell death was determined by measurement of lactate dehydrogenase (LDH) released into the incubation medium as described previously (Abe, 2000; Samai et al., 2007). Results were calculated as percentage of the total LDH released from control cells (i.e. those not exposed to paraquat) which were incubated with 1% (w/v) Triton X-100 for 30 min and expressed as cell death (%TX-100 controls). Based on the data obtained from the LDH assays, two concentrations of paraquat (1 and 30

mM) were chosen which produced a submaximal, but significant cell death over a 24 or 4 h incubation periods, respectively (approximately 75% of the LDH released by cells treated with Triton X-100).

Measurement of ROS Generation
The production of superoxide anion was determined using the nitroblue tetrazolium (NBT) assay (Falasca et al., 1993; Samai et al., 2007). Subsequent hydroxyl radical generation was assessed by deoxyribose assay as described previously (Halliwell et al., 1988; Samai et al., 2007). Briefly, NRK-52E cells in 6-well plates were incubated for 4 or 24 hrs with DMEM containing 25µg/ml NBT or 3 mM deoxyribose in the presence of either 30 or 1mM paraquat respectively. In addition, NRK-52E cells were co-incubated for the pre-determined time with DMEM containing 25µg/ml NBT or 3 mM deoxyribose and 30 or 1mM paraquat in the presence of either SOD (1,000 U/ml) or CAT (1,000 U/ml). The concentrations of SOD and CAT were based on previous work by Scheid and colleagues (Scheid et al., 1996). Superoxide anion levels were expressed as the absorbance of NBT reduction recorded at 700 nm wavelength and hydroxyl radical content expressed in nmoles/mg protein after determination of the protein content of the samples as described previously (Bradford, 1976; Samai et al., 2007)

Measurement of Lipid Peroxidation
Lipid peroxidation was determined by measuring malondialdehyde (MDA) using the thiobarbituric acid method as described previously (Uchiyama and Mihara, 1978). Briefly, NRK-52E cells were incubated with DMEM only and DMEM containing 30 or 1mM paraquat for 4 and 24 hours respectively. Cells were then harvested in ice cold 1.15% KCl containing 3 mM EDTA and the mixture sonicated on ice for 60 seconds. The cell suspension was spun at 500g for 10 mins at 4°C and the supernatant was used for the determination of MDA and protein. The amount of MDA was expressed as MDA levels (nmoles/mg protein).

Measurement of DNA and de novo Protein Synthesis
The incorporation of [3H]-thymidine and [3H]-leucine into NRK-52E cells was performed as described previously for renal cell cultures (Chan et al., 1996; Chatterjee et al., 1997) and used for the estimation of DNA and de novo protein synthesis respectively. Briefly, confluent NRK-52E cells grown in 6-well plates were incubated with DMEM only and DM

containing 30 or 1mM PQ for 4 and 24 hours respectively. At the end of the incubation periods, the culture medium was replaced with warm DMEM containing [³H]-thymidine or [³H]-leucine (final concentration of 2µCi/well) and the cells were incubated at 37°C for 1 hour. Cells were then washed 5 times with 5% (w/v) trichloroacetic acid and scraped into 500 µL NaOH. The radioactivity of the samples from each well was determined by scintillation counting and cellular incorporation expressed as disintegrations per minute (dpm) per microgram (µg) protein. Results were expressed as a percentage of the control value (i.e. the measurement obtained from untreated cells).

Measurement of DNA Single Strand Breaks

DNA single strand breaks were measured by the DNA precipitation assay as described previously (Olive *et al.*, 1988; Min *et al.*, 2000). Briefly, confluent NRK-52E cells grown and maintained in 6-well plates were incubated with DMEM containing [³H]-thymidine (at final concentration of 0.25 µCi/ml) for 24 hours. Thereafter, the cell monolayers were rinsed 3-4 times with Hank's balanced salt solution (HBSS) {containing 115 mM NaCl, 5 mM KCl, 25 mM NaHCO₃, 2 mM NaH₂PO₄, 1mM MgSO₄, 1 mM CaCl₂, and 5 mM glucose (pH 7.4)} and incubated with DMEM only or DMEM containing 30 or 1mM paraquat for 4 and 24 hours respectively. The amount of DNA single-strand breaks was computed by dividing the dpm value of the supernatant by the total dpm value of the pellet plus supernatant and multiplying by 100.

Measurement of Antioxidant Enzyme Activities

SOD activity was determined as described previously (Oberley and Spitz, 1984) using a method which utilizes NBT as the indicator agent. A colorimetric method for assessing hydrogen peroxide by the addition of ferrous ions and thiocyanate as previously described (Cohen *et al.*, 1996) was used to determine CAT activity. GSH-Px activity was assessed by the dithionitrobenzoic method as previously described [Paglia and Valentine, 1967), which measures the rate of oxidation of reduced glutathione (GSH) by GSH-Px in the presence of hydrogen peroxide. Briefly, confluent NRK-52E cells were incubated with DMEM containing 1 or 30mM paraquat for 24 and 4 hours respectively. At the end of the incubation period, the cell monolayer was rinsed 3-5 times with phosphate buffer (PB), harvested in ice cold PB and were sonicated in cold for 60 seconds. Thereafter, 50µL of the cell suspension was

removed for protein determination using the Bradford assay (Bradford, 1976). An aliquot of 220µL of the cells sonicate was transferred into a micro centrifuge tube into which 25µL of 1% (v/v) Triton X-100 was added and the mixture gently mixed with a low speed vortex and centrifuged at 18,000 x g for 15 minutes. Following centrifugation, the supernatant was assayed for the enzymes activities.

Measurement of the Expression of Antioxidant Enzymes

The expression of MnSOD, Cu/ZnSOD or CAT by NRK-52E cells incubated with DMEM containing 1 or 30 mM paraquat for 24 and 4 hours respectively was determined by Western blot analysis as described previously (Rinaldi *et al.*, 2006). At the end of the incubation period the cell monolayers were rinsed 3-5 times with ice cold PBS followed by the addition of "RIPA buffer" (150mM NaCl, 50mM Tris-HCl (pH 7.4), 1mM ethylenediaminetetraacetic acid, 1mM phenylmethylsulfonylfluoride, 1% Triton-X100, 1% deoxycholic sodium salt and 0.1% SDS) containing cocktail inhibitor and the cells were kept on ice for 30 minutes. The cells were subsequently scraped and transferred into microcentrifuge tubes and were spun at 10,000g for 10 minutes at 4°C. The supernatant was carefully transferred into microcentrifuge tube from which 50µL was used to quantify the protein content using Bradford assay (Bradford, 1976). Equal amounts of protein extract (30µg) were dissolved in Laemmli sample buffer, boiled for 5 minutes and subjected to SDS-PAGE (10% polyacrylamide) and then transferred to nitrocellulose membranes.

For MnSOD determination, the blots were blocked for 1 hour with 3% non-fat dry milk reagent dissolved in PBS. Membranes were incubated overnight with anti Mn-SOD polyclonal antibody (1:1000, Upstate, CA). After further washing in PBS, a goat anti-rabbit was used as secondary polyclonal antibody. Cu/ZnSOD and CAT were blocked with 5% non-fat milk dissolved in TTBS (1 x Tris buffered saline with 0.05% Tween-20) for 1 hour. Membranes were incubated with a polyclonal antibody against Cu/Zn-SOD protein (1:1000, Stress Gen, Biotechnologies Corp), or with monoclonal antibody against catalase protein (1:2000 Sigma, Milan, Italy) overnight. After further washing in 0.05% TTBS, a conjugated goat anti-rabbit polyclonal IgG HRP, and a conjugategoat anti-mouse monoclonal IgG HRP served as secondary antibody for Cu/Zn-SOD and CAT,

respectively. Anti-actin polyclonal antibody (Sigma, Milan, Italy) was used as internal standard. The blots were visualized with ECL Western blotting detection system (Amersham) and autoradiographed.Protein levels were quantified by scanning densitometry (Gel Doc-2000, Bio-Rad) and the results represented as Arbitrary Units (AU).

Statistical Analysis

Results are expressed as mean ± SEM. Means were obtained from multiple experiments performed in triplicate. Data were analyzed and graphed with the commercially available software (Graphpad Prism, version 3.0, Graphpad software, San Diego, CA, USA). Differences between mean values within the groups were determined using a Student's t-test or one way analysis of variance (ANOVA) followed by a Dunnett's test for comparison of multiple means. A *P*-value of less than <0.05 was taken to indicate significance.

RESULTS

The release of LDH by the confluent NRK-52E cells (target cells) upon exposure to paraquat was evaluated using the LDH assay and was used as a measure of cell death. Exposure of the target cells to 1-100mM paraquat for 4 hours resulted in a statistically significant increased LDH release from 23% for the control (untreated) cells to 49-79% ($p<0.05$) (Figure 1a). Further exposure of the target cells to lower concentrations of paraquat (0.003-1 mM) for 24 hours also resulted in a significant increased LDH release from 23% for the control cells to 78% ($p<0.05$) (Figure 1b).

Once the renocytotoxic effect of paraquat was confirmed, the hypotheses that paraquat increases the generation of ROS, specifically, superoxide anion and hydroxyl radical, which may mediate its cytotoxic effect via oxidative stress injury, particularly lipid peroxidation, DNA and protein synthesis inhibition and increased DNA single strand breaks were tested. Exposure of the target cells to 1 or 30mM paraquat for 24 or 4 hours significantly increased the production of MDA (a biochemical index of lipid peroxidation) by 2.7- and 1.8-fold respectively ($p<0.05$) (Figure 2a). This was also accompanied by a statistically significant reduction in the synthesis of DNA from 100% for the control cells to 3 and 10% respectively and protein synthesis from 100% to 15% (p<0.05) (Figures 2b and 2c); with a significant increased DNA single strand breaks by 15 and 25% respectively (Figure 2d).
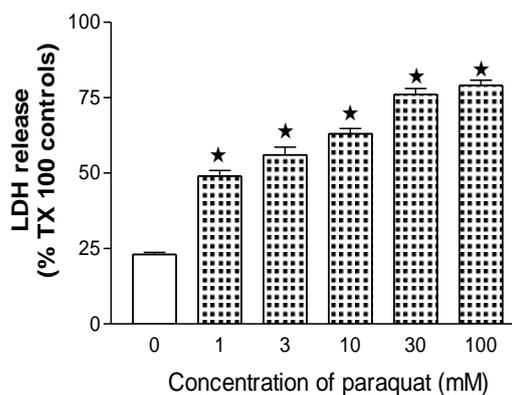

Figure 1a: 4-hour Concentration Response Effect of Five Doses of Paraquat on the Release of LDH by Confluent NRK-52E Cells
(N=10 for each concentration of paraquat at each time point).
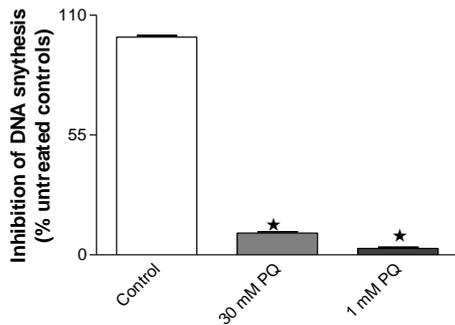*P<0.05 vs. control cells (cells treated with DMEM only).
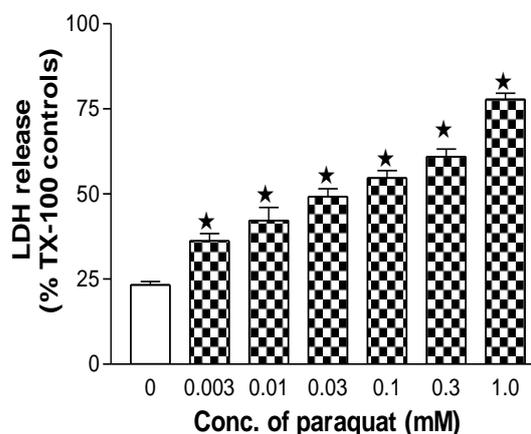
Figure1b: The 24- hour Concentration Response Effect of Five Doses of Paraquat on the Release of LDH by Confluent NRK-52E Cells (N=10 for each concentration of paraquat at each time point). *P<0.05 vs. control cells (cells treated with DMEM only)
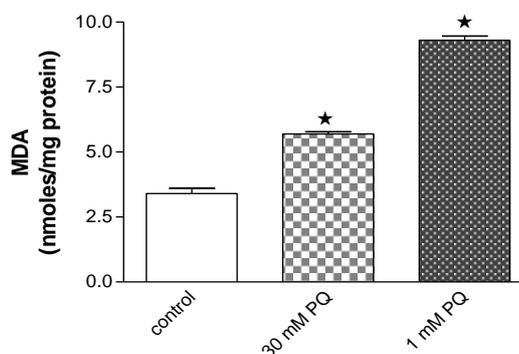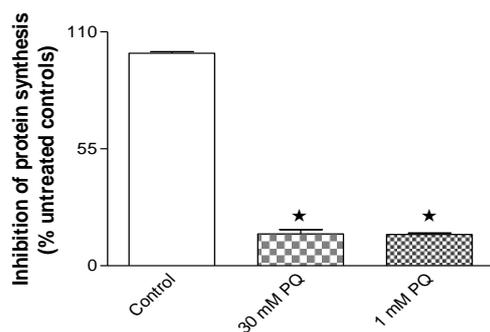

Figure 2a: Effect of Paraquat on the Synthesis of MDA in Confluent NRK-52E Cells Incubated with DMEM Containing 1 or 30mM PQ fo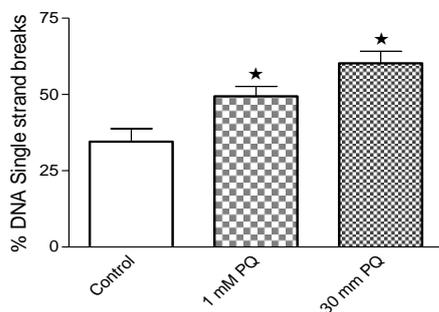r 4 or 24-hours respectively. (N=6 for each subgroup).*p<0.05 vs. untreated control (N=6 for each subgroup).*p<0.05 vs. untreated controls.

Figure 2b: The Inhibitory Effect of Paraquat on DNA Synthesis in Confluent NRK-52E Cells Incubated with DMEM Containing 1 or 30mM PQ for 4 or 24-hours Respectively *(N=9 for each subgroup). *p<0.05 vs. untreated controls.*

**Figure 2c: The Inhibitory Effect of Paraquat on Protein Synthesis in Confluent NRK-52E Cells Incubate with DMEM Containing 1 or 30mM PQ for 4 or 24-hours Respectively** *(N=9 for each subgroup). *p<0.05 vs. untreated controls*

Figure 2d: **Effect of paraquat on DNA Single Strand Breaks in Confluent NRK-52E Cells Incubated with DMEM Containing 1 or 30mM PQ for 4 or 24-hours respectively** *(N=9 for each subgroup). *p<0.05 vs. untreated controls.*

Moreover, during the 24 and 4 hour incubation periods, paraquat significantly increased NBT absorbance by 52 and 51% respectively ($p<0.05$). However, co-incubation of the target cells with paraquat and SOD (1,000 U/ml) for 4 and 24 hours

significantly reduced the paraquat-induced changes in NBT reduction by 46 and 40% respectively ($p<0.05$). On the other hand CAT (1,000 U/ml) did not significantly alter the paraquat-induced increase in NBT reduction during both incubation periods ($P>0.05$) (Figures 3a and 3b). This suggests that the production of superoxide anion is responsible for the reduction of NBT. Furthermore, exposure of the target cells to paraquat resulted in a 34- and 10-fold increase hydroxyl radical generation during the 4 and 24 hours incubation periods respectively ($p<0.05$). Unlike SOD (1,000 U/ml) which had no significant effect on the paraquat-induced increase hydroxyl production during both exposure periods ($p>0.05$), CAT (1000 U/ml) significantly reduced the paraquat-induced increase hydroxyl radical production by 70 and 68% during the 4 and 24 hour incubation periods respectively ($p<0.05$) (Figures 3c and 3d).

If the cytotoxic effect of paraquat is mediated by oxidative stress injury then paraquat may have either reduced the expression and/or activity of the antioxidant enzymes or its action may have overwhelmed their activities. Thus, the hypothesis that during paraquat-induced oxidative stress injury the expression and/or activity of the antioxidant enzymes maybe reduced was further tested. Exposure of NRK-52E cells to 30 or 1mM paraquat for 4 or 24 hours significantly reduced the activity of SOD and catalase by 30 and 24% and 64 and 52% respectively ($P<0.05$). Unlike SOD and CAT activities, paraquat significantly increased the activity of GSH-Px in the target cells by 97 & 89% after the 4 and 24 hour exposure periods respectively ($p<0.05$) (Figure 4a and 4b).
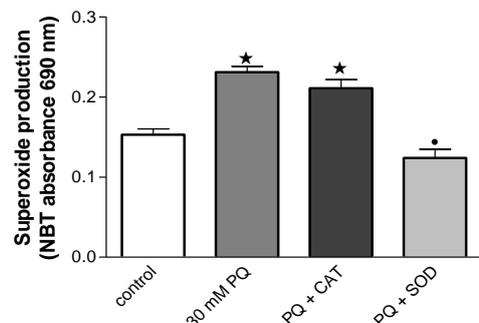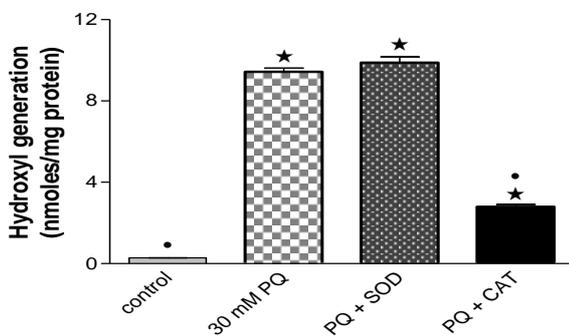

Figure 3a: Shows the Effect of Paraquat on Superoxide Generation and the relative effect of SOD and CAT on Paraquat-mediated Increased Superoxide Production (NBT absorbance) by Confluent NRK-52E cells during the 4 hour Incubation Period (N=8). *P<0.05 vs. the untreated control group and ●p<0.05 vs. PQ only treated group.

The reduction in SOD activity was accompanied by a significant reduction in the expression of MnSOD during the 4 and 24 hours paraquat exposure periods by 23 and 27% respectively ($p<0.05$). However, the expression of CuZnSOD by the target cells was significantly reduced by 25% after exposure to 1 mM paraquat for 24 hours ($p<0.05$) but was not significantly altered after the 4 hour incubation with 30 mM paraquat ($p>0.05$). Interestingly, the reduction in CAT activity was not accompanied by any concomitant significant effect on its expression ($p>0.05$) (Figure 4c).
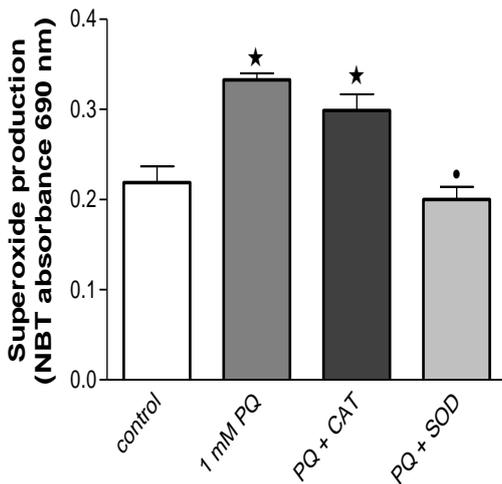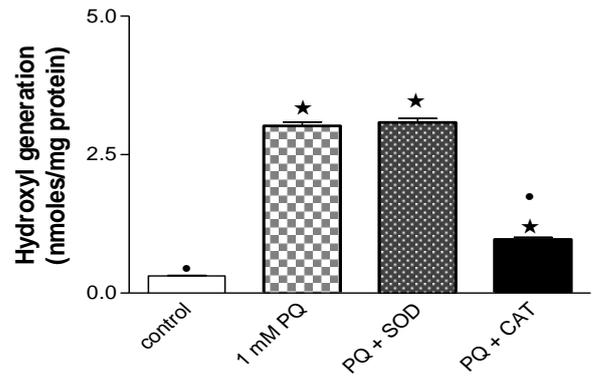

Figure 3b: The Effect of Paraquat on Superoxide Generation and the Relative Effect of SOD and CAT on Paraquat-mediated Increased Superoxide Production
(NBT absorbance) by confluent NRK-52E cells during the 24 hour incubation period (N=8). *$P<0.05$ vs. the untreated control group and ●$p<0.05$ vs. PQ only treated group


Figure 3c: The Effect of Paraquat on Hydroxyl Radical Generation and the Relative Effect of SOD and CAT on the Paraquat-mediated Increased Hydroxyl Radical Production by the Confluent NRK-52E Cells during the 4-hour Incubation Period (N=8). *$P<0.05$ vs. the untreated control group and ●$p<0.05$ vs. the PQ only treated group
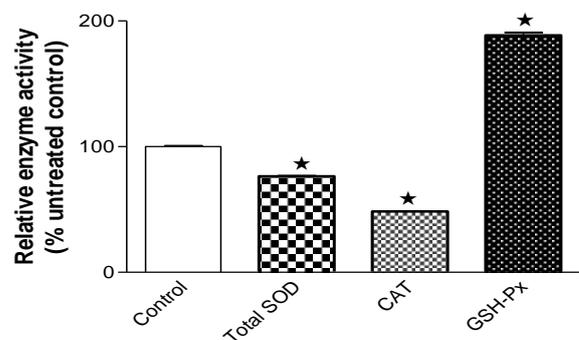

Figure 3d: Shows the Effect of Paraquat on Hydroxyl Radical Generation and the Relative Effect of SOD and CAT on the Paraquat-mediated Increased Hydroxyl Radical Production by the Confluent NRK-52E Cells during the 24 hour Incubation Period (N=8). *$P<0.05$ vs. the untreated control group and ●$p<0.05$ vs. the PQ only treated group
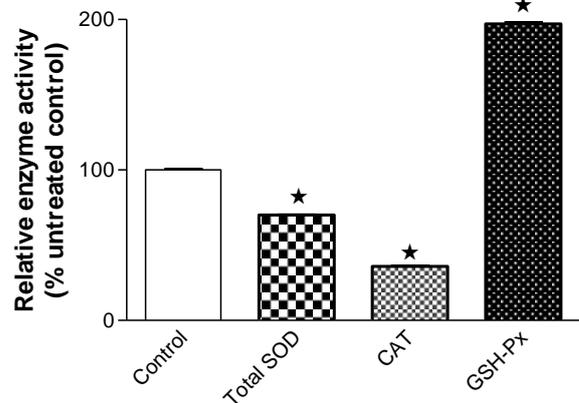
Figure 4a: The Effect of 30 mM Paraquat on Enzyme Activity of SOD, CAT and GSH-Px in Confluent NRK-52E Cells after 4 hours of Incubation (N=8 for each subgroup). *$p<0.05$ vs. the control (cells treated with DMEM only).
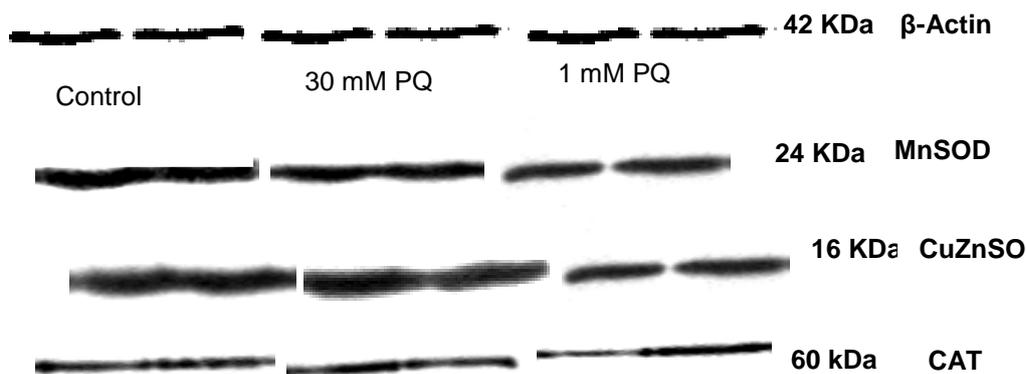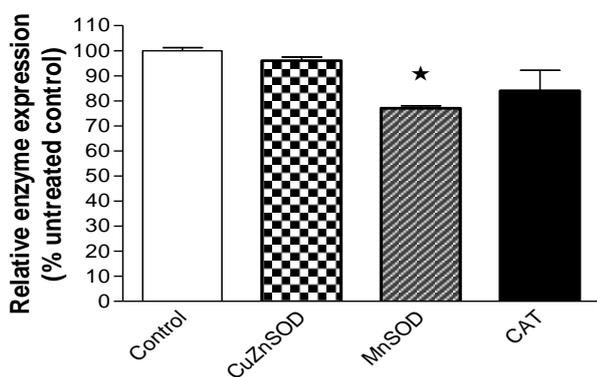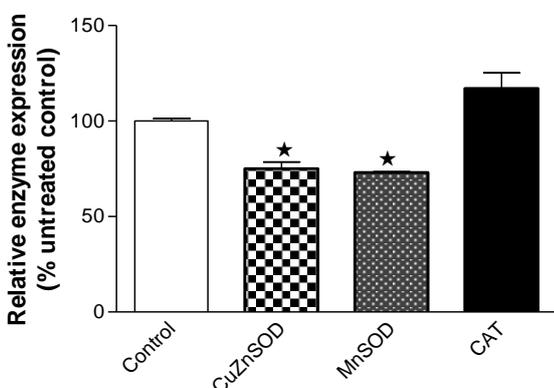

Figure 4b: Shows the Effect of 1mM Paraquat on Enzyme Activity of SOD, CAT and GSH-Px in Confluent NRK-52E Cells after 24-hours of Incubation (N=8 for each subgroup). *$p<0.05$ vs. the control (cells treated with DMEM only).

42 KDa β-Actin

Control   30 mM PQ   1 mM PQ

24 KDa MnSOD

16 KDa CuZnSO

60 kDa CAT

Figure 4c: Western Blots Showing the Effects of 1 & 30 mM Paraquat on Enzyme Expression of MnSOD, CuZnSOD and CAT in Confluent NRK-24E cells after Incubation for 24 hours and 1 hour respectively.

Figure 4d: Effect of 30mM Paraquat on Enzyme Expression of CuZnSOD, MnSOD and CAT in Confluent NRK-52E Cells after 4 hours of Incubation (N=6 for each subgroup). *$p<0.05$ vs. the untreated control (cells treated with DMEM only). The results depicted are normalized to levels of β-actin protein expression*

Figure 4e: Shows the Effect of 1mM Paraquat on Enzyme Expression of CuZnSOD, MnSOD and CAT in Confluent NRK-52E Cells after 24-hours of Incubation (N=6 for each subgroup). *$p<0.05$ vs. the untreated control (cells treated with DMEM only). The results depicted are normalized to levels of β-actin protein expression

DISCUSSION

The results of this study indicate that: (i) paraquat is renocytotoxic, specifically, producing a concentration- and time-dependent increase in cell death; (ii) paraquat increases ROS generation, specifically, significant superoxide anion and hydroxyl radical generation; (iii) the renocytotoxic effect of paraquat is mediated in part by increased lipid peroxidation, inhibition of DNA and protein synthesis and increased DNA single strand breaks; and (iv) paraquat reduces the activity and expression of SOD; reduces the activity of catalase with no significant effect on its expression; and increases the activity of GSH-Px.

Increased superoxide anion generation due to paraquat and its contribution to paraquat toxicity have been documented over many years (Inal *et al.*, 1999; Mimc-Oka *et al.*, 1999; Modlinger *et al.*, 2004; Sindhu *et al.*, 2005) and in this study, its role including that of hydroxyl radical in the development of renocytotoxicity was confirmed *in vitro*. As expected, the paraquat-induced increase in the generation of superoxide anions and hydroxyl radicals was significantly reduced by superoxide dismutase and catalase respectively. Moreover, its renocytotoxic effect was noted to be mediated by oxidative stress injury as evidenced in part by a significant increased in lipid peroxidation. This finding supports earlier reports in which lipid peroxidation secondary to an increase production of lipid hydroperoxide has been documented to be responsible for the cytotoxic effect of paraquat (Krall *et al.*, 1988; Sommerburg *et al.*, 2002; Ray *et al.*, 2007). Furthermore, the effect of oxidative stress by paraquat on DNA damage and inhibition of DNA and protein synthesis has been

demonstrated in primary cultures of porcine aortic endothelial cells (Garcia-Alfonso *et al.*, 1995) and confluent LLC-PK1 cells (Matkovics *et al.*, 1982) respectively; and these effects were further confirmed in confluent NRK-52E cells in this study.

Oxidative stress remains an elusive phenomenon which may be difficult to define. However, it arises either as a result of excess generation of ROS in the tissues under consideration and/or a failure to metabolize ROS. Interestingly, prokaryotic and eukaryotic organisms have evolved antioxidant defences to protect against ROS, predominant amongst which is the enzymatic antioxidant pathway. The major pathways for metabolism are superoxide dismutase (SOD), which is expressed as extracellular (EC),intracellular, and mitochondrial isoforms that metabolize superoxide anion ($O_2^-\cdot$) to hydrogen (H_2O_2). Peroxidases such as catalase and glutathione peroxidise (predominantly intracellular) further metabolize H_2O_2 to O_2 and water. Theoretically at least, the balance between the first and second step antioxidant enzymes may be critical: on the one hand too little SOD relative to GSH-Px and/or CAT could lead to an accumulation of O_2^- which are toxic to macromolecules; and on the other hand, too much SOD relative to GSH-Px and/or CAT could lead to increased production of the H_2O_2 intermediate, which in itself may be responsible for the genesis of hydroxyl radical ($OH^\cdot$) that are even more toxic than O_2^- (Senator *et al.*, 2004).

Unfortunately, there are conflicting reports regarding the activity and/or expression of the antioxidant enzyme defence systems during oxidative stress injury. For instance, a significant increase in GSH-Px (Godin *et al.*, 1988; Kakkar *et al.*,1995; Duncan *et al.*,1996; Ray *et al.*, 2007), CAT (Matkovics *et al.*, 1982; Yadav *et al.*,1997; Ray *et al.*, 2007), and SOD activity (Garcia-Alfonso *et al.*, 1995; Ray *et al.*, 2007) and decrease in GSH-Px (Senator *et al.*, 2004; Sindhu *et al.*, 2005), CAT (Kakkar *et al.*,1995, Sommerburg *et al.*, 2002) and SOD activity (Kakkar *et al.*, 1995; Senator *et al.*, 2004) has been noted during oxidative stress injury depending on the experimental conditions. However, in the current study, paraquat significantly reduced the activity and expression of SOD, the activity of CAT with no significant change in CAT expression. This was accompanied by a significant increase in the activity of GSH-Px.

It should be noted that whilst CAT is specifically responsible for the degradation of H_2O_2, GSH-Px has a broader spectrum as it also reduces lipid peroxides. Thus, during oxidative stress injury, the activity of GSH-Px activity is usually increased in order to scavenge lipid peroxide production at the early stages of lipid peroxidation (Taylor *et al.*, 1993; Kiyomiya *et al.,* 2000). Moreover, tissues in which CAT activity is decreased may be critically dependent on increased GSH-Px activity for protection (Kakkar *et al.*, 1997). Furthermore, a significant increase in GSH-Px and CAT gene expression with a respective increase and decrease in their activities has been noted during oxidative stress injury of the kidney (Limaye *et al.*, 2003). Thus, the increase in GSH-Px activity with a concomitant decrease in CAT activity indicates that there could be compensatory mechanisms between these antioxidant enzymes in response to oxidative stress.

In conclusion, paraquat mediates its nephrotoxic effect in part via oxidative stress secondary to an increase generation of ROS with a reduction in CAT activity and a concomitant reduction in the expression and activity of SOD.

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