Induction of IL-8(CXCL8) and MCP-1(CCL2) with oxidative stress and its inhibition with N-acetyl cysteine (NAC) in cell culture model using HK-2 cell

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Abstract:

Renal transplantation can often be complicated due to delayed graft function, which is a direct sequel of ischemia reperfusion injury. The adverse outcome of delayed graft function is not only short term but the long term function of the graft is also affected. Therefore, it is important to understand the mechanisms of ischemia reperfusion injury. Reactive oxygen species are the key mediators in ischemia reperfusion injury causing direct cell damage which also initiate inflammation by inducing chemokines. Presence of Inflammation is a marker of severe delayed graft function. However, the effect of oxidative stress on the expression of key chemokines has not been fully established yet.

Therefore, the aim of this study was to measure the oxidative stress response and the secretion of chemokines in a cell culture model that mimics the effects of ischemia reperfusion injury in immortalised human renal proximal tubular epithelial cells, HK-2.

Cells were treated with varying concentrations of hydrogen peroxide and markers of oxidative stress response and chemokine release were measured. Exposure to hydrogen peroxide induced a significant increase in the activity of the antioxidant enzyme glutathione peroxidase and the levels of the chemokines Interleukin-8 (IL-8; CXCL8) and MCP-1 (CCL2). A dose related increase of chemokine secretion was also observed. The cytokine Interleukin-1β (IL-1β) at 1ng/ml significantly potentiated the expression of both IL-8 (CXCL8) and MCP-1 (CCL2) which showed synergistic response in the presence of hydrogen peroxide. Pre-incubation of the cells with the anti-oxidant N-acetyl cysteine (NAC) strongly suppressed the induction of both IL-8 and MCP-1 when stimulated with hydrogen peroxide and IL-1β.

This study demonstrates the potential of anti-oxidants like N-acetyl cysteine in ameliorating the effects of ischemia reperfusion injury thus suggesting a new therapeutic approach in renal transplantation. These findings can have potential implications for clinical use to prevent ischemia reperfusion injury in renal transplantation.

Key words:

Oxidative stress, N-acetyl cysteine (NAC), Interleukin-8 (IL-8, CXCL8), Monocyte chemo attractant protein (MCP-1, CCL2), HK-2 (human kidney-2)

Abbreviations:

BCA (Bicinchoninic acid assay), DGF (Delayed Graft Function), IRI (Ischemia reperfusion injury), GM (Growth Media), GPx (Glutathione Peroxidase), IFN-γ (Interferon – gamma), IL-1β (Interleukin 1 beta), NAC (N-acetyl cysteine), NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells), ROS (Reactive Oxygen species), SFM (Serum Free Media)

1. Introduction:

Compared with dialysis, renal transplantation offers patients with end stage renal failure improved survival and quality of life. Delayed graft function (DGF) is a well-known complication affecting the kidney allograft in the immediate post-transplantation period. The incidence of DGF has been increasing over time from 14.7% to 21.3% perhaps due to the use of expanded criteria donors (1). Patients with DGF require prolonged hospitalisation and rehabilitation, which increase the overall cost of transplantation. Meta-analysis by Yarlagadda et al had shown that patients with DGF had a 41% increased risk of graft loss and a higher mean serum creatinine. DGF was also associated with a 38% relative increase in the risk of acute rejection (2). Halloran et al have reported that the half-life of cadaveric kidneys with no delayed graft function was 11·5 years, compared with 7.2 years for those with delayed function (3). This makes a compelling case to investigate DGF further and to find novel solutions to ameliorate this phenomenon.

The proximal tubules located in the outer medulla of the kidney are the primary site of injury in renal ischemia and reperfusion (4). Under normal physiological conditions this region is marginally oxygenated and has a high basal metabolic demand (5),(6). Therefore, hypoxic or ischemic injury can cause a significant damage to the proximal tubules.

The Ischemia-reperfusion injury (IRI) is a highly complex cascade of events that includes interactions between the vascular endothelium, interstitial compartments, circulating immune cells, and other biochemical factors. Inflammation is known to be a key mediator of IRI and a considerable body of data demonstrates the significance of the innate immunity in IRI (7),(8). The absence of oxygen and nutrients during ischemia creates a condition in which the restoration of circulation results in

production of Reactive Oxygen Species (ROS) in the proximal tubules. Oxidative damage from the ROS increases the local generation of pro-inflammatory cytokines and chemokines which recruit neutrophils and lymphocytes at the site of injury and lead to an inflammation-like state. The neutrophils themselves increase the generation of ROS and also cause direct cellular injury rather than restoration of normal function.

Reactive oxygen species (ROS) are known to stimulate the production of key transcription factors, growth factors and inflammatory mediators like Interleukin-8 (IL-8; CXCL8), Monocyte chemoattractant protein-1 (MCP-1; CCL2) and Regulated on normal T cell expressed and secreted (RANTES; CCL5)(9). These chemokines recruit neutrophils and lymphocytes thus leading to subsequent cellular injury (10).

The chemokines are a family of chemotactic cytokines that act as directional signals in the migration of effector leukocytes (11). In addition, chemokines have also been shown to activate leukocytes, influence haematopoiesis, and modulate angiogenesis (12),(13).

Interleukin-8 (CXCL8) represents a classical CXC chemokine which is a potent inducer of neutrophil chemotaxis (14). It is produced early in inflammation in response to stimuli such as endotoxin and by the early, alarm cytokines such as TNFα and IL-1. Monocyte Chemoattractant Protein-1 (MCP-1/CCL2) is a member of the C-C chemokine family, and a potent chemotactic factor for monocytes. CCL2 is produced by a variety of cell types it is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages (15).

Neutrophil activation in IRI has been gaining attention in the past several years, primarily due to the re-evaluation of the role of excessive inflammatory response in kidney IRI. In models of renal IRI, depletion of neutrophils, blockade of neutrophil

adhesion to the endothelium, and inhibition of the complement system have shown to decrease kidney damage (16). Land et al have demonstrated that changes in the expression of chemokine and chemokine receptors following transplantation regulate the migration of leukocytes from the peripheral circulation into the allograft (17). The role of chemokines in IRI known but its behaviour in proximal tubules is less known.

2. Objectives:

1. To measure oxidative stress response in a cell culture system (immortalised human renal proximal tubular epithelial cells hRPTEC) in response to simulated pathological condition of ischemia reperfusion injury.

2. To determine the ability of human tubular cells to produce chemokines in a similar setting.

3. To establish the role of oxidative stress on the expression of IL-8 (CXCL8) and MCP-1 (CCL2)

3. Materials and Methods

Cell culture

HK-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, CRL-2190) and were cultured until 90–95% confluent in six-well plates or T75 flasks in DMEM/Ham's F12 growth medium supplemented with 5.5 mM glucose, 2 mM L-glutamine, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, 0.4 µg/ml hydrocortisone, 5 ng/ml epidermal growth factor, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 mM HEPES, and 10% FCS. The growth medium was changed on alternate days and the cells were used for no more than 10 passages. HK-2 cells cultured with serum were growth arrested for 48h in serum free media (SFM).

Subculture was performed weekly or when the monolayers reached 80% confluence as assessed by phase-contrast microscopy. Cells were maintained in an incubator at 37°C and 5% CO_2. Cell viability was assessed using the Trypan blue exclusion assay using 30, 300, 1000 µM H_2O_2 (18).

Model for Ischaemia Reperfusion Injury

HK-2 cells were seeded in 6 well culture plates as described previously. Once 80% confluence was reached, growth was arrested by incubation in SFM for 24 hrs prior to all experiments. The cells were then incubated with 0.5 mM hydrogen peroxide for 60 minutes at 37°C in an incubator. Cells were then washed with PBS at room temperature three times and incubated in growth media for 6,12,24, or 36 hours. The control group were not exposed to hydrogen peroxide but were given PBS wash to keep the conditions similar in both groups. The cells were then harvested at the above stated time intervals to assess markers of oxidative stress catalase and glutathione peroxidise.

Model for Chemokine Induction

HK-2 cells were grown as described previously. Oxidative stress was induced by exposure to 30 or 300μM hydrogen peroxide for 60 minutes. The cells were washed then three times with PBS and were incubated in SFM for 24hrs. Supernatants were collected for IL-8 (CXCL8) and MCP-1 (CCL2) analysis. The cells were harvested in sodium hydroxide to determine the protein content which was analysed by BCA (Sigma).

Model for Chemokine Suppression with NAC

HK-2 cells were grown as described previously. Cells were incubated with 10mM NAC for 60 minutes and washed three times with PBS. The control cells were not exposed to NAC but received a wash with PBS to keep all conditions similar in both sets. Cells were then incubated in 30 and 300μM H_2O_2 for 60 min. The cells were then washed three times with PBS and incubated in SFM for 24 hrs. Supernatants were collected and analysed for IL-8 and MCP-1.

Model for Chemokine Induction with IL-1β and NAC Inhibition:

HK-2 cells were cultured as described previously. Cells were incubated with 10mM NAC for 60 minutes and washed three times with PBS. The control cells were not exposed to NAC but however received a wash with PBS to keep all conditions similar in both sets. Following this some cells were pre-incubated with 1ng/ml IL-1β for 24 hrs. Other cells remained in SFM. Cells were then washed three times with SFM and incubated in 30 or 300μM H_2O_2 for 60 min. The cells were washed three times with SFM and incubated in SFM for 24 hrs. Supernatants were collected and analysed for IL-8 and MCP-1 using ELISAs.

Glutathione Peroxidase and Catalase activity assays

Cells were harvested in 550µl of PBS/well and sonicated for 10sec at 15 microns (Soniprep 150, Sanyo Scientific). The cell extracts were clarified by centrifugation, and the protein content was measured by the bicinchoninic acid method (BCA).

Glutathione peroxidase (GPx) activity was measured using the method of Flohe and Gunzler (19).

Catalase activity was measured spectrophotometrically using the assay developed by Beers and Sizer (20).

IL-8 and MCP-1 ELISA

The media supernatants were collected, clarified from cell debris by centrifugation and stored at -20°C prior to analysis.

IL-8 and MCP-1 levels were measured using sandwich ELISA according to the manufacturer's protocol (R&D Systems). The ELISA plates were read using a micro plate reader (Powerwave X340, Bio-tech Instruments Inc, Vermont,USA) at 450nm using background subtraction at 570nm. The concentrations of IL-8 and MCP-1 in the supernatants were determined from a standard curve and were normalised using the total protein values of the respective cell monolayers.

Data analysis

Statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS version 11.01, Surrey, UK). All data were expressed as means ± SE. Data were analysed using a one-way repeated measures ANOVA. Where a significant value was observed, Tukey's HSD post hoc analysis was performed to identify where the significant differences occurred. A P value of <0.05 was considered significant.

4. **Results:**

Cell viability was assessed using the Trypan blue exclusion assay. Adherent and floating cells were harvested at 24 and 48 hrs after exposure to either SFM alone (control) or to Hydrogen peroxide preparation. The pooled adherent and non-adherent cells remained viable both at 24 and 48 hours after exposure to 30,300,1000 μM hydrogen peroxide with no significant difference between the three groups.(Figure not shown)

Glutathione peroxidase and catalase enzyme activity in response to H_2O_2

Glutathione peroxidase activity was measured at 2,4,8,12,24 and 36 hours. There was a significant rise in glutathione peroxidase activity in the samples collected after 36 hours of injury (181.8+/110.61mU/mg compared to 95.34+/-23.88mU/mg in control; ($p<0.05$)), as shown in Figure 1. The experiments were repeated on at least three separate occasions.

There was no significant change in the activity of catalase at any time interval (n=12; figure not shown).

Figure 1: Glutathione peroxidase (Gpx) activity in cell exposed to 0.5 mM hydrogen peroxide (n=6)

Basal production of IL-8 (CXCL8)

IL-8 (CXCL8) was detected within 6 hours of incubation in SFM 70.3+/-36.86pg/ml and the concentration increased with time, 456.8+/-94.28pg/ml at 12 hours, 925+/-139pg/ml at 24 hours and 1337+/-158pg/ml at 48 hours (results not shown). Basal IL-8 production could be due to the stress of cell culture due to transfer of cells and change in media. The progressive rise with time is possibly the effect of accumulation.

Effect of growth media and hydrogen peroxide on IL-8 (CXCL8):

When exposed to 30 or 300 μM of hydrogen peroxide in growth media or SFM, no change in IL-8 (CXC) was observed in cells cultured in growth media. In contrast, cells incubated in SFM showed a significant rise in IL-8 (CXCL8) with 300 μM of hydrogen peroxide measured at 24 hrs (Figure 2).

The possible explanation for this could be the albumin in foetal calf serum may have scavenged the free radicals resulting in reduced stimulation of cells in growth media group. Other explanation can be that the cells are less stressed in growth media as compared to SFM thus resulting in less stimulation in growth media group.

Figure 2: Induction of IL-8 with 30 and 300μM H_2O_2 in serum free media but not in growth media

Effect of Interleukin-1beta (IL-1β) on IL-8 (CXCL8) production

HK-2 cells when stimulated with 1ng/ml IL-1β showed an increase in IL-8 (CXCL8) production (Figure 3). In the same experiment 30 and 300 μM of hydrogen peroxide also increased the production of IL-8 significantly. A further increase in IL-8 (CXCL8) was observed in cells exposed to IL-1β and then incubated with 30 and 300μM hydrogen peroxide, (Figure 3). There was no statistical difference between the exposure to 30 and 300μM hydrogen peroxide alone. However, there was a significant rise between cells exposed to 30 or 300 μM hydrogen peroxide and further stimulated with IL-1β which was more than expected suggesting synergism between IL-1β and hydrogen peroxide.

Figure 3: IL-8 induction with IL-1 alone and in combination with H_2O_2 (n=6) * P <0.05

Effects of N-Acetyl cysteine (NAC) and hydrogen peroxide on IL-8 production
In HK-2 cells were pre-incubated with NAC 10mM and then stimulated with hydrogen peroxide 30 and 300 μM, IL-8 (CXCL8) was inhibited in both groups. 4464+/-425pg/ml and 3993+/-1464pg/ml respectively as compared to cells not pre-incubated with NAC 5919+/-659pg/ml and 6865+/-105pg/ml, although this was not statistically significant (p>0.05; data not shown).

Effects of N-acetyl cysteine (NAC) and Interleukin-1beta (IL-1β) on IL-8 production

HK-2 cells when stimulated with 1ng/ml IL-1β significantly increased the production of IL-8 to 7990+/-972pg/ml when compared with control cells that were incubated with culture medium alone at 5631+/-154pg/ml ($p<0.05$). Further, in cells pre-incubated with NAC the effect of IL-1 β was suppressed significantly (5669+/-798pg/ml; $p<0.05$) as shown in Figure 4.

Figure 4: IL-1β alone stimulation of IL-8 (CXCL8) and its inhibition by NAC pre-incubation

Stimulation of IL-8 (CXCL8) with IL-1β and hydrogen peroxide and its inhibition with NAC:

To study the effect of antioxidant treatments on the production of chemokines, the above experiments were repeated on cells that were induced with IL-1β and H_2O_2 (30 or 300 μM). A significant increase of IL-8 was observed in cells exposed to IL-1β alone. However, this effect was partially abolished by NAC (Figure 5). The IL-8 generation in cells stimulated with 30μM of hydrogen peroxide was moderately inhibited in response to 10mM NAC pre-incubation (2495+/1924pg/ml vs 3222+/-

904pg/ml). In contrast, the IL-8 levels in cells stimulated with 300μM of hydrogen peroxide were significantly inhibited by NAC (1961+/-322pg/ml vs 4837+/-414pg/ml) as shown in Figure 5. The results demonstrate the role of oxidative stress induced by hydrogen peroxide in mediating IL-8 (CXCL8) release and show a dose related response.

Figure 5: Suppression of H_2O_2 and IL-1β mediated production of IL-8 (CXCL8) by pre-incubation with NAC (N)

Oxidative stress and MCP-1 (CCL2) production

Effect of Hydrogen peroxide and IL-1β on MCP-1 (CCL2)

MCP-1(CCL2) was investigated similar to previous experiments on IL-8 (CXCL8). There was no change in MCP-1 (CCL2) levels in response to either 30 or 300 μM of hydrogen peroxide (Figure 6). When cells were incubated with IL-1β they showed a significant increase in MCP-1 (CCL2) (2902+/-68pg/ml) compared to the control group (1616+/-54pg/ml) as shown in Figure 6. These results indicate that hydrogen peroxide has less involvement in MCP-1 (CCL2) production as compared to IL-8 (CXCL8), however IL-1β was able to potentiate its production suggesting different mechanisms maybe involved for hydrogen peroxide and IL-1β.

Figure 6: Induction of MCP-1 (CCL2) with IL-1β but not with hydrogen peroxide

Effect of pre-incubation with NAC and induction with hydrogen peroxide and IL-1β on MCP-1 (CCL2)

Pre-incubation with NAC resulted in a significant reduction of MCP-1 (CCL2) levels in cells exposed to 300 μM of hydrogen peroxide (793.7+/-57pg/ml) but not in response to 30 μM hydrogen peroxide (1172+/-109pg/ml) (Figure 7). These findings again suggest oxidative stress is involved, although pathways may possibly be more complex. Pre-incubation of cells with NAC also resulted in inhibition of IL-1β induction although this was not statistically significant as shown in Figure 7.

Figure 7: Effect of pre-incubation with NAC and induction with Hydrogen peroxide and IL-1β on MCP-1 (CCL2) levels

Combined effect of H_2O_2, IL-1β, and NAC on MCP-1 (CCL2)

The above experiments were repeated similar to the experiment with IL-8 (CXCL8) with the addition of cells stimulated with both IL-1β and hydrogen peroxide (30 and 300 μM) and cells pre-incubated with 10mM NAC. Results were similar to those seen with IL-8 (CXCL8). IL-1β significantly increased the MCP-1 (CCL2) to 2902+/-68pg/ml and NAC was able to inhibit this to a level of 2818+/-261pg/ml although again this was not statistically significant. In the subset which were induced with hydrogen peroxide and IL-1, NAC pre-incubation caused a reduction in MCP-1 (CCL2) in 300 μM group to 1044+/-303pg/ml when compared with the set which did not get NAC, 2636+/-177pg/ml ($p<0.05$) as shown in Figure 8.

Figure 8: Combined effect on MCP-1 production after NAC pre-incubation on IL-1β and H_2O_2 stimulated cells

5. DISCUSSION

Oxidative stress resulting from IRI has been shown to have a major role in causing DGF (21). Chemokines mediate the inflammatory response but it is difficult to establish a detailed role of proximal tubules. Therefore, a simulated condition of oxidative stress was attempted within the constraints of an *in vitro* model. HK-2 cells are a suitable model system for the purpose of these experiments because of their thorough characterisation, durability and consistent physiological response.

IRI was simulated by incubating HK-2 cells with hydrogen peroxide. Hydrogen peroxide introduced exogenously in high concentration is known to permeate through cell membranes cannot be detoxified to water by the intra-cellular antioxidant enzymes and produce oxidative stress (22).

Hydrogen peroxide is converted by catalase and glutathione peroxidase to water. Glutathione peroxidase has been mainly shown to be produced in kidneys and HK-2 cells have been shown to express glutathione peroxidase (23,24). Several other studies have also reported involvement of glutathione peroxidase in defence responses to oxidative stress in proximal tubules (25,26).Our study has shown significant increase in glutathione peroxidase from HK-2 cells after induction with hydrogen peroxide.

Miguel et al have utilised hydrogen peroxide in a cell culture model of HeLa cells to study its effect on antioxidant enzymes. Hydrogen peroxide induced oxidative stress in a time- and concentration- dependent manner which was prevented with the use of the anti-oxidants ascorbic acid and N-acetyl cysteine (27).

There was no change in catalase activity in this model. There could be several reasons for this but catalase has shown to be less responsive in experimental oxidative stress conditions due to differential inhibition (27). Catalase is also reported

to be inactivated by hydroxyl radicals, while glutathione peroxidase and superoxide dismutase (SOD) are considerably less affected by these radicals (28).

The observed basal secretion of IL-8 (CXCL8) by HK-2 cells is possibly the response of the cells to normal stress of growth inherent in cell culture systems. Bioprocess forces are known to be encountered during cell culture and include hydrodynamic shear and stresses resulting from transfer through capillaries or by pipetting to suspend cell pellets (29). The progressive rise with time is the effect of accumulation. Andreucci et al also found a rise in IL-8 (CXCL8) after hydrogen peroxide stimulation in HK-2 cells (30). Shimada et al showed a similar rise from gastric epithelial cells when stimulated with hydrogen peroxide (31). DeForge et al showed the stimulatory effect of hydrogen peroxide on IL-8 (CXCL8) production by HepG2 cells, A549 pulmonary type II epithelial cells, and human skin fibroblasts (32).

Cytokines like IL-1β, TNF or the bacterial product LPS in both a time- and dose-dependent manner are known to increase expression of IL-8 (CXCL8) mRNA and secreting IL-8 (CXCL8) peptide (33). Ivison et al proved the link and mechanism of hydrogen peroxide mediated IL-8 (CXCL8) induction. This was dose dependent and associated with synergistic phosphorylation of p38 MAP kinase and with prolonged I-κB degradation and NF-κB activation (34).

Enesa et al observed that H_2O_2 combined with cytokines (IL-1β, TNFα) induced IL-8 (CXCL8) synergistically in cultured epithelial cells. They also observed that the capacity of H_2O_2 to enhance IL-8 (CXCL8) induction in response to TNFα was suppressed in cultures that were pre-treated with NAC (35).

Inhibition of MCP-1 (CCL2) signalling in ischemic renal injury has been shown to have a protective role (36). Takahashi et al have suggested JNK- and IκB-dependent

pathways regulate MCP-1 (CCL2) synthesis and also found NAC could suppress MCP-1 (CCL2) production (37).

NAC has also been shown to protect kidneys against oxidative damage from in vitro simulated reperfusion injury (38). Antonicelli et al investigated the effect of Nacystelyn, a recently developed lysine salt of NAC upon interleukin IL-8 (CXCL8) release and the activation of the re-dox-sensitive transcription factors AP-1, NF-κB, and C/EBP in a human alveolar epithelial cell line (A549)(39).

Ayvaz et al have reported the benefit of NAC in intestinal ischemic injury as well as, reperfusion in rat model (40). Similarly Nitescu et al have shown NAC improves kidney function, and reduces renal interstitial inflammation, in rats subjected to renal IRI (41). Zhang et al found that NAC exerts its protective effect in part by directly scavenging ROS and in part via ERK1/2 activation in HK-2 cells (42) while Lee et al have shown NAC reduced Colistin induced nephrotoxicity in human proximal tubules (43).

Despite many favourable results in the *in vitro* models, NAC has not been used widely in the clinical world. So far it has shown some clinical benefit in preventing DGF and contrast nephropathy. (44-46). Briguori et al have reported that NAC was better than fenoldopam mesylate to prevent contrast agent-associated nephrotoxicity (47).

Danilovic et al have shown clinical benefit of NAC in renal transplant recipients. Use of NAC resulted in reduced rate of DGF and better renal function was seen for up to one year (48). NAC administration has also showed an improvement in the histopathological findings of ischemia/reperfusion damages (40). Donor pre treatment with NAC has been shown to preserve renal metabolism and improve outcomes of IRI injured kidney transplants (49). Fuentes et al have shown NAC

treatment in patients with stable renal function after transplantation increased high-density lipoprotein cholesterol and antioxidant molecules in relation to glutathione peroxidase, with a positive influence on renal function (50). However clinical trials have failed to show any benefit of using NAC as pre-treatment in deceased donors (51).

Although there is widespread involvement of ROS but there is a lack of widespread use of anti oxidants in clinical use. *N*-acetylcysteine, despite the growing evidence of benefit, has very limited utility used only for treating over-doses of acetaminophen and for improvement of bronchial mucous fluidity (52).

Many anti-oxidants show strong positive effects in the laboratory, but only a few of these drugs are used as anti-oxidants in humans. Some plausible reasons for this is that because ROS production occurs ubiquitously in aerobic cells and chemical reactivity of ROS takes place in a non-specific manner; to find statistically significant differences in different cells or tissues is difficult. Because ROS production is very common and the human body has an abundant capacity for producing anti-oxidants, it is difficult to obtain statistical differences in trials designed to evaluate an anti-oxidative drug.

Also even if expression of enzyme or protein is seen it may not necessarily result in an increase in activity (53).

Conclusion:

This study has shown that HK-2 cells are responsive to various physiological stimuli like hydrogen peroxide and cytokines. IL-1β mediated IL-8 production is partly mediated via oxidative radicals as NAC, a powerful antioxidant, was able to inhibit this reaction. Oxidative stress is possibly the underlying mechanism involved in the

various responses seen. The challenge remains in using these novel targets and their translation from bench to bedside (54).

DGF is an important clinical outcome after kidney transplantation, and one that needs to be addressed by funding agencies and clinical trials. In an era of tremendous shortage of kidneys for transplantation, every effort should be made to improve the survival of the transplanted kidneys in the recipient. Therefore, it is imperative that we implement strategies to reduce the incidence of DGF in an effort to improve long-term graft survival. Cold storage phase is indeed an underused therapeutic window. High dosage of NAC can be delivered in cold perfusion fluids. The present results should be interpreted keeping in mind the constraints of cell culture model.

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