International Journal of Research in Chemistry and Environment
Vol. 1 Issue 1 July 2011(95-100)
ISSN 2248-9649

Repercussion of isocyanates exposure on different cellular proteins in human pulmonary arterial endothelial cells

Panwar Hariom *, Mishra Pradyumna Kumar
Department of Zoology, Dr. HS Gour University, Sagar, (M.P.), INDIA.
Division of Translational Research, Tata Memorial Centre, Navi Mumbai, INDIA.

Available online at: www.ijrce.org

(Received 15th April 2011, Accepted 28th April 2011)

Abstract- *The endothelium is the thin interior surface of blood vessels normally involved in many aspects of vascular biology; including vasoconstriction, vasodilatation, and angiogenesis can be injured by physical or chemical stress, particularly by the drugs and other environmental toxins. Although, isocyanates have drawn significant attention in the recent past as they react with DNA to produce toxicogenomic insults due to high degree of reactivity and low molecular weight having functional group–N=C=O. Moreover, patho-physiological implications resulting from occupational and accidental exposures of these compounds are yet too elusive. On this premise, a strenuous effort was made to assess the cellular response of methyl isocyanate on cultured human pulmonary arterial endothelial cells (HPAE-26). Study was conducted in different time intervals to evaluate cellular response after exposure to isocyanates using N-succinimidyl N-methylcarbamate, a surrogate chemical substitute to methyl isocyanate (MIC). The role of different cell cycle regulatory proteins: cyclin A and E & cdk2; cell cycle inhibitory proteins: p53, p21& GADD45; DNA damage responsive proteins; ATM, ATR & γH2AX and repair proteins: Mre11, Nbs1 & Rad50 were evaluated through immuno-blotting. Our results demonstrate that isocyanates induced toxicity involved in vascular damage of endothelial cells with subsequent perturb expression of different tumor suppressor and repair proteins concurrently higher expression of Cdk2 and cyclins unveiled the progression of oncogenesis in the cells in-vitro.*

Key words: endothelial damage, oncogenesis, cell cycle, methyl isocyanate, ATM, p53, cyclin E.

Introduction

Endothelial cells wrap the entire internal circulatory system; dysfunction, or the loss of proper endothelial function, is a hallmark for many vascular diseases. However, understanding the function of different cellular proteins is necessary to comprehend the processes of angiogenesis, vasoconstriction, vasodilation, and tumorigenesis in vital system like circulatory system. Cellular response to genotoxic stress is a very complex process, and it usually starts with the sensing of the DNA damage, followed by a series of events that include signal transduction and activation of transcription factors [1] but, mutational inactivation or loss of heterozygocity of various cellular regulatory genes leads to predisposition to cancer. The imperative mobilizers of the DSB response are two nuclear protein kinases ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related); they are emerged as potential sensors of DNA damage [2], [3].

The histone H2A variant H2AX also play significant role in response to DNA Damage specifically controls the recruitment of DNA repair proteins to the sites of DNA damage and this activity is phosphorylation dependent. ATM, ATR and DNA-PK are primarily responsible for the phosphorylation of H2AX (γH2AX- S139) at DSB for MRN foci formation [4], [5]. Ascertaining the damage response cascade of these proteins might contribute in explicating molecular mechanisms after genotoxic exposure to isocyanates. MIC was well known chemical entity since the worst chemical disaster of Bhopal.

Mre11 complex/MRN complex is twisted form of Mre11, Rad50, and Nbs1/Xrs2 is prominently involved in the signaling pathways of checkpoint activation and DNA repair. In genotoxic exposure to DNA it play vital role in initiating kinase activation and ability to tether DNA strands [6]. Mre11 is the core complex member and it interacts with itself and both Rad50 and Nbs1/Xrs2. Mre11 has endonuclease and 3□-5□ exonuclease activities that are important in the processing of DNA ends for recognition by DNA repair and cell cycle checkpoint proteins [7] - [9]. However, Rad50 gene encodes a 153kd polypeptide involved in many DNA metabolic pathways that maintain genomic integrity and highly conserved DNA double-strand break (DSB) repair factor [10]-[12]. Another member of this complex is Nbs1 bind γH2AX

after phosphorylation, resulting in a second wave of Mre11 complex recruitment to sites of damage and thus further amplification of the checkpoint signal [6].

As p53 is such an important mediator in cellular response to genotoxic stress, it is no wonder that ATM/ATR can regulate p53 activity at multiple levels. The most straightforward way to manage p53 is through direct interaction, e.g., phosphorylation of p53. Both ATM and ATR have been shown to phosphorylate p53 protein at serine 15 to enhance its transactivating activity [13]-[16]. One critical response is the activation of p53, is in regulation of the mammalian cellular stress response, activation of genes involved in cell cycle control, DNA repair, and apoptosis [17]. Regulation of the cell cycle involves processes crucial to the survival of a cell, including the detection and repair of genetic damage as well as the prevention of uncontrolled cell division. The two main families of proteins involved in cell cycle regulation are cyclin-dependent protein kinases (cdks) and cyclins. Cyclins play a vital role in the orderly progression of the cell division cycle through their timed expression and their ability to bind, activate and enhance substrate affinity of their associated cdks, these multiple cdks control the cell cycle and have been long considered essential for normal proliferation, development and homeostasis [18], [19].

The cyclin-dependent kinase inhibitor 1A (CDKN1A), also known as p21 (WAF1/CIP1) modulates cell cycle, senescence and differentiation via specific protein–protein interactions with the cyclins, cyclin-dependent kinase (Cdk), and many others [20]. GADD45, a growth arrest and DNA damage inducible protein involved in important processes such as regulation of DNA repair, cell cycle control. It is a nuclear protein and bind to multiple important cellular proteins such as proliferating cell nuclear antigen (PCNA), p21 protein, core histone proteins, and MTK/MEKK4 an upstream activator of the JNK pathway, and cdc2 protein kinase [21]-[25]. Understanding how these proteins interact to regulate the cell cycle and other critical cellular processes has become increasingly important to researchers.

The present study is first of its kind to expound the genotoxic potential of MIC with explicating the dynamic role of different cellular proteins in cultured endothelial cells following in-vitro exposure to *N*-succinimidyl *N*-methylcarbamate a surrogate chemical to MIC [26]. The study was carried out in cultured human pulmonary arterial endothelial cells (HPAE -26) to address the cellular response by quantitative analysis of different cell cycle regulatory proteins: cyclin A and E & cdk2; cell cycle inhibitory proteins: p53, p21& GADD45; DNA damage responsive proteins; ATM, ATR & γH2AX and repair proteins: Mre11, Nbs1 & Rad50 expression states through Western Blot.

Material and Methods

Reagents and antibodies

N-succinimidyl *N*-methylcarbamate [CAS No. 18342-66-0] (Sigma Aldrich Laboratories, St. Louis, USA) stock dissolved in 2mM dimethylsulfoxide (DMSO) with final concentration of 0.005 μM was used for investigations. The culture petri-dishes were procured from BD Falcon Discovery Labware (Franklin Lakes, NJ, USA). ATCC complete growth medium for HPAE culture were procured from ATCC, Manassas, VA, USA. Bradford reagent for protein estimation was procured from Amresco Inc. (Solon, OH, USA). For assessing the cell cycle regulatory proteins - cyclins (cyclin A and E) and cdk (cdk2); tumor suppressor proteins- (p53, p21 and GADD45); DNA damage responsive proteins-(ATM, ATR & γH2AX) and repair proteins- (Mre11, Nbs1 & Rad50) primary antibodies from Abcam Inc. (Cambridge, UK) and Calbiochem (Nottingham, UK) and secondary antibodies from Promega (Madison, WI, USA) were used with appropriate dilutions.

Cell lines and Culture Conditions

HPAE-26 (ATCC No: CRL-2598) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Further, they were seeded at the density of 10 to 15 x 10^5 cells/100 mm culture dishes for western blot in ATCC complete growth medium (Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 0.1 mg/ml heparin and 0.03 mg/ml endothelial cell growth supplement (ECGS), 90%; fetal bovine serum, 10%) at 37°C in the humidified atmosphere of 5% CO_2 in 95% air as per ATCC catalogue instructions. After optimum confluency, the cells were treated with the experimental agent, *N*-succinimidyl *N*-methyl carbamate. At the onset of the experiments, the cells were at an exponential and asynchronous phase of growth.

Study Design

Experiments were conducted at different time intervals ranging from 6 h to 96 h with controls cells. The cells were treated using a constant 0.005 μM concentration of *N*-succinimidyl *N*-methylcarbamate. The selection of the dose was done on the basis of author's previous observations, in which they found that this concentration of *N*-succinimidyl *N*-methylcarbamate was optimum to induce DNA damage, oxidative stress and inflammation in cultured mammalian cells [27] - [29]. Controls were untreated normal HPAE-26 cells.

Western Blot

10 to 15x 10^5 *N*-succinimidyl *N*-methylcarbamate treated cells were harvested at different time intervals, and untreated cells were harvested as control. Briefly, cells were rinsed twice in 1X- PBS and lysed in the buffer (10% SDS, 1M Tris pH-7.6, 0.5mM EDTA). The obtained cell lysates were centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was collected. Protein concentrations were determined from the supernatant by Bradford assay. An amount of 100 μg protein was analyzed through 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane in 25 mM Tris, 194 mM glycine, and 20% methanol at 4°C with semi-dryer transfer unit of Hoefer (Holliston, MA, USA). Membranes were blocked with 5% non-fat milk in 0.1% PBST and incubated for overnight at 4 °C with primary mouse monoclonal antibody specific for cyclins (cyclin A & E) and cdk (cdk2); tumor suppressor proteins- (p53, p21 and GADD45) DNA damage responsive proteins- (ATM, ATR & γH2AX) and repair proteins- (Mre11, Nbs1 & Rad50) (1:1000 dilution). Membranes were washed two to three times

for 20 min with 0.1% PBST and incubated for 2 h at room temperature with alkaline phosphatase conjugated secondary antibody (1:2500 dilution). Membranes were washed, and band was visualized by reacting the membrane with the substrate 5–bromo–4–chloro–3indolyl phosphate/nitroblue tetrazolium (BCIP/NBT).

Results

Western Blot

To study the mechanisms underlying the cellular response after exposure to isocyanates using *N*-succinimidyl *N*-methylcarbamate, we analyzed the expression of various cellular proteins from 6h to 96h with corresponding control cells. Analysis of these modulators through western blot revealed interesting results. ATM, ATR and γH2AX (fig. 1) are principal sensor of DNA Damage response they shows their aberrant expression in different progressive time course, also different tumor suppressor and growth arrested protein p53, p21 and GADD-45 showed reduced or distress expression in *N*-succinimidyl *N*-methylcarbamate treated cells during the different time intervals denoting their incompetence to regulate cell cycle due to profused genetic alteration in comparison to controls cells. Software band density analysis of p53 and p21 proteins shows high percentage of expression at 6 h but subsequently show down regulation in different progressive time intervals while growth arrested protein GADD-45 show its down regulation from 12 h (fig. 3). Besides, sensor and tumor suppressors, repair complex (Mre11/Rad50/Nbs1) also been abruptly disappear in corresponding time scales (fig. 2). Interestingly on the other hand, both the cyclins show high fold of expression from 12 h to 96h, whereas cdk2 expresses higher at 24 h and 96 h. Over-expression of cyclin A, E and cdk2 proteins were observed in different progressive time intervals in contrast to controls implicating their role in tumorigenesis due to inactivation of cell cycle checkpoints that, in turn, resulting in proliferation of abnormal cells (fig. 4). The modifications in cell cycle progression caused by *N*-succinimidyl *N*-methylcarbamate exposure might be apprehended to the carcinogenic property of the isocyanate substitute used.

Discussion

The endothelium lining serve as a selective barrier between the vessel lumen and surrounding tissue, it also involve in controlling the passage of materials and the transit of white blood cells into and out of the bloodstream. However, the pulmonary airways are exposed to various chemical irrespective of the route of administration, and at high dose levels both toxicity and cancer have been observed in laboratory animals [30], [31]. The present study is first of its kind and imparts a sign of vascular damage by the exposed chemical substitute of MIC with the expression of damage responsive proteins and other cascade machinery. Author's previous studies have also suggested that the exposure to isocyanates can lead to DNA Damage, alteration in cell cycle regulation and chromosomal abnormalities in-vitro [27] - [29]. Our present study reveal that isocyanate cause genotoxic stress in mammalian cells and a concentration as low as 0.005 μM can trigger alteration in the expression of different cellular proteins.

DNA damage response commence with a multichannel signaling pathways that includes cell cycle checkpoints, cell cycle arrest, DNA repair, and modulation of many other processes [27] - [3], [32] & [33]. After double strand breaks induction, ATM and ATR are phosphorylated and activated [2], [3] & [34], further they phosphorylates numerous substrates playing different roles in various damage response pathways. MRN complex co-localizes with proliferating cell nuclear antigen (PCNA) throughout S phase and the replication fork is a site of MRN complex chromatin loading, there is limited co-localization of the MRN complex with BRCA-1 and γH2AX [35]. However, aberrant expression of these proteins (ATM/ATR/γH2AX) in following subsequent time hours in our study shows their incompetence in progressive cellular response (see figure 1). Perturbed expression of repair proteins generate halt in number of DNA metabolic processes, such as HR, NHEJ, telomere maintenance, the formation of meiotic DSBs, the removal of Spo11 from meiotic DSBs, the processing of DSBs, checkpoint regulation leads to uncontrolled proliferation of cells [36]. Similar expression of repair proteins observed after isocyanates induced toxicity in endothelial cells (see figure 2).

Earlier chemical stress studies with human pulmonary-artery endothelial cells showed that subtle quantity of toxins can exert severe effects [37], [38]. The over expression of cyclin A and E has been observed in a broad spectrum of human malignancies, and altered cyclin E is thought to endorse early loss of heterozygosity of p53 and tumorigenesis [39], [40] Also, loss or mutational inactivation of p53 leads to uncontrolled activation/expression of cdk2 in cells arrested for cell cycling due to physiological stress as well as damaged DNA [39], [41] - [44].

Although very few alterations in p21 are found in human cancers, it is implicated in tumorigenesis through its regulation by the p53 tumor suppressor protein. The p53 gene is the most frequently mutated gene in human cancer. An important role for p53 is as a cell cycle checkpoint regulator [45], [46]. p53 stabilization in response to DNA damage results in either a G1 or G2 phase arrest, which may allow DNA repair to occur. In cells lacking p53, genome stability is compromised leading to increased mutations, amplifications, and chromosomal abnormalities. There is strong evidence to suggest that p21 is the key mediator of the ability of p53 to regulate these cell cycle checkpoints [47]. However, our results imply the cancerous competence of isocyanates with down regulation of p53, p21 with Gadd45 (see figure 3) and up-regulation of cyclin A, cyclin E and cdk2 in progressive time intervals (see figure 4).

In conclusion, the present investigation notifies the tumorigenic capability of MIC substitute with deregulation of ATM/ATR/γH2AX/cyclins and decreased expression of different tumor suppressor and repair proteins. The toxicant may affect the cascade signaling mechanisms of sensors ATM/ATR/γH2AX, p53 and p21 protein expression involve in cell cycle arrest with perturbation of GADD45 expression. Further, the altered over expression of cyclins and cdk2 expression unveiled the progression of cancer in the cells. We also apprise about gene–environment interactions in generation of site-specific cancers with vascular damage in implemented cultured endothelial cells. The study would

specify out the new toolbox to address cancer resulting from the common modifier genes and multiple environmental exposures.

Acknowledgement

The authors are thankful to the Bhopal Memorial Hospital Trust, India for financial support.

References

1. Yang, J., Xu, Z., Huang, Y., Hamrick, H.E., Duerksen-Hughes, P.J., Yu, Y. ATM and ATR: Sensing DNA damage. *World J. Gastroenterol.* 10, 155-160(2004).
2. Kurz, E.U. & Lees-Miller, S. P. DNA damage-induced activation of ATM and ATM-dependent signaling pathways. *DNA Repair (Amst).* 3, 889–900 (2004).
3. Shiloh, Y. ATM and related protein kinases: safeguarding genome integrity. *Nat. Rev. Cancer.* 3, 155–168 (2003).
4. Furuta, T., Takemura, H., Liao, Z.Y., Aune, G.J., Redon, C., Sedelnikova, O.A., Pilch, D.R.., Rogakou, E.P., Celeste, A., Chen, H.T., Nussenzweig, A., Aladjem, M.I., Bonner, W.M., Pommier, Y. Phosphorylation of histone H2AX and activation of Mre11, Rad50 and Nbs1 in response to replication-dependent DNA-double-strand breaks induced by mammalian DNA topoisomerase I cleavage complexes. *J. Biol. Chem.* 278, 20303-20312 (2003).
5. Jowsey, P.A., Williams, F.M., Blai, P.G. DNA damage, signalling and repair after exposure of cells to the sulphur mustard analogue 2-chloroethyl ethyl sulphide. *Toxicology*. 257, 105–112 (2009).
6. Borde, V. and Cobb, J. Double functions for the Mre11 complex during DNA double-strandbreak repair and replication. *Int. J. Biochem. Cell. B.* 41, 1249–1253 (2009).
7. Jazayeri, A., Balestrini, A., Garner, E., Haber, J.E., Costanzo, V. Mre11–Rad50–Nbs1- dependent processing of DNA breaks generates oligonucleotides that stimulate ATM activity. *EMBO J*. 27, 1953–62 (2008).
8. Lee, J.H., Paull, T.T. ATM activation by DNA double-strand breaks through the Mre11–Rad50–Nbs1 complex. *Science* 308, 551–4 (2005).
9. Paull, T.T., Gellert, M. The 3´ to 5´ exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks. *Mol. Cell.* 1, 969–79 (1998).
10. Constantinesco, F., Forterre, P., Koonin, E.V., Aravind, L., and Elie, C. A bipolar DNA helicase gene, herA, clusters with rad50, mre11 and nurA genes in thermophilic archaea. *Nucleic Acids Research*. 32, 1439-1447 (2004).
11. Wu, G., Lee, W.H., Chen, P. NBS1 and TRF1 colocalize at promyelocytic leukemia bodies during late S/G2 phases in immortalized telomerase-negative cells. Implication of NBS1 in alternative lengthening of telomeres. *J. Biol. Chem*. 275, 30618–30622 (2000).
12. Zhu, X.D., Kuster, B., Mann, M., Petrini., J.H., deLange, T. Cell-cycle-regulated association of RAD50/MRE11/NBS1 with TRF2 and human telomeres. *Nat. Genet*. 25, 347–352 (2000).
13. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y., Ziv, Y. Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science*. 281, 1674-1677 (1998).
14. Canman, C.E., Lim, D.S., Cimprich, K.A., Tayam, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B., Siliciano, J.D. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science*. 281, 1677-1679 (1998).
15. Nakagawa, K., Taya, Y., Tamai, K., Yamaizumi, M. Requirement of ATM in phosphorylation of the human p53 protein at serine 15 following DNA double-strand breaks. *Mol. Cell Biol.* 19, 2828-2834 (1999).
16. Tibbetts, R.S., Brumbaugh, K.M., Williams, J.M., Sarkaria, J.N., Cliby, W.A., Shieh, S.Y., Taya, Y., Prives, C., Abraham, R.T. A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes. Dev*. 13, 152-157 (1999).
17. Amundson, S.A., Myers, T.G., Fornace, Jr. A.J. Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. *Oncogene*. 17, 3287- 3299 (1998).
18. Chulu, J.L., Liu, H.J. Recent patents on cell cycle regulatory proteins. *Recent. Pat. Biotechnol.* 3, 1-9 (2009).
19. Schmidt, E.E., Ichimura, K., Reifenberger, G., Collins, V.P. CDKN2 (p16/MTS1) gene deletion or CDK4 amplification occurs in the majority of glioblastomas. *Cancer Res.* 54, 6321-6324 (1994).
20. Ouellet, S., Vigneault, F., Lessard, M., Leclerc, S., Drouin, R. Guerin, S.L Transcriptional regulation of the cyclin-dependent kinase inhibitor 1A (p21) gene by NFI in proliferating human cells. *Nucleic Acids Research.* 34, 6472–6487 (2006).
21. Carrier, F., Georgel, P.T., Pourquier, P., Blake, M., Kontny, H.U., Antinore, M.J., Gariboldi, M., Myers, T.G., Weinstein, J.N., Pommier, Y., Fornace, A.J. Jr. Gadd45, a p53-responsive stress protein, modifies DNA accessibility on damaged chromatin. *Mol. Cell. Biol.* 19, 1673-1685 (1999).
22. Chen, I.T., Akamatsu, M., Smith, M.L., Lung, F.D., Duba, D., Roller, P.P., Fornace, A.J. Jr. O'Connor, P.M. Characterization of p21Cip1/Waf1 peptide domains required for cyclin E/Cdk2 and PCNA interaction. *Oncogene*.12, 595-607 (1996).
23. Hall, P.A., Kearsey, J.M., Coates, P.J., Norman, D.G., Warbrick, E., Cox, L.S. Characterisation of the interaction between PCNA and Gadd45. *Oncogene.* 10, 2427-2433 (1995).
24. Takekawa, M., Saito, H. A family of stress-inducible GADD45-like proteins mediates activation of the stress-responsive MTK1/MEKK4 MAPKKK. *Cell.* 95, 521-530 (1998).
25. Peretz, G., Bakhrat, A., Abdu, U. Expression of the Drosophila melanogaster GADD45 homolog (CG11086) affects egg asymmetric development that is mediated by the c-Jun N-terminal kinase pathway. *Genetics*. 177, 1691-702 (2007).
26. Martinez, J., Oiry, J., Imbach, J.L., Winternitz, F. Activated N-nitrosocarbamates for regioselective synthesis of N-nitrosoureas. *J. Med. Chem.* 25, 78-182 (1982).
27. Mishra, P.K., Panwar, H., Bhargava, A., Gorantla, V.R., Jain, S.K., Banerjee, S., Maudar, K.K. Isocyanates induces DNA damage, apoptosis, oxidative stress, and

inflammation in cultured human lymphocytes. *J. Biochem. Mol. Toxicol.* 22, 429-440 (2008).
28. Mishra, P.K., Khan, S., Bhargava, A., Panwar, H., Banerjee, S., Jain, S.K., Maudar, K.K. Regulation of isocyanate-induced apoptosis, oxidative stress, and inflammation in cultured human neutrophils: Isocyanate-induced neutrophils apoptosis. *Cell Biol. Toxicol.* 26(3):279-91(2010).
29. Mishra, P.K., Raghuram, G.V., Panwar, H., Jain, D., Pandey, H., Maudar, K.K. Mitochondrial oxidative stress elicits chromosomal instability after exposure to isocyanates in human kidney epithelial cells. *Free Radic. Res*. 9, 1-11 (2009).
30. Green, T. Pulmonary Toxicity and Carcinogenicity of Trichloroethylene: Species Differences and Modes of Action. *Environmental Health Perspectives*. 108, 261-264 (2000).
31. Reznik-Schuller, H. Ultrastructural alterations of nonciliated cells after nitrosamine treatment and their significance for pulmonary carcinogenesis. *Am. J. Pathol.* 85, 549-552 (1976).
32. Bakkenist, C. J. & Kastan, M. B. Initiating cellular stress responses. *Cell*. 118, 9–17 (2004).
33. Norbury, C.J., Hickson, I. D. Cellular responses to DNA damage. *Annu. Rev. Pharmacol. Toxicol.* 41, 367–401 (2001).
34. Bakkenist, C. J. & Kastan, M.B. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature*. 421, 499–506 (2003).
35. Mirzoeva, O.K. Petrini, J.H. DNA replication-dependent nuclear dynamics of the mre11 complex. *Mol. Cancer. Res*. 1, 207-218 (2003).
36. Van den Bosch, M., Bree, R.T., Lowndes, N. F. The MRN complex: coordinating and mediating the response to broken chromosomes. *EMBO Rep*. 4, 844–849 (2003).
37. Spaggiari, L., Carbognani, P., Rusca, M., Cattelani, L., Tecchio, T., Salcuni, P.F., Bobbio, P. Morphological changes of human pulmonary-artery endothelial cells after 12-hours hypothermic storage in organ-preservation solutions. *Tokushima J. Exp. Med.* 43, 61-68 (1996).
38. Kachel, D.L., Moyer, T.P., Martin, W.J. Amiodarone-induced injury of human pulmonary artery endothelial cells: protection by alpha-tocopherol. *American Society for Pharmacology and Experimental Therapeutics*. 254, 1107-1112 (1990).
39. Ekholm-Reed, S., Spruck, C.H., Sangfelt, O., VanDrogen, F., Mueller-Holzner, E., Widschwendter, M., Zetterberg, A., Reed, S.I. Mutation of hCDC4 leads to cell cycle deregulation of cyclin E in cancer. *Cancer Res*. 64,795–800 (2004).
40. Smith, A.P., Henze, M., Lee, J.A., Osborn, K.G., Keck, J.M., Tedesco, D., Bortner, D.M., Rosenberg, M.P., Reed, S.I. Deregulated cyclin E promotes p53 loss of heterozygosity and tumorigenesis in the mouse mammary gland. *Oncogene*. 25, 7245–7259 (2006).
41. Fukasawa, K. p53, cyclin-dependent kinase and abnormal amplification of centrosomes. *Biochim. Biophys. Acta*. 1786, 15-23 (2008).
42. Erlandsson, F., Wahlby, C., Ekholm-Reed, S., Hellström, A.C., Bengtsson, E., Zetterberg, A. Abnormal expression pattern of cyclin E in tumour cells. *Int. J. Cancer*. 104, 369-75 (2003).
43. Bahnassy, A.A., Zekri, A.R., El-Houssini, S., El-Shehaby, A.M., Mahmoud, M.R., Abdallah, S., El-Serafi, M. Cyclin A and cyclin D1 as significant prognostic markers in colorectal cancer patients. *BMC Gastroenterol*. 23, 4:22 (2004).
44. Habermann, J., Lenander, C., Roblick, U.J., Kruger, S., Ludwig, D., Alaiya, A., Reitag, S., Dumbgen, L., Bruch, H.P., Stange, E., Salo, S., Tryggvason, K., Auer, G., Schimmelpenning, H. Ulcerative colitis and colorectal carcinoma: DNA profile, laminin-5 gamma 2 chain and cyclin A expression as early markers for risk assessment. *Scand. J. Gastroenterol.* 36, 751-758 (2001).
45. el-Deiry WS. p21/p53, cellular growth control and genomic integrity. *Curr. Top. Microbiol. Immunol*. 227, 121-37 (1998).
46. Kamb, A. Cyclin-dependent kinase inhibitors and human cancer. *Curr. To. Microbiol. Immunol*. 227, 139-48 (1998).
47. Deng, C., Zhang, P., Harper, J.W., Elledgem, S.J., Leder, P. Mice lacking p21 CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell*. 82, 675–84 (1995).

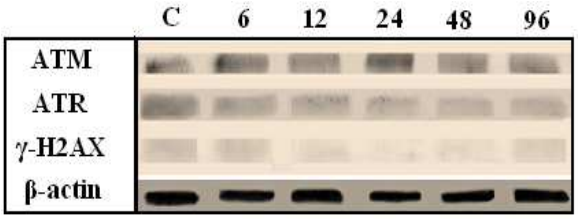

Figure 1- Status of DNA damage sensors. Western blot analysis showing expression of different DNA Damage sensors (ATM/ATR/γH2AX) proteins in control (C) and following treatment with 0.005 µM *N*-succinimidyl *N*-methylcarbamate at different time intervals (6 h to 96 h) in human pulmonary arterial endothelial cells (HPAE-26) cells.

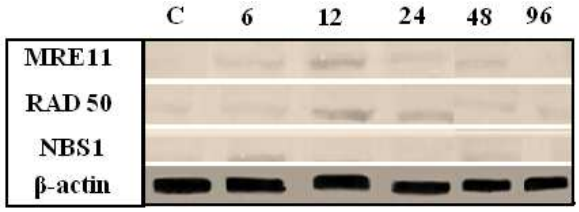

Figure 2: Status of DNA repair proteins. Western blot analysis of MRN complex members in control (C) and following treatment with 0.005 µM *N*-succinimidyl *N*-methylcarbamate at different time intervals (6 h to 96 h) in human pulmonary arterial endothelial cells (HPAE-26) cells.

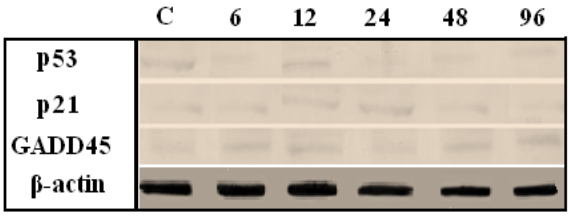

Figure 3: Status of cell cycle modulators. Western blot analysis shows expression of different tumor suppressor (p53, p21 and GADD45) proteins in control (C) and following treatment with 0.005 µM *N*-succinimidyl *N*-methylcarbamate at different time intervals (6 h to 96 h) in human pulmonary arterial endothelial cells (HPAE-26) cells.

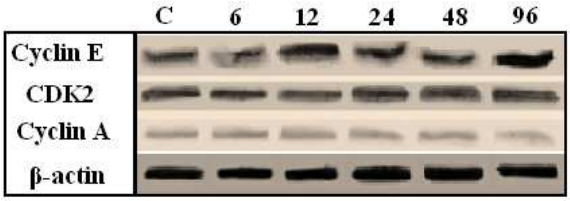

Figure 4: Status of cell cycle regulators. Western blot analysis of cyclins (cyclin A and E) and cdk (cdk2) in control (C) and following treatment with 0.005 µM *N*-succinimidyl *N*-methylcarbamate at different time intervals (6 h to 96 h) in human pulmonary arterial endothelial cells (HPAE-26) cells.