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The Expression of DNA Polymerase-β and DNA Damage in Jurkat Cells Exposed to Hydrogen Peroxide under Hyperbaric Pressure

Donggeun Sul[1,2], **Sangnam Oh**[1] **& Eunil Lee**[2,3]

[1]Graduate School of Medicine, Korea University, Seoul 136-705, Korea
[2]Environmental Toxico-Gemomic and Proteomic Center, College of Medicine, Korea University, Seoul 136-705, Korea
[3]Department of Preventive Medicine, College of Medicine, Korea University, Seoul 136-705, Korea
Correspondence and requests for materials should be addressed to E. I. Lee (eunil@korea.ac.kr)

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Abstract

Long term exposure of Jurkat cells to 2 ATA pressure resulted in the inhibition of cell growth. Under a 2 ATA pressure, the morphological changes in the cells were visualized by electron microscopy. The cells exhibited significant inhibitory responses after three passages. However, short-term exposure study was carried out, 2 ATA pressure may have beneficial effects. The Jurkat cells were exposed to H_2O_2 (25 and 50 μM) in order to induce DNA damage, and then incubated under at either normal pressure or 2 ATA for 1 or 2 hours in order to recover the DNA damage. The extent of DNA damage was determined via Comet assay. More recovery from DNA damage was observed at 2 ATA than at normal pressure. The activity of the DNA repair enzymes, DNA polymerase-β, was also evaluated at both normal pressure and 2 ATA. The activity of DNA polymerase-β was observed to have increased significantly at the 2 ATA than at normal pressure. In conclusion, the effects of hyperbaric pressure from 1 ATA to 2 ATA on biochemical systems can be either beneficial or harmful. Long term exposure to hyperbaric pressure clearly inhibited cell proliferation and caused genotoxic effects, but short-term exposure to hyperbaric pressure proved to be beneficial in terms of bolstering the DNA repair system. The results of the present study have clinical therapeutic application, and might prove to be an useful tool in the study of genotoxicity in the future.

Keywords: DNA damage, DNA repair, Hydroxyl radical, Hyperbaric pressure, Repair enzyme

Hyperbaric oxygen therapy has been used to treat many diseases, including carbon monoxide and cyanide poisoning, acute traumatic ischemia, decompression sickness, osteomyelitis and also to speed the healing of skin grafts or flaps. However, most studies concerning hyperbaric oxygen therapy have dealt mainly with their clinical or toxic effects, as many other complications, including barotraumas, oxygen toxicity, reversible visual changes, and claustrophobia have been observed as the result of hyperbaric treatment[1]. Most hyperbaric oxygen therapy treatments are performed at hyperbaric pressures of 2 to 3 ATA at 100% of O_2. It has been reported that the hyperbaric oxygen treatment on the human subjects (exposure to 100% oxygen at a pressure of 2.5 ATA for a total period of 3×20 minutes) caused clear and reproducible DNA damage in the lymphocytes, as detected by comet assay[2]. It appeared that the significant genotoxic effects of 2 ATA hyperbaric oxygen therapy treatments were caused by the oxygen toxicity.

On the other hand, many studies concerning the effects of low pressure (1 to 2 ATA) on cells and humans have also been reported. Watase *et al*. reported that low pressure, 105 mmHg or 120/90 mmHg induced increases in the proliferative rate of smooth muscle cells, with no concomitant cytotoxicity[3]. It was also reported that pressures of 40 to 120 mmHg promoted the cell proliferation and DNA synthesis in rat intestinal epithelial cells[4]. Similar results were obtained earlier, from human aortic endothelial cells immortalized with the simian virus 40 (SE-1) at ambient pressure. In addition, pressure from 70 to 90 mmHg, activates the mitogen-activated protein kinase, which then in turn induces the activation of the tyrosine kinases, and enhances the proliferation of mesangial cells, probably through the expression of cyclin D1 expression[5].

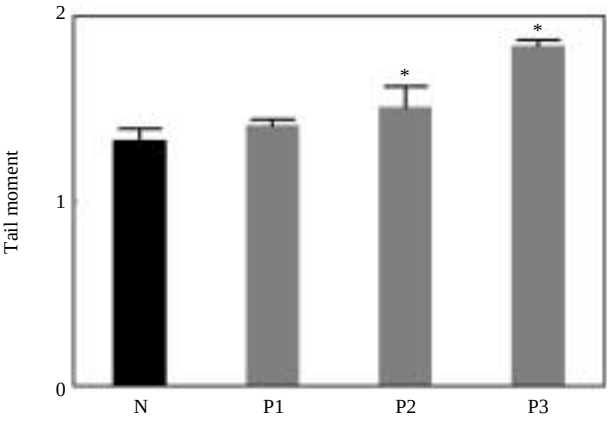

Figure 1. DNA damage of the cells grown under a 2 ATA hyperbaric pressure. DNA damage was analyzed at 3 days in the first passage (P1), 2 days in the second passage (P2) and 1 day in the third passage (P3) by comet assay.

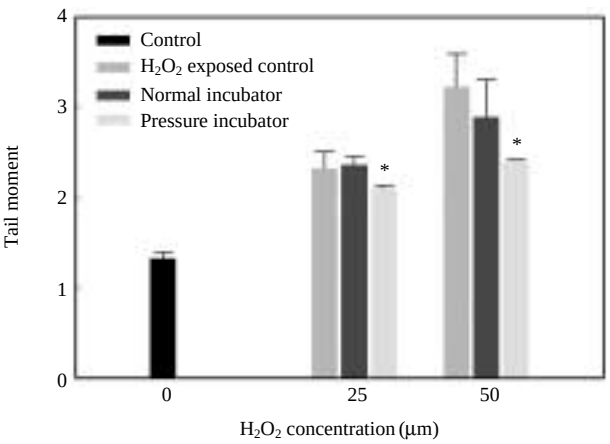

Figure 2. The recovery of the DNA damage in the Jurkat cells that were treated with 25 and 50 μM of H_2O_2 for 5 minutes. The cells were then incubated in a normal and pressure chamber (2 ATA) for 1 or 2 hours. The DNA damage was analyzed by comet assay.

However, in another study, the amount of [^{3}H]-thymidine incorporated into SE-1 reached a maximum at 150 mmHg, while incorporation was decreased at 200 mmHg pressure[6]. In addition, mesangial cell proliferation, as well as apoptosis, were evaluated under simulated normal glomerular pressure (30-35 mmHg) as well as simulated glomerular hypertension (55-60 mmHg) and it was found that simulated glomerular hypertension (55-60 mmHg) promoted mesangial cell apoptosis, and induced the generation of cathepsin-B and clusterin[7].

In conclusion, the effects of hyperbaric pressure from 1 ATA to 2 ATA on biochemical systems can, depending on conditions of exposure, prove to be either beneficial or harmful in view of medical and environmental aspects. Therefore, we hypothesizes that long-term exposure to hyperbaric pressure of 2 ATA inhibits cell proliferation and induces genotoxic effects, but short term exposure to hyperbaric pressure may be beneficial in terms of cell proliferation or the DNA repair system, because DNA synthesis was stimulated by low pressure, as explained above.

Therefore, in the present study, we evaluated the following factors, in order to determine the conditions under which beneficial or harmful effects of hyperbaric pressure could be imposed on *in vitro* biochemical systems, in this case, using Jurkat cells. Primary in this endeavor was the construction of a novel hyperbaric pressure chamber, the pressure inside of which could be controlled between 1 and 3 ATA, with an automatic system to supply 5% CO_2. In our chamber, the temperature could also be controlled, and we could achieve temperature up to 70˚C. We also attempted to determine the geno- and cytotoxic effects of a hyperbaric pressure of 2 ATA on Jurkat cells, using both Comet and MTS assays. Finally, we evaluated the effects of pressure on cell growth and DNA damage at 2 ATA pressure and also assessed repair enzyme activities after short-time incubation under pressure of 2 ATA after a H_2O_2 treatment for the induction of DNA damage.

Determination of DNA Damage in the Cells Pressurized at a 2 ATA

Comet assays were performed to evaluate the DNA damage in Jurkat cells cultured for a third passage. Figure 2 shows the Olive tail moments observed in the Jurkat cells cultured in a normal and 2 ATA pressure. At the normal pressure, the Olive tail moment was 1.33 ± 0.06. The Olive tail moments of the cells pressurized for 3 days in the first passage, 2 days in the second passage and 1 day in the third passage were 1.41 ± 0.03, 1.51 ± 0.11, and 1.84 ± 0.03, respectively (Figure 2).

Recovery of DNA Damage in the Cells Pressurized under 2 ATA for a Short Time

The cells were treated with 25 and 50 μM of H_2O_2 for 5 minutes and incubated for 1 or 2 hours in a normal CO_2 incubator and in a hyperbaric pressure chambers at 2 ATA. Figure 3 shows the recovery of the DNA damage in the cells under normal and hyperbaric pressures. In the cells treated with 25 and 50 μM H_2O_2, the DNA damages was significantly reduced in the hyperbaric pressure condition than in the normal pressure condition. The Olive tail moments of the cells treated with 25 and 50 μM H_2O_2 were shifted

significantly from 2.37±0.08 and 2.97±0.40 to 2.11 ±0.02 and 2.41±0.01, respectively ($P < 0.01$).

Determination of the DNA Repair Enzyme Expression

The expression of the DNA repair enzyme, DNA polymerase-β, was measured by Western Bolt analysis using a monoclonal antibody. Figure 3 the result of DNA repair enzyme expression in the cells treated with 25 and 50 μM of H_2O_2 for 5 minutes and then incubated at 1 ATA and 2 ATA pressure conditions for 1 and 2 hours. The hyperbaric pressure increased DNA polymerase-β activity by almost 2.5 fold that observed at the normal pressure (Figure 4).

Discussion

In this study, a novel hyperbaric pressure chamber was constructed, and we attempted to determine the effects of hyperbaric pressure with respect to biochemical and DNA repair systems. In practice, many types of pressure chambers have been established and used for study. In 1950, the first pressure chamber was constructed by Zobell and Oppenheimer, in order to evaluate the effects of high pressure on the growth of ocean bacteria[8]. During the last five decades, many pressure chambers have been constructed for high or low pressure studies. However, the pressure chamber designed by Watase *et al*. had advanced functions, which regulated the CO_2 levels automatically, using solenoid valves and cultured cells[3]. Our hyperbaric pressure chamber was created according to their design, but with other additional functions, which provided suitable conditions, including CO_2 concentration, temperature, and moisture, for examining the effects of pressure on cells.

In the present study, cell growth was completely inhibited, and the level of DNA damage increased with increasing incubation time under a hyperbaric 2 ATA pressure. The 2 ATA pressure condition induced significant morphological changes in the cell surface, and these changes appeared to have severe effects on

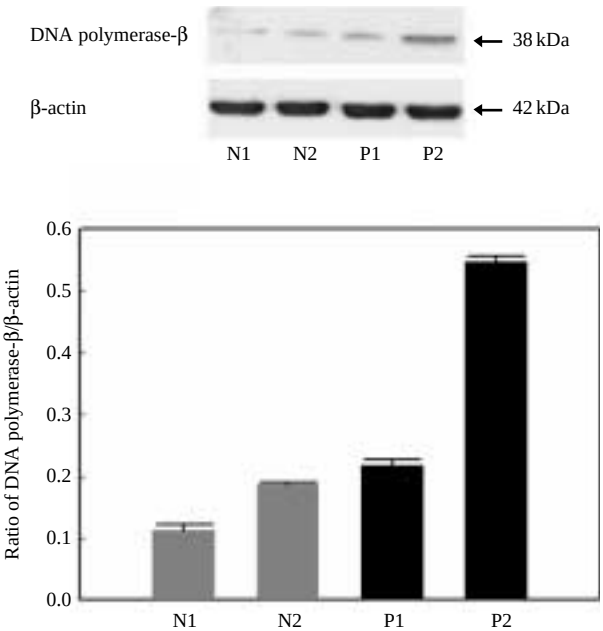

Figure 3. The activity of DNA polymerase-β in the Jurkat cells that treated with 50 μM of H_2O_2 for 5 minutes. The cells were then incubated in a normal and pressure chamber (2 ATA) for 1 or 2 hours. A western blot assay was performed to determine the activity of DNA polymerase-β. The β-actin was used as an internal standard. The quantitation was measured by a densitometry. N1 and N2: 1 hour and 2 hours incubation in a normal pressure chamber respectively, P1 and P2: 1 hour and 2 hours incubation in a pressure chamber.

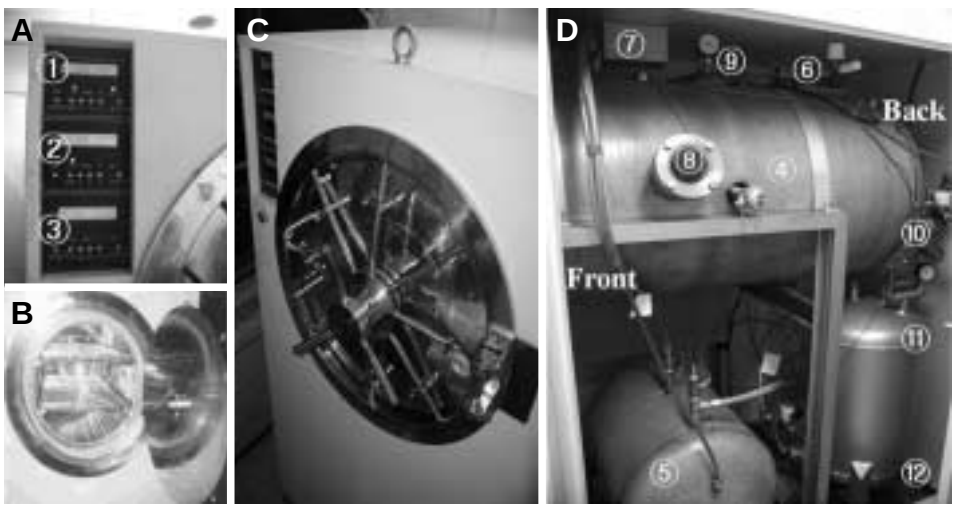

Figure 4. Hyperbaric pressure chamber: A1: Temperature control pad, A2: CO_2 control pad, A3: Pressure control pad, B: Camber inside, C: Chamber out side, D4: Incubation chamber, D5: Temperature control water tank, D6: CO_2 control solenoid valve, D7: CO_2 sensor, D8: Incubation chamber window, D9: Pressure gauge, D10: Pressure control solenoid valve, D11: Second pressure tank, D12: Air compressor.

intracellular biochemical interactions. The time required to achieve maximum cell growth at each passage under hyperbaric 2 ATA pressure was gradually reduced from 3 days to 1 day. The pressure conditions, in three passages, caused severe DNA damage leading to cell death. In the mesangial cells, the stimulated glomerular hypertension resulted in DNA fragmentation[7]. In addition, many high-pressure studies have demonstrated the effects of pressure on biological macromolecules, which, in general, involve the generation of structural changes or alterations in the cellular process. These changes are characterized by a wide variety of reactions, including ligand-protein interactions, redox reactions, protein-protein interactions, interactions between proteins and nucleic acids, etc[9].

In contrast, a hyperbaric pressure over short periods of time provided a very different set of beneficial effects on the cells treated with H_2O_2, which can react with reduced transition metals (especially Fe^{2+} or Cu^{1+}) to form hydroxyl radicals (· OH), which are the proximal agents responsible for the vast overbalance of oxidative damage to DNA. After DNA damage was induced by the H_2O_2 treatment, the cells were incubated under either normal or hyperbaric 2 ATA pressure for 1 or 2 hours, in order to assess any recovery from DNA damage which might take place. A higher rate of DNA recovery was found to be occurring under the 2 ATA hyperbaric pressure condition than under normal pressure. At 150 or 160 mmHg pressure, DNA synthesis increased in the rat epithelial cells[4,6]. The present study evaluated the involvement of a repair enzyme of DNA polymerase-β, which functions specifically in *in vivo* base-excision repair in the cells treated with H_2O_2 under a hyperbaric pressure[10]. A hyperbaric pressure of 2 ATA was found to induce an increase in repair enzyme activity, resulting in a recovery of previous the DNA damage over a short time.

It has already been established that an appropriate level of pressure can provide beneficial effects on cell growth, but not high pressure. In this study, a hyperbaric pressure of 2 ATA had genotoxic effects under conditions of long-time exposure, but short-time exposure to a pressure 2 ATA resulted in increased recovery of DNA damage. These results have potential clinical applicatons, and might prove to be a useful tool in the future study of genotoxicity. However, much fundamental research is required in order to further characterize the effects of hyperbaric pressure, involving various repair enzymes, which mitigate oxidative damages to DNA, and help to counteract the potential cytotoxic, mutagenic, and carcinogenic effects of this damage[11] will be performed, in oredr to determine the effects of pressure in a hyperbaric pressure CO_2 chamber that can be controlled automatically by a digital system.

Methods

Chemicals

Hydrogen peroxides and hexamethyldisilazane were purchased from Sigma Chemical Co. (St Louis, MO, USA). Absolute ethanol, osmium tetraoxide and glutaraldehyde solution were obtained from Merck (Darmstadt, Germany).

Cell Line and Culture

Jurkat cells were obtained from American Type Culture Collection (TIB-152) and were maintained in RPMI 1640 containing 10% fetal bovine serum albumin (Gibco BRL, Grand Island, USA), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a 5% CO_2 in air atmosphere. Cells cultured for 3 passages and growth curve was determined by MTS assay. Cells were treated with 0, 25 and 50 μM of hydrogen peroxides for 5 min in ice and then incubated under normal and hyperbaric pressures for the recovery of DNA damage. The recovery of DNA damage was analyzed by Comet assay.

Construction of Hyperbaric Pressure Chamber

The hyperbaric pressure chamber was designed according to the pressure chamber made by Watase *et al.* and was manufactured with several modifications by the Daeil Chemical Co. (Seoul, Korea) (Figure 1)[12]. Briefly, the hyperbaric pressure chamber was equipped several systems where the pressure was controlled from 1 to 3 ATA and CO_2 and temperature were regulated automatically. These systems are visualized and operated digitally in front of the hyperbaric chamber (Figure 1). The pressure chamber consisted of the following four major parts: digital control pads (Figure 4A 1, 2, 3), a chamber box (Figure 4D 4), a CO_2 control system (Figure 4D 6 and 9), a temperature control water tank (Figure 4D 5), and a second pressure tank (Figure 4D 11). The digital control pads regulated the temperature, CO_2 concentration and the pressure. The second pressure tank was connected to an air compressor installed on the outside, and the pressure tank was also connected to a chamber box with a pressure control solenoid valve (Figure 4D 10). The pressure in the second pressure tank was maintained up to 4 ATA and the pressure in incubation chamber was then controlled from 1 to 3 ATA. The equilibration of the CO_2 concentration up

to 5% takes 5 minutes. The dissolved oxygen and the pH were determined in the culture medium. In order to determine any changes of the pH in the medium, a serum conditioned medium, which had been incubated in the hyperbaric chamber under 2 ATA pressure with 5% CO_2, was collected and its pH was measured using an expandable ion Analyzer EA920 (Orion Research, MA). These changes in the pressures led a shift of pH from 7.40 to 7.35 ($P < 0.05$). The dissolved oxygen concentration in the medium was measured with a Sension Dissolved Oxygen Electrode (HACH Co., Loveland, Colorado). The concentrations of DO in the media under 1 ATA and 2 ATA were 2.53 and 2.40, respectively ($P < 0.05$).

Comet Assay

DNA damages were determined using the comet assay in cells that were cultured under normal and pressure conditions. The comet assay was performed according to Singh with minor modification[13]. Normal melting point agarose (Ameresco, NMA) and low melting point agarose (Ameresco, LMA) were dissolved in PBS (Gibco BRL) using microwave. In brief, 100 μL of 1% NMA was added onto a fully frosted slides precoated with 50 μL of 1% NMA for a firm attachment and the slides were allowed to solidify with cover slips in the refrigerator for 5 min. After solidification of the gel, the cover slips were removed and lymphocytes in 50 μL mixed with 50 μL of 1% LMA was added. The cover slips were added on the layer and the slides were allowed to solidify in the refrigerator for 5 min. After removing cover slips, 100 μL of 0.5% LMA was added on the third layer and the slides were placed with cover slips again in the refrigerator for 5 min. The slides were submersed in the lysing solution (2.5 M NaCl, 100 mM EDTA-2Na, 10 mM Tris-HCl, pH 10; 1% Triton X-100 and 10% DMSO, pH 10 were added fresh) for 1 hour. The slides were then placed in unwinding buffer (1 mM EDTA and 300 mM NaOH, pH 13) for 20 min and electrophoresis was carried out using the same solution for 20 min at 25 V and 300 mA (0.8 v/cm). After electrophoresis, the slides were neutralized by washing three times with neutralization buffer (400 mM Tris-HCl, pH 7.4) 5 min each and were stained with 50 μL of 10 μg/mL ethidium bromide. The slides were examined using a Komet 4.0 image analysis system (Kinetic Imaging, Liverpool, UK) fitted with an Olympus BX50 fluorescence microscope equipped with an excitation filter of 515-560 nm and a barrier filter 590 nm. For each treatment group, two slides were prepared and each 50 randomly chosen cells (total 100 cells) were scored manually. The parameter of Olive tail moment (=(Tail.mean-Head.mean) ∗Tail%DNA/100) was calculated automatically using the Komet 4.0 image analysis system.

Western Blot Analysis

Jurkat cells were grown in 25 mL dishes at 1 ATA. After reaching confluence, cells was treated with 0, 25 and 50 μM of hydrogen peroxides for 5 min in ice and then incubated under normal and hyperbaric pressures for the recovery of DNA damage. After incubation for 1 and 2 hours under 1 ATA and 2 ATA, cells were washed twice with ice-cold PBS, lysed in 1 mL of lysis buffer (PBS, 1% Triton X-100, 1% protease inhibitor cocktail), sonicated, and centrifuged at 15,000 rpm. The supernatant was then subjected Western blotting, which was performed on 12.5% SDS-PAGE with equal amounts of protein (50 μg) loaded for each sample. The separated proteins on gel were transferred to PVDF membrane (Millipore, Bradford, MA) and probed with mouse monoclonal anti-DNA polymerase-β (Santa Cruz Biotechnology, Inc.) and mouse monoclonal anti-β-actin (Upstate Biotechnology, Inc.) at a 1 : 1,000 dilution in PBST (0.1%) at 4˚C overnight. The protein-antibody complexes were visualized with horseradish peroxidase-goat anti-mouse IgG conjugate at a 1 : 2,000 dilution. Immuno-reactivities were detected using ECL plus kit (Amersham Biosciences) and quantitative data were obtained using a densitometry (CAMAG TLC SCANNER 3, Camag Scientific, Inc. German)

Statistical Analysis

All results are expressed as Mean ± SD of 6 experiments. Statistic comparisons carried out using Student's unpaired t test. Differences among groups were evaluated by one-way analysis of variance (ANOVA). Values of $P < 0.05$ were considered significant.

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