Pattern Recognition Analysis of Proton Nuclear Magnetic Resonance Spectra of Extracts of Intestinal Epithelial Cells under Oxidative Stress

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Abstract

Background: Mesenteric ischemia-reperfusion induces gut mucosal damage. Intestinal mucosal wounds are repaired by epithelial restitution. Although many different molecular mechanisms have been shown to affect cell metabolism under oxidative conditions, these molecular mechanisms and metabolic phenotypes are not well understood. Nuclear magnetic resonance (NMR) spectroscopic data can be used to study metabolic phenotypes in biological systems. Pattern recognition with multivariate analysis is one chemometric technique. The purpose of this study was to visualize, using a chemometric technique to interpret NMR data, different degrees of oxidant injury in rat small intestine (IEC-6) cells exposed to H₂O₂.

Methods: Oxidant stress was induced by H₂O₂ in IEC-6 cells. Cell restitution and viability were assessed at different H₂O₂ concentrations and time points. Cells were harvested for pattern recognition analysis of ¹H-NMR data.

Results: Cell viability and restitution were significantly suppressed by H₂O₂ in a dose-dependent manner compared with control. Each class was clearly separated into clusters by partial least squares discriminant analysis, and class variance was greater than 90% from 2 factors.

Conclusion: Pattern recognition of NMR spectral data using a chemometric technique clearly visualized the differences of oxidant injury in IEC-6 cells under oxidant stress.

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Key words: pattern recognition, partial least squares discriminant analysis, nuclear magnetic resonance, oxidant stress

Introduction

Intestinal ischemia-reperfusion (I/R) injury is often seen after hemorrhagic shock, septic shock, and small-bowel transplantation. It causes histological evidence of mucosal injury and physiological evidence of loss of normal digestive and barrier functions, which can lead to development of postinjury multiple organ dysfunction syndrome¹. I/R injury is the tissue damage that is caused when blood supply returns to the tissue after a period of ischemia. Because of the absence of oxygen supplied by blood during the ischemic period, the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress.

In experimental models used to investigate oxidative stress responses of cells, cultured cells are often exposed to hydrogen peroxide (H_2O_2) added to the culture medium^{2,3}. IEC-6 cells are derived from normal rat intestine, are nontumorigenic, and retain the undifferentiated character of epithelial stem cells⁴. These cells are a well-studied *in vitro* model of restitution following mechanical injury to cell monolayers^{5,6}. Scraped wounding was used as an *in vitro* model of injury that mimics the early cell-division-independent stages of epithelial restitution.

Studies *in vitro* and *in vivo* indicate that healing of superficial damage to the mucosa occurs by rapid migration of normal adjacent intestinal epithelial cells over the denuded area, with reestablishment of epithelial continuity and integrity, a process termed restitution. The repair process is regulated in a cell-specific manner by various growth factors, cytokines, peptides, and matrix components⁷⁻⁹. Many different molecular mechanisms have also been shown to influence cell metabolism with up-regulation or down-regulation of target genes, enzymes, and functional proteins under oxidative conditions. However, these molecular mechanisms and metabolic phenotypes are not well understood.

Nuclear magnetic resonance (NMR) spectroscopic data can be used to study metabolic phenotypes in biological systems¹⁰. Chemometrics is a chemical discipline that uses mathematics, statistics, and formal logic to design or select optimal experimental

procedures, to provide maximum relevant chemical information by analyzing chemical data, and to obtain knowledge about chemical systems¹¹. Pattern recognition with multivariate analysis is one chemometrics technique. The goal of this study was to visualize different degrees of oxidant injury in IEC-6 cells exposed to H_2O_2 by using a chemometric technique to interpret NMR data.

Materials and Methods

Cell Preparation and Reagents

IEC-6 cells purchased from the American Tissue Culture Collection (Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine, 4,500 mg/L glucose, 1,500 mg/L sodium bicarbonate, 10% fetal bovine serum, 0.1 U/mL insulin (Sigma-Aldrich, St. Louis, MO, USA), and 1% streptomycin (Gibco BRL, Gaithersburg, MD, USA). Cells were cultured in a humidified incubator with an atmosphere of 5% CO_2 at 37°C and were given fresh medium every 2 days. Hydrogen peroxide was purchased from Wako Pure Chemicals (Osaka, Japan), and 0.4% trypan-blue stain was purchased from Invitrogen (Carlsbad, CA, USA).

Experiment 1. Different Dose Responses Following Oxidant Stress on IEC-6 Cells

The IEC-6 cells were cultured to confluence then subjected to oxidant stress induced by H_2O_2 (control, 0.25 mM, 0.5 mM) for 24 hours. Cell migration and cell viability were assessed. Cells were harvested after 24 hours for pattern recognition of NMR spectra.

Experiment 2. Different Time Points Following Oxidant Stress on IEC-6 Cells

IEC-6 cells were cultured to confluence, and then oxidant stress was induced by H_2O_2 (0.5 mM). Cell migration was assessed. Cells were harvested after 6, 12, and 24 hours for pattern recognition of NMR spectra.

Wound Restitution Measurement

The restitution of IEC-6 cells was measured with a modified version of a previously described

technique^{12,13}. The IEC-6 cells were plated in 6-well polystyrene plates to reach confluence in normal growth medium. A denuded epithelial wound was created in a standardized fashion by scraping the IEC-6 monolayers with a plastic blade. After being scraped, the wounded monolayers were washed, and complete medium was then added. Wound areas were viewed under a microscope at various times after scrape wounding and were photographed with a microscope equipped with a camera (Spot Insight, Spot Imaging Solutions, Sterling Heights, MI, USA). The mean restitution length was calculated as the migration area as a region of interest.

Cell Viability

Cell viability was measured with a trypan blue dye exclusion assay, as has been described previously¹⁴. Briefly, cells were grown to confluence and incubated with different concentrations of H₂O₂. Viable cells and nonviable cells were counted after trypan blue staining. The percentage of viable cells was calculated as $100 \times (\text{number of viable cells}) / (\text{total number of cells})$.

Sample Preparation

Cells were grown to confluence and incubated with different concentrations and times of H₂O₂ exposure. Cells were washed twice in cold phosphate-buffered saline, collected with a scraper on the attached cells, and stored at -80°C until further analysis. Neutral extraction was performed according to the protocol of Yoshioka¹⁵. The basic extraction method was designed by Folch¹⁶ and was originally designed for extraction of total lipids from animal tissues. However, this method is also useful for extracting polar organic compounds together, separately from nonpolar lipids and essentially without any *in vitro* modifications. The frozen cells were crushed and mixed thoroughly in 1.5 mL of a 2 : 1 chilled chloroform : methanol mixture. The mixture was extracted and separated with an extractive device (CentractorTM, Uniflows, Tokyo, Japan). The mixture was spun in a centrifuge at 3,000 revolutions per minute (rpm) for 10 minutes. A total of 0.5 mL of distilled water was added to the mixture and then extracted under the same

conditions described above. The mixture was separated into 2 layers of solutions and insoluble residues. Finally, the extract was centrifuged at 3,000 rpm for 5 minutes, and the upper phase solution was evaporated in an evacuated centrifuge for 3 hours. Dried extracts were reconstituted in 180 μL of deuterium oxide (D₂O) (ISOTEC, Sigma-Aldrich) and pipetted into 3-mm NMR tubes (Wilmad-LabGlass, Vineland, NJ, USA). The tubes were placed in 5-mm NMR tubes (Wilmad-LabGlass) that contained 300 μL of D₂O containing 25 μM sodium (3-trimethylsilyl) tetradeuteriopropionate-2,2,3,3-d₄ (TMSP) (MSD Isotopes, Montreal, Canada) for subsequent NMR measurements. The D₂O provided a deuterium field frequency lock for the NMR spectrometer, while TMSP provided an internal chemical shift reference ($\delta=0.00$).

Chemical shift δ (ppm) $\times 10^{-6}$

$$= \frac{\text{difference between a resonance frequency and that of a reference substance (Hz)}}{\text{operating frequency of the spectrometer (Hz)}}$$

Acquisition of Proton Nuclear Magnetic Resonance Data

Solution state ¹H NMR spectroscopy was performed at a proton resonance frequency of 300 MHz using a ECX NMR spectrometer interfaced with a TH5 probe equipped with an automatic 16-position sample changer and with DeltaTM NMR processing and control software (version 4.3.2) (JEOL Ltd., Tokyo, Japan).

The ¹H NMR spectra were acquired automatically at a probe temperature of 23°C using the macro program in DeltaTM systems for automatic measurement supplied by JEOL. The observation range of the NMR signal was 4,500 Hz. The water resonance was suppressed with a conventional presaturation pulse sequence for water (HDO) proton signal suppression based on homogated irradiation and the delays alternating with nutation for tailored excitation (DANTE) pulse sequence (presaturation time=2 seconds, danTE pulse=8 microseconds, DANTE interval=0.1 milliseconds, DANTE loop=185, ante attenuator=24 dB). Carr-Purcell-Meiboom-Gill (CPMG) spin-echo spectra were measured with a spin-echo loop time of 19.2

milliseconds, a relaxation delay of 2.0 seconds, and 8,000 transients.

Data Processing and Reduction

The resultant spectra were processed with Alice2TM, ver. 5.5 (JEOL DATUM Ltd., Tokyo, Japan). Free induction decays were subjected to an exponential weighting function of 0.1 Hz, Fourier transformed from the time to the frequency domain, phased manually, and followed by linear baseline correction and referencing to the TMSP singlet at 0.00 Hz (chemical shift value=0.00 ppm).

The signal intensity distribution of ^1H NMR spectral data of each cell was calculated with an macro program written within the Alice2 software for MetabolomeTM, ver. 1.0 (JEOL DATUM Ltd.) software package, which integrated the spectral data between 150 Hz (0.5 ppm) and 2,850 Hz (9.5 ppm) into 225 segments (bins) with 12-Hz (0.04 ppm) integral regions. Spectral regions containing resonances of residual water (1,380 Hz [4.6 ppm] to 1,500 Hz [5.0 ppm]) were excluded before integration. Different bin sizes can reveal different features of the data. Depending on the actual data distribution and the goals of the analysis, different bin widths may be appropriate, so experimentation is usually needed to determine an appropriate width. Finally, all the spectral data were reduced into 174 bins, and to account for the bulk mass differences between samples, each spectral region was normalized to the sum of all of the integrals of each bin.

Multivariate Analysis

The calculated results obtained from all measured spectra were exported to a spreadsheet as a text file that was used as the input into a pattern recognition/multivariate statistics software package (Unscrambler XTM version 10.2, Camo Software AS, Oslo, Norway). Datasets were imported, and partial least squares discriminant analysis (PLS-DA) was performed^{17,18}. In brief, PLS-DA with cross-validation was performed to investigate the relationship between different classes. PLS models use both the x- and y-matrices simultaneously to find the latent variables in x that best predict the y. In other words, PLS-DA was performed to sharpen the

separation between groups by rotating components for principal components analysis with the goal of finding a maximum separation. In this study PLS-DA was performed between the spectral profiles and experimental classes, such as concentration and different time points. Each PLS-DA score plot has a correlation loading profile, which helps identify the spectral regions responsible for the observed sample clustering. The horizontal axis in correlation loadings represents chemical shift, and the vertical axis represents contribution degree. Positive contribution areas and negative contribution areas in correlation loadings have an opposite power to separate the plus or minus direction in the score plot.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Data were analyzed with one-way analysis of variance, and individual group means were compared by means of Tukey's multiple-group comparison tests. P values <0.05 were considered to indicate significance. Means with different letters are significantly different.

Results

Experiment 1.

Effect of different concentrations of H_2O_2 on IEC-6 cells wound restitution

Restitution (**Fig. 1**) was significantly suppressed by H_2O_2 at 0.25 mM ($33.1\pm 2.0\ \mu\text{m}$) and by H_2O_2 at 0.5 mM ($19.8\pm 1.5\ \mu\text{m}$) in a dose-dependent manner compared with control ($40.8\pm 3.4\ \mu\text{m}$).

Effect of different concentrations of H_2O_2 on IEC-6 cell viability

Cell viability (**Fig. 2**) was significantly decreased by H_2O_2 at 0.25 mM ($88.1\pm 1.2\%$) and H_2O_2 at 0.5 mM ($68.1\pm 2.3\%$) in a dose-dependent manner compared with control ($95.4\pm 1.0\%$).

Visualization of ^1H NMR spectral data of IEC-6 cells exposed to different concentrations of H_2O_2 using PLS-DA analysis

The PLS-DA score plot is shown in **Figure 3A**. Each dot on the score plot represents the score of factor 1 or of factor 2 of the ^1H NMR spectral data of the IEC-6 cell extract calculated by the PLS-DA

Fig. 1 Effect of different concentrations of H₂O₂ on IEC-6 cell wound restitution

- (A) Confluent monolayers of IEC-6 cells were wounded with a scraper, and the cells were then exposed to H₂O₂ at 0.25 mM or H₂O₂ at 0.5 mM for 24 hours. The denuded areas were photographed immediately after addition of H₂O₂ and at 24 hours after wounding. A black dot marker was oriented at the same place for photomicrographs between times. The denuded areas are highlighted by a black line.
- (B) The average length of the restitution area is presented. P values <0.05 were considered to indicate significance. Means with different letters are significantly different between groups.

Fig. 2 Effect of different concentrations of H₂O₂ on IEC-6 cell viability

The cell viability is presented. Confluent monolayers of IEC-6 cells were exposed to H₂O₂ at 0.25 mM or H₂O₂ at 0.5 mM for 24 hours. P values <0.05 were considered significant. Means with different letters are significantly different between groups.

algorithm installed in Unscrambler X. On the plot, the color of each dot indicates the class. The classes are as follows: control at 24 hours (blue), H₂O₂ 0.25 mM at 24 hours (green), and H₂O₂ 0.5 mM at 24 hours (red). The dots for each class were clearly separated into separate clusters by means of PLS-

DA analysis. This is a 2-dimensional scatter plot of scores for 2 specified factors from PLS regression. The scores plots for factor 1 and factor 2 give information about ¹H NMR spectral data, and the class data in the samples and these 2 components summarize the variation of all the data. The 2 values (X and Y) in the brackets of the axis labels indicate the variance by each corresponding factor. "X" is the spectral variance that was captured by the factor, and "Y" is the class variance captured by the factor. Spectral variance and class variance were 87% and 48%, respectively, for factor 1, and 5% and 43%, respectively, for factor 2. Consequently, these 2 factors together described 92% (87%+5%) of spectral variance and 91% (48%+43%) of class variance.

Corresponding correlation loadings for factor 1 are shown in **Figure 3B**, and those for factor 2 are shown in **Figure 3C**. The correlation loadings plot shows the relationship between spectral variables of the samples analyzed, which are responsible for the clustering in the score plots of the samples.

Fig. 3 PLS-DA score plot and correlation loadings of different concentration of H_2O_2 on IEC-6 cells
 (A) Each dot represents the score of an IEC-6 cell sample calculated with PLS-DA analysis. Classes are delineated as follows: control 24 hours (blue), H_2O_2 at 0.25 mM at 24 hours (green), H_2O_2 at 0.5 mM at 24 hours. The dots for each class were clearly separated into clusters with PLS-DA analysis.
 (B) Corresponding loadings of factor 1 are present.
 (C) Corresponding loadings of factor 2 are present.

Experiment 2.

Effect of different time points of H_2O_2 on IEC-6 cell wound restitution

Restitution was gradually increased by H_2O_2 at 0.5 mM at 6 hours ($4.4 \pm 0.5 \mu\text{m}$), at 12 hours ($13.3 \pm 1.4 \mu\text{m}$), and at 24 hours ($19.8 \pm 1.5 \mu\text{m}$) in a time-dependent manner, but restitution was significantly suppressed compared with control at each time point: 6 hours ($11.1 \pm 1.2 \mu\text{m}$), 12 hours ($28.0 \pm 1.6 \mu\text{m}$), and 24 hours ($40.8 \pm 3.4 \mu\text{m}$) (**Fig. 4**).

Effect of different time points of H_2O_2 on IEC-6 cell viability

Cell viability was similar between H_2O_2 at 0.5 mM at 6 hours ($90.2\% \pm 0.5\%$) and at 12 hours ($89.5\% \pm 1.2\%$) and was significantly decreased between H_2O_2 at 0.5 mM at 24 hours ($68.4\% \pm 1.2\%$). The viability of cells exposed to H_2O_2 at 0.5 mM was significantly different from control at each time point: 0 hours ($97.3\% \pm 0.2\%$), 6 hours ($97.1\% \pm 0.9\%$), 12 hours ($96.6\% \pm 0.8\%$), and 24 hours ($96.6\% \pm 0.4\%$) (**Fig. 5**).

Visualization with PLS-DA analysis of ^1H NMR spectral data of IEC-6 cells at different time points after exposure to H_2O_2

The dots for each class were clearly separated into separate clusters by means of PLS-DA analysis. Two-dimensional scatter plots of scores for the 2 specified factors from PLS regression were created. The score plot for factor 1 and factor 2 gives information about ^1H NMR spectral data and the class data in the samples, and these 2 components summarize the variation in all of the data.

The PLS-DA score plot for exposure to H_2O_2 is shown in **Figure 6A**. Each dot on the score plot represents the score of factors 1 and 2 of the ^1H NMR spectral data of the IEC-6 cell extract calculated with the PLS-DA algorithm that is installed in Unscrambler X. On the plot, the color of each dot indicates the class. The classes are as follows: control at 0 hours (blue), H_2O_2 at 0.5 mM at 6 hours (green), H_2O_2 at 0.5 mM at 12 hours (orange), and H_2O_2 at 0.5 mM at 24 hours (red). The dots of the

Fig. 4 Effect of different time points of H₂O₂ on IEC-6 cell wound restitution

(A) Confluent monolayers of IEC-6 cells were wounded with a scraper, and the cells were then exposed to H₂O₂ at 0.5 mM for 6 hours, H₂O₂ at 0.5 mM 12 hours, H₂O₂ at 0.5 mM for 24 hours, and without H₂O₂ as control. The denuded areas were photographed immediately after addition of H₂O₂ or without H₂O₂ at 6, 12, and 24 hours after wounding. A black marker dot is oriented at the same place in photomicrographs between times. The denuded areas are highlighted by a black line.

(B) The average length of the restitution area is presented. P values 0.05 were considered significant. Means with different letters are significantly different between groups. □ was control and ■ was H₂O₂.

classes at 6 hours and at 12 hours were not separated into clusters. The dots were clearly distinguished into 3 separate clusters between control, at 6 and 12 hours, and at 24 hours on the score plot calculated with PLS-DA analysis. Spectral variance and category variance were 95% and 32%, respectively, for factor 1 and 2% and 28%, respectively, for factor 2. Consequently, factor 1 and factor 2 together described 97% (95%+2%) and 60% (32%+28%) of the variation, respectively. Correlation loading of factor 1 is shown in **Figure 6B**, and that of factor 2 is shown in **Figure 6C**.

The PLS-DA score plot for exposure to H₂O₂ and control is shown in **Figures 7A and 7B**. Each dot on the score plot represents the score of factors 1, 2, and 3 of the ¹H NMR spectral data of the IEC-6 cell

extract calculated with the PLS-DA algorithm of Unscrambler X. On the plot, the color of each dot indicates the class. The PLS-DA score plot in **Figure 7A** is from factors 1 and 2, and that in **Figure 7B** is from factors 1 and 3. The classes are as follows: control at 0 hours (blue), control at 6 hours (green ring), control at 12 hours (orange ring), control at 24 hours (red ring), H₂O₂ at 0.5 mM at 6 hours (green), H₂O₂ at 0.5 mM at 12 hours (orange), and H₂O₂ at 0.5 mM at 24 hours (red). The dots of the control class at 0 hours and 24 hours were not separated into clusters (**Fig. 7A**). The dots of the control at 6 hours and at 12 hours were broadly distinguished into separate clusters but were in different areas from those of control at 0 hours and at 24 hours (**Fig. 7A**). On the score plot calculated with PLS-DA analysis,

Fig. 5 Effect of different time points of H_2O_2 on IEC-6 cell viability

Cell viability is presented. Confluent monolayers of IEC-6 cells were exposed to H_2O_2 at 0.5 mM for 6 hours, H_2O_2 at 0.5 mM for 12 hours, or H_2O_2 at 0.5 mM for 24 hours. Cell viability was also assessed without H_2O_2 as control. P values <0.05 were considered significant. Means with different letters are significantly different between groups. □ was control and ■ was H_2O_2 .

the dots were separated into 5 clusters: control at 0 and 24 hours, control at 6 hours, control at 12 hours, H_2O_2 at 6 and 12 hours, and H_2O_2 at 24 hours. The dots of control at 0 hour and at 24 hours were not separated into clusters (**Fig. 7B**). The dots of control at 6 and at 12 hours were not separated into clusters but were in areas different from those of control at 0 hour and at 24 hours (**Fig. 7B**). The dots of the H_2O_2 at 6, 12, and 24 hours were clustered in similar areas but were clearly distinguished from those of the control groups (**Fig. 7B**). Spectral variance was and category variance were 93% and 92%, respectively, for factor 1, and 3% and 3%, respectively, for factor 2, and 1% and 1%, respectively, for factor 3. Consequently, factors 1 and 2 together described 96% (93%+3%) and 95% (92%+3%) of the variation, and factors 1 and 3 together described 94% (93%+1%) and 93% (92%+1%) of the variation. Correlation loadings of factor 1, 2, and 3

Fig. 6 PLS-DA score plot and correlation loadings of different time points of exposure to H_2O_2 0.5 on IEC-6 cells

- (A) Each dot represents the score of an IEC-6 cell sample calculated with PLS-DA analysis. Classes are delineated as follows: control at 0 hours (blue), H_2O_2 at 0.5 mM at 6 hours (green), H_2O_2 at 0.5 mM at 12 hours (orange), and H_2O_2 at 0.5 mM at 24 hours (red).
- (B) Correlation loadings of factor 1 are presented.
- (C) Correlation loadings of factor 2 are presented.

Fig. 7 PLS-DA score plot and correlation loadings of different time points of H₂O₂ and without H₂O₂ as control on IEC-6 cells

- (A) Each dot represents the score of an IEC-6 cell sample calculated with PLS-DA analysis for factors 1 and 2. Classes are delineated as follows: control at 0 hours (blue), control at 6 hours (green ring), control at 12 hours (orange ring), control at 24 hours (red ring), H₂O₂ at 0.5 mM at 6 hours (green), H₂O₂ at 0.5 mM at 12 hours (orange), and H₂O₂ at 0.5 mM at 24 hours (red).
- (B) Each dot represents the score of an IEC-6 cell sample calculated with PLS-DA analysis for factors 1 and 3. Classes are delineated as follows: control at 0 hours (blue), control at 6 hours (green ring), control at 12 hours (orange ring), control at 24 hours (red ring), H₂O₂ at 0.5 mM at 6 hours (green), H₂O₂ at 0.5 mM at 12 hours (orange), and H₂O₂ at 0.5 mM at 24 hours (red).
- (C) Correlation loadings of factor 1 are presented.
- (D) Correlation loadings of factor 2 are presented.
- (E) Correlation loadings of factor 3 are presented.

are shown in **Figures 7C, 7D, and 7E**, respectively.

Discussion

The gut is crucial in the development of acute lung injury and multiple organ dysfunction syndrome after initial injuries, such as major trauma,

hemorrhagic shock, severe septic shock, superior mesenteric occlusion, and small intestine transplantation. All these conditions can disturb the splanchnic blood flow and lead to I/R mucosal injury in the gut¹. Early studies focused on translocation of intestinal flora to the systemic circulation following gut I/R, with the portal vein as the main cause^{19,20}.

Although initial animal models offered evidence supporting bacterial translocation, diversion of the mesenteric lymph duct before hemorrhagic shock appears to be attenuated by neutrophil priming, endothelial adhesion, and inflammatory molecular expression in animal models¹⁹. On the basis of this evidence, the release of bacteria or nonmicrobial factors or both from the gut through the portal vein or the intestinal lymphatic route or both potentiate the development of acute lung injury and multiple organ dysfunction syndrome. The gut-mucosa repair process is absolutely necessary. The initial phase of the repair process is rapid migration of surviving cells over the denuded area to reestablish a continuous epithelial layer. The second phase is proliferation and differentiation of cells to replace the lost cells to build up mucosal villi. The last phase is the functional phase, in which the mucosa returns to providing its normal absorptive function and gut immunity. Although all these phases are significant, the initial phase is especially important for preventing further injury to distant organs.

In this experimental model of intestinal epithelial cell injury, IEC-6 cells were subjected to H_2O_2 oxidant stress. Cell viability and wound restitution were examined as visible final phenomenon after exposure to different levels of H_2O_2 oxidant stress. Experiment 1 of the present study showed that H_2O_2 oxidant stress inhibited wound restitution and decreased cell viability in a dose-dependent manner (**Fig. 1, 2**). Experiment 2 of this study also demonstrated that IEC-6 cells exposed to H_2O_2 at 0.5 mM gradually migrated to the denuded area in a time-dependent manner, inhibited wound restitution compared with control, and decreased cell viability (**Fig. 4, 5**). Many investigators have subjected intestinal epithelial cells to H_2O_2 oxidant stress and showed similar cell damage^{22–25}. Studying cell damage produced by various degrees of H_2O_2 oxidant stimuli can provide insight into molecular biological mechanisms of the response to stress.

We have also investigated cyclooxygenase (COX)-2 messenger RNA levels as a stress marker in our model. Levels of COX-2 messenger RNA were significantly higher in cells exposed to H_2O_2 at 0.25 mM or 0.5 mM than in control cells (data not shown).

Although many stress markers for evaluating oxidative stress have been proposed, portions of the molecular pathways have gradually been clarified. However, numerous molecular pathways are activated at the same time and interact with each other via stimuli, and different stimuli lead to different molecular pathways. Thus, pursuing all pathways is extremely difficult. We believe that the most important indicator is cell status, which reflects cell viability and cell growth. We believe that evaluation methods can still be greatly improved.

Recent advances in systems biology have allowed for the study and characterization of genes, transcripts, proteins, and metabolites on a global level. Pattern recognition analysis of NMR spectra has been applied to global metabolic studies^{26,27}.

In the present study, differences in oxidant injury were successfully visualized in IEC-6 cells exposed to different concentrations of H_2O_2 using the chemometrics technique to interpret the NMR data of samples. Although the classes could not be separated with unsupervised principal components analysis²⁸, they were clearly separated with supervised PLS-DA. Score plots generated with PLS-DA methods can provide a visual representation of information-rich spectral data by means of dimensionality reduction²⁹. A score plot shows biological samples clustering into either similar or different groups. The closer the dots are in the samples in the score plot, the more similar they are with respect to the 2 components concerned. On the other hand, dots that are far away from each other are different from each other. In the present study the PLS-DA score plot (**Fig. 3A**) showed a clear separation between the different classes. The spectral variance of factor 1 was 87%, and that of factor 2 was 5%. Factor 1 contributed to separation with or without H_2O_2 . Factor 2 contributed to important separations between exposure to different concentrations of H_2O_2 . These combined attributes explain 92% of the variation in **Figure 3A**. Similarly, the PLS-DA score plot in **Figure 6A** shows 3 clusters clearly separated between time differences of exposure to H_2O_2 . Score plots for 6 and 12 hours' exposure were nearby, but score plots for 24 hours' exposure were clustered in a different area (**Fig.**

6A).

The PLS-DA score plot combining all control groups and H₂O₂ groups are shown in **Figures 7A and 7B**. The score plot of **Figure 7A**, which was from factors 1 and 2, contributed to sufficient cluster separation. However, separation was unclear between control at 6 hours and 12 hours and H₂O₂ at 6 and 12 hours. The score plot of **Figure 7B**, which was from factors 1 and 3, contributed to a clear separation with or without H₂O₂. **Figure 7B** shows superior separation between the control groups and H₂O₂ groups. The PLS-DA score plot in **Figure 6A** shows a spectral variance of 97% (95% for factor 1 and 2% for factor 2), that in **Figure 7A** shows a spectral variance of 96% (93% for factor 1 and 3% for factor 2), and that in **Figure 7B** shows a spectral variance of 94% (93% for factor 1 and 1% for factor 3). The PLS-DA score plot in **Figure 6A** shows a category variance of 61% (32% for factor 1 and 28% for factor 2), that in **Figure 7A** shows a category variance of 95% (92% for factor 1 and 3% for factor 2), and that in **Figure 7B** shows a category variance of 93% (92% for factor 1 and 1% for factor 3). All these PLS-DA score plots successfully showed clear cluster separation. Correlation loadings in **Figures 6B, 7B, and 7C** showed good separation of many spectral-region-related clusters in these PLS-DA score plots. The IEC-6 cells exposed to H₂O₂ showed dramatically changed cellular status, which included molecular changes, intermolecular forces, structural changes, and metabolic changes. Indeed, NMR spectra reveal whole cellular information that contains these changes. We speculate that our use in the present study of whole NMR spectra data rather than several assigned peaks allowed us to accurately reveal cell status. We successfully visualized cell phenomena, such as cell viability and restitution, using PLS scores calculated from whole NMR spectral data.

In summary, pattern recognition of NMR spectral data using a supervised PLS-DA method clearly visualized differences in oxidant injury in IEC-6 cells exposed to H₂O₂. With this method it is possible to evaluate cellular status, such as cell viability and cell growth, of intestinal epithelial cell extracts under oxidative stress. In addition, this method is useful for

further elucidating complicated pathophysiology.

Conflict of Interest: The authors declare that they have no conflict of interest.

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