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Detection of reactive oxygen species by flow cytometry after spinal cord injury

Jian Luo [a], Nianyu Li [b], J. Paul Robinson [b], Riyi Shi [a,*]

[a] *Department of Basic Medical Sciences, Center for Paralysis Research, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907, USA*

[b] *Department of Basic Medical Sciences, Cytometry Laboratories, Purdue University, West Lafayette, IN 47907, USA*

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Abstract

The monitoring of reactive oxygen species (ROS) levels in injured nervous tissue is critical for both studying the mechanism of secondary damage and evaluating the effectiveness of antioxidants. Flow cytometry is an excellent method to detect ROS in cultured cells and naturally suspended individual cells. However, its use in nervous tissue is limited due to the difficulties in obtaining single cells in suspension. We have developed a new method which minimizes the error during conventional dissociation. Specifically, we introduced a fixation step (with formaldehyde) between the dye loading and dissociation. As a result, the post-injury ROS signals detected by flow cytometry increase significantly when using hydroethidine as superoxide indicator. The injury-induced elevation of ROS obtained from this new method was also in better agreement with the two other standard ROS detection methods, fluorescence microscopy and lipid peroxidation assay. Furthermore, more pronounced decrease of ROS was found in this improved method in response to treatment with a superoxide scavenger, manganese(III)tetrakis(4-benzoic acid)porphyrin. Based on these observations, we suggest that the data obtained from the cells by this new method are more accurate than those from the classic cell dissociation method that dissociates cells directly from fresh tissues.

Keywords: Flow cytometry; Reactive oxygen species; Scavenger; Lipid peroxidation; Spinal cord injury; Formaldehyde

1. Introduction

Increasing evidence suggests that reactive oxygen species (ROS) are important mediators of secondary injury in CNS trauma (Hall, 1989; Coyle and Puttfarcken, 1993). It is well established that ROS increase significantly following CNS mechanical trauma in live animals (Hall, 1989). The inhibition of ROS has been shown to provide behavioral and functional recovery following various types of injuries (Hall, 1995). Therefore, it is important to develop reliable, accurate assays to monitor ROS levels in different compartments of the tissue for both studying the mechanism of injury and evaluating the effectiveness of antioxidants. Currently, there are many methods available to evaluate ROS, including lipid peroxidation (LPO) assay, electron spin resonance (ESR), and image analysis-based fluorescence microscopy using ROS-specific fluorescent dyes.

In recent years, flow cytometry has emerged as an effective rapid method to specifically detect intracellular ROS. The major advantage of flow cytometry is that, unlike other available methods, intracellular ROS levels can be evaluated on a cell-by-cell basis in the absence of tissue background. Despite the frequent use of such method in detecting ROS in neutrophils, lymphocytes or cultured cells (Robinson, 1993; Carter et al., 1994), its ability to detect ROS in nervous tissue is limited. The main reason is that in order to utilize flow cytometer, the neurons in CNS tissue must be dissociated into single cells in suspension. This dissociation process, unfortunately, can introduce error by eliminating fragile cells which usually have higher level of ROS or causing ROS production. The purpose of this study is to introduce a new procedure to reduce the error accrued during the dissociation process and facilitating the use of flow cytometry in research involving nervous tissue.

* Corresponding author. Tel.: +1-765-496-3018; fax: +1-765-494-7605

E-mail address: riyi@vet.purdue.edu (R. Shi).

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We have found that a fixation step introduced after the dye loading and before the dissociating procedure significantly increased the post-injury ROS signals detected by flow cytometry compared to that without the fixation step. The data obtained from this new method was in overall better agreement with two other standard ROS detection methods, image analysis-based fluorescence microscopy and LPO assay. This method may facilitate the more frequent use of flow cytometry in free radical research in nervous tissue.

2. Methods and materials

2.1. In vitro spinal cord injury model

All animals used in this study were handled in strict accordance with the NIH guide for the Care and Use of Laboratory Animals and the experimental protocol was approved by the Purdue Animal Care and Usage Committee. In these experiments, every effort was made to reduce the number and suffering of the animals used. Adult Hartley guinea pigs, weighing 300–500 g were anesthetized deeply with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg). They were then perfused through the heart with 500 ml of oxygenated, cold Krebs' solution to remove blood and lower core temperature. The vertebral column was excised rapidly and a complete laminectomy was performed. The spinal cord was removed from the vertebrae and immersed in cold Krebs' solution. The cord was separated into two halves by midline sagittal division. The composition of the modified Krebs' solution was as follows: NaCl, 124 mM; KCl, 2 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.3 mM; $CaCl_2$, 2 mM; dextrose, 20 mM; $NaHCO_3$, 26 mM, equilibrated by bubbling with 95% O_2/5% CO_2 to produce a pH of 7.2–7.4. The spinal cords were maintained in oxygenated Krebs' solution at 37 °C for 1 h before the onset of the experiment to ensure recovery from dissection before experimentation. All the samples were bubbled with 95% O_2/5% CO_2 throughout the duration of the experiment.

The spinal cord strips were randomly divided into three groups: control-uninjured (control), compression-untreated (injury), and compression-treated (treated). The control-uninjured group did not receive any compression. The compression-untreated group received the compression injury which was induced by a constant displacement of 5 s compression of the spinal cord using a modified forceps possessing a spacer. The compression-treated group was placed in the modified Krebs' solution containing 100 μM manganese(III)tetrakis(4-benzoic acid)porphyrin (MnTBAP) immediately after compression. MnTBAP is a cell-permeable superoxide scavenger. All experiments were performed at 37 °C.

One hour after injury, the spinal cord strips were taken out of the Krebs' bath and the center of the injury site (about 6 mm long) was obtained and the ROS measurements were performed.

2.2. Detecting ROS using flow cytometry

Hydroethidine (HE) was used as a superoxide indicator. HE is oxidized selectively by superoxide to ethidium, whose fluorescence intensity within the cell is proportional to the total production of superoxide (Rothe and Valet, 1990; Bindokas et al., 1996). The oxidation of HE is not accomplished by hydroxyl radical, singlet O_2, H_2O_2 or nitrogen radicals (Bindokas et al., 1996).

2.2.1. Isolating cells for flow cytometry

2.2.1.1. Method A. This method was optimized for injured spinal cord from the cell dissociation methods used in most literature (Grogan and Collins, 1990; Watson, 1991; Visscher and Crissman, 1994; Diamond et al., 2000). The isolation procedure was performed on fresh tissues using a mixture of enzymes after mechanical mincing. Briefly, spinal cord strips, from the center of the injury site, were minced with a surgical blade. The minced tissue was disaggregated first by an automatic mechanical procedure using BD Medimachine (BD Biosciences, San Jose, CA) for 15 s. The resultant tissue was then incubated in 1 ml of previously oxygenated (95% O_2/5% CO_2) dissociation solution for 20 min and kept oxygenated during the dissociation process. The dissociation solution contained 0.5 mg/ml collagenase and 0.1 mg/ml trypsin in Krebs' solution. The concentrations of the enzymes and duration of incubation were optimized to enable effective dissociation of cells and at the same time cause minimal cell damage based on the observation of production of subcellular debris. During dissociation, the tissue was gently triturated with a 2 ml pipette (10 strokes). After 20 min dissociation, the remaining tissue was removed and the suspensions were spun at $300 \times g$ for 10 min, the dissociation solution was aspirated and the cells were washed twice with PBS before dye loading. Pelleted cells were washed with 2 ml PBS and centrifuged as above. Undisaggregated tissue debris and doublets were removed by filtration through a mesh (60 μm). The resultant cells (10^6) were then incubated in 1 ml of 1 μM HE solution at 37 °C for 5 min in the dark. Cells were then washed twice in PBS by two additional spinning/washing cycles and resuspended in 1 ml of PBS for flow cytometric analysis.

2.2.1.2. Method B. The major differences between methods A and B were that the fluorescence dye was loaded before the dissociation procedure and the tissue

was immediately fixed after dye loading; therefore, the dissociation was performed on the fixed tissues. The spinal cord strips were incubated with 1 ml of PBS with HE at final concentrations of 1 μM for 5 min at 37 °C in the dark. The strips were then fixed with 3.7% formaldehyde for 2 h. After fixation, the spinal cord strips were subjected to rehydration by incubation in PBS overnight. The aim of the rehydration procedure was to remove the formaldehyde to prevent the inactivation of enzymes used to digest the tissue (Gibellini et al., 1995). After rehydration, the spinal cord strips were minced manually. The minced tissue was disaggregated using BD Medimachine (BD Biosciences, San Jose, CA) for 25 s and incubated in enzymatic dissociation solution for 20 min. The dissociation mixture contained 5 mg/ml collagenase and 1 mg/ml trypsin in Krebs' solution. During dissociation, the tissue was gently triturated with a 2 ml pipette (maximum of 20 strokes). After the remaining tissue was removed, the suspensions were spun at $300 \times g$ for 10 min and the dissociation solution was aspirated. Pelleted cells were washed with 2 ml PBS and centrifuged as above. Undisaggregated tissue debris and doublets were removed by filtration through a 60 μm metal mesh. The final cells (10^6) were resuspended in 1 ml PBS and were analyzed by flow cytometry immediately.

2.2.2. Flow cytometry analysis

Flow cytometric data acquisition and analysis were performed on an EPICS XL cytometer (Beckman-Coulter, Miami, FL). The flow cytometer was equipped with an argon ion laser with excitation at 488 nm and a 15 mW output power. The red fluorescence of ethidium was monitored using a 610 nm long pass filter. Electronic gating was used to exclude doublets and subcellular debris. Specifically, a forward scatter (FSC) threshold was set to eliminate most of the subcellular debris. For each parameter investigated, at least 2×10^4 events (cells) were analyzed per sample. The following data were collected: forward scatter, 90° scatter, and the mean fluorescence intensity of ethidium signals (as logarithmically amplified data).

Since we introduced a fixing step in method B, we wanted to know the effect of the fixation procedure on the fluorescence intensity of HE. This was done by comparing the fluorescence intensity in the same cell population with and without fixation.

As compared with unfixed samples, the fixed samples showed an average 6–10% increase of fluorescence intensity in both control and injured groups (data not shown). The fixation did not significantly affect the mean coefficient variation (data not shown), and the increase was cancelled out when expressed as a percentage of the control.

2.3. Morphological studies using fluorescence microscopy

In order to observe the morphology change after isolation, the isolated single cells were observed under a fluorescence microscope. This was done immediately when the cells were ready for flow cytometry (before resuspension) by dropping the pellets directly onto the glass slides and observing immediately under an Olympus Vanox fluorescence microscope (excitation filter: BP 545 nm; barrier filter: 0–590 nm).

2.4. Detecting ROS using fluorescence microscopy

In order to compare the accuracy of the flow cytometry data obtained from the cells dissociated by methods A and B, we used fluorescence microscopy to measure the fluorescence intensity. For the fluorescence microscopy experiment, the dye loading and fixation was the same as described above in method B. After fixation, the segments were cut into sections of 40 μm, using an EMS-4000 Tissue Slicer (Electron Microscopy Sciences, Fort Washington, PA). The sections were dehydrated and mounted on glass slides with DPX. The sections were then observed with an Olympus Vanox fluorescent microscope (excitation filter: BP 545 nm; barrier filter: 0–590 nm). HE fluorescence images were captured through a 2× objective, using a high-resolution CCD camera (DEI-750, Optronic Eng., Goleta, CA). At this magnification, we were able to capture the whole section with just one image. The images were saved for later analysis and quantification. Camera exposure and light settings were kept constant during each experiment.

Quantification was performed from coded slides by an individual who did not know the treatment history of the specimen. The fluorescence intensity measurements were carried out by an image analysis program, Sigmascan Pro (SPSS Science, Chicago, IL). For each tissue sample, which was about 6 mm long, five sections were chosen randomly and the fluorescent intensity was measured separately. An average of the values from these five sections was calculated and used as the final fluorescent value of the cord sample. For each section image, the area occupied by the spinal cord tissue was carefully delineated manually along the edge and the fluorescent intensity of the tissue was taken. Four smaller areas (5% of the total field) were randomly selected at each corner (outside of the spinal cord tissue) and the average fluorescence intensities of these four areas were used as background intensity. The fluorescence intensity of the spinal cord samples was obtained by subtracting the background fluorescence from that of the spinal cord image and expressed in arbitrary units.

2.5. Lipid peroxidation assay

LPO was analyzed using a Lipid Hydroperoxide Assay Kit from Cayman Chemical Company (Ann Arbor, MI). The spinal cord strips obtained from the center of the injury site were weighed and homogenized. The homogenization was carried out in a Teflon–glass homogenizer in the modified Krebs' solution to obtain a 10% (w/v) whole homogenate. Immediately after homogenization, lipid hydroperoxides were extracted from the sample into chloroform. The measurement was then performed immediately upon extraction. The chromogenic reaction was conducted at 37 °C for 5 min. The absorbance was read at 500 nm using a 96-well plate reader on a spectrophotometer (SLT spectra plate reader, SLT Labinstrument, Salzburg, Austria). 13-HpODE (13-hydroperoxy octadecadienoic acid) was used as the standard. Tissue lipid peroxide was calculated and expressed as nanomoles per 100 mg of wet tissue (nmol/100 mg).

2.6. Chemicals and reagents

HE was purchased from Molecular Probe (Eugene, OR). Lipid Hydroperoxide Assay Kit and MnTBAP were purchased from Cayman Chemical Company (Ann Arbor, MI). DPX Mountant for histology and all other chemicals used were obtained from Sigma Chemical Co. (St. Louis, MO).

2.7. Statistics

All data are represented as means ± SD. Statistical analysis was performed using the Student's t-test. Values were accepted as being statistically significantly different if a P-value was < 0.05.

3. Results

3.1. Overall cell yields and morphology after isolation

In this study, the mean weight of the fresh spinal cord strips, about 6 mm from the injury center, was 272 mg. The mean cellular yield of method A was 3.5×10^6 cells, while that of method B was 4.1×10^6. The standard protocol requires a final cell concentration of 1×10^6 cells suspended in 1 ml. Therefore, both methods provide sufficient cells. Furthermore, under the fluorescence microscope, there is no difference between the morphology of the cells isolated by these two methods (Fig. 1).

3.2. Histogram resolution and light scatter properties

The mean coefficient of variation (CV) was 3.68% in method A, and 5.19% in method B. Representative histograms of the two different methods were shown in Fig. 2.

The light scattering properties of the isolated cells were determined by forward angle light scatter (FALS) and 90° angle light scatter (90LS) as indicators of cell size and complexity (granularity), respectively. Fig. 2A and D shows the light scattering properties of the isolated cells. As can be observed, the light scattering properties of the cell suspension of both methods are identical. Also, both cell suspensions have high cellular complexity and these samples contain a wide variety of cell size and granularity.

3.3. Spinal cord superoxide is significantly increased after compression injury

HE is selectively oxidized to ethidium by superoxide. The intensity of ethidium fluorescence reflects the level of O_2^- produced in the cell since O_2^- cannot easily transverse the cell membrane. Fig. 2B displays three flow cytometry histograms of the data obtained from the cells dissociated by method A. Each histogram represents a different group: control-uninjured, compression-untreated, and compression-treated, respectively. As can be observed from Fig. 2B, as a result of compression injury, the peak of fluorescent intensity had shifted to the right from a to c, indicating an increase of fluorescent intensity. The overall intensity was reduced from c to b due to the MnTBAP treatment. Fig. 2C shows the summary of the data from five experiments. The fluorescence intensity in compression-untreated groups was significantly higher as compared with that of controls (135.1% of control, $P < 0.01$, $n = 5$). The increase was significantly inhibited by MnTBAP treatment (reduced to 113.4% of control, $P < 0.05$, $n = 5$).

Fig. 2E displays three representative flow cytometry histograms of the data obtained from the cells dissociated by method B. Significant shifts were observed between control and injury, and injury and treated groups. This was further confirmed by the summary of the results from five experiments (Fig. 2F). As shown in Fig. 2F, the fluorescent intensity of the samples in the injured group increased 208.2% of control ($P < 0.01$, $n = 5$), while MnTBAP treatment reduced the increase to 132.8% of control ($P < 0.01$, $n = 5$) (Fig. 2F).

Quantitative analysis of fluorescence images obtained from the fluorescent microscope shows that the increase of ROS following injury is statistically significant as is the decrease of ROS by the treatment of MnTBAP. Specifically, the fluorescent intensity of ethidium of the samples in the injured group increased about 101.2% 1 h after injury (201.2% of control, Fig. 3) ($P < 0.01$, $n = 5$),

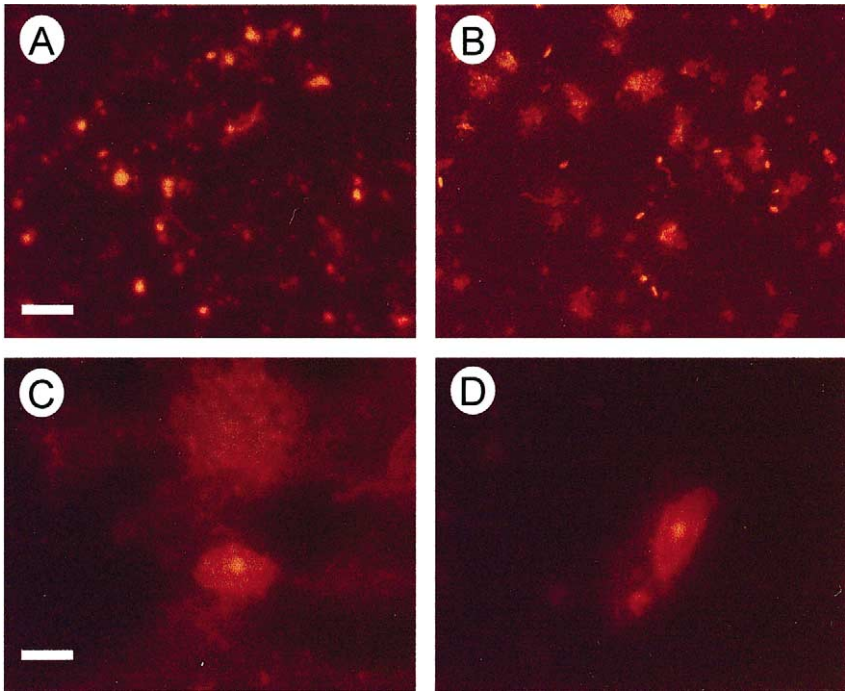

Fig. 1. Representative micrographs showing the morphology of the isolated cells. The observation was performed immediately after the cells were ready for flow cytometry. The pellets were dropped to the glass slides and observed using an Olympus Vanox fluorescence microscope. Note that there is no difference between the morphology of the cells isolated by method A (A and C) and method B (B and D). Scale bars: A = B = 50 μm; C = D = 10 μm.

while MnTBAP treatment reduced the increase to 141.3% of control ($P < 0.01$, $n = 5$).

In the LPO experiment, a standard compression produced an LPO level of 21.23 ± 3.22 nmol/100 mg at 1 h after injury, a 209.5% increase from the control-uninjured group (6.86 ± 1.26 nmol/100 mg) ($P < 0.01$, $n = 5$, Fig. 4). Treatment with MnTBAP reduced the LPO level to 16.57 ± 3.15 nmol/100 mg, significantly lower than those measured in the compression-untreated group ($P < 0.01$, $n = 5$, Fig. 4).

3.4. Comparison of ROS detected using three different methods

Table 1 clearly shows that the flow cytometry data obtained from the cells by both methods A and B are comparable to those of two other methods, fluorescence microscopy and LPO assay. However, by comparing the extents of SCI-induced ROS increase and MnTBAP treatment-induced ROS decrease, the data obtained from method B were found to be better in agreement with those of fluorescence microscope and LPO assay.

4. Discussion

There are a number of methods currently available for quantifying the generation of ROS (de Zwart et al., 1999). The most commonly used one is the measurement of ROS products such as lipid peroxides (LPO assay) or protein carboxyl adducts (de Zwart et al., 1999). The LPO assay is useful in evaluating the overall level of tissue LPO. An alternative method is the use of spin trap molecules, whose behavior in response to ROS may then be detected by ESR (Valgimigli et al., 2001). One obvious limitation of these methods is that they cannot be used to discern events in subcellular compartments. Further, LPO is a result of a combinatory effect of all ROS; the effect of each individual ROS (i.e. superoxide or hydroxyl radical) cannot be dissected out. Techniques using specific fluorescent dyes have the ability to detect the level of each ROS. For example, by using superoxide-specific HE, the fluorescence microscopy method used in the current study gives an approximation of superoxide levels in both intracellular and extracellular compartments. Flow cytometry, on the other hand, measures only intracellular superoxide levels.

Flow cytometry combines the advantages of microscopy and biochemical analysis in a single sensitive technique for a rapid examination of numerous individual cells. However, the samples for flow cytometry have to be single particles in suspension. Hence, the cells in CNS tissue must be dissociated into single cells in order to be measured by flow cytometry. One main problem of isolating single cells from whole tissue is that the dissociation procedure itself may introduce unwanted damage to the cells. The dissociation process may affect the final results of ROS measurements in the

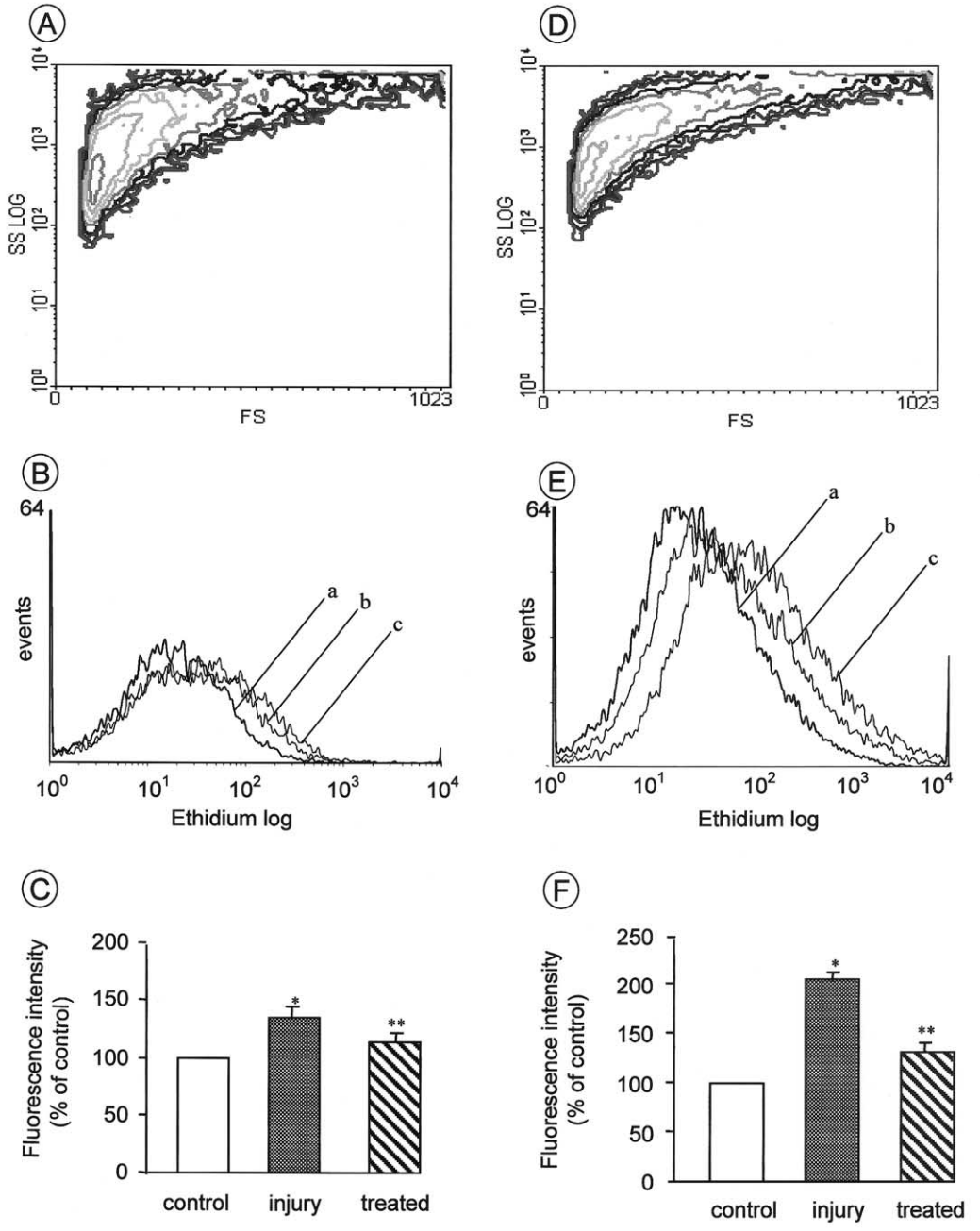

Fig. 2. Flow cytometry analysis of intracellular superoxide anion (O_2^-) generation after compression injury and treatment of MnTBAP, a superoxide scavenger. Intracellular O_2^- levels were evaluated after a period of 5 min incubation with HE solution: (A–C) data obtained by method A; (D–F) data obtained by method B. (A, D) Light scattering properties of the cell suspensions obtained by the two dissociation methods. Note that there is no difference between these two methods. The cells of both methods show high cellular complexity and a wide range of cell sizes. (B, E) Typical experiments showing the level of O_2^- detected by flow cytometry in control-uninjured (a), injured-untreated (c), and injured-treated (b) groups, respectively. Data were collected at 1 h after injury or injury and treatment. Note the significant shifts in E. (C, F) Quantitative analysis showing fluorescence intensity changes following compression and treatment. Compression injury resulted in significant increases of ethidium fluorescence intensity 1 h after injury ($n = 5$, $P < 0.01$). The treatment of MnTBAP significantly lowered the injury-mediated elevation of ethidium fluorescence ($n = 5$, $P < 0.01$). Note the significant differences between control and injury, injury and treated groups in F. * Indicates significant difference ($P < 0.01$) between control-uninjured (control) and injured-untreated (injury) groups. ** Indicates significant difference ($P < 0.01$) between injured-untreated (injury) and injured-treated (treated) groups.

following two ways. First, severely injured cells (possibly with high levels of ROS) in the injured samples may be destroyed and become debris; such cells with high levels of ROS are excluded from the flow cytometry samples, therefore, an underestimation of ROS may result. Second, the dissociation procedure could cause a certain degree of cell damage and ROS generation. This may damage the original healthy cells as well as worsen the

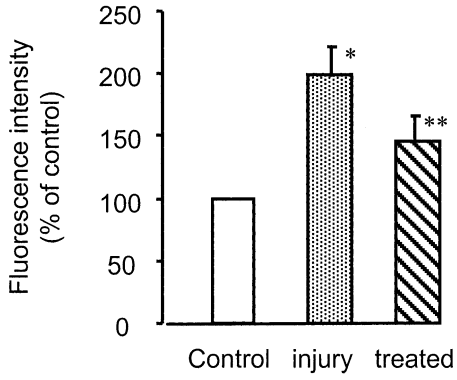

Fig. 3. Quantification of ethidium fluorescence intensity and its inhibition at 1 h after injury as observed by the fluorescence microscope. The fluorescence intensity of the sample area was then obtained by background subtraction. Values were expressed as percentages of control groups with control as 100%. Compression injury caused significantly ethidium fluorescence intensity ($n = 5$, $^{*}P < 0.05$ as compared with control). Treatment of MnTBAP reduced ethidium fluorescence intensity ($n = 5$, $^{**}P < 0.05$ as compared with injury).

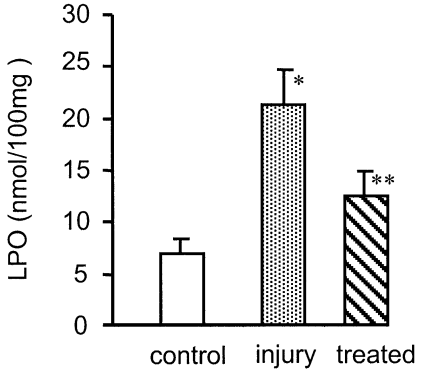

Fig. 4. The change of LPO following compression and its inhibition 1 h post-injury by MnTBAP. LPO (nmol/100 mg wet tissue) was expressed as mean ± SD ($n = 5$ in each group, $P < 0.01$). * Indicates significant difference ($P < 0.01$) between control-uninjured (control) and injured-untreated (injury) groups. ** Indicates significant difference ($P < 0.01$) between injured-untreated (injury) and injured-treated (treated) groups.

injured cells. Both can lead to increase of ROS, which may obscure the ROS generation caused by deliberate injury. Obviously, the optimal dissociation method is the one that causes only minimal cell damage. Currently, the commonly used methods are mechanical, enzymatic (biochemical), and the combination of both. These methods have been widely and successfully applied in many kinds of studies to dissociate single cells from fresh, uninjured tissues (Kay and Wong, 1986; Grogan and Collins, 1990; Watson, 1991; Visscher and Crissman, 1994; Brewer, 1997; Diamond et al., 2000). In preliminary studies, we found that the combination method works best with respect to the maximal cell yields and minimal cell damage during isolation (data not shown). We also found that a relatively speedy dissociation was achieved when using enzyme mixtures, as suggested by others (Pretlow and Pretlow, 1991). In this study, we used a mixture of trypsin and collagenase, considering trypsin to break intercellular linkage and collagenase to break up stromal components (Pretlow and Pretlow, 1991). We also identified the optimal concentrations of the enzymes to use and the proper mechanical forces to be applied. Using this optimized dissociation method, we are able to reduce the damage caused by the dissociation process to some degree. However, it was still not certain that the damage caused by the dissociation process was minimal. It was clear to us that an extra step was needed to make a significant improvement.

In an attempt to solve this problem, we developed a new dissociation method (method B). In this method, we introduced a fixation step after HE loading and before dissociation. It is evident that this method is better than method A in terms of ROS detection sensitivity. Since the cells are fixed before dissociation, the actual ROS level before dissociation is preserved and the possibility of ROS generation during dissociation is greatly minimized. Further, since all the cells, including injured and healthy ones, in the samples are fixed and cellular structures are preserved, they may have equal susceptibility to the damage caused by the dissociation process. This reduces the possibility that more severely injured cells with higher ROS levels are destroyed and excluded from flow cytometry analysis. Therefore, the results obtained by method B are presumably more accurate.

Table 1
Comparison of the data of ROS detected by different methods

	Flow cytometry		Fluorescence microscopy	LPO
	Method A	Method B		
SCI-induced ROS increases	35.1	108.2	101.2	209.5
MnTBAP-treatment-induced ROS decreases	16.06	32.13	29.77	28.12

The 'SCI-induced ROS increases' were expressed as $[(\text{data}_{\text{injury group}} \times \text{data}_{\text{control group}})/\text{data}_{\text{control group}}] \times 100\%$; the 'MnTBAP-treatment-induced ROS decreases' were expressed as $[(\text{data}_{\text{injury group}} - \text{data}_{\text{treated group}})/\text{data}_{\text{injury group}}] \times 100\%$. Note that the flow cytometry data obtained from method B are in better agreement with those of fluorescence microscopy and LPO assays.

The fixative used in this study was formaldehyde. It is possible that the fixation procedure may affect the fluorescence intensity of ethidium. This effect may come from two aspects. One is the contribution of the fixation process itself, which may simulate cell injury by cell permeabilization. The other is that the fixative (formaldehyde) may directly affect the fluorescence intensity of ethidium (Larsen et al., 1986). However, we believe that this did not affect our ROS measurement significantly since all the samples were fixed with an identical concentration of formaldehyde and the results were expressed as percentages of control.

One significant application of ROS detection is to screen effective antioxidants. In this study, we tested the effect of MnTBAP, a known cell-permeable superoxide scavenger, on the superoxide level. The fact that MnTBAP treatment significantly decreased the superoxide level measured by flow cytometry, as well the other two methods, suggests the feasibility of using flow cytometry to detect superoxide and that this method can be used to screen antioxidants. Further, compared to method A, MnTBAP-induced ROS decrease measured by method B, was more pronounced, and was in better agreement with two other methods (Table 1). It is clearly more valuable to test the efficiency of antioxidants in tissue than in cultured cells (Ostrovidov et al., 2000). This is because the artificial conditions in cell culture usually do not fully mimic an in vivo situation. In particular, it lacks the mechanical interactions between the cells and extracellular matrix that could affect the access of the antioxidants to their targets. The in vitro model used in this study has the advantage of preserving the local environment seen in vivo as compared to cell culture. Therefore, the conclusions drawn from this model would be more likely to be applicable in in vivo situations.

In conclusion, the critical components of this improved method are rapid fixation of the spinal cord and rehydration of the tissue to make it amenable to enzymatic digestion. By fixing the tissue before dissociation, the original ROS level is preserved and the effect of the dissociation process on ROS generation is greatly decreased. As a result, the accuracy and sensitivity are greatly improved.

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