Protective Effect of *Ninjin-yoei-to* on Damage to Isolated Hepatocytes Following Transient Exposure to *tert*-Butyl Hydroperoxide

Fusako Takayama*, Toru Egashira and Yasumitsu Yamanaka

Department of Pharmacology, Oita Medical University, 1-1, Idaigaoka, Hasama-machi, Oita 879-5593, Japan

Received July 5, 2000 Accepted December 7, 2000

ABSTRACT—To establish a simple screening system for estimating efficacy of an agent for an oxidative-related lesion, we investigated the damage in isolated rat hepatocytes exposed to 75 μ M *tert*-butyl hydroperoxide (*t*-BuOOH) and then subsequently incubated the cells in fresh medium. By electron spin resonance spectroscopy analysis using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), DMPO adducts of *tert*-butoxyl radicals and carbon center radicals were detected during the *t*-BuOOH exposure, and DMPO-OH formation was detected after *t*-BuOOH removal. In *t*-BuOOH-exposed cells, the level of phosphatidylcholine hydroperoxide (PCOOH), a peroxidative product of biomembranes in the hepatocytes, and the leakage of enzymes into the culture medium were significantly increased. An increase in acid phosphatase (AP) activity representing lysosome destabilization preceded the aspartate oxoglutarate aminotransferase (AST), alanine oxoglutarate aminotransferase (ALT) and lactate dehydrogenase (LDH) leakage. *Ninjin-yoei-to* added to the culture medium following the *t*-BuOOH exposure significantly inhibited the PCOOH formation and the leakage of AP, AST, ALT and LDH, concentration-dependently. *Ninjin-yoei-to* at 1 mg/ml in culture medium completely diminished these increases in enzyme activities down to the background levels found in control experiments and this reduction was greater than the most effective α -tocopherol concentration of 20 μ mol/ml. Considering all of these results, it is likely *Ninjin-yoei-to* may exert its protective effect by anti-oxidative action and membrane stabilization.

Keywords: *Ninjin-yoei-to*, Biomembrane peroxidation, Oxidative stress, Lysosome, Isolated hepatocyte

Oxidative damage is widely recognized as being involved in the development of many pathological conditions (1–3). The mechanisms behind oxidative stress, considered to begin by lipid peroxidation in biomembranes, subsequently can cause structural and functional degeneration, when it is severe, and may end in cell death. Recently it has been reported that the leakage of destructive lysosomal contents is closely related to the ensuing cell rupture and necrosis following oxidative stress (4, 5). As one of the countermeasures in searching for a therapeutic agent for an oxidative-related disorder, it is useful to establish an experimental system in which cellular injury is induced in vitro using isolated cells by contact with a generator of reactive oxygen radicals and to pay attention to the occurrence of phosphatidylcholine hydroperoxide (PCOOH), an oxidative product of membrane phospholipid, and the leakage of lysosomal contents (6).

Recently, we demonstrated the strong ability of *Ninjin-yoei-to* to scavenge reactive oxygen species in in vitro

systems including the suspension of isolated hepatocytes (7). *Ninjin-yoei-to* scavenged 1-diphenyl-2-picrylhydrazyl radicals, superoxide anion and hydroxyl radicals. The hydroxyl radicals formation induced by *tert*-butyl hydroperoxide (*t*-BuOOH) in free hepatocytes or brain homogenate was also inhibited by *Ninjin-yoei-to*. Furthermore, we demonstrated that *Ninjin-yoei-to* administration decreased the amounts of reactive oxygen species released from leukocytes in contact with phorbol myristate acetate (8). Besides, *Ninjin-yoei-to* is applicable to athrepsia states after surgery and after a long-term illness, because it potentiates immunological competence and hematopoietic function and regulates the endocrine system balance (9). In addition, the anti-inflammatory and anti-allergic effects are also found in its constitutional herbs such as angelicae radix, citrus unshiu peel, rehmanniae radix, Chinese cinnamon and glycyrrhiza (9). Incidentally, lysosomal enzymes are involved in inflammation and allergic reactions. Therefore, *Ninjin-yoei-to* was expected to be favorable for the treatment of complications related to oxidative stress and lysosomal lysis.

In the present study, we examined the cellular damage

*Corresponding author. FAX: +81-97-586-5729

E-mail: takayam@oita-med.ac.jp

of isolated hepatocytes exposed to oxidative stress with *t*-BuOOH and then evaluated the effect of *Ninjin-yoei-to* on the damage induced by oxidative stress.

MATERIALS AND METHODS

Materials

Powdered extract of *Ninjin-yoei-to* (the lot number: 250181010) was supplied by Tsumura & Co., Tokyo. The chemicals used and their sources were as follows: butylated hydroxytoluene (BHT), *Clostridium histolyticum* collagenase, Daigo's T Medium®, ethylenediaminetetraacetic acid disodium salt (EDTA), ethylene glycol-*O,O'*-bis(2-aminoethyl)-*N,N,N',N'*-tetraacetic acid (EGTA), luminol, disodium *p*-nitrophenyl phosphate hexahydrate, *p*-nitrophenol and tris(hydroxymethyl)aminomethane (Tris), from Wako Pure Chemical Industries, Ltd., Osaka; *t*-BuOOH, horse heart cytochrome *c* and type II crude trypsin inhibitor, from Sigma Chemical Co., St. Louis, MO, USA; 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) from Labotec, Tokyo. HPLC-grade reagents were used for the mobile phase of the HPLC-chemiluminescence (CL) assay, and all other reagents were of analytical grade.

Animals

Male Wistar rats (Seack Laboratory Animals, Inc., Fukuoka) weighing 200–250 g were used. They were kept in an environmentally controlled room (23 ± 2°C, 50–60% humidity, illuminated from 7:00 to 19:00 h) with food and water available ad libitum.

Isolation and loading of rat hepatocytes with oxidative stress

Isolated hepatocytes were prepared from rat liver by recirculating collagenase perfusion with trypsin inhibitor in situ, according to the procedure of Ichihara et al. (10) with minor modifications. Nonparenchymal cells and debris were removed by 60 × *g* centrifugation for 2 min at 4°C and discarding of the supernatant, three times over. The precipitated hepatocytes were resuspended in Daigo's T Medium®, pH 7.4, which is referred to as the "culture medium or media" in the text. Viability was more than 95% as assessed by the trypan blue exclusion test. Prior to loading the oxidative stress, the hepatocytes were incubated for 1 h at a density of 1% (by wet weight/volume) in culture media. The culture medium was renewed by 60 × *g* centrifugation for 2 min at 4°C and the supernatant was discarded, followed by resuspension of the cells in substituted fresh medium. The oxidative stress was applied to the hepatocytes by a 15-min incubation in the changed culture medium to which 75 μM *t*-BuOOH had been added. Also, as a negative control, hepatocytes were incubated for 15 min only in fresh culture medium. The oxidative stress was terminated

by changing the culture medium. Then, the hepatocytes were resuspended and continuously incubated in fresh culture media (negative and positive controls) or in medium to which 0.001–1 mg/ml of *Ninjin-yoei-to* or 20 μmol/ml of α -tocopherol had been added. Each experiment under the same conditions was performed in triplicate. Incubation was executed consistently with 1% hepatocytes at 37°C with gentle shaking and aliquots of hepatocyte suspensions were collected to assess cell damage.

The hepatocyte suspensions were centrifuged at 60 × *g* for 1 min at 4°C and fractionated into culture medium (supernatant) and hepatocytes (precipitate). As shown below, the oxidative degeneration of biomembranes was examined in the precipitate, while the leakage of enzymes such as acid phosphatase (AP), aspartate oxoglutarate aminotransferase (AST), alanine oxoglutarate aminotransferase (ALT) and lactate dehydrogenase (LDH) from the hepatocytes was investigated to determine the activity of each of these in the supernatant fraction.

Evaluation of free-radical generation during and after oxidative stress

The generation of free radicals in hepatocyte suspensions was investigated by electron spin resonance (ESR) spectroscopy measurements, using DMPO as a spin-trapping agent.

To examine reactive oxygen radical generation during the *t*-BuOOH exposure, 45-μl aliquots of a 1% hepatocyte suspension in culture medium containing 420 mM DMPO and 75 μM of *t*-BuOOH was placed into a capillary glass tube (i.d. 1 mm), and this was in turn introduced into a quartz tube (i.d. 4 mm, o.d. 5 mm; LST-5HG; Labotec, Tokyo). The recording of the ESR spectrum of the sample was initiated 50 s after the *t*-BuOOH addition, at room temperature with an X-band ESR spectrometer (JES-RE1X; Jeol, Tokyo). The instrument conditions were the following: about 9.4 GHz with 100-kHz modulation frequency, central field ± sweep width of 335.3 ± 5 mT, microwave power of 10 mW, field modulation width of 0.079 mT, time constant of 0.1 s and sweep speed of 5 mT/min. The magnetic field was calculated from the splitting of divalent manganese (Mn²⁺), in which the distance from the third to fourth signal is 8.69 mT.

To examine free-radical generation after washing out *t*-BuOOH, the hepatocytes were resuspended in a quarter volume of fresh medium and subsequently incubated for 1 h. Then, the aliquots of hepatocyte suspensions were taken and DMPO was added. ESR measurements were also performed 45 s after the DMPO addition, with 45-μl aliquots of a 3% hepatocyte suspension in culture medium containing 420 mM DMPO, in the same manner as described above.

Determination of oxidative degeneration in biomembranes of hepatocytes

In order to examine the lipid peroxidation of biomembranes, the PCOOH content in hepatocytes was quantified by HPLC-CL analysis (11). In the HPLC-CL measurement, total phosphatidylcholine (PC) content including nascent, hydroperoxide and hydroxy derivatives in hepatocytes was monitored by UV detection at 205 nm. Immediately after collecting the hepatocyte suspensions, the culture media were substituted by physiological saline containing 0.0025% BHT and 1 mM EDTA and stored at -80°C until use in the lipid extraction procedure for the HPLC-CL assay, as previously described (12). The CL reagent containing $1.5\text{ }\mu\text{g/ml}$ of luminol and $15\text{ }\mu\text{g/ml}$ of cytochrome c in 10 mM borate-sodium carbonate buffer, pH 10.5, was delivered to the post-column. Hydroperoxides were monitored by the detection of CL from luminol oxidation caused by the reaction between cytochrome c in the post-column CL reagent and each hydroperoxide that was separated and eluted through the column. The PCOOH quantity corrected by the hepatocyte protein concentration was expressed according to the moles of active oxygen from hydroperoxide ($[\text{O}]$ nmol/mg protein).

AP activity

In the present study, the AP activity was examined, instead of β -glucuronidase activity, recognized as the general marker enzyme for lysosomes, as these two are localized in lysosomes and as the supernatant activities of these were confirmed to change proportionally following oxidative stress in preliminary experiments. Therefore, the AP activity was determined according to the method of Barret and Heath (13) with minor modifications. Each sample was preincubated with acetate buffer, pH 4.9, for 10 min to destroy glucose-6-phosphatase, and then *p*-nitrophenyl phosphate, a substrate for AP, was added. The mixture ($400\text{ }\mu\text{l}$) consisting of the preparation ($240\text{ }\mu\text{l}$), 0.2 M acetate buffer pH 4.9 ($60\text{ }\mu\text{l}$) and 32 mM *p*-nitrophenyl phosphate ($100\text{ }\mu\text{l}$) was incubated at 37°C for 1 h. Then ice-cold 1 M Tris-0.4 M phosphate buffer pH 8.5 ($400\text{ }\mu\text{l}$) was added to stop the enzyme reaction and for coloration of *p*-nitrophenol released from *p*-nitrophenyl phosphate. The *p*-nitrophenol concentration was estimated by comparing the absorbance at 420 nm of the experimental solution with standard solutions of known concentrations, by spectrophotometric detection. Then, the AP activity was expressed by the released *p*-nitrophenol corrected by the supernatant protein concentration (mM/mg protein per hour).

AST, ALT and LDH activities

The activities of AST, ALT and LDH in culture medium were measured using a CL20 Auto Analyzer (Shimadzu Co., Kyoto) to estimate the hepatocyte injury. These enzyme

activities (indicated in Karmen units: K.U. and Wroblewski units: W.U.) were corrected by the supernatant protein concentration and were expressed by the supernatant protein concentration (K.U./mg protein or W.U./mg protein).

Protein concentration

Protein concentration was determined according to the method of Lowry et al. (14).

Statistics

Quantitative results are reported as the mean $\pm$ S.D. for triplicate determinations. The difference induced by oxidative stress was clarified by comparison between the positive control and negative control preparations by one-way analysis of variance (ANOVA) techniques with Dunnett's multiple comparison procedure. The effect of *Ninjin-yoei-to* or $20\text{ }\mu\text{M}$ α -tocopherol was statistically analyzed by ANOVA techniques with William's multiple comparison procedure or with Dunnett's method, respectively (15). Significance was established at the $P < 0.05$ level.

RESULTS

Free radical generation in hepatocyte suspensions after incubation with *t*-BuOOH

The ESR spectra of the DMPO spin adducts observed in hepatocyte suspensions are shown in Fig. 1. The recording of spectrum was begun 45 s after mixing with DMPO. As shown in Fig. 1A, no signal was observed in the suspension of control hepatocytes. When the hepatocytes were incubated in the culture medium with $75\text{ }\mu\text{M}$ *t*-BuOOH for 15 min, several signals appeared (Fig. 1B). They were tentatively assigned by computer simulation to the quartet signal with intensities 1:1:1:1 and hyperfine splitting constants $a_N = 1.48\text{ mT}$, $a_H = 1.63\text{ mT}$ for DMPO spin adducts of *tert*-butoxyl radicals (16) and to the sextet signal with $a_N = 1.55\text{ mT}$, $a_H = 2.27\text{ mT}$ for DMPO spin adducts of carbon center radicals (17).

During subsequent incubation at onset and at 30 min (Fig. 1, C and D, respectively) after termination of *t*-BuOOH exposure by renewing the culture medium, the characteristic ESR signal appeared due to the formation of DMPO-OH, DMPO hydroxyl radical adducts (quartet signal with intensities 1:2:2:1 and $a_N = 1.49\text{ mT}$, $a_H = 1.49\text{ mT}$) (18) in the 3% hepatocyte suspension. With the hepatocyte suspension during the subsequent incubation following the period of oxidative stress, the DMPO-OH formation was detected. Its signal intensity was stronger at shorter time of the subsequent incubation.

Oxidative degeneration in hepatocyte biomembranes

The oxidative degeneration of hepatocyte membranes was detected with the formation of PCOOH, a primary

Fig. 1. Reactive oxygen radical formation observed as DMPO adducts in hepatocyte suspension. ESR measurements were performed with suspensions of hepatocytes exposed to 0 (A) or 75 μM (B) of *t*-BuOOH for 15 min and during subsequent incubation at onset (C) or 30 min (D) after termination of *t*-BuOOH exposure by renewing the culture medium. The former two were detected in the mixture consisting of 1% hepatocyte suspension in culture medium containing 0 or 75 μM *t*-BuOOH and 420 mM DMPO. The latter two were detected in the mixture consisting of 3% hepatocyte suspension in culture medium containing 420 mM DMPO. The quartet signal with intensities 1:1:1:1 ($\circ$: $a_N = 1.48$ mT, $a_H = 1.63$ mT) was assigned to the DMPO adducts of *t*-BuO $\cdot$. The sextet signal ($\triangle$: $a_N = 1.55$ mT, $a_H = 2.27$ mT) was assigned to the DMPO spin adducts of carbon center radicals. The quartet signal with intensities 1:2:2:1 ($\blacksquare$: $a_N = 1.49$ mT, $a_H = 1.49$ mT) was assigned to DMPO-OH.

peroxidative product of PC. Figure 2 shows chromatograms detected by HPLC-CL analysis of lipid extracts from hepatocytes. PCOOH and total PC were monitored by the detection of CL intensity (solid line) and UV absorbance at 205 nm (broken line), respectively. The CL peak at about 8 min was identified as PCOOH; this increased in the hepatocytes after 15 min of 75 μM *t*-BuOOH exposure (Fig. 2B), in comparison with the negative-control hepatocytes at the same time (Fig. 2A), and increased much more in the hepatocytes incubated for an additional hour in fresh culture medium following washout of *t*-BuOOH (Fig. 2C). The UV peak at about 8 min was identified as total PC and did not change remarkably in hepatocytes after 15 min of 75 μM *t*-BuOOH exposure compared to the negative-control hepatocytes, but was then reduced in hepatocytes incubated for an additional hour.

Ninjin-yoei-to and α -tocopherol effects on PCOOH content in hepatocytes exposed to *t*-BuOOH

The PCOOH content in hepatocytes kept at 4°C was 0.029 ± 0.003 [O] nmol/mg hepatocyte-protein. After the *t*-BuOOH exposure at 37°C for 15 min, it increased to 0.291 ± 0.045 ($P < 0.01$), which was significantly higher than the 0.046 ± 0.008 found in negative-control hepatocytes at the same time. The PCOOH content after 1 h of subsequent incubation in the positive-control hepatocytes, which were continuously incubated only in fresh culture medium following washout of the medium containing *t*-

Fig. 2. Oxidative degeneration of hepatocyte biomembranes due to *t*-BuOOH exposure. The solid line represents chemiluminescence (CL) detection and the broken line represents UV detection at 205 nm following HPLC separation. Each peak at about 8 min was identified as PCOOH and total PC, respectively. PCOOH increased in hepatocytes after 15 min of 75 μM *t*-BuOOH exposure (B) in comparison with the control hepatocytes incubated in the same manner without BuOOH (A), and increased much more in hepatocytes incubated for an additional hour in fresh culture medium following washout of *t*-BuOOH (C).

BuOOH, was increased to 0.696 ± 0.097 , which was significantly ($P < 0.01$) higher than the 0.105 ± 0.015 found in negative-control hepatocytes treated in the same manner except for the *t*-BuOOH addition. When the hepatocytes were treated with *Ninjin-yoei-to* and α -tocopherol added to fresh culture medium after washout of the medium containing *t*-BuOOH, the increase in PCOOH content in hepatocytes after subsequent 1-h incubation was significantly lowered (Fig. 3). The *Ninjin-yoei-to* reduced the PCOOH elevation concentration-dependently, compared to that in the positive control.

Ninjin-yoei-to and α -tocopherol effects on AP release from hepatocytes exposed to *t*-BuOOH

In the present study, the lysosomal enzyme release from cells was assessed by the AP activity in the culture medium obtained as the supernatant by $60 \times g$ centrifugation as described above. The AP activity in the culture medium of the hepatocyte suspension collected just before oxidative stress was 0.160 ± 0.006 mM/mg protein per hour. The AP

Fig. 3. Effects of *Ninjin-yoei-to* and α -tocopherol treatments following oxidative stress on PCOOH levels in hepatocytes exposed to *t*-BuOOH. Following *t*-BuOOH exposure, the hepatocytes were treated with *Ninjin-yoei-to* and α -tocopherol added to fresh culture medium, which had been exchanged for the medium containing *t*-BuOOH. The final concentration is denoted on the vertical axis. Positive-control hepatocytes were subsequently incubated in fresh culture medium after washout of the oxidative stress agent. Negative-control hepatocytes were handled in the same manner but with simple culture medium throughout. The PCOOH levels were examined after an additional hour of incubation following transient exposure of *t*-BuOOH for 15 min. Each column and bar represents the mean $\pm$ S.D. for PCOOH levels corrected by hepatocyte protein from triplicate experiments. $^{##}P<0.01$ vs negative control, $^{††}P<0.01$ vs positive control (ANOVA with Dunnett's test). $^{*}P<0.05$, $^{**}P<0.01$ vs positive control (ANOVA with William's test).

activity at the endpoint of *t*-BuOOH exposure was 0.220 ± 0.021 , which was lower, but did not differ from that of negative-control preparations, 0.262 ± 0.017 . After 1 h of subsequent incubation following *t*-BuOOH removal, it increased to 0.447 ± 0.021 , which was significantly ($P<0.01$) higher than 0.239 ± 0.040 , the value of the negative-control preparation collected at the same time. This significant increase of AP activity was suppressed concentration-dependently by the *Ninjin-yoei-to* or α -tocopherol added after *t*-BuOOH exposure. (Fig. 4). The *Ninjin-yoei-to* of 1 mg/ml in the final concentration dramatically protected the AP release, yielding a value of 0.213 ± 0.016 , which was below the value of the negative-control experiment.

Ninjin-yoei-to and α -tocopherol effects on leakage of marker enzymes due to hepatocyte damage by *t*-BuOOH exposure

The leakage of AST, ALT and LDH from cells into the culture medium was examined in order to monitor hepatocyte damage. The activities of these enzymes in the culture medium fraction obtained from the hepatocyte suspension at the endpoint of the *t*-BuOOH exposure period were almost the same as those in the negative-control experiment (data not shown). As shown in Fig. 5, these activities in

Fig. 4. Effects of *Ninjin-yoei-to* and α -tocopherol treatments following oxidative stress on lysosomal lysis in hepatocytes exposed to *t*-BuOOH. The *Ninjin-yoei-to* and α -tocopherol treatments were performed as described in the legend to Fig. 3. The AP activity in each culture medium (supernatant fraction) was examined after an additional hour of incubation following transient *t*-BuOOH exposure and was corrected by the supernatant protein concentration. The lysosomal lysis is expressed as a percentage of the background AP activity found in the Negative-control experiments. Each column and bar represents the mean $\pm$ S.D. from triplicate experiments. $^{##}P<0.01$ vs negative control, $^{††}P<0.01$ vs positive control (ANOVA with Dunnett's test). $^{*}P<0.05$, $^{**}P<0.01$ vs positive control (ANOVA with William's test).

the supernatant obtained from positive-control hepatocyte suspensions significantly increased ($P<0.01$) after 2 h of subsequent incubation following termination of oxidative stress, when compared to those of the negative control. These increases were significantly suppressed by *Ninjin-yoei-to* and α -tocopherol added to the fresh medium after termination of oxidative stress. The *Ninjin-yoei-to* effect was concentration-dependent.

DISCUSSION

The occurrence of reactive oxygen radicals in the present experiment when isolated hepatocytes were exposed to *t*-BuOOH was demonstrated by ESR detection of the DMPO spin adducts of *tert*-butoxyl radicals and carbon center radicals during the *t*-BuOOH exposure. The oxidative stress, which was detected by DMPO-OH formation, continued after termination of the *t*-BuOOH exposure. The *t*-BuOOH exposure for 15 min increased PCOOH in hepatocytes, but did not bring about the PC release. Then, during subsequent incubation after washout of *t*-BuOOH, lipid peroxidation progressed further and membrane destabilization was observed as decreased PC in hepatocytes after an additional hour of incubation. There occurred a significant elevation of AP activity in the culture medium after 1 h of incubation, exhibiting lysosomal lysis followed by cell rupture as

Fig. 5. Effects of *Ninjin-yoei-to* and α -tocopherol treatments following oxidative stress on ALT, AST and LDH leakage from hepatocytes exposed to *t*-BuOOH. The *Ninjin-yoei-to* (NYT) and α -tocopherol treatments were performed as described in the legend to Fig. 3. The enzyme activities in each culture medium (supernatant fraction) were examined after 2 additional hours of incubation following transient *t*-BuOOH exposure and were corrected by the supernatant protein concentration. Each column and bar represents the mean $\pm$ S.D. from triplicate experiments. $^{\#}P < 0.01$ vs negative control, $^{**}P < 0.01$ vs positive control (ANOVA with Dunnett's test). $^{*}P < 0.05$, $^{**}P < 0.01$ vs positive control (ANOVA with William's test).

shown by the leakage of AST, ALT and LDH after 2 h of incubation. The ESR data and the changes in biochemical markers confirmed the validity of the present experiment inducing hepatocyte damage for screening drug efficacy on complications induced by oxidative stress and subsequently released lysosomal enzymes.

Some members of the herbal medicinal system known as "*Kampo* medicines" are expected to be effective for oxidative-related damage due to their scavenging abilities for reactive oxygen radicals (7, 19) because they consist of many herbs containing compounds such as flavonoids, saponins, tannins, polyphenol and others (9). These scavenging abilities (7) contribute in part to the effects of *Kampo* medicines including *Ninjin-yoei-to*. *Ninjin-yoei-to* consists of angelicae radix, astragali radix, atractylodis rhizoma, cinnamon, citrus unshiu peel, ginseng radix, glycyrrhiza, hoelen, paeonia albiflora, rehmanniae radix and schizandrae fructus (9). In addition to the scavenging ability of reactive oxygen radicals (7), *Ninjin-yoei-to* can stabilize biomembranes and suppress inflammation and allergic reactions (9) in which lysosomal enzymes are involved. Therefore, using the present system for inducing hepatocyte damage by transient *t*-BuOOH exposure, we estimated the efficacy of *Ninjin-yoei-to* in comparison to α -tocopherol, a general anti-oxidant. The treatments of hepatocytes with *Ninjin-yoei-to* and α -tocopherol were performed by adding each of these to fresh medium after washout of the oxidative stress agent. Therefore, the following effects on cellular injury were not based on direct interference with the added *t*-BuOOH.

Ninjin-yoei-to added to the culture medium following *t*-BuOOH exposure significantly inhibited the PCOOH

formation and the leakage of AP, AST, ALT and LDH, concentration-dependently. The protective effect of *Ninjin-yoei-to* seemed complete at a concentration of 1 mg/ml in the culture medium as increases in injury markers were diminished down to the background levels found in control experiments. Although only the most effective result with 20 μ mol/ml is presented in this report, α -tocopherol, which can scavenge most reactive oxygen radicals except for hydroxyl radicals, also concentration-dependently reduced the increases in injury, but could not abolish them completely, like *Ninjin-yoei-to* did. When α -tocopherol was tried at a higher concentration, conversely, its effect was minimized (data not shown).

Recently, attention has been paid to the lysosome enzymes in necrosis behind oxidative stress (5, 6). The superiority of *Ninjin-yoei-to* could not be explained only by its free-radical scavenging capacity, but also by its interference in biomembrane stabilization and lysosomal lysis (20).

This study was aimed at establishing a system for assessing the efficacy of agents to counteract complications due to triggering oxidative reactions and the ensuing lysosomal lysis. In conclusion, the present system and biochemical markers allow us to discuss the role of reactive oxygen radicals, membrane destabilization and lysosomal lysis in the pathological process and in the mechanisms of therapeutic agents.

The present study shows satisfactory results by the in vitro treatment of *Ninjin-yoei-to* for the damage of isolated hepatocytes exposed to transitory oxidative stress. We recently demonstrated the suppressive effect of *Ninjin-yoei-to* on the sensitivity of leukocytes obtained from the mouse with its oral administration (8). It was also observed that

the oral administration of *Ninjin-yoei-to* could elevate the plasma abilities to scavenge reactive oxygen species (21). These observations give strong support to the *Ninjin-yoei-to* efficacy in vivo.

REFERENCES

- 1 Pacifici EH, McLeod LL, Peterson H and Sevanian A: Linoleic acid hydroperoxide-induced peroxidation of endothelial cell phospholipids and cytotoxicity. *Free Radic Biol Med* **17**, 285 – 295 (1994)
- 2 Asano M, Adachi J and Ueno Y: Cholesterol-derived hydroperoxides in alcoholic liver disease. *Lipids* **34**, 557 – 561 (1999)
- 3 Fallon PG, Richardson EJ, Smith P and Dunne DW: Elevated type 1, diminished type 2 cytokines and impaired antibody response are associated with hepatotoxicity and mortalities during *Schistosoma mansoni* infection of CD4-depleted mice. *Eur J Immunol* **30**, 470 – 480 (2000)
- 4 Öllinger K and Brunk UT: Cellular injury induced by oxidative stress is mediated through lysosomal damage. *Free Radic Biol Med* **19**, 565 – 574 (1995)
- 5 Nirmala C, Anand S and Puvanakrishnan R: Curcumin treatment modulates collagen metabolism in isoproterenol induced myocardial necrosis in rats. *Mol Cell Biochem* **197**, 31 – 37 (1999)
- 6 Takayama F, Egashira T and Yamanaka Y: Oxidative biomembrane destruction with ensuing cell rupture associated with free radical species generation. *J Toxicol Sci* **22**, 351 (1997)
- 7 Egashira T, Takayama F and Yamanaka Y: Studies on pharmacological properties of *Ninjin-yoei-to*. Report 1. Free radical scavenging ability of *Ninjin-yoei-to*. *J Trad Med* **16**, 108 – 115 (1999) (text in Japanese with English abstract)
- 8 Egashira T, Ikebe A, Inoue M, Ohyama M, Nariai M, Narisako K, Tanaka S, Takayama F and Yamanaka Y: Studies on pharmacological properties of *Ninjin-yoei-to*. Report 2. Effects of long-term treatment with *Ninjin-yoei-to* on free radicals and lipid peroxidation in mouse. *J Trad Med* **16**, 116 – 122 (1999) (text in Japanese with English abstract)
- 9 Zhu YP: Chinese Materia Medica. Chemistry, Pharmacology and Applications. Harwood Academic Publishers, Amsterdam (1998)
- 10 Ichihara A, Nakamura T, Tanaka K, Tomita Y, Aoyama K, Kato S and Shinno H: Biochemical functions of adult rat hepatocytes in primary culture. *Ann NY Acad Sci* **349**, 77 – 84 (1980)
- 11 Takayama F, Egashira T and Yamanaka Y: Effect of diclofenac, a non-steroidal anti-inflammatory drug, on lipid peroxidation caused by ischemia-reperfusion in rat liver. *Jpn J Pharmacol* **64**, 71 – 78 (1994)
- 12 Takayama F, Egashira T and Yamanaka Y: ESR measurement of endogenous nitric oxide in liver and blood of mice subjected to hepatic ischemia-reperfusion. *Pathophysiology* **6**, 45 – 51 (1999)
- 13 Barret AJ and Heath MF: Lysosomal enzymes. In *Lysosomes. A Laboratory Hand Book Second Edition*, Edited by Dingle JT, pp 113 – 145, Elsevier/North-Holland Publishing, Amsterdam (1977)
- 14 Lowry OH, Rosebrough NF, Farr AL and Randall RJ: Protein measurement with the Foline phenol reagent. *J Biol Chem* **193**, 265 – 275 (1951)
- 15 Bruning JL and Kintz BL: *Computational Handbook of Statistics Third Edition*. Harper Collins Publisher, Glenview (1987)
- 16 Akaike T, Sato K, Ijiri S, Miyamoto Y, Kohno M, Ando M and Maeda H: Bactericidal activity of alkyl peroxy radicals generated by heme-iron catalyzed decomposition of organic peroxides. *Arch Biochem Biophys* **294**, 55 – 63 (1992)
- 17 Nishi J, Ogura R, Sugiyama M, Hidaka T and Kohno M: Involvement of active oxygen in lipid peroxide radical reaction of epidermal homogenate following ultraviolet light exposure. *J Invest Dermatol* **97**, 115 – 119 (1991)
- 18 Britigan BE, Rosen GM, Thompson BY, Chai Y and Cohen MS: Stimulated human neutrophils limit iron-catalyzed hydroxyl radical formation as detected by spin-trapping techniques. *J Biol Chem* **261**, 17026 – 17032 (1986)
- 19 Egashira T, Takayama F, Yamanaka Y and Komatsu Y: Monitoring of radical scavenging ability of peroral administration of *Kampo* medicine *Sho-saiko-to* in rats. *Jpn J Pharmacol* **80**, 379 – 382 (1999)
- 20 Takayama F, Egashira T and Yamanaka Y: Effects of glycyrrhizin and glycyrrhetinic acid on damage to isolated hepatocytes by transient exposure to *tert*-butyl hydroperoxide. *Jpn Pharmacol Ther* **28**, 763 – 770 (2000) (text in Japanese with English abstract)
- 21 Egashira T, Takayama F and Yamanaka Y: Free radical scavenging activity of *Kampo* medicine *Ninjin-yoei-to*. *Jpn J Pharmacol* **82**, Suppl I, 253P (2000)