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Effects of ascorbic acid and sodium selenite on growth and redifferentiation in human hepatoma cells and its mechanisms

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After being treated with ascorbic acid (AA) 3 mM + sodium selenite (SS) 1.5 μM, the growth rate and mitotic index of human hepatoma cells BEL-7402 decreased remarkably. The indexes related to cell malignancy were improved, such as cell surface charge obviously decreased, the electrophoresis rate fell from 1.76 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$ to 0.93, the average of α-fetoprotein (α-FP) content decreased from 341 $\mu g \cdot g^{-1}$ protein to 92, and γ-glutamyl-transpeptidase (γ-GT) activity from 0.76 $U \cdot g^{-1}$ protein to 0.19. The indexes related to cell differentiation were affected favourably, such as the level of tyrosine-α-ketoglutarate transaminase (TAT) activity increased from 14.2 $\mu mol \cdot g^{-1}$ protein to 49.0, and the colonogenic potential decreased 95.3%. These results indicated that hepatoma cells had been successfully induced to redifferentiation by AA + SS. The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) were significantly higher, while the activity of catalase (CAT) was slower in the treated group than in the control group. The malondialdehyde (MDA) content decreased slightly, reduced glutathione (GSH) decreased sharply, and H_2O_2 content increased dramatically. In conclusion, these results indicate that the combination of ascorbic acid and sodium selenite may induce the redifferentiation of hepatoma cells and inhibit cell growth by virtue of enhancing the activities of antioxidative enzymes and reducing the formation of H_2O_2, and altering the cell redox status. The combination of ascorbic acid and sodium selenite may be a potent anticancer treatment option for human hepatoma cells.

1. Introduction

A great many studies have shown that differentiation can be induced in malignant tumor cells. Many inducers such as retinoic acid [1], dimethyl sulfoxide [2], superoxide dismutase (SOD) [3], verbascoside [4], and DL-α-tocopherol [5] are effective antioxidants. In our laboratory, we have successfully induced human hepatoma cells to differentiate with several antioxidants [4, 6, 7]. An increasing amount of experimental and epidemiological evidence implicates free radicals in every step of cell carcinogenesis, such as initiation, promotion and metastasis.

Ascorbic acid (AA), an essential nutrient in humans is involved in many cellular functions. The epidemiological evidence and laboratory studies have shown that AA is a powerful plasma antioxidant [8] that can protect lipids, proteins, and cell membranes from oxidative damage by scavenging oxygen radicals. AA has been found to be able to inhibit cell growth and induce cell differentiation by both our own [6] and other laboratories [9], and used as a leukemia therapeutic agent. There has been a considerable discussion on AA and cancer over recent years. Although some authors believe that AA could protect against cancers, or, at least, against some types of cancers, others seem to be inclined to doubt such a function. Pauling et al. [10] found that an overload of AA (600 mg per day) could prevent against and cure cancers. But physiological mechanisms of AA absorption, tissue uptake, metabolism, and elimination support the theory that an overload of AA is unlikely to occur in man [11]. Podmore et al. [12] recently found that AA administered as a dietary supplement to healthy humans at a high dose (500 mg/day) exhibited a prooxidant effect and led to DNA damage *in vivo*. Therefore it becomes necessary to find a way to treat cancer with a relatively low dose of AA.

Sodium selenite ($NaSeO_3$) (SS) is an effective free-radical scavenger [13], and is able to enhance the activity of glutathione peroxidase (GPX) [14]. It has also been found that $NaSeO_3$ can induce differentiation of tumor cells [14]; however at high-concentrations SS is cytotoxic. By this means a general clinically viable method could be offered to combat cancer by normalizing tumor cells with a low combination dose of AA and SS instead of a high dose of AA or SS alone. In order to find out whether the differentiation induced by AA + SS is connected with a decrease in reactive oxygen species (ROS), regulating the activities of antioxidant enzymes and altering the cell redox status, the activities of SOD, catalase (CAT), and GPX and the content of malondialdehyde (MDA), H_2O_2, and reduced glutathione (GSH) were assayed.

2. Investigations, results and discussion

2.1. Inhibition of human hepatoma cell proliferation

2.1.1. Effect on cell proliferation

The cell doubling time was retarded from 21.6 h in control to 36.1 h in the AA + SS group, and to 33.7 h in the AA 6 mM group. However, the cell doubling time was retarded slightly in both the AA 3 mM group and the SS 1.5 μM group. The cell inhibition was 54.8% in the AA 6 mM group and 63.3% in the AA + SS group, but only 18.9% in the AA 3 mM group and 22.5% in the SS 1.5 μM group (Table 1).

Table 1: Effect of ascorbic acid and sodium selenite on proliferation of human hepatoma cells

	Cell doubling time (h)	$10^5 \times$ Number of cells		Inhibition (%)
		Original	Final	
Control	21.6 ± 1.5	1.8	137 ± 21	
AA (3 mM) (a)	22.5 ± 1.3	1.8	115 ± 15*	18.9
AA (6 mM)	33.7 ± 1.2	1.8	63 ± 6**	54.8
SS (1.5μM) (b)	28.4 ± 1.7	1.8	112 ± 13*	22.5
a + b	36.1 ± 1.5	1.8	57 ± 7**	63.3

n = 3 experiments, each experiment contains 3 cultures (mean ± SD)
* $P < 0.05$, ** $P < 0.01$ *vs* control group

Table 2: Effect of ascorbic acid and sodium selenite treatment for 48 and 72 h on the survival rate of hepatoma cells (total cells = 1000 in each group)

	Dead cells/1000cells		Survival rate (%)	
	48h	72h	48h	72h
Control	20 ± 3	22 ± 4	98.0	97.8
AA (6 mM)	48 ± 7	47 ± 5	95.2	95.3[d]
a + b	62 ± 8	60 ± 7	93.8	94.0[d]

a + b: see Table 1
n = 3 experiments, each experiment contains 3 cultures (mean ± SD)
[d] $P > 0.05$ *vs* control group

2.1.2. Effect on the survival rate of human hepatoma cells

In order to find out whether the inhibitory effect on cell growth was due to cytotoxicity, we evaluated the survival rate of treated cells by the trypan blue dye exclusion method. Although the inhibitory effect on human hepatoma cell proliferation was exacerbated by the AA + SS combination, the survival rate of cells showed no obvious difference between different groups (Table 2). Therefore the inhibitory effect of the AA + SS combination was not due to its cytotoxicity.

2.1.3. Effect on the mitotic index

To study further the nature of inhibition and proliferation, we investigated the mitotic index in various groups. The mitotic index shows that untreated hepatoma cells exhibited vigorous proliferating capability with a division peak on the fifth day. After treatment with AA 6 mM or AA + SS, the mitotic index declined from 5.1 (control) to 2.7% and 2.1% respectively, while after treatment with AA 3 mM or SS 1.5 μM alone, the mitotic index declined to 4.6% and 3.7% respectively, and the division peak in the SS alone, AA alone and AA + SS groups shifted to the fourth day (Fig. 1).

Continuous division and constant multiplication are essential characteristics of a malignant tumor. Therefore, the inhibitory effect on the multiplication of tumor cells is a significant indicator of induced differentiation. The results in Tables 1, 2 and Figure 1 confirm that the AA + SS combination inhibited multiplication of hepatoma cells without exhibiting any cytotoxicity.

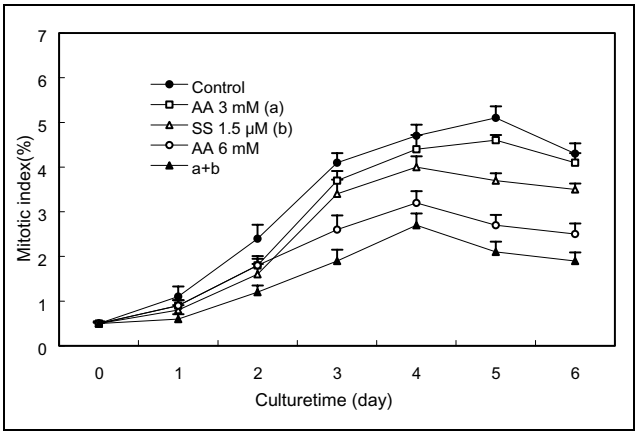

Fig. 1: Effect of ascorbic acid and sodium selenite on the mitotic index of hepatomacytes. Cells were cultured at a density of 1×10^8 cells/ml. The cells were cultured in medium without (●) or with AA 3 mM alone (□), or AA 6 mM alone (○), or SS 1.5 μM alone (△), or AA 3 mM + SS 1.5 μM (a + b) (▲). Means of three parallel experiments. n = 3 experiments × 3 cultures (mean ± SD)

Table 3: Effect of AA and SS on cell electrophoresis rate

	Electrophoresis time (s)	Electrophoresis Rate ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$)	Retardation (%)
Control	9.2 ± 0.34	1.76	
AA (6 mM)	18.2 ± 1.3	1.17**	35.4
a + b	21.4 ± 0.67	0.93**	44.2

a + b: see Table 1
n = 3 experiments × 3 cultures (mean ± SD)
** $P < 0.01$ *vs* control group

2.2. AA and SS alleviate the malignancy of human hepatoma cells

2.2.1. Effect on cell surface charge

The net charges at the surface of tumor cells are in general more than those in corresponding normal cells, thus, cell electrophoresis rate of tumor cells should be higher than that of normal cells. Therefore the decrease in the cell electrophoresis rate has been taken as a measure of tumor cell differentiation [15]. After treatment with AA 6 mM alone or AA 3 mM + SS 1.5 μM (a + b) for 6 days, the electrophoresis rate apparently slowed down. The percentage retardation was as high as 35.4% and 44.2% (Table 3).

2.2.2. Effect on α-FP content and γ-GT activity

The increase of the content of α-FP and the activity of γ-GT are related to hepatocyte malignancy, and they have been taken as markers to distinguish malignant and benign hepatoma [16]. After treatment with AA 6 mM alone or 3 mM + SS 1.5 μM (a + b), both the α-FP content and the γ-GT activity decreased markedly (Table 4).

2.3. AA and SS induce differentiation of human hepatoma cells

2.3.1. Effect on the activity of TAT

The activity of TAT is higher in normal hepatocytes but reduced during carcinomatous degeneration, and the increase of TAT activity has been regarded as an index related to hepatoma cell differentiation [17]. After treatment with AA 6 mM or AA 3 mM + SS 1.5 μM (a + b) the average values of TAT activity significantly increased (Table 4).

2.3.2. Effect on the colonogenic potential

The colonogenic potential (CP) is a general index of cell differentiation, and the CP of culture cells examined in soft agar is a very important marker for distinguishing maligant, benign and normal cells [18, 19]. The colonogenic potential (CP) of treated cells in soft agar decreased sig-

Table 4: Effect of ascorbic acid and sodium selenite on α-FP content, γ-GT and TAT activities of hepatoma cells

	α-FP ($\mu g \cdot g^{-1}$ protein)	γ-GT (unit $\cdot g^{-1}$ protein)	TAT ($\mu mol \cdot g^{-1}$ protein)
Control	341 ± 27	0.76 ± 0.06	14.2 ± 1.1
AA (6 mM)	137 ± 14**	0.15 ± 0.02**	38 ± 2.1**
a + b	92 ± 6**	0.19 ± 0.01**	49 ± 3.2**

a + b: see Table 1
n = 3 experiments × 3 cultures (mean ± SD)
** $P < 0.01$ *vs* control group

Table 5: Effect of ascorbic acid and sodium selenite on colonogenic potential of hepatomacytes

	Number of colonies	Colonogenic potential (%)
Control	278 ± 21	100
AA (6 mM)	$42 \pm 6^{**}$	13.3
a + b	$14 \pm 3^{**}$	4.7

a + b: see Table 1
n = 3 experiments × 3 cultures (mean ± SD)
** $P < 0.01$ *vs* control group

nificantly (Table 5). Thus, AA + SS showed inhibition of cell colony formation.

In short, our results showed that with AA 3 mM + SS 1.5 μM treatment, the malignant characteristics of human hepatoma cells were alleviated, while the properties related to cell normalization were strengthened. All these changes were suggested that the hepatoma cells inclined towards normalization, and confirmed that AA + SS combination has the ability of inducing human hepatoma cell redifferentiation and causing reversion of the cells against the malignant phenotype.

2.4. Influence of AA and SS on the activities of antioxidant enzymes

From the data above, we conclude that hepatoma cells can be successfully induced to redifferentiation by AA + SS. AA and SS are both antioxidants, so we are interested in whether the differentiation of hepatoma cells induced by AA and SS is related to minimizing the generation of ROS directly or through regulating the activities of antioxidant enzymes. The results have shown that the activities of SOD and GPX in treated groups are significantly higher than those in controls, while the activity of CAT is lower (Fig. 2).

2.5. Effect on the contents of H_2O_2, MDA, and GSH

In order to find whether the changes in activities of SOD, CAT and GPX were due to the alteration of the H_2O_2, MDA and GSH contents, we made a further examination. After being treated with AA + SS, the content of MDA decreased slightly, but the difference is not significant. H_2O_2 increased dramatically, and GSH was sharply reduced (Fig. 3).

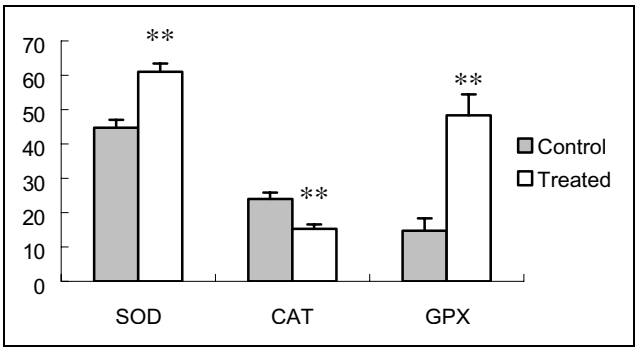

Fig. 2: Effect of ascorbic acid and sodium selenite on the activities of SOD, CAT and GPX in hepatomacytes. Cells were incubated at a density of 1×10^8 cells/ml. The cells were cultured in medium without (control) or with AA 3 mM + SS 1.5 μM (treated). The activity of SOD is expressed by $U \cdot mg^{-1}$ protein, while CAT is $K \cdot 10^4 \cdot mg^{-1}$ protein and GPX is $nmol \cdot min^{-1} \cdot mg^{-1}$ protein. Means of three parallel experiments. n = 3 experiments × 3 cultures (mean ± SD). ** $P < 0.01$ *vs* control group

Reactive oxygen species (ROS), represented by superoxide, hydrogen peroxide and hydroxyl radicals, have been implicated in many diseases including cancer. ROS have been known to play an important role in the initiation and promotion of multistep carcinogenesis. The cellular antioxidants play a crucial role in protection against neoplastic disease. Both AA and SS are well-known antioxidants, and sodium selenite can enhance the activity of glutathione peroxidase (GPX), so their induction of hepatoma cell differentiation may be related to their abilities to regulate the activities of antioxidant enzymes and alter the cell redox status.

The effects of AA and trace element Se on the activities of antioxidant enzymes and lipid peroxide levels in chicken erythrocytes have been investigated. SOD activity in the AA group was increased by 20 per cent. GPX activity in the Se group was raised by 35 per cent. Also, GPX activity in the AA group was increased by 33 per cent [20]. An increase in GPX activities was also found in the Se and AA alone groups, while CAT activities in the liver and heart of the AA group were significantly decreased (by 32%) [21]. Another study has shown that the induction of SOD activity can led to cell differentiation [22]. All these are consistent with our results. Another study showed that GPX activity rises during *in vitro*-induced monocytic or granulocytic differentiation of myeloid cell lines and that the increased expression of the cellular GPX gene occured through complex mechanisms that include transcriptional up-regulation [23].

In vitro treatment of PC3 and MLL cells with sodium ascorbate (0–10 mM) resulted in a decrease in cell viability and thymidine incorporation into DNA [25]. Ascorbate induced these changes through the production of hydrogen peroxide since the addition of catalase (100–300 units/ml), an enzyme that degrades hydrogen peroxide, inhibited the effects of ascorbate on these cell lines. In contrast, superoxide dismutase, an enzyme that dismutates superoxide and generates hydrogen peroxide did not prevent ascorbate-induced changes, emphasizing the involvement of ROS in cellular damage. Singlet oxygen scavengers such as sodium azide and hydroquinone, and hydroxyl radical scavengers such as D-mannitol and DL-alpha-tocopherol did not counteract the effects of ascorbate on thymidine incorporation. We also have observed an increase in H_2O_2 in our studies (Fig. 3). The results suggest that AA in-

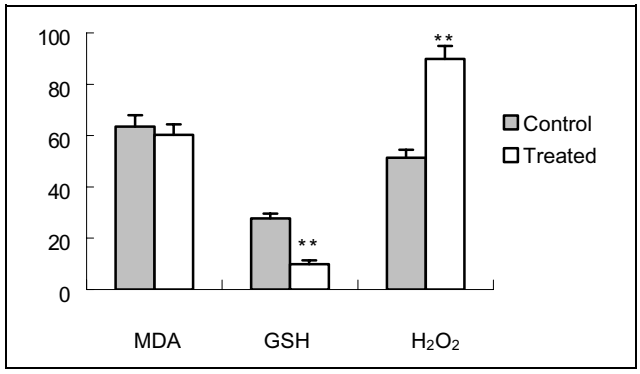

Fig. 3: Effect of ascorbic acid and sodium selenite on the contents of MDA, GSH and H_2O_2 in hepatomacytes. Cells were incubated at a density of 1×10^8 cells/ml. The cells were cultured in medium without (control) or with AA 3 mM + SS 1.5 μM (treated). The content of MDA is expressed as $ng \cdot mg^{-1}$ protein, while GSH is expressed $10 \times nmol \cdot mg^{-1}$ protein and H_2O_2 is expressed $\mu mol \cdot mg^{-1}$ protein. Means of three parallel experiments. n = 3 experiments × 3 cultures (mean ± SD). ** $P < 0.01$ *vs* control group

hibits tumor growth and induces human hepatoma cell differentiation by virtue of producing reactive oxygen species.

Glutathione is the major redox buffer in the secretory pathway [26], and reduced glutathione, a natural thiol antioxidant, maintains the redox potential and thus protects against oxidative damage to the cells [27]. During cell differentiation induced by AA + SS, the activities of SOD and GPX and the amount of H_2O_2 increased, while the activity of CAT and the amount of GSH decreased, thus leading to redox changed. These results indicate that redox status must be involved in cell differentiation.

Lee et al. [28] recently determined that AA induces lipid hydroperoxide decomposition to the DNA-reactive bifunctional electrophiles 4-oxo-2-nonenal, 4,5-epoxy-2 (*E*)-decenal, and 4-hydroxy-2-nonenal. The compound 4,5-epoxy-2 (*E*)-decenal is a precursor of etheno-2′-deoxyadenosine, a highly mutagenic lesion found in human DNA. At low concentrations of AA, the major products were *trans*-4,5-epoxy-2 (*E*)-decenal, 4-hydroperoxy-2-nonenal, and 4-oxo-2-nonenal. As the concentration of AA increased, the amount of 4-hydroperoxy-2-nonenal decreased, with a concomitant increase in *trans*- and *cis*-4,5-epoxy-2 (*E*)-decenal, 4-oxo-2-nonenal, and 4-hydroxy-2-nonenal. Maximal yields of 4-oxo-2-nonenal, 4,5-epoxy-2(*E*)-decenal, and 4-hydroxy-2-nonenal were obtained with an excess of AA [28]. Thus, a relatively low dose of AA should be used for preventing and curing cancers.

AA-mediated formation of genotoxins from lipid hydroperoxides in the absence of transition metal ions could help explain why AA has not demonstrated substantial efficacy in cancer chemoprevention trials [29]. The finding that AA generates bifunctional electrophiles explains why hydroperoxide-dependent lipid peroxidation is enhanced by AA *in vitro* [30].

Although as an important antioxidant, AA has many physiological functions, it also has potential risk of DNA damage, so we must find a way to treat cancers with a relatively low dose of AA.

These results suggest that the combination of AA and SS may be a potent anticancer treatment option for human hepatoma cells.

3. Experimental

3.1. Reagents

RPMI-1640 was purchased from Gibco Laboratories (Santa Clara, CA). Bovine serum was obtained from Si-Ji-Qing Biotechnology Co(Hangzhou, China). Trypsin was obtained from Sigma (St Louis, MO, USA). All other reagents were analytical reagent grade.

3.2. Cell culture

Human hepatoma cell line BEL-7402 was set up by the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were grown in RPMI-1640 medium containing 10% inactivated bovine serum, streptomycin (100 μg/ml), penicillin (100 units/ml), and $NaHCO_3$ 2.0 g/l, and were maintained at 37 °C in a humidified atmosphere of 5% CO_2. After the cells (1×10^8 cells/ml) were cultured for 24 h, the culture medium was aspirated and replaced with culture medium containing AA 3 or 6 mM alone, or sodium selenite 1.5 μM alone, or AA 3 mM combined with sodium selenite 1.5 μM. After adding AA to the medium, the pH was adjusted to 7.1 with 1 N NaOH.

3.3. Determinations of cell growth curve and mitotic index

The viable cells were counted every day in the first 8 days by the trypan blue dye exclusion method. The cell doubling time is calculated as follows: $T_D = 0.693(T_2-T_1)/\ln(N_2/N_1)$, where T_D is the cell doubling time from T_1 to T_2, and N_1 and N_2 are the cell numbers at T_1 and T_2 [31]. Three cover slips, which cells cultured on, were fixed in Bouin-Hollonde solution and stained with hematoxylin-eosin. The mitotic cells from 1000 cells were counted every day. Mitotic index is defined here as the fraction of hepatoma cells in metaphase, anaphase, or telophase.

3.4. Cell electrophoresis

The cells were collected and washed with D-Hanks' solution twice, then resuspended at a density of 1×10^9 cells/l. The cell electrophoresis determination was performed with a round plastic tube electric-bridge filled with NaCl 10%-agar 1% and Ag−AgCl electrodes at a direct current voltage of 40 V, at room temperature of 24 °C, using sucrose 9% as the electrophoretic medium. The results were expressed as the average time (s) during which a cell moves over a distance of 120 μm and 40 cells in each group were determined. The experiments were repeated three times with similar results. Thus, the result of one experiment will be used as the criterion.

3.5. Assays for α-FP and γ-GT

Cell suspension (5×10^8 cells) 0.2 ml was transferred into 0.3 ml of ice-cold lysis buffer containing Tris 5 mM, edetic acid 20 mM and Triton X−100 0.5% (vol/vol), pH 8.0, and left to lyse on ice for 30 min before centrifugation at 3000 × g for 10 min. The α-FP in the supernatant was determined by α-FP reagent kit (Biological Reagent Research Institute, Lanzhou, China) using ELISA. γ-GT was determined by γ-GT reagent kit (Chemical Reagent Research Institute, Lanzhou, China). Protein content was measured with Folin phenol reagent by Lowry's method.

3.6. Assay for TAT

TAT activity in whole cells was detected by the method of Diamondstone [32]. Cell suspension 0.2 ml (in KCl 0.14 mM,1×10^8 cells/ml) was transferred into 0.3 ml of ice-cold lysis buffer as described above, and samples were allowed to lyse at 0 °C, before centrifugation at presumably 31000 × g for 30 min. *p*-Hydroxybenzaldehyde 1 μmol produced in the reaction system at 30 °C for 30 min was defined as 1 unit of TAT.

3.7. Colonogenic assay

After 48-h treatment, cells were washed with RPMI-1640 medium containing 10% heat-inactivated bovine serum. Cell counts were performed by hemocytometer, and viable cells were assayed by the trypan blue exclusion method. The cells were plated in a 24-well culture plate in a double layer nutrient agar system [33]. The medium was RPMI-1640 with a final concentration of 10% bovine serum. Cells were routinely plated at a concentration of 6×10^6 cells/ml (0.5 ml for each well in 24-well culture plate). The plates were incubated for 21 days at 37 °C in a humidified air with 5% CO_2. A colony was defined to be an aggregate of > 50 cells [34].

3.8. Assays for SOD, GPX and CAT

Cellular lysates were prepared by sonication of scraped cells in phosphate-buffered saline (PBS), pH 7.4, containing 0.05% deoxycholate. An aliquot of cell lysate was used for protein estimation. CAT activity was measured by the method of Aebi [35] with some modification [36]. SOD activity was measured according to Sun and Zigman [37]. GPX activity was measured by the method of Flohe and Gunzler [38]. Protein content was measured with Folin phenol reagent by Lowry's method, with BSA as a standard.

3.9. Assays for the content of MDA and H_2O_2

Cell lipid peroxides were determined by measuring malondialdehyde (MDA) formation according to Wong et al. [39]. H_2O_2 in cells was determined fluorimetrically on 0.15 ml aliquots of the supernatant, using 25 μg *p*-hydroxyphenyl acetate (PHPA) and 80 μg horseradish peroxidase (HRP)[40] with excitation and emission wavelengths of 300 and 420 nm. The nonfluorescent substrate PHPA was oxidized to the stable fluorescent product, tentatively named as 2,2′-dihydroxybiphenyl-5,5′-diacetate [$(PHPA)_2$], via the enzymatic reduction of H_2O_2 by horseradish peroxidase (HRP).

3.10. Assay for intracellular reduced glutathione (GSH)

GSH was assessed with DTNB (5,5′-dithio-bis(2-nitrobenzoic)) based on the method described by Jocelyn [41]. The cells (1×10^8/ml) were sonicated for 30 s in 300 μl 5% 5-sulfosalicylic acid and centrifuged for 10 min at 1000 × g. The resultant acid thiol extract was assayed for non-protein sulfhydryls by quantifying the reduction of DTNB through its conversion to 5-thiol−2-nitrobenzoic acid (TNB) at 412 nm using a spectrophotometer. Sample values were then calculated from a standard curve generated with known amounts of GSH.

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