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Cytotoxic Effect of Adriamycin in Cultured Skin Cells of Fetal Rat

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ABSTRACT

Local extravasation during intravenous administration of adriamycin (doxorubicin HCl) can cause severe skin ulceration and necrosis. To investigate the mechanism of adriamycin-induced skin toxicity, effects of adriamycin on reactive oxygen radical metabolism using cultured skin cells of fetal rat. Adriamycin produced significant release of lactic dehydrogenase from cultured skin cell preparations dose- and time-dependently. The production of superoxide anion in sonicated suspensions of cultured skin cells was significantly increased by adriamycin under the presence of NADPH and NADH. The drug also stimulated malondialdehyde (MDA) production, an index of lipid peroxidation, in NADPH- and NADH-supported cell preparations. The increased production of MDA was significantly inhibited by oxygen radical scavengers (superoxide dismutase, catalase, thiourea) and antioxidants (butylated hydroxytoluene, α-tocopherol). Treatment of cultured skin cells with 1, 3,-bis (2-chloroethyl)-1-nitrosourea (BCNU), an inhibitor of glutathione reductase, enhanced the lipid peroxidation induced by adriamycin. The present study suggests that lipid peroxidation which is resulted from the stimulated production of reactive oxygen radical causes cellular damage in adriamycin-treated skin cells of rat.

Key Words: Adriamycin, Skin toxicity, Cultured skin cell, Oxygen free radical, Lipid peroxidation

INTRODUCTION

Adriamycin (Doxorubicin HCl) is an anthracycline anticancer chemotherapeutic agent which is widely used against variety of malignancies. The drug intercalates with cellular DNA to destroy helical structure and thus interferes with the processes of DNA replication and RNA synthesis (Blum & Carter, 1974; Tewey *et al.*, 1984). The clinical use of adriamycin is limited by its toxicity to normal cells. The most serious one is a cumulative dose-dependent cardiac toxicity. Complex acute and chronic cardiovascular changes which develop ultimately into serious heart failure have been described in patients with adraimycin therapy (Bossa *et al.*, 1978; Kim *et al.*, 1989; Prage *et al.*, 1979). The most prominent and consistent ultrastructural alterations observed in the toxicity are loss of myocardial fibrils, damages of sarcoplasmic reticulum and mitochondria (Lefrak *et al.*, 1973; Singal *et al.*, 1987).

Adriamycin gives damages to the normal skin tissue too. Local extravasation during parenteral administration of adriamycin is associated with major morbidity and has become an increasingly common complication occuring in 1 to 6 percent of patients (Olver & Schwarz, 1983). The extravasated area undergoes an evolution of tissue destruction. An erythematous reaction develops immediately and frequently progresses to necrosis and ulcer formation. The local reaction is proportional to the amount and extent of the ex-

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travasation into the dermis and subcutaneous tissue. The progression of the ulcer is indolent and often requires weeks to establish the limits of necrosis in untreated (Bower & Lynch, 1978; Olver & Schwarz, 1983; Snyderman & Krasna, 1986). Many attempts to treat the adriamycin-induced skin injuries with pharmacological agents have been proposed, but with no consistent beneficial effects. Surgical therapy, therefore, still remains the mainstay of teatment for this condition. The key successful surgical treatment is early excision of involved tissues with wide margins and some form of skin grafting or covering (Larson *et al.*, 1985; Linder *et al.*, 1983).

The mechanism of adriamycin skin toxicity has not been firmly established yet. In in vitro studies, quinone-containing adriamycin has been shown to from oxygen free radicals during its metabolism undergoing reduction-oxidation cycle (Trush *et al.*, 1982). The formation of reactive oxygen radicals and subsequent lipid peroxidation of cellular membranous structures are well documented in adriamycin-induced cardiac toxicity (Bachur *et al.*, 1977; Doroshow, 1983; Mimnaugh *et al.*, 1985). Considering this oxygen radical-mediated mechanism in the adriamycin cardiac toxicity, it may be possible that the adriamycin-induced cellular damages in skin tissues are also associated with similar actions involving oxygen free radicals. In an attempt to elucidate this possibility, we investigated the mechanism of adriamycin-induced skin toxicity, and measured effect of the drug on oxygen radical production and lipid peroxidation in vitro systems using cultured skin cells of fetal rat.

MATERIALS AND METHODS

Skin cell culture

CRL 1213 skin cell line isolated from Sprague-Dawley fetal rat at 18-day of gestation period was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). The cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 15 mM N-2-hydroxyethyl piperazine N'-2-ethane sulfonic acid (HEPES), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. They were plated on the plastic 75 cm^2-culture flasks or 6-well culture plates (Falcon, New Jersey, U.S.A.) at a concentration of 2×10^4 cells/cm^2 and incubated in a humidified CO_2 incubator (5% CO_2: 95% air) maintained at constant temperature of 37°C (Forma Scientific, OH, U.S.A.).

Adriamycin-induced skin cell toxicity

The degree of lactic dehydrogenase (LDH) release from the cultured cells was measured as an index of cellular toxicity. Confluent monolayers of cells in the 6-well plates were washed three times with minimum essential medium (MEM) containing 15 mM HEPES, but without FBS and antibiotics. One ml of the MEM with adriamycin (10~100 μM) was added to each well. After 24~48 hr incubation in CO_2 incubator at 37°C, a 0.5 ml aliquot of supernatant medium was sampled from each well to measure the activity of LDH leaked from the cells. For determining the total LDH activity, the remaining cells were treated with 0.1% Triton X-100 for 30 min to cause complete leakage of the enzyme from the cells. The degree of cell injury was assessed by the percent activity of the released LDH in the supernatant medium to the total LDH. The activity of LDH was assayed by UV-spectrophotometric method (Bergmeyer & Bernt, 1974). A 0.1 ml aliquot of sample was added into a cuvette containing 2 ml of reaction mixture consisted of 50 mM Na-phosphate buffer (pH 7.5), 0.67 mM Na pyruvate and 0.24 mM NADH. The rate of absorbance change was measured with UV-spectrophotometer (Hewlett-Packard, 8452A) at 340 nm and 25°C.

Lipid peroxidation

The degree of lipid peroxidation in cultured skin cell was estimated from the malon-dialdehyde (MDA) concnetration measured by thiobarbituric acid method (Bidlack & Tappel, 1973). The confluent monolayer of cells in 75 cm^2-flask was washed three times with Hank's balanced salt solution (HBSS). The cells were scraped in 1 ml of 150 mM KCl-50 mM Tris HCl (pH 7.4) with a rubber policeman and were then sonicated for 30 sec (Heat Systems-Ultrasonics, W-385). The sonic disrupted cell suspension was incubated in a reaction mixture containing 50 mM Tris HCl (pH 7.4), 150 mM KCl, 0.58 mM NADPH, 0.58 mM NADH and 10~100 μM adriamycin for 0.1~

1 hr under the environment of 100% O_2 and 37°C. The incubated mixture was mixed with equal volume of 0.75% thiobarbituric acid (TBA)-15% trichloroacetic acid and then was placed on boiling water bath for 30 min. After cooling to room temperature, the pink-colored TBA reacting substance (MDA) was extracted with n-butanol. The absorbance of the extract was recorded at 535 nm with a spectrophotometer (Hewlett-Packard, 8452-A). The MDA concentration was calculated as nmoles/mg prot by using the molar extinction coefficient of 1.52×10^5/M/cm (Placer *et al.*, 1966). The protein concentration was determined by the method of Bradford (1976).

Effcts of oxygen radical scavengers and antioxidants on adriamycin-induced lipid peroxidation were observed. The scavengers and antioxidants studied were superoxide dismutase (SOD, 100 μM), catalase (10 μM), thiourea (50 mM), butylated hydroxytoluene (BHT, 50 μM), α-tocopherol (500 μM). The drugs were added to the sonic disrupted cell suspension and were incubated in the same way. In addition, the effect of a glutathione reductase inhibitor, 1, 3,-bis (2-chlorlethyl)-1-nitrosourea (BCNU, 75 μM), was also examined. BCNU was added to the culture flask from 1 hr prior to scraping cells.

Assay of superoxide anion

The production of superoxide anion was observed by measuring the SOD-inhibitable oxidation of epinephrine to adrenochrome (Misra & fridovich, 1972). Cultured skin cells in 75 cm^2-flasks were washed, scraped and disrupted by sonication in the same way as in the assay of lipid peroxidation. A 0.1 ml aliquot of sonic disruped cell suspension (2~4 mg prot/ml) was added to a cuvette containing 2 ml of reaction mixture consisted of 50 mM K-phosphate buffer (pH 7.4), 150 mM KCl, 0.5 mM EDTA, 0.25 mM NADPH, 0.25 mM NADH, 1 mM epinephrine and 10~200 μM adriamycin. The absorbance change was recorded at a wavelength pair of 485/575 nm with a dual-wavelength spectrophotometer (Hitachi, Model 557). The rate of adrenochrome formation under the presence or the absence of SOD (1 μM) was calculated by using molar extinction coefficient of 2,860/M/cm.

RESULTS

Adriamycin toxicity to cultured skin cell

The LDH leagkage from the cultured skin cells was increased by adriamycin concentration-and time-dependently. During 24 hrs of incubation, LDH relased from the control cells was 15.1±2.3 % of the total enzyme. In the cells treated with 10 ~100 μM of adriamycin, the LDH release was increased by 2~4 time of control value during the same incubation period. Prolonged incubation of the cells for 48 hr increased the enzyme release upto 82% of the total, irrespective of adriamycin concentration applied (Fig. 1). The concentration and time-dependent LDH release indicated that adriamycin causes cellular injury directly to the cultured skin cells of fetal rat in this experimental condition.

Adriamycin-induced superoxide readical production

The production of superoxide radical was estimated by measuring the adrenochrome formation from epinephrine. Adriamycin increased the adrenochrome formation from epinephrine in sonic disrupted cell suspensions. The adriamycin-induced adrenochrome formation was almost completely inhibited by SOD. The SOD-inhi-bitable portion of adrenochrome formation was increased by adriamycin treatment dose dependently. The additions of 10~200 μM adriamycin induced the adrenochrome formation 2~6 times higher than that of the control (0.97 nmole/mg/min) (Fig. 2). The adriamycin-induced formation of adrenochrome was dependent on the presence of NADH and NADPH. NADH caused to increase the rate of adrenochrome formation more rapidly than NADPH (Fig. 3). These results strongly suggest that adriamycin produces oxygen free radical in cultured skin cells of fetal rat.

Adriamycin-induced lipid peroxidation

Adriamycin caused a increase in the lipid peroxidation of cultured skin cells of fetal rat. In the cells treated with 100 μM adriamycin, MDA production was increased by 40% (13.6±1.0 nmole/mg prot/hr) compared to the control value (9.7±0.2 nmole/mg prot/hr) (Fig. 4). The

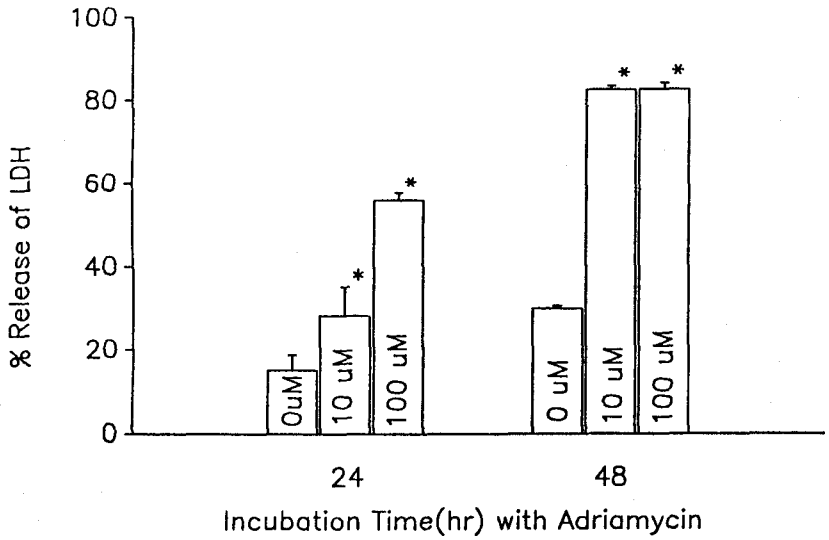

Fig. 1. Adriamycin-induced LDH release from cultured skin cells of rat. Cells were plated onto 6-well plates at a concentration of 3.5×10^5 cells/dish in MEM, and incubated with 10 μM and 100 μM of adriamycin at 37°C in a CO_2 incubator(5 % CO_2-95 % air) for the indicated times. Lactic dehydrogenase(LDH) activity was determied enzymatically from both culture medium and cells as described in 'Materials and Methods'. Figures in the columns indicate concentrations of adriamycin added in the culture well. Mean±S.E. of 5-9 experiments.
*$p<0.01$ vs 0 μM.

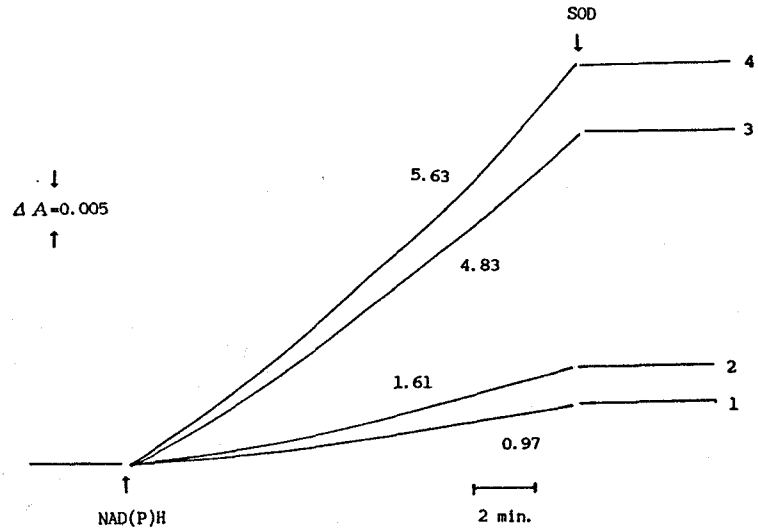

Fig. 2. Effect of adriamycin on SOD-inhibitable adrenochrome formation in cultured skin cells of rat. Cells harvested from the culture flasks were disrupted by ultra-sonication for 30 sec. Sonicated cell preparation(0.2 mg prot/ml) was incubated in a cuvette containing 50 mM K-phosphate buffer(pH 7.4), 150 mM KCl, 0.5 mM EDTA, 1 mM epinephrine and 100~200 μM adriamycin(ADR). Starting with additions of 0.25 mM each of NADPH, NADH, changes in absorbance was recorded by Dual-wavelength spectrophotometer at 485/575 nm. Superoxide dismutase(SOD, 1 μM) was added at indicated arrow. Numbers on the tracings reprensent the rate of SOD-inhibitable adrenochrome formation in nmole/mg prot/min.
1: ADR. 0, 2: ADR. 10 μM, 3: ADR. 100 μM, 4: ADR. 200 μM.

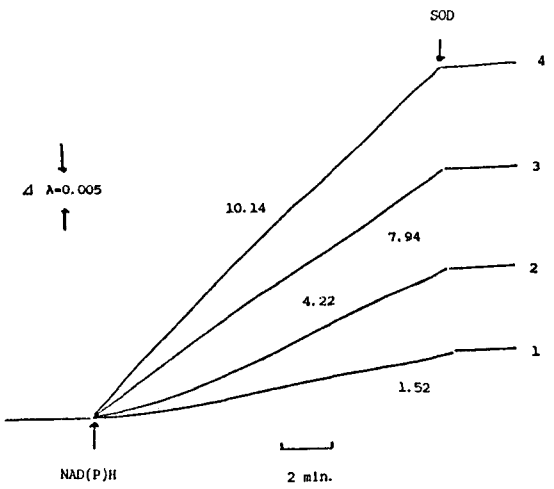

Fig. 3. Differential effects of NADPH and NADH on adriamycin-induced SOD-inhibitable adrenochrome formation in cultured skin cells of rat. Conditions and methods of cell preparation, incubation and measurement of SOD-inhi-bitable adrenochrome formation were same as in Fig. 2. Adriamycin(ADR) was contained in the reaction medium at a concentration of 100 μM. NADPH or NADH was added with a concentration of 0.5 mM each at the first arrow, and SOD(1 μM) at the second arrow. Protein concentration in the reaction mixture was 0.1 mg/ml. Numbers on the tracings represent the rate of SOD-inhibitable adrenochrome formation in nomle/mg prot/min.
1: ADR.−, NAD(P)H,−, 2: ADR.+, NAD(P)H.−, 3:ADR.+, NADH.+, 4: ADR.+, NADPH.+.

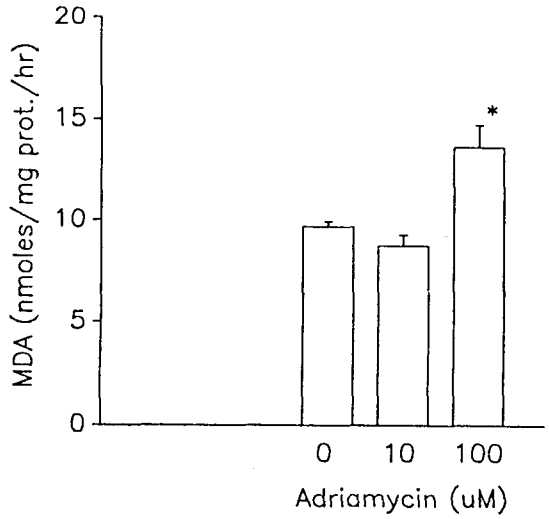

Fig. 4. Adriamycin-induced lipid peroxidation in cultures skin cells of rat. Cells harvested from the culture flasks were disrupted by ultrasonication for 30 sec in 150 mM KCl, 50 mM Tris(pH 7.4). Sonicated cell suspensions were incubated with adriamycin at 37°C for 1 hr under the environment of 100% O_2. Malondialdehyde(MDA) was measured by thiobarbituric acid as described in Materials and Methods. Figures in the columns indicate concentrations of adriamycin in the reaction medium.
Mean±S.E. of 4~5 experiments.
* $p<0.05$ VS 0 μM.

Table 1. Effects of oxygen radical scavengers on adriamycin-induced lipid peroxidation in cultured skin cells of rat

Condition	MDA* (nmoles/mg prot/hr)	% inhibition
Control(ADR, 100 μM)[1]	13.63±1.04	−
+ SOD(100 μM)[2]	8.08±0.92**	−40.7
+ Catalase(10 μM)	3.98±0.31**	−70.8
+ Thiourea(50 mM)	4.43±1.04**	−67.5

1: Sonicated cell suspensions were incubated with 100 μM adriamycin(ADR) as described in the method of Fig. 4.
2: SOD: superoxide dismutase
* Malondialdehyde(MDA) was measured by thiobarbituric acid method as described in Methods. Mean±S.E. of 4~5 experiments
** $p<0.05$ vs Control.

adriamycin-induced MDA production was significantly suppressed by oxygen radical scavengers, SOD (100 μM), catalase (10 μM) and thiourea (50 mM) (Table 1), and also by antioxidants (BHT, 50 μM; α-tocopherol, 500 μM) (Table 2). In addition, the pretreatment of BCNU (1, 3-bis-(2-

Table 2. Effects of antioxidants on adriamycin-induced lipid peroxidation in cultured skin cells of rat

Condition	MDA* (nmoles/mg prot/hr)	% inhibition
Control(ADR, 100 μM)[1]	13.63±1.04	−
+ BHT(50 μM)[2]	6.53±0.68**	−52.1
+ α-Tocopherol(500 μM)	10.06±0.91**	−26.3

1: Sonicated cell suspensions were incubated with 100 μM adriamycin(ADR)
2: BHT: butylated hydroxytoluene
* Malondialdehyde(MDA) was measured by thiobarbituric acid method as described in Methods. Mean±S.E. of 4~5 experiments
** $p<0.05$ vs Control.

Table 3. Effect of BCNU on adriamycin-induced lipid peroxidation in cultured skin cells of rat[1]

Conditions		MDA*(nmoles/mg prot/30 min)	% stimulation
BCNU(75 μM)	ADR(100 μM)		
−(Control)	−	4.85±0.12	−
−	+	6.82±0.52**	+40.6
+	−	7.03±1.16**	+44.9
+	+	8.62±0.92**	+77.7

1: Skin cells pretreated with or without 75 μM 1.3-bis(2-chloroethyl)-1-nitrosourea(BCNU) for 1 hr at 37°C were disrupted by ultra-sonication for 30 sec. The sonicated cell suspensions were incubated with or without 100 μM adriamycin(ADR) for 30 min at 37°C as described in Materials and Methods.
* Malondialdehyde(MDA) was measured by thiobarbituric acid method. Mean±S.E. of 4~5 experiments.
** $p<0.05$ Vs Control

Table 4. Effect of antioxidant on adriamycin-induced lipid peroxidation in BCNU-treated cultured skin cells of rat[1]

Conditions			MDA*(nmoles/mg prot/30 min)	% inhibition
BCNU(75 μM)	ADR(100 μM)	Antioxidant		
+	+	−(control)	8.62±0.92	−
+	+	+ BHT[2]	6.69±1.36**	−22.4
+	+	+ α-Tocop[3]	6.53±0.58**	−24.2

1. BCNU(1.3-bis(2-choroethyl)-1-nitrosourea treatment and adriamycin(ADR) incubation of skin cells were as same as in Table 3.
2. BHT: butylated hydroxytoluene, 50 μM
3: α-Tocopherol, 500 μM
*: Malondialdehyde(MDA) was determined by thiobarbituric acid. Mean±S.E. M of 5 experiments
**: $p<0.01$ VS Control.

chlorethyl)-1-nitrosourea), which inhibits glutathione reductase, stimulated the production of MDA in the control as well as in the adriamycin-treated cells (Table 3). The BCNU-stimulated MDA production was also reduced by antioxidants (Table 4).

DISCUSSION

Among the drugs undergoing an oxidation-reduction cycle, there are many compounds of which intermediate metabolites are free radicals, which exert the wanted pharmacological actions or the unwanted side effects. Of these radical forms of intermediates, oxygen free radicals are very important in their biological significances (Pryor *et al.*, 1976; Trush *et al.*, 1982). Adriamycin is a quinone-containing tetracylic aglycone to which an amino sugar is attached through a glycoside linkage. In in vitro studies, the quinone moieties of adriamycin have been shown to form semiquinone free radical intermediates in the presence of flavin enzymes. Under aerobic conditions, this semiquinone free radical can readily donate its unpaired electron to moleuclar oxygen so as to form oxygen free radicals (Trush *et al.*, 1982). Bachur *et al.* (1977, 1979), Doroshow & Daives (1983) and Kim *et al.* (1989) have demonstrated that adriamycin stimulates NADPH-and NADH-dependent formation of oxygen radicals in cardiac microsomes and mitochondria. They have identified that the NADPH: cytochrome P450 reductase of sarcoplasmic reticulum and the NADH dehydrogenase complex of mitochondria are capable of activating adriamycin to free radical intermediates in the heart cell. Similarly in the present study, adriamycin stimulated the rate of superoxide anion production in the sonic disrupted cultured skin cell preparations supplemented with either NADPH or NADH. This was in accordance with the reports in cardiac cells and was indicative that adriamycin could produce oxygn radicals intracellularly in skin cells by the actions of NADPH-and NADH-dependent enzyme systems.

The unpaired electron of oxygen free radical makes the radical highly reactive, ready to donate an electron or abstract one hydrogen atom from the methylene groups of polyunsaturated fatty acids which are abundunt in membrane phospholipids. This event can initiate and propagate a chain reaction in which there is formation of organic lipid peroxides and their intermediates (Halliwell & Gutteridge, 1989). The accumulations of lipid peroxides and the intermediates introduce hydrophilic moieties into the membrane hydrophobic phase and thus alter membrane permeability and cellular functions leading to eventual irreversible cell death (Freeman & Crapo, 1982). In the present study, the increased release of intracellular LDH, which represents with increased membrane permeability and damage was demonstrated in the skin cells incubated with adriamycin. Some of the identified intermediates of the lipid peroxidation process include peroxide radicals, conjugated dienes and malondialehyde (MDA). the biochemical determination of MDA has been used as an indicator of the lipid peroxidation induced by oxygen radical. In the previous studies with cardiac cells and intracellular membranous organelles, adriamycin stimulated the MDA production under the presence of NADPH and NADH (Kim *et al.*, 1989; Mimnaugh *et al.*, 1981, 1985). Similar to these studies, we observed the increased MDA production by adriamycin in cultured skin cell preparations incubated with NADPH or NADH. This increased production was inhibited by oxygen radical scavengers, SOD, catalase, thiourea and antioxidants, α-tocopherol and BHT. This result seems to demonstrate that adriamycin-induced oxygen radical cause membrane lipid peroxidation which provokes structural and functional derangements in the skin cells. This was also in accordance with the earlier reports that oxygen radical scavengers and antioxidants had some beneficial effects in preventing the adriamycin-induced skin necorsis in experimental animal (Sevigen *et al.*, 1981; Upton *et al.*, 1986).

Enzymatic and non-enzymatic antioxidative defensive systems against oxygen radicals are existed endogenously. In spite of increased oxygen radical production, if cellular defensive systems are intact, the radicals may not exert any harmful effects upon the cells. Glutathione peroxidase-reductase system is an enzymatic mechanism which degrades H_2O_2 and organic peroxides (Halliwell & Gutteridge, 1989). It was demonstrated in the liver cells that the inhibition of this enzyme system stimulated the lipid

peroxidation in normal as well as in adriamycin-treated cells (Bobson, 1981; Meredith & Reed, 1983). In the present study, the pretreatment of BCNU, which inhibits glutathione reductase, also promoted the lipid peroxidation both in normal and adriamycin-treated skin cells of rat. This result suggests that the reduction of endogenous defensive systems against oxygen radicals may also contribute to the development of adriamycin induced skin toxicity.

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= 국문초록 =

백서 태자의 배양 피부세포에서 Adriamycin의 세포독성에 관한 연구

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Adriamycin (Doxorubicin HCl)의 혈관밖 유출에 따른 조직의 손상, 특히 피부괴양 및 괴사 기전을 규명하기 위한 연구의 일환으로 흰쥐 피부세포를 이용한 in vitro 실험에서 adriamycin에 의한 산소라디칼 생성 및 그와 관련된 세포독성 기전으로 지질과산화를 검토하였다. Adriamycin은 흰쥐 태자 피부의 배양세포에서 lactic dehydrogenase(LDH) 유리를 용량 및 시간 의존적으로 증가 시켰으며, NADPH 및 NADH 첨가 조건에서 superoxide anion($O_2^{-\bullet}$)생성을 현저히 증가시켰다. Adriamycin은 지질과산화 반응의 척도인 malondialdehyde(MDA) 생성을 역시 NADPH, NADH 존재하에서 용량의존적으로 증가시켰고, 산소라디칼 제거물질들인 superoxide dismutase (SOD), catalase 및 thiourea와 항산화물질인 butylated hydroxytoluene(BHT), α-tocopherol은 MDA 생성증가를 현저히 억제하였다. 1, 3,-bis(2-chloroethyl)-1-nitrosourea(BCNU)를 처리하여 산화성 공격에 대한 방어기전의 하나인 glutahione 체계를 억제할 경우 adriamycin에 의한 MDA 생성은 더욱 현저히 증가하였고, 이는 역시 항산화 물질들에 의하여 억제되었다.

이상의 연구성적에서 adriamycin은 산소라디칼 생성의 증가와 그에 따른 지질과산화를 촉진하므로서 피부세포에 손상을 줄 것으로 사료되었다.