A Study of a Selection of Antidotes for Paraquat-induced Skin Damage

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***Background*: Paraquat is a widely used herbicide, known to cause lethal toxicity in humans. Most studies about paraquat have concentrated on systemic toxicity, however several cases of paraquat-induced dermatitis have been reported.**

***Objective*: The purpose of this study was to confirm the cutaneous toxic effect of paraquat and to select potential antidotes in paraquat-induced dermatitis.**

***Methods*: Keratinocyte toxicity due to paraquat and the toxicity reduction capacity of several drugs were investigated *in vitro*. Topical effects of these drugs on paraquat-induced dermatitis in guinea pig skin was also investigated.**

***Results*: Over 50% of keratinocytes failed to survive at a concentration of $2X10^{-4}M$ paraquat by a neutral red uptake assay. Skin irritation by paraquat was observed at 2% concentration by non-invasive methods as well as a skin biopsy. Dexamethasone, glutathione and tocopherol showed some capacity to reduce paraquat-induced keratinocyte toxicity *in vitro*. Only dexamethasone, however, showed a reduction of cutaneous blood flow volume and dermal inflammatory cell infiltration in the guinea pig study.**

***Conclusion*: This result indicates the possible *in vitro* protective effect of paraquat toxicity in glutathione and tocopherol. Dexamethasone was capable of reducing paraquat-induced cytotoxicity and dermatitis both *in vitro* and *in vivo*. (Ann Dermatol 10:(1) 13~19, 1998).**

Key Words : Animal study, Antidotes, Keratinocyte, Paraquat, Skin irritation

Paraquat is a herbicide widely used in the agricultural industry to aid in the harvesting of a wide variety of crops. Despite its usefulness, paraquat is a well-known pulmonary toxicant and approximately 600 deaths had been reported globally in the literatures by 1977[1]. The major cause of systemic toxicity leading to death is accidental or intentional oral ingestion. It is generally believed that paraquat is safe when used correctly. However, systemic poisoning resulting from cutaneous exposure has been reported[2]. In a field study, many paraquat-spraying agricultural workers suffered from associated skin rash[3]. In view of the widespread use of paraquat and the poor report system in developing countries, paraquat-induced dermatitis may be much more frequent than generally believed. Even though percutaneous absorption of paraquat through intact skin is poor, it may easily be absorbed through diseased skin[2]. Thus, it is important to recognize and treat paraquat-induced dermatitis.

No specific antidote against paraquat-toxicity has been available currently[4]. Some compounds, however, appear to show promising results in experimental animals. Most potential antidotes have been directed toward compounds that detoxify the superoxide radical or the subsequently formed toxic reactants, and have included tocopherol, glutathione, carotene, superoxide dismutase, ascorbic

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acid, desferrioxamine, selenium, clofibrate, N-acetylcysteine, riboflavin and niacin[5].

In this study, keratinocyte toxicity due to paraquat and the toxicity reducing capacity of several antidotes (dexamethasone, tocopherol, glutathione and carotene) were investigated. Whether the application of these antidotes lowers the degree of inflammation of paraquat-induced dermatitis in guinea pig skin was also investigated.

MATERIALS AND METHODS

In vitro paraquat cytotoxicity

Normal human keratinocytes, derived from neonatal foreskins and cultured for several passages as previously described[6], were used as target cells. Prior to use in an assay, the cells were transferred to 96-well tissue culture plates at a concentration of 1×10^5 cells/ml in 100μl of KGM (keratinocyte growth medium, Clonetics) and allowed to be incubated at 37℃ with 5% CO_2 for 24 h.

Paraquat (methyl viologen, Sigma) was diluted in KGM at concentrations ranging from 10^{-2}M to 10^{-8}M. Medium was aspirated from target cells and replaced with 200μl of KGM containing paraquat. The target cells were then incubated for 24 h at 37℃ with 5% CO_2.

After incubation, the paraquat-containing KGM was aspirated and replaced with KGM containing NR (neutral red, Sigma), and incubated for 3 h at 37C. The wells were washed with phosphate buffered saline and replaced with an extraction solution containing 1% acetic acid and 50% ethanol. After 5-10 min of extraction, the O.D. (optical density) of each well was measured at 560 nm using an enzyme-linked immunosorbent assay reader. The O.D. value was corrected for the adherence of NR to the plastic well, which consistently averaged 0.067 absoption units in determinations made with empty wells.

Protective effects of several antidotes in paraquat-induced cytotoxicity

A concentration of paraquat which showed approximately 50% reduction of absorption from that of the control was obtained (2×10^{-4}M paraquat).

The stock solutions (10^{-2}M) of antidotes (dexamethasone, α-tocopherol, ß-carotene and glutathione) were diluted in KGM at concentrations ranging from 10^{-4}M to 10^{-8}M. The target cell medium was replaced with 100μl of KGM containing antidotes. After incubation for 24 h, 100μl of KGM containing paraquat (4×10^{-4}M) was added to each well. The target cells were then incubated with 2×10^{-4}M of paraquat and various concentrations of each antidote for an additional 24 h. An NR uptake assay was carried out on each plate.

Skin irritation caused by paraquat on guinea pig skin

Albino guinea pigs of the Hartley strain weighing 250-300g were purchased from the Daehan experimental animal center (Seoul, Korea). Animals used in this experiment were cared for in accordance with guidelines recommended by the Council on Animal Care of the Seoul National University Hospital.

For dermal testing the backs and flanks of the animals were clipped with an electric clipper and shaver, with the usual emphasis on avoiding skin damage. Solutions (40μl) of 0.5%, 1.0% and 2.0% of paraquat in distilled water and distilled water as a control were applied on four sites of each animal (n=10) using large Finn chambers (Epitest, Sweden). The chambers were fixed with Tegaderm® and microporous tape (3M, USA). After 24 h the tested sites were marked and air-dried for 1 h. Then, irritation of the skin was measured by non-

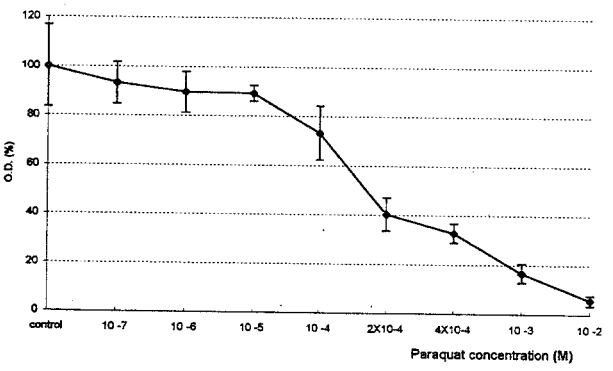

Fig. 1. The toxic effect of paraquat on cultured human keratinocytes measured by the neutral red uptake assay. The value represents the mean and standard deviation of each well (n=12) and was expressed as a percentage of O.D. compared to the controls. There is a constant decrease of keratinocyte viability on increasing paraquat concentration. Over 50% of keratinocytes failed to survive at 2×10^{-4}M of paraquat.
O.D. : optical density measured at 560 nm with ElLISA reader

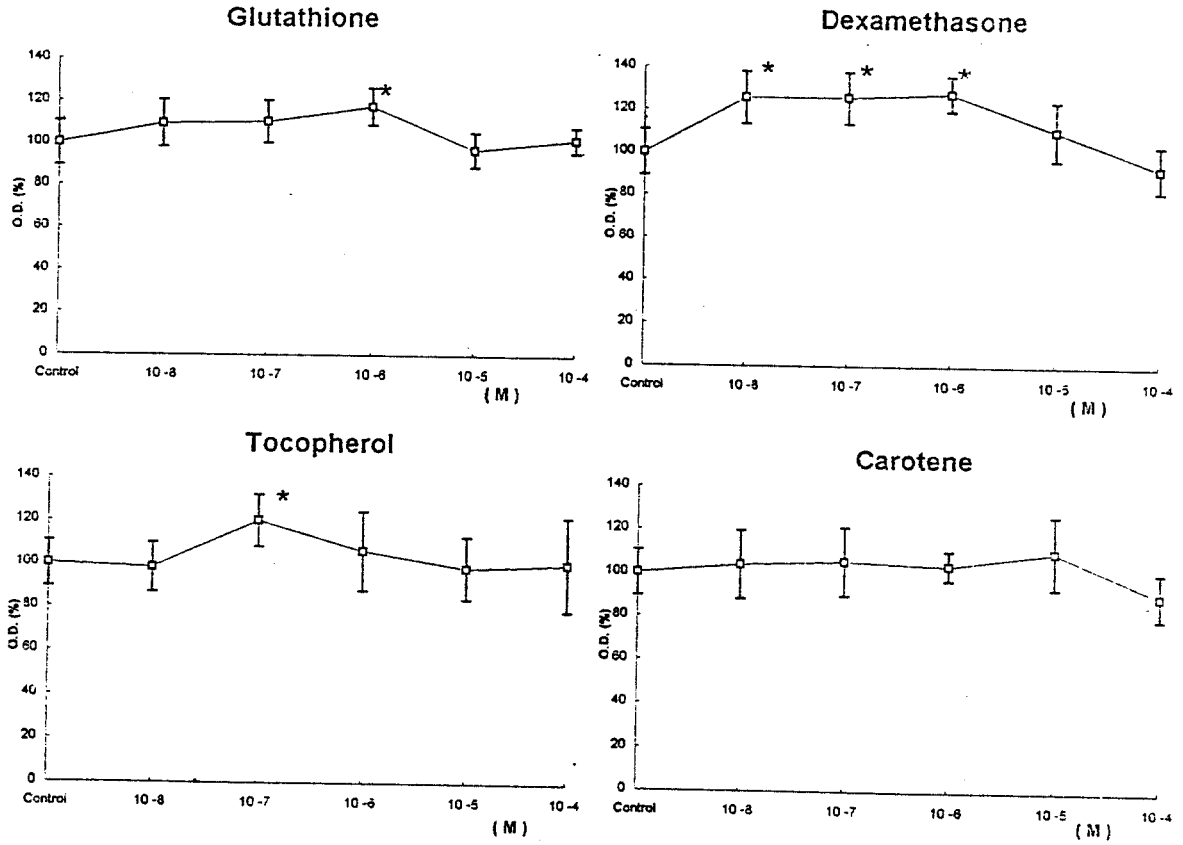

Fig. 2. The effect of various antidotes on paraquat damaged human keratinocytes. The value represents the mean and standard deviation of each well (n=12) and was expressed as a percentage of O.D. compared to controls. Only 2×10^{-4}M of paraquat, without antidotes, was incubated with the cells in the control wells. Glutathione, tocopherol and de xamethasone showed the capacity to reduce paraquat-induced cytotoxicity.
* statistically significant difference compared to control ($p<0.05$).

invasive methods using a dermatospectrophotometer (DermaSpectrometer®, Cortex Technology, Sweden), a laser Doppler flowmeter (Periflux PF2®, Perimed, Sweden) and an evaporimeter (EP1®, Servomed, Sweden). These procedures were undertaken at temperatures of 19-24℃ and relative humidity of 30-50%. The measuring technique has previously been described in detail[7-9].

After the measurements, a skin biopsy was taken at the central part of each test site with a 6-mm punch. The specimens were stained in the usual way with hematoxylin and eosin for light microscopic examination. Various histological parameters, namely epidermal necrosis, pustular epidermitis, intercellular edema, dermoepidermal seperation and dermal inflammation were used to evaluate the skin irritation and graded as 0 (no change), 1 (mild), 2 (moderate) and 3 (severe). All the evaluation of the *in vivo* experiments (including histological evaluation) were carried out by two different observers.

Protective effects of several antidotes on paraquat-induced skin irritation

A 2% concentration of paraquat was chosen; this had shown marked changes when evaluated by non-invasive methods and histology. Antidotes which had shown protective effects against paraquat-induced cytotoxicity *in vitro* were selected (glutathione, tocopherol and dexamethasone). After shaving and anesthetizing, 1% of each antidote and solvent as a control were applied for 30 min on paraquat application sites using cotton balls (n=5 for each antidote). The 2% solution of paraquat was then applied on each site using a large Finn chamber, as described above. After 24 h, the chambers were

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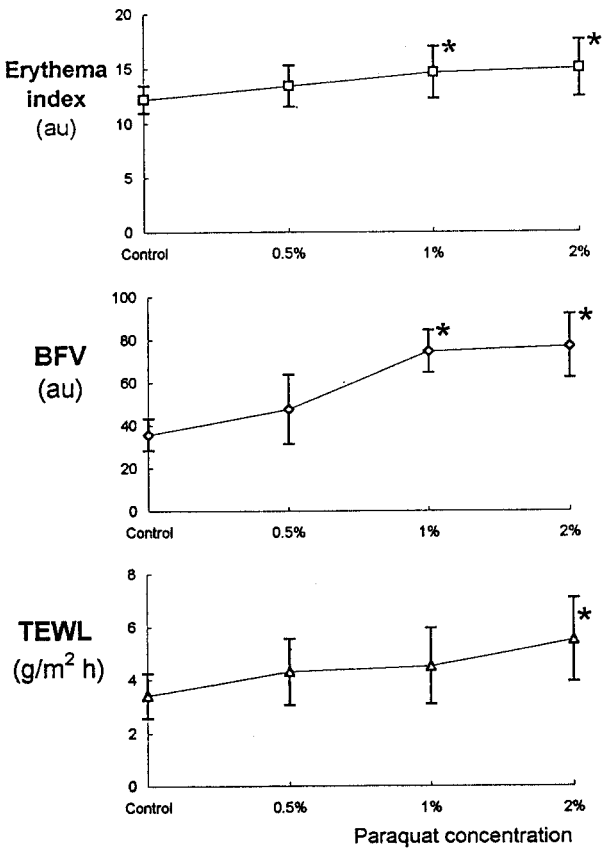

Fig. 3. The effect of paraquat on guinea pig skin, measured by non-invasive methods. The value represents the mean and standard deviation of the result for each animal (n=10). Compared with the controls, all the parameters used in non-invasive methods significantly increased when 2% paraquat was applied.
BFV: blood flow volume; TEWL: transepidermal water loss; au: arbitrary unit
* statistically significant difference compared to the controls ($p<0.05$).

removed and antidotes were applied again for 30 min. Non-invasive methods and skin biopsies were undertaken at each site after 1 h of air-drying. The procedures were the same as above.

Statistical analysis

The Mann-Whitney U test, a non-parametric analysis, was used to compare differences between the group to which antidote had been applied and the control group. Values were considered significantly different if $p<0.05$.

RESULTS

In vitro paraquat cytotoxicity

Over 50% of keratinocytes failed to survive at a concentration of 2×10^{-4}M of paraquat by the NR uptake assay (Fig. 1).

Protective effects of antidotes in paraquat-induced cytotoxicity

Dexamethasone showed the capacity to reduce paraquat-induced cytotoxicity at concentrations of 10^{-8}M, 10^{-7}M and 10^{-6}M, glutathione at 10^{-6}M and tocopherol at 10^{-7}M. However, carotene did not show any such capacity (Fig. 2). These antidotes, when administered in keratinocytes without paraquat, produced cytotoxicity in a dose-dependent manner (data not shown).

Skin irritation caused by paraquat

Compared with controls, the erythema index, cutaneous blood flow volume and transepidermal water loss were increased significantly at sites where 2% paraquat had been applied (Fig. 3). On histological examination, epidermal necrosis, pustular epidermitis, intercellular edema and dermal in-

Table 1. Histological evaluation of paraquat-induced irritation of guinea pig skin. The value represents the mean and standard deviation of the result for each animal (n=10): 0= no change; 1= mild; 2= moderate; 3=severe. Epidermal necrosis, pustular epidermitis, intercellular edema and dermal inflammation were more frequently seen at sites where 2% paraquat had been applied than in controls

	Control	0.5%	1%	2%
Intercelluar edema	0.2 ± 0.42	0.3 ± 0.48	0.9 ± 0.74*	1.9 ± 0.74*
Epidermal necrosis	0.0 ± 0.00	0.2 ± 0.42	0.7 ± 0.67	2.5 ± 0.53*
Pustular epidermitis	0.1 ± 0.32	0.5 ± 0.71	0.7 ± 0.82	1.0 ± 0.82*
Dermoepidermal separation	0.0 ± 0.00	0.0 ± 0.00	0.3 ± 0.48	0.2 ± 0.42
Dermal inflammation	0.2 ± 0.42	0.3 ± 0.48	1.6 ± 0.52*	2.0 ± 0.67*

* statistically significant difference compared to controls ($p<0.05$)

Table 2. The protective effect of several antidotes on paraquat-induced guinea pig skin irritation, assessed by non-invasive methods. Only dexamethasone showed a reduction of cutaneous blood flow volume

	Control	Glutathione	Tocopherol	Dexamethasone
Erythema index(au)	13.0 ± 1.9	13.6 ± 1.1	13.6 ± 1.5	11.8 ± 1.1
BFV(au)	67.0 ± 13.5	55.2 ± 18.8	62.0 ± 32.9	40.0 ± 12.7*
TEWL(au)	4.0 ± 0.7	3.8 ± 1.3	3.0 ± 1.4	3.4 ± 1.1

* statistically significant difference compared to controls ($p<0.05$)

Table 3. The protective effect of several antidotes on paraquat-induced guinea pig skin irritation, assessed by various histological parameters. Only dexamethasone showed a reduction of dermal inflammation

	Control	Glutathione	Tocopherol	Dexamethasone
Intercelluar edema	1.2 ± 0.45	1.0 ± 0.71	0.8 ± 0.84	0.8 ± 0.45
Epodermal necrosis	1.6 ± 0.55	1.6 ± 0.55	1.4 ± 0.55	1.6 ± 0.55
Pustular epidermitis	1.0 ± 0.71	1.0 ± 0.71	0.6 ± 0.55	0.8 ± 0.84
Dermal inflammation	1.8 ± 0.84	1.4 ± 0.55	1.0 ± 0.71	0.6 ± 0.55*

* statistically significant difference compared to controls ($p<0.05$)

flammation were more frequently seen at sites where 2% paraquat had been applied. (Table 1).

Protective effects of antidotes on paraquat-induced skin irritation

Applications of glutathione and tocopherol at 1% concentration showed no change in either non-invasive methods or histology. Only dexamethasone showed a reduction of cutaneous blood flow volume (Table 2) and dermal inflammation (Table 3).

DISCUSSION

Percutaneous absorption of paraquat is very low in normal skin. Human studies have shown an *in vivo* absorption rate of 0.03μg/cm^2 which is negligible if safety guidelines are adhered to[10]. However, at least 14 cases of systemic poisoning resulting from dermal exposure were reported[4]; in all cases, one or more of the following features were present: previous skin damage, concentrated paraquat solution, or prolonged skin contact. Although paraquat is poorly absorbed through intact skin, it may have deleterious effects on epidermal morphology in the absence of significant percutaneous absorption[11]. Only 0.1% of paraquat remained after routine decontamination (dried in air and washed with water) at the application site[12]. A small amount of paraquat may, however, remain on the skin for a long period after dermal application and this may explain the development of delayed dermatitis[2]. In fact, skin irritation or rashes associated with paraquat spraying have been reported in 40% of agricultural workers[3].

In plants, paraquat has been shown to interfere with intracellular electron transfer systems, inhibiting the reduction of NADP to NADPH during photosynthesis, at which time superoxide is formed. This stimulates lipid peroxidation and leads to cell death. In humans, toxicity is felt to follow a similar mechanism, which includes depletion of the enzyme superoxide dismutase. Paraquat also competes with natural oligoamines in the body for its uptake and accumulation[13].

We used a keratinocyte culture system to evaluate the toxicity of paraquat. The toxic effect was measured by the NR uptake assay in this experiment. This assay is based on the active uptake of NR dye into lysosomes of viable cells[14]. In fact, we measured the cytotoxicity of paraquat using the MTT method, which is based on the cleavage of the tetrazolium ring by active mitochondria of viable cells[15]. The toxicity profile of paraquat was similar using both methods (data not shown). However, the MTT method was not suitable for this study since antioxidants used in this experiment (glutathione, tocopherol and carotene) cleaved the tetrazolium

ring without the presence of cultured cells. It has been reported that the *in vitro* cytotoxicity of certain test substances measured by the NR uptake assay correlated with human skin patch test data[16].

Studies using paraquat on humans may raise ethical problems, because a small amount of absorbed paraquat could have serious effects on volunteers. The standard test for evaluating the irritancy potential of a chemical in humans from the results obtained with animal models was published by Draize et al. and many modifications have been made to improve interpretations. We used a large Finn chamber and Tegaderm® instead of a one square inch surgical gauze and rubberized cloth. This modification seemed to be simpler and faster than the standard skin test recommended by the Code of Federal Regulation[17]. The scoring system used in the Draize method uses the naked eye, resulting in great interobserver variation, and so we replaced it with several non-invasive methods and skin biopsy.

Corticosteroids has been used in the management of paraquat intoxication to reduce the extent of pulmonary inflammation and fibrosis[18]; most cases of paraquat-induced dermatitis also responded to steroid cream[3]. In the present study, dexamethasone showed the capacity to reduce paraquat-induced keratinocyte toxicity. It is believed that the cytotoxicity of paraquat is due to its generation of oxygen free radicals and the anti-inflammatory mechanism of corticosteroid in skin is thought to be a vascular constriction and blocking of the prostaglandin pathway. From this result, we assumed that corticosteroids may block the toxic pathway of the oxygen free radicals or that paraquat has another unknown pathomechanism which could be interrupted by corticosteroids.

Antidotes used in this study are thought to block the pathomechanism of paraquat in the cell. Tocopherol stabilizes the cell membrane and interferes with lipid peroxidation[19]. The concentration of glutathione peroxidase increased in paraquat-resistant cell lines[20]. Carotene quenches oxygen free radicals and inhibits lipid peroxidation by interacting with the peroxiradical[21]. The capacity of antioxidants to reduce paraquat-induced cytotoxicity in the present study raises the possibility that these antioxidants may exert protective and therapeutic effects on paraquat-induced dermatitis in actual usage. However, glutathione and tocopherol failed to prevent paraquat-induced dermatitis *in vivo*. We thought this might be explained in one of two ways. First, the concentration of glutathione and tocopherol might have been too low; when, in a preliminary study, we applied 10%, 1% and 0.1% of glutathione and tocopherol without application of paraquat, skin irritation was observed at 10% concentration (data not shown). We considered that the application of 1% of glutathione and tocopherol would be sufficient to exert their protective effects. Second, the drug delivery system might have been inappropriate. Certain antioxidants have been tested in paraquat-exposed humans and animals as a pharmacological treatment. Results have shown that these antioxidants are not able to eliminate the *in vivo* toxic actions of paraquat[5], an observation perhaps attributable to their inability to cross the cell membrane barrier. A recent study, however, has demonstrated that the encapsulation of tocopherol in liposomes promotes their therapeutic potential against paraquat-induced lung damage, presumably by the ability of liposome to facilitate the intracellular uptake of macromolecules[22]. This may also apply to the skin, and the use of liposome or other methods to increase the cellular delivery of antidotes should be studied further.

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