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Original Research Article

Effects of Trifoliate Orange (*Poncirus trifoliata*) Extract on Inflammatory Responses in LPS-induced Shock Rats and RAW 264.7 Cells

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Abstract - This study examined the effects of trifoliate orange extract (TOE) on inflammatory reactions at the time of an LPS shock by performing experiments on rats injected with trifoliate orange extract and in Raw 264.7 cell cultures, with the aim of developing a new anti-inflammatory medicine. The IL-1β, IL-6, and TNF-α concentrations were lower in all of the groups treated with TOE than in the control group after 5 h of LPS treatment. The IL-10 concentration was higher in the 300-㎎/㎏ TOE group than in the control group after 2 h and 5 h of LPS treatment. The liver concentrations of cytokines IL-1β and IL-6 decreased more in the groups treated with TOE than in the control group and the IL-6 concentration did not differ significantly between the 100-㎎/㎏ TOE group than in the control group. The TNF-α and IL-10 concentrations did not differ significantly between the TOE groups and the control group. In the experiments involving Raw 264.7 macrophage cultures subjected to LPS shock, the productions of IL-1β, IL-6, and TNF-α decreased in all of the groups treated with TOE compared to the control group. The IL-10 concentration did not differ significantly between the groups treated with TOE and the control group. Together the findings of this study suggest that TOE contains functional substances that can influence inflammatory reactions.

Key words - Ttrifoliate orange extracts, Lipopolysaccharide, Anti-inflammatory, Cytokine

Introduction

New anti-inflammatory medicines that exert fewer adverse effects and better curative effects are needed. Several investigators are working to develop effective cytokine-modulating anti-inflammatory therapies for treating sepsis (Surh, 2002; Byun, 2005; Yoon *et al*., 2007; Yun *et al*., 2008), and numerous natural products (e.g., *Scutellariae radix*, *chrysanthemum indicum* L., *Pulsatilla koreana*, and *Allium victorialis*) have been investigated for their anti-inflammatory effects in this laboratories (Lee, 2007; Lee, 2009; Lee *et al*., 2012; Lee, 2014), but no satisfactory results have been reported.

Poncirus trifoliata belongs to the Rutaceae family, and produces trifoliate orange fruits. The main active components of trifoliate orange are flavonoids, hesperidin, neohesperidin, naringin, and cumalin (Park and Chun, 1969; Oh *et al*., 1989; Chung *et al*., 2004). The main biological functions of trifoliate orange are involvement in immune function and lipid lowering (Patkar *et al*., 1979; Fewtrell *et al*., 1982; Sagi-Eisenberg *et al*., 1985; Kanemoto *et al*., 1993; Lee *et al*., 1996; Lee *et al*., 2005).

Lipopolysaccharides (LPS) are structural components of the outer membranes of Gram-negative bacteria, and they are associated with tissue injury and fatal outcome in septic shock. Proinflammatory cytokines, such as interleukin (IL)-1 β, tumor necrosis factor (TNF-α), IL-8, and IL-6, and anti-inflammatory cytokines, such as IL-1 receptor antagonist and IL-10, are produced in response to LPS (Luster *et al*., 1994; Ayala *et al*., 1994; Aono *et al*., 1997). Moreover, since macrophages are the immune cells responsible for innate immunity, such as Raw 264.7 cells and monocytes increase the production of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β, they are frequently applied as an experimental model when studying inflammatory reactions (Binetruy *et al*., 1991; Funk *et al*., 1991; Willeaume *et al*., 1996; Mathiak *et al*., 2000; Bhatta -charyya *et al*., 2002).

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This study investigated the anti-inflammatory effects of TOE on rats and *in-vitro*-cultured Raw 264.7 cells subjected to LPS shock, with the aim of facilitating the development of a new anti-inflammatory medicine.

Materials and Methods

Animals and Treatment

Twenty-eight Sprague-Dawley male rats with a body weight of 181.53±6.72 g (Orient Bio co., mean±SD, 7 weeks) were used in this study. They were housed in a temperature and humidity controlled environment and were allowed free access to a basal diet for 1 week before the experiment, and had free access to water ad libitum throughout experiment. The rats were randomly assigned to one of the following four groups(n =7/group) according to the administered concentration of trifoliate orange extract (Control-saline 100 ㎎/㎏, trifoliate orange extract 100 ㎎/㎏/day, trifoliate orange extract 200 ㎎/㎏/day, trifoliate orange extract 300 ㎎/㎏/day group).

Diet and water

Dietary (Table 1) and water were *ad libitum* provided for 6 weeks of experiment period.

Table 1. Composition of experimental diet

Ingredients	Composition (%)
Casein	20.0
α-Corn starch	35.5
Sucrose	11.0
Lard	4.0
Corn oil	1.0
Mineral mix[z]	3.5
Vitamin mix[y]	1.0
Cellurose powder	23.7
DL-methione	0.3

[z]Mineral mix. (g/kg diet): $CaCO_3$, 29.29; $CaHPO_4{\cdot}2H_2O$, 0.43; KH_2PO_4, 34.30; NaCl, 25.06; $MgSO_4{\cdot}7H_2O$, 9.98; Feric citrate hexahydrate, 0.623; $CuSO_4{\cdot}5H_2O$, 0.516; $MnSO_4{\cdot}H_2O$, 0.121; $ZnCl_2$, 0.02; KI, 0.005; $(NH_4)_6\ MO_7O_{24}{\cdot}4H_2O$, 0.0025.
[y]Vitamin mix (㎎/㎏ diet): Thiamine-HCl, 12; Riboflavin, 40; Pyridoxin-HCl, 8; Vitamin-B_{12}, 0.005; Ascorbic acid, 300; D-biotin, 0.2; Menadione, 52; Folic acid, 2; D-calcium pantothenate, 50; P-aminobenzoic acid, 50; Nicotinic acid, 60; Cholin choloride, 2000 (IU/㎏ diet); Rethinyl acetae, 5000 (IU/㎏ diet); Cholecalciferol, 250 (IU/㎏ diet).

Trifoliate orange extract (TOE)

The trifoliate orange was used as collected, dried to a sample in this laboratory and the air-dry trifoliate orange 500 g (dried weight) was divided and extracted 3 times at 5 hours each in cooling water reflux cistern, and decompression concentrated, and made EtOH extract 80g, and kept under refrigeration at 2℃. TOE administration was placed orally using Jones tube at 5 pm every day. The control group was given normal saline in the same form.

LPS injection

After 6 weeks of trifoliate orange extract administration, the LPS (*Escherichia coli* 026: B6; Difco, Detroit, MI, U.S.A., Sigma) was injected into abdominal cavity in same method to all groups at the level of 5 ㎎/㎏ (Marriot *et al* ., 1998).

Collecting blood and livers

The blood samplings were done at the end of 6 weeks for each group of rats, just before LPS injection and 2 h and 5 h after LPS injection. Each group of rats was blood sampled under ether anesthesia using the cardiac puncture method. Five hours after LPS injection, all experimental groups underwent a mid-abdominal incision, and livers were harvested from all the experimental groups of rats.

Raw 264.7 cells culture, trifoliate orange extracts and LPS treatment

Raw 264.7 cells were purchased from Korea Cells Bank (Seoul), and cultured by using a culture medium with Dulbecco's modified Eagle's medium (DMEM) added with 10% fetal bovine serum (FBS), penicillin (100 U/㎖) and streptomycin (100 ㎍/㎖) in an incubator set at 37℃ and 5% CO_2. All the cells in the testing process were experimented on at 80~90% confluency and only such cells that had not exceeded 20 passages were used. For TOE and LPS treatment, after cells have been divided into 4 well dishes (10^6/㎖), TOE were processed through 4 steps of 0 ㎍, 10 ㎍, 30 ㎍, and 100 ㎍/㎖, LPS 1 ㎍/㎖ was added after 1 h and the specimens were collected at 6 h after LPS shock.

Analysis

Blood samples were immediately centrifuged at 3000 rpm for 10 min. Sera were collected, frozen, and kept at -80 °C. Liver cytokine samples were prepared as follows: 1 g of liver particles were homogenized on ice in 5 ㎖ of cold phosphate-buffered saline (PBS) with a pH of 7.4 and containing a protease inhibitor cocktail (Tablete Complete Roche, Germany). The samples were centrifuged at 15,000 rpm for 15 min at 4℃. Supernatants were filtered through a 0.45 ㎛ filter (Millex-HA, Millipore, France) and again centrifuged at 15,000 rpm for 15 min at 4℃. Liver extracts were removed and kept at -80℃ until cytokine analysis was performed.

In the Raw 264.7 cells experiment, after cytokines quantified has undergone centrifugation at 4℃, 15,000 rpm for 15 minutes, Supernatants were filtered through a 0.45 ㎛ filter (Millex-HA, Millipore, France) and kept under refrigeration at -80℃.

Cytokine (IL-1β, TNF-α, IL-6 and IL-10) concentrations were determined by enzyme-linked immunosorbent assay (ELISA), using commercial kits (Biosource International, USA). The minimum detectable concentration of TNF-α was 0.7 pg/㎖, and the remaining cytokines were 3-8 pg/㎖. Hepatic amounts of cytokines were calculated per 1 g of wet tissue in 5 ㎖ of PBS. Plasma and raw cell cytokine concentrations were expressed as picograms per milliliter, and hepatic cytokine amounts were measured as picograms per milligram of tissue.

Statistical analysis

Results were one-way ANOVA examined by using SPSS package, and each group's significance examination was done in the level of P<0.05 by Duncan's multiple range test.

Results and Discussion

The plasma proinflammatory cytokine concentration in rats treated with LPS will vary over time. Therefore, this study collected specimens for measuring plasma cytokines prior to applying LPS treatment (0 h) and after 2 h and 5 h, taking into account the results of previous studies and those conducted by other researchers (Mathiak, *et al*., 2000; Eduard *et al*., 2004). Specimens for measuring cytokines in the liver were collected at 5 h after LPS injection, when the experiment ended. The LPS treatment concentration was set at 5 ㎎/㎏ based on other researchers finding that this could cause endotoxin shock in rats and mice over a short period of time, raising the cytokine concentration level in the liver and blood (Corral *et al*., 1996; Aono *et al*., 1997; Harry, *et al*., 1999; Sang *et al*., 1999).

Plasma IL-1β

Table 2 lists the plasma IL-1β concentration by treatment group over time during LPS treatment. Before LPS treatment (0 h) there were no significant differences between the four test groups. After 2 h of LPS treatment, the concentrations had increased markedly in all treatment groups, but it was significantly lower in the TOE groups than in the control group. After 5 h of LPS treatment, the concentrations had increased linearly, and they remained lower in the TOE groups than in the control group.

These results are similar to those found by other researchers confirming that the plasma IL-1β concentration peaked after 4-6 hours of LPS treatment (Mathiak, *et al*., 2000; Eduard *et*

Table 2. Effect of TOE ext. on plasma IL-1β concentration in lipopolysaccharide-exposed rats

Treatment	IL-1β (pg/㎖), Time (h)[z]		
	0h	2h	5h
Control (saline, 100 ㎎/㎏)	13.11 ± 3.17[NSy]	164.22 ± 12.74[bx]	291.53 ± 21.49[bx]
Trifoliate orange ext. (100 ㎎/㎏)	13.67 ± 3.55[NS]	131.38 ± 10.11[a]	242.46 ± 18.55[a]
Trifoliate orange ext. (200 ㎎/㎏)	12.19 ± 3.28[NS]	125.15 ± 12.83[a]	227.21 ± 21.39[a]
Trifoliate orange ext. (300 ㎎/㎏)	13.38 ± 3.12[NS]	127.79 ± 12.44[a]	218.79 ± 25.35[a]

[z]0h, 2h and 5h after LPS injection.
[y]Not significantly different ($P > 0.05$).
[x]Means in the same column with different superscripts are significantly different ($P < 0.05$).

al., 2004), and suggest that functional substances in trifoliate orange are involved in LPS-induced acute inflammatory reactions.

Plasma IL-6

Table 3 indicates that after 2 h and 5 h of LPS treatment there were marked increases in IL-6 in all of the treatment groups. This result is similar to those found in experiments on LPS-induced inflammatory reactions conducted by other researchers (Mathiak, *et al*., 2000; Eduard *et al*., 2004).

The IL-6 concentration was significantly lower in the 300-㎎/㎏ trifoliate orange extract group than in the control group after 2 h and 5 h of LSP treatment. This result and the observed changes in IL-1β confirm that functional substances in trifoliate orange are involved in LPS-induced acute inflammatory reactions.

Plasma TNF-α

The TNF-α concentration increased markedly after 2 h of LPS treatment, and remained elevated after 5 h (Table 4). The concentrations were significantly lower in the 200-㎎/㎏ and 300-㎎/㎏ TOE groups than in the control group after 2 h of LPS treatment, and significantly lower in all of the TOE groups than in the control group after 5 h of LPS treatment. TNF-α caused damage and necrosis in liver cells in the LPS-shock experiments (Chamulitrat *et al*., 1995), and the additional TNF-α released can cause a pathogenic state over a wide scale. Therefore, it has become an important task to adjust the production of TNF-α in inflammatory reactions (Hamada et al., 1999; Abul *et al*., 2007). The present results show that TOE exerts positive anti-inflammatory effects.

Plasma IL-10

The plasma IL-10 concentration increased more in the groups treated with TOE than in the control group after both 2 h and 5 h of LPS treatment (Table 5). The concentration was significantly higher in the 200-㎎/㎏ and 300-㎎/㎏ TOE groups than in the control group after 2 h of LPS treatment,

Table 3. Effect of TOE on plasma IL-6 concentration in lipopolysaccharide-exposed rats

Treatment	IL-6 (pg/㎖), Time (h)[z]		
	0h	2h	5h
Control(saline, 100 ㎎/㎏)	24.78 ± 4.52[NSy]	259.19 ± 22.57[bx]	592.15 ±2 9.54[bx]
Trifoliate orange ext. (100 ㎎/㎏)	26.11 ± 4.81[NS]	247.33 ± 25.12[ab]	509.75 ± 31.29[a]
Trifoliate orange ext. (200 ㎎/㎏)	25.85 ± 5.11[NS]	231.46 ± 21.92[ab]	481.23 ± 28.51[a]
Trifoliate orange ext. (300 ㎎/㎏)	26.34 ± 4.83[NS]	211.76 ± 22.58[a]	463.29 ± 27.32[a]

[z]0h, 2h and 5h after LPS injection.
[y]Not significantly different ($P > 0.05$).
[x]Means in the same column with different superscripts are significantly different ($P < 0.05$).

Table 4. Effect of TOE on plasma TNF-α concentration in lipopolysaccharide-exposed rats

Treatment	TNF-α (pg/㎖), Time (h)[z]		
	0h	2h	5h
Control(saline, 100 ㎎/㎏)	17.84 ± 2.97[NSy]	727.54 ± 41.95[cx]	809.24 ± 47.58[cx]
Trifoliate orange ext. (100 ㎎/㎏)	16.21 ± 3.51[NS]	675.88 ± 39.51[bc]	711.37 ± 42.55[b]
Trifoliate orange ext. (200 ㎎/㎏)	17.08 ± 3.44[NS]	634.19 ± 37.15[b]	647.24 ± 48.11[ab]
Trifoliate orange ext. (300 ㎎/㎏)	17.92 ± 3.38[NS]	547.27 ± 35.19[a]	627.95 ± 48.58[a]

[z]0h, 2h and 5h after LPS injection.
[y]Not significantly different ($P > 0.05$).
[x]Means in the same column with different superscripts are significantly different ($P < 0.05$).

Table 5. Effect of TOE on plasma IL-10 concentration in lipopolysaccharide-exposed rats

Treatment	IL-10 (pg/ml), Time (h)[z]		
	0h	2h	5h
Control (saline, 100 mg/kg)	23.81 ± 3.17^{NSy}	51.36 ± 3.18^{ax}	121.31 ± 12.35^{ax}
Trifoliate orange ext. (100 mg/kg)	21.58 ± 3.75^{NS}	51.27 ± 3.95^{a}	129.65 ± 13.17^{ab}
Trifoliate orange ext. (200 mg/kg)	23.63 ± 3.44^{NS}	67.58 ± 5.11^{b}	137.93 ± 12.25^{ab}
Trifoliate orange ext. (300 mg/kg)	22.57 ± 3.89^{NS}	69.45 ± 4.29^{b}	147.28 ± 13.19^{b}

[z]0h, 2h and 5h after LPS injection.
[y]Not significantly different ($P > 0.05$).
[x]Means in the same column with different superscripts are significantly different ($P < 0.05$).

Table 6. Effects of TOE on liver cytokine levels in liver tissue in lipopolysaccharide-exposed rats

Treatment	IL-1β (pg /mg)	IL-6 (pg /mg)	TNF-α (pg /mg)	IL-10 (pg /mg)
Control (saline, 100 mg/kg)	27.95 ± 3.41^{bz}	14.27 ± 2.89^{cz}	1.85 ± 0.91^{NSy}	1.64 ± 0.72^{NSy}
Trifoliate orange ext. (100 mg/kg)	22.11 ± 3.29^{ab}	13.51 ± 2.74^{bc}	1.61 ± 0.72^{NS}	1.88 ± 0.53^{NS}
Trifoliate orange ext. (200 mg/kg)	21.37 ± 3.58^{ab}	9.15 ± 1.77^{ab}	1.69 ± 0.51^{NS}	1.93 ± 0.81^{NS}
Trifoliate orange ext. (300 mg/kg)	18.44 ± 3.12^{a}	8.71 ± 1.94^{a}	1.58 ± 0.72^{NS}	1.97 ± 0.68^{NS}

[z]Means in the same column with different superscripts are significantly different($P < 0.05$).
[y]Not significantly different ($P > 0.05$).
[z]Means in the same column with different superscripts are significantly different($P<0.05$). [y]Not significantly different ($P>0.05$).

and significantly higher in the 300-mg/kg trifoliate orange extract group than in the control group after 5 h of LPS treatment.

IL-10 suppresses the synthesis of proinflammatory cytokines such as IL-6 and TNF-α, and was found to reduce T-cell revitalization in *in-vitro* and *in-vivo* experiments (Clerici *et al*., 1994; Pender *et al*., 1999; Schotte *et al*., 2004). This suggests that trifoliate orange extract improves the production of IL-10 suppressed by proinflammatory cytokines.

Liver cytokines

The liver concentrations of the cytokines IL-1β and IL-6 decreased more in the groups treated with TOE than in the control group (Table 6). However, the IL-1β concentration did not differ between the 100-mg/kg and 200-mg/kg TOE groups and the control group, while that of IL-6 concentration did not differ between the 100-mg/kg TOE group and the control group. The TNF-α and IL-10 concentrations did not differ significantly between the TOE groups and the control group.

These results suggest that TOE affect the LPS-shock inflammatory reactions and the degree of this effect may be substantially related to the degree to which cytokines synthesized in the liver are drained into the bloodstream.

Raw 264.7 cell culture experiments

In the experiments involving Raw 264.7 macrophage cultures subjected to LPS shock, the productions of IL-1β (Fig. 1), IL-6 (Fig. 2), and TNF-α (Fig. 3) decreased in all of the groups treated with TOE compared to the control group. The IL-10 concentration did not differ significantly between the groups treated with TOE and the control group (Fig. 4). These results were similar to the results for proinflammatory cytokines in the *in-vivo* experiments, suggesting that TOE could have influenced the inflammatory reactions.

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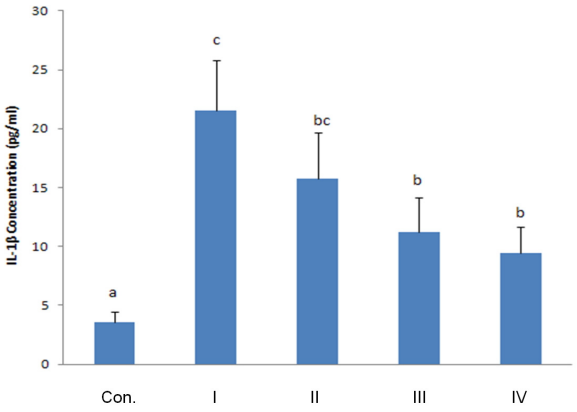

Fig. 1. Effect of TOE on IL-1b concentration in lipopolysaccharide induced Raw 264.7 macrophages.
[a,b,c]: Means with different superscripts are significantly different ($P < 0.05$).
Con: Control, Ⅰ: LPS (1 ㎍/㎖), Ⅱ: LPS (1 ㎍/㎖)+10 ㎍/㎖ TOE., Ⅲ: LPS (1 ㎍/㎖)+30 ㎍/㎖ TOE., Ⅳ: LPS (1 ㎍/㎖) +100 ㎍/㎖ TOE.

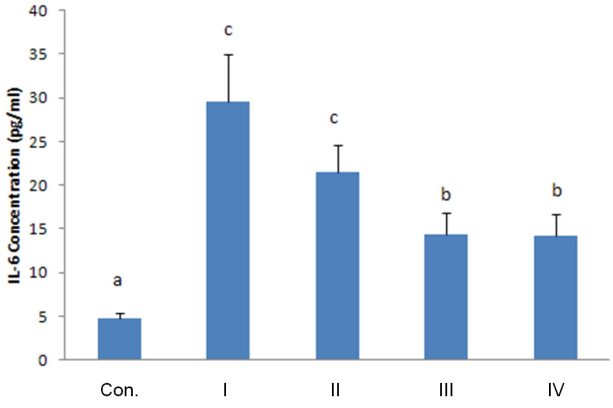

Fig. 2. Effect of TOE on IL-6 concentration in lipopolysaccharide induced Raw 264.7 macrophages.
[a,b,c]: Means with different superscripts are significantly different ($P < 0.05$).
Con: Control, Ⅰ: LPS (1 ㎍/㎖), Ⅱ: LPS (1 ㎍/㎖)+10 ㎍/㎖ TOE., Ⅲ: LPS (1 ㎍/㎖)+30 ㎍/㎖ TOE., Ⅳ: LPS (1 ㎍/㎖) +100 ㎍/㎖ TOE.

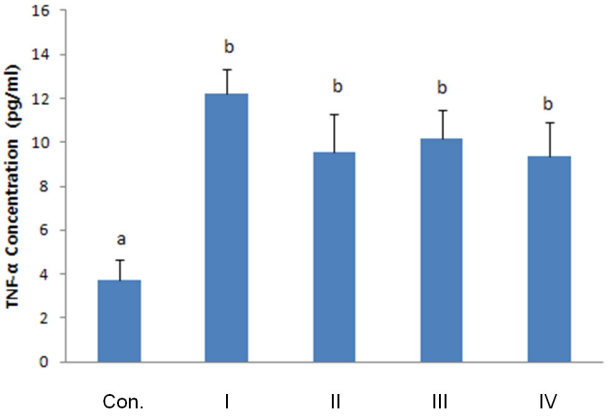

Fig. 3. Effect of TOE on TNF-α concentration in lipopolysaccharide induced Raw 264.7 macrophages.
[a,b]: Means with different superscripts are significantly different ($P < 0.05$).
Con: Control, Ⅰ: LPS (1 ㎍/㎖), Ⅱ: LPS (1 ㎍/㎖)+10 ㎍/㎖ TOE., Ⅲ: LPS (1 ㎍/㎖)+30 ㎍/㎖ TOE., Ⅳ: LPS (1 ㎍/㎖)+100 ㎍/㎖ TOE.

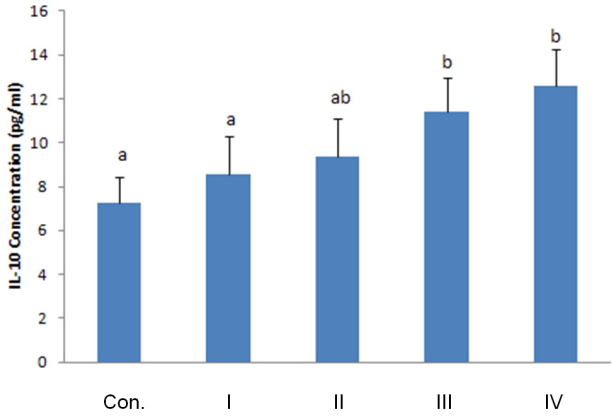

Fig. 4. Effect of TOE on IL-10 concentration in lipopolysaccharide induced Raw 264.7 macrophages.
[a,b]: Means with different superscripts are significantly different ($P < 0.05$).
Con: Control, Ⅰ: LPS (1 ㎍/㎖), Ⅱ: LPS (1 ㎍/㎖)+10 ㎍/㎖ TOE., Ⅲ: LPS (1 ㎍/㎖)+30 ㎍/㎖ TOE., Ⅳ: LPS (1 ㎍/㎖)+100 ㎍/㎖ TOE.

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