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Synergistic immunostimulatory effect of pidotimod and red ginseng acidic polysaccharide on humoral immunity of immunosuppressed mice

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We investigated the synergistic effect of pidotimod and red ginseng acidic polysaccharide (RGAP) from *Panax ginseng* C.A. Meyer on humoral immune response challenged by lipopolysaccharide (LPS) and sheep red blood cells (SRBC) in immunosuppressed mice. Combined treatment with pidotimod and RGAP significantly increased the number of plaque-forming cells in the spleen in response to both LPS and SRBC, while treatment with either pidotimod or RGAP individually had no such effect. IgG levels in serum were augmented for secondary responses to SRBC in co-treated mice, but not in mice treated with either drug alone. Microscopic studies revealed that architecture of the spleen, thymus, and lymph nodes was conserved. GPT and creatinine in serum as indicators of hepatic and renal functions showed no difference compared to the control group. These results indicate that combined treatment with pidotimod and RGAP has an immunostimulatory effect in a synergistic manner on antibody response to challenge with LPS and SRBC without toxic changes.

1. Introduction

Ginseng, the dry extract prepared from *Panax ginseng* C.A. Meyer, has occupied an important place among the tonic remedies of oriental medicine for thousands of years. Ginseng contains many active components such as ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, fatty acids and mineral oils. Among these components, ginsenosides have been shown to be responsible for the pharmacological and immunological actions (Lee et al. 2004; Lee and Han 2006). However, non-saponin components have also received great attention. Polysaccharides isolated from ginseng root have been found to have mitogenic (Eun et al. 1989), hypoglycemic (Konno et al. 1983; Konno et al. 1985), and antitumor activities (Moon et al. 1983; Lee et al. 1997). Among the polysaccharides isolated from ginseng root, it is thought that acidic polysaccharides are more effective than neutral polysaccharides with regard to their immunostimulating activities. They have been found to activate macrophage and NK cells, induce the action of nitric oxide synthase (NOS), and increase the number of macrophages (Kim et al. 1990).
An essential facet of traditional oriental medicine is “combination”. The modern concept of “combination” relates to molecules possessing the same or similar functions, which are combined as a group and which show particular activities. The advantages of using a combination would be to reduce drug dosages, thereby lowering the potential risk of toxicity, and also to reduce the development of drug resistance. Pidotimod ((*R*)-3-[(*S*)-(5-oxo-2-pyrrolidinyl)carbonyl]thiazolidine-4-carboxylic acid) is a biological immunoregulatory modifier synthesized by Poli Industria Chimica (Milan, Italy) following a screening of molecules endowed with an immunostimulating activity. It is the first compound of a new class of biological response modifiers with peptide-like structures, and it is active in both innate and acquired immunity (Migliorati et al. 1992; Coppi and Manzardo 1994). Earlier studies in our laboratory demonstrated the synergistic effect of combined treatment with pidotimod and red ginseng acidic polysaccharide (RGAP) from *Panax ginseng* C.A. Meyer on cell-mediated immunity in immunosuppressed mice. In this study, we focused on the effect of the combined therapy on the humoral immune response using lipopolysaccharides (LPS) as a thymus-independent (TI) antigen and sheep red blood cells (SRBC) as a thymus-dependent (TD) antigen, respectively. The histopathology of spleen, thymus, and lymph nodes and also GPT (glutamic pyruvic transminase) and creatinine in serum as indicators of hepatic and renal functions were studied after a six-week treatment to test possible synergistic toxicity. The purpose of this study was to investigate the effect of the combination of pidotimod and RGAP to provide a reference for the combined use of western and oriental medicines.

2. Investigations and results

2.1. Effect of pidotimod and RGAP on white blood cell (WBC) count and spleen cellularity against cyclophosphamide

WBC count and spleen cellularity were determined after treatment with cyclophosphamide (Table 1). There was no difference when SRBC-treated groups were compared to

Table 1: Effect of pidotimod and RGAP on WBC ($\times 10^6$/ml) and spleen cellularity ($\times 10^7$) against cyclophosphamide

Group	WBC number ($\times 10^6$/ml)	Spleen cellularity ($\times 10^7$)
Control	3.5 ± 0.3	26.0 ± 4.6
SRBC	3.4 ± 0.4	26.4 ± 3.0
SRBC + Cy	$0.5 \pm 0.2^{\#\#\#}$	$4.6 \pm 0.5^{\#\#}$
SRBC + Cy + Pi	1.0 ± 0.1	4.2 ± 0.4
SRBC + Cy + RGAP	1.0 ± 0.0	8.8 ± 1.6
SRBC + Cy + Pi + RGAP	$1.4 \pm 0.1^{\$\$}$	$9.8 \pm 1.4^{\$}$

Each value represents mean ± SEM of 10 mice
Cy=cyclophosphamide, Pi=pidotimod
$^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs. SRBC group
$^{\$}P<0.05$; $^{\$\$}P < 0.01$; $^{\$\$\$}P < 0.001$ vs. (SRBC + Cy) group

the control group. Administration of cyclophosphamide resulted in a significant reduction in WBC count and spleen cellularity. The combination of pidotimod with RGAP showed significant enhancement in the WBC count of cyclophosphamide-treated mice. The combined therapy also increased splenocyte count to $(9.8 \pm 1.4) \times 10^7$ compared to $(4.6 \pm 0.5) \times 10^7$ in the cyclophosphamide-treated group.

2.2. *Effect of pidotimod and RGAP on the number of plaque-forming cells (PFC) in spleen challenged with LPS*

The aim of this experiment was to examine the immune response using LPS-coated SRBC as a TI antigen. LPS-coated SRBC was able to boost the number of PFCs in the spleen to 68.9/10^6 splenocytes (Fig. 1). The number of PFCs was decreased significantly to 20.7/10^6 in methotrexate-treated mice. There was no significant change with monotherapies. However, this reduced response was enhanced significantly to 49.0/10^6 by combined treatment with pidotimod and RGAP.

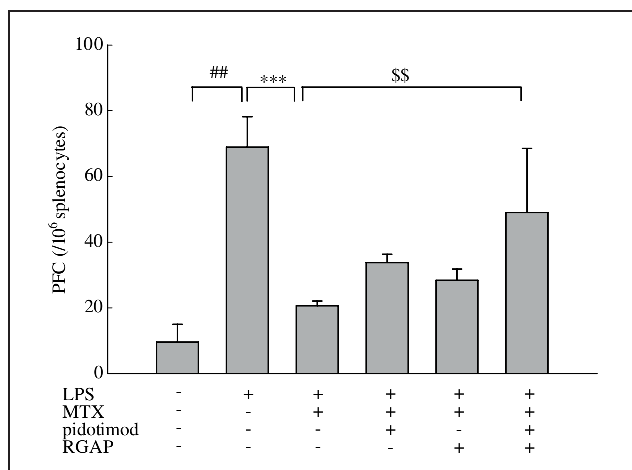

Fig. 1: Effect of pidotimod and RGAP on PFC count in spleen challenged with LPS. Immunization induced by LPS at a dose of 10 µg/mouse i.p. except for negative control group. Immunodepression induced by methotrexate (MTX) i.p. at a dose of 20 mg/kg on the day after immunization. Different groups treated p.o. starting two weeks prior to LPS injection with: pidotimod 200 mg/kg/day; RGAP 300 mg/kg/day; and pidotimod (200 mg/kg/day) plus RGAP (300 mg/kg/day) combination. Mice sacrificed four days after immunization. Each bar represents mean ± SEM of 10 mice. $^{\#\#}P < 0.01$ vs. control group; $^{***}P < 0.001$ vs LPS group; $^{\$\$}P < 0.01$ vs. (LPS + MTX) group

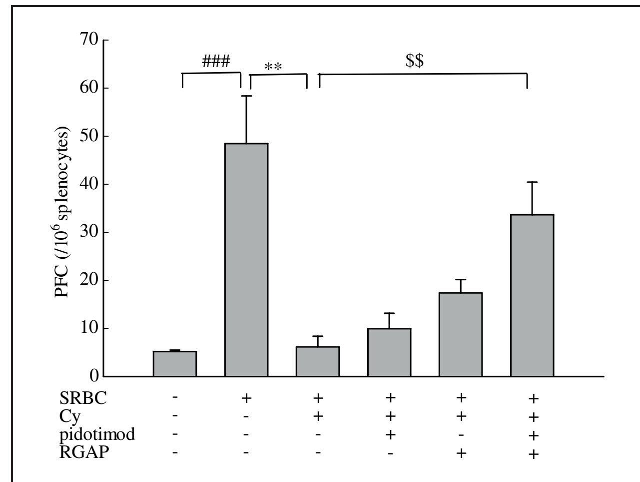

Fig. 2: Effect of pidotimod and RGAP on PFC count in spleen in response to initial challenge of SRBC. Immunization induced by SRBC i.p. at 4×10^8/mouse on Day 0 to all mice except negative control group. Immunodepression induced by cyclophosphamide (Cy) i.p. at 50 mg/kg on Days 3, 4 and 5. Different groups were treated p.o. starting from third day prior to SRBC challenge with: pidotimod 200 mg/kg/day; RGAP 300 mg/kg/day; and pidotimod (200 mg/kg/day) plus RGAP (300 mg/kg/day) combination. Mice sacrificed six days after immunization. Each bar represents mean ± SEM of 10 mice. $^{\#\#\#}P < 0.001$ vs. control group; $^{**}P < 0.01$ vs Cy group; $^{\$\$}P < 0.01$ vs. (SRBC + Cy) group.

2.3. *Effect of pidotimod and RGAP on the PFC count in the spleen for the primary response to SRBC*

Results of PFC responses to the initial challenge with SRBC are shown in Fig. 2. Cyclophosphamide was able to reduce the number of PFCs in the spleen significantly, showing a count of $(6.2 \pm 2.2)/10^6$ splenocytes compared to $(48.5 \pm 9.9)/10^6$ in SRBC-treated mice. The number of PFCs was significantly elevated when mice were co-treated with pidotimod and RGAP.

2.4. *Effect of pidotimod and RGAP on serum IgG and IgE levels*

As shown in Fig. 3A, the IgG level in serum was increased to 970.6 ± 123.3 µg/ml by the second challenge of SRBC, but significantly decreased to 688.7 ± 54.3 µg/ml by treatment with cyclophosphamide. It was restored by the combination of pidotimod and RGAP in cyclophosphamide-treated mice. As regards IgE levels, cyclophosphamide induced a significant increase to 1419.7 ± 94.1 ng/ml (Fig. 3B). Monotreatment with RGAP (300 mg/kg) markedly inhibited the IgE level to 792.9 ± 99.7 ng/ml, while the combination of pidotimod with RGAP was unable to improve it.

2.5. *Toxicity tests*

Bodyweights and organ weights were measured after mice were treated with pidotimod and RGAP alone or in combination for 6 weeks. There was no significant change between any of two groups (data not shown).
None of the groups showed mortality or toxic changes on histopathology examinations of spleen, thymus, and lymph nodes. No significant differences were observed in serum GPT and creatinine levels in the various groups of mice (Table 2).

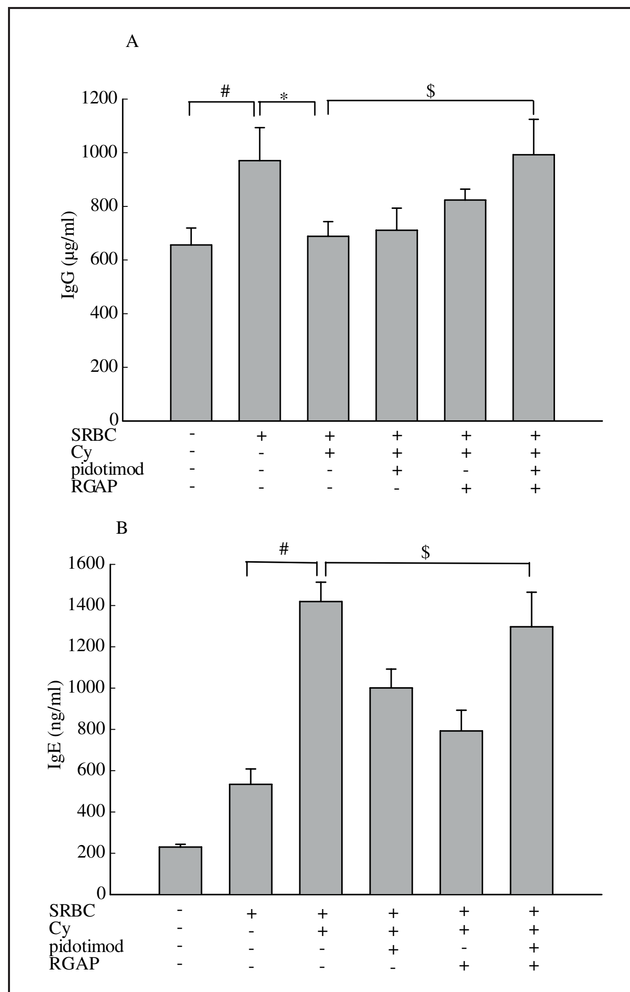

Fig. 3: Effect of pidotimod and RGAP on serum IgG (A) and IgE (B). Immunization induced by SRBC at 4×10^8/mouse i.p. on Day 0 to all mice except negative control group. Immunodepression was induced by cyclophosphamide (Cy) i.p. at 50 mg/kg on Days 3, 4 and 5. Different groups treated p.o. with: pidotimod 200 mg/kg/day; RGAP 300 mg/kg/day; and pidotimod (200 mg/kg/day) plus RGAP (300 mg/kg/day) combination. Mice received a second challenge with SRBC four weeks after the initial one and were sacrificed another two weeks later. Each bar represents mean ± SEM of 10 mice. $^{###}P < 0.001$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Cy group; $^{\$}P < 0.05$ vs. (SRBC + Cy) group

Table 2: Effect of pidotimod and RGAP on serum GPT (IU/l) and creatinine (mg/ml)

Group	GPT (IU/l)	Creatinine (mg/ml)
Control	11.8 ± 1.2	69.0 ± 2.0
SRBC	13.1 ± 0.5	70.0 ± 0.6
SRBC + Cy	13.6 ± 0.7	64.3 ± 1.3
SRBC + Cy + Pi	10.9 ± 1.3	68.0 ± 2.3
SRBC + Cy + RGAP	10.1 ± 1.2	59.8 ± 1.7
SRBC + Cy + Pi + RGAP	9.7 ± 0.9	63.8 ± 1.7

Each value represents mean ± SEM of 10 mice.
Cy = cyclophosphamide, Pi = pidotimod

3. Discussion

Integrative medicine, combining the theories and treatments of both western and traditional medicine, has become a growing trend in medicine in parallel with social developments since the 1970's (Feng 1977; Liu 2003). Previous reports have indicated some progress in pre-clinical and clinical research (Chen et al. 2003; Wang and Zhou 2003). Both pidotimod and RGAP have been regarded as possessing immunostimulating activity. This concept led us to evaluate the synergistic effect of pidotimod and RGAP on antibody response.

Cyclophosphamide and methotrexate are chemotherapeutic drugs used to treat various types of cancer and some autoimmune disorders by slowing or stopping cell growth. Both of them interfere with the growth of certain body cells, especially cells that reproduce quickly such as lymphocytes, which decrease the immune system's response to various diseases (Choudhury et al. 2000; Bafna and Mishra 2006). As expected, cyclophosphamide or methotrexate-treated mice showed immunosuppression in WBC count, spleen cellularity, and antibody production, which were restored to different extents by the administration of RGAP and pidotimod alone or in combination.

The humoral immune response was evaluated by measuring the antibody response to challenge with SRBC and *E. coli* LPS. The first antigen is thymus T cell-dependent, since co-operation of T helper cells with B cells is necessary for the generation of an antibody response. The antibody response to challenge by SRBC was assessed by PFC assay for the primary immune response and by serum IgG assay for the secondary immune response. The thymus-independent immune response to LPS is well established for the mouse (Andersson and Blomgren 1971; Moller and Michael 1971). Antibody production against LPS is a function of B lymphocytes only (Andersson and Blomgren 1971; Ward et al. 1984), as was determined by PFC assay in this study.

B cells in the spleen produce antibodies, predominantly the IgM isotype, for the primary antigen that then bind to SRBC membrane antigens and produce lysis of the SRBC and the subsequent formation of plaques. PFC assay has been a widely used method of assessing the humoral immune response. The secondary response to SRBC has a large IgG component. The production of antigen-specific antibodies represents a major defense mechanism of humoral immune responses and protects against infectious agents by neutralizing viruses, destroying targets by antibody-dependent cellular cytotoxicity and complement-mediated lysis, or by enhancing phagocytosis through opsonization. Our results demonstrated that combined treatment with pidotimod and RGAP markedly elevated the primary PFC in the spleen which was suppressed by cyclophosphamide, whereas treatment with pidotimod or RGAP individually did not exert any effect. Memory function was increased by co-treatment with pidotimod and RGAP because the IgG production of the secondary response of SRBC was restored, which was an indicator of enhancement of the function of innate B cells and memory T cells (Luster et al. 1982).

Animals treated with cyclophosphamide showed elevated IgE levels indicating the potential for hypersensitivity as cyclophosphamide damages the short-lived suppressor T-cells in immune regulatory systems (Mitsuoka et al. 1976). It has also been reported that cyclophosphamide worsened features of allergic pulmonary inflammation in ovalbumin-immunized BALB/c mice in association with increased production of IgE and T helper type 2 (Th2) cytokines (Su et al. 2006). Our results showed that only RGAP inhibited the boost of IgE production, whereas neither monotreatment with pidotimod nor combined treatment showed any change in the IgE increase induced by cyclophosphamide. B-Lymphocytes can switch expression of Ig class (isotype) from IgM to IgG, IgE, or IgA during

an immune response (Kracker and Radbruch 2004). Cytokines such as interleukin (IL)-4, IL-5, IL-6, and tumor necrosis factor alpha (TNF-α) (mostly Th2 cytokines) can direct murine class switching to IgG1 and IgE, and human class switching to IgG4 and IgE, while IL-2, interferon (IFN)-γ, IFN-α, and transforming growth factor-β (TGF-β) (mostly Th1 cytokines) promote murine IgG2a production but inhibit IgE production (Ayoub et al. 2003; Packard and Khan 2003; Kracker and Radbruch 2004). IgE production is critically dependent on the Th1/Th2 ratio in which Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-5) cytokines are elicited *in vivo* (Lin et al. 2006). Recent studies have demonstrated that Korean red ginseng saponin fraction and its genuine ginsenosides potently inhibit the passive cutaneous anaphylaxis reaction induced by IgE, probably by regulating expression of IL-1β and IFN-γ (Bae et al. 2006). In our previous study, RGAP alone or combined with pidotimod significantly restored IFN-γ levels in immunosuppressed mice, whereas pidotimod alone did not. Hence, we assumed that RGAP enhanced the secretion of Th1 cytokines, secondary inhibited the isotype switching to IgE, and maintained the lower serum IgE levels *in vivo*.
Furthermore, cytotoxicity using these drugs was investigated simultaneously by assessing hematological and pathological parameters. Macroscopic and microscopic examination of the spleen, thymus, and lymph nodes of mice which were treated with pidotimod and RGAP did not show any differences compared with control animals. Commonly used serum biomarkers of hepatotoxicity and nephrotoxicity: GPT and creatinine were tested. No increased levels in serum indicating functional injury in liver and kidney were found in treated mice.
On the basis of the results obtained in the present study, it can be concluded that combined treatment with pidotimod and RGAP possesses a potent immunostimulating action on the humoral immune system challenged with TD and TI antigen in immunosuppressed mice. The combination showed good results and in almost all cases was better than the respective monotherapies. The combination tested here could offer alternative therapies for immunosuppression. Further studies on molecular mechanisms related to the synergistic action have already been started and will follow in our future communications.

4. Experimental

4.1. Red ginseng acidic polysaccharide (RGAP)

RGAP was provided by Korean Tobacco and Ginseng Company (Korea). It was isolated from *Panax ginseng* C.A. Meyer as described previously (Kyeong 2000). RGAP was confirmed to be the acidic polysaccharide fraction composed of about 56.9% uronic acid, 28.3% neutral sugar and less than 1% protein. The analysis of the component sugars in RGAP by GC revealed that the polysaccharides in RGAP contained about 51.8 mol% glucuronic acid, 26.1 mol% glucose and 5.1 mol% galacturonic acid as major components, and arabinose, rhamnose, and galactose as minor components. One milligram of RGAP contained less than 0.006 EU (endotoxin units) of endotoxin, which did not affect the experimental results obtained with RGAP.

4.2. Animals

Inbred female BALB/c mice (4 weeks) weighing 14 ~ 16 g were purchased from Koatech Company, Korea. They were housed under standard conditions of temperature (23 ± 3 °C), relative humidity (60 ± 10%), and 12/12 h light/dark cycles and fed with Certified Rodent Diet (Koatech Company, Korea) and tap water *ad libitum*. The experimental protocol was approved by our institutional animal care and use committee.

4.3. Study design

Groups of 30 mice were established for each treatment. Ten mice were used for the TI antigen study and twenty for the TD antigen study.
For the TI antigen study, immunization was induced by LPS (Sigma-Aldrich, USA) at a dose of 10 μg/mouse i.p. (Coppi and Manzardo 1994). Immunosuppression was induced by methotrexate (Calbicchem, USA) at a dose of 20 mg/kg i.p. on the day after immunization. The different groups were treated p.o. with the following: pidotimod (Yungjin Pharmaceutical Company, Korea) at a dose of 200 mg/kg/day; RGAP at a dose of 300 mg/kg/day; and the combination of pidotimod (200 mg/kg/day) plus RGAP (300 mg/kg/day), starting two weeks prior to LPS injection. Mice were sacrificed four days after immunization and the spleen was processed to a single cell suspension and used for the determination of PFC response as follows.
For the TD antigen study, immunization was induced by SRBC at a dose of 4×10^8/mouse i.p. on day 0 to all the mice except for the negative control group (Thejass and Kuttan 2007). Immunosuppression was induced by cyclophosphamide (Choongwe Pharmaceutical Company, Korea) at a dose of 50 mg/kg i.p. on days 3, 4 and 5 (Coppi and Manzardo 1994). The treatments were at the same doses as described above and began from the third day prior to the challenge with SRBC. The immunological response was evaluated *in vitro* by the PFC test in response to SRBC on day 6. WBC and spleen cells were counted. Mice received a second challenge with SRBC four weeks after the initial one and were sacrificed a further two weeks later. Serum antibody response was evaluated by IgG and IgE level. GPT and creatinine in serum were measured to test the hepatic and renal functions. Spleen, thymus and lymph nodes (mediastinal, mesenteric, and mandibular) were fixed immediately in 10% neutral buffered formalin and subjected to histopathological examination. Processed tissues were embedded in paraffin, cut to a nominal thickness of 5 μm, and stained with hematoxylin and eosin (Sigma-Aldrich, USA).

4.4. PFC assay

The method described by Ladics (2007) to detect antibody-forming spleen cells was adopted with some modifications. Single splenocyte suspensions were obtained in RPMI-1640 medium (Sigma-Aldrich, USA). Naturally occurring antibodies to SRBC were removed by absorption with SRBC.
Appropriate amounts of splenocyte suspension, LPS-coated SRBC as a TI antigen or SRBC as a TD antigen, and guinea pig complement (Samtako Bio Korea Inc, Korea) were added to 0.5 % agar (Becton, Dickinson and Company, USA) which was made in EBSS (WelGENE, Inc. Korea) with 0.05% diethylaminoethyl dextran (Becton, Dickinson and Company, USA). LPS-coated SRBC were prepared as previously described (Blankwater et al. 1975). The mixture was poured into the petri dish and covered by a glass cover and placed in a 37 °C incubator for 3 h. The plaques were counted with a dissecting microscope. Data were typically expressed as PFC/10^6 splenocytes.

4.5. Assay of immunoglobulin in serum

Blood was collected from the posterior vena cava and antibody levels in serum were analyzed by a sandwich enzyme-linked immunoabsorbent assay (ELISA) using the mouse Immunoglobulin ELISA Quantitation Kit (Bethyl, USA). The absorbance at 450 nm was measured with an ELISA reader (Jas.Co, V-530) to calculate the result in relative units using a four parameter logistic regression analysis.

4.6. Assessment of GPT and creatinine in serum

Serum GPT and creatinine levels in serum were determined with commercial kits (Asan Pharmaceutical Company, Korea) using the methods of Rietman and Frankel, and Jaffe, respectively.

4.7. Statistics

Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 13.0. For multiple comparisons, data were analyzed by one-way ANOVA followed by Dunnett's test when significant differences were detected. Comparison between two groups was made using the Student t-test (two-tailed). Differences were considered statistically significant if $P < 0.05$.

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