Methotrexate (MTX) is a folate antagonist that exerts anti-proliferative effect via inhibition of nucleotide synthesis. Its effects were first reported in acute leukemic children, and it is now used to treat patients with malignant and autoimmune diseases. However, the side effects of long-term MTX therapy could increase infection rates due to its myelosuppressive activity. Thus, MTX has some limitations in chemotherapy, even though MTX has replaced the more powerful and toxic antifolate, aminopterin. In addition, it has been suggested that the immunosuppressive actions of MTX, such as myelosuppression, may be related to cytokine expression change, increased IL-4 and IL-10 gene expression and decreases in proinflammatory cytokines such as IL-2 and IFN-γ.

Panax ginseng is categorized as either cultivated or wild growing naturally in forest. A previous study found that the immunomodulating effects of wild...
Panax ginseng (WPG) may be substantially stronger than those of domesticated Panax ginseng. Recently, our lab also observed that WPG appears to have the potential to defend against benzo[a]pyrene-induced carcinogenic risk. Red-mold rice (RMR), also known as ‘Koji’ in Japan and ‘Hong Qu’ or ‘Ang Khak’ in China, has been used in many kinds of foods and folk medicines for thousands of years in Eastern Asia. It has various beneficial effects such as anti-hypertension, antibacterial activity and macrophage stimulating activity.

The main objective of this study was to examine whether the mixture of wild Panax ginseng and red-mold rice extracts (MPR) could potentiate immune response in RAW264.7 macrophage cells.

Here we report that MPR potently protects against MTX-induced cytotoxicity and induces IL-1α, IL-1β, and IL-6 mRNA expression. In addition, the co-treatment of MPR with MTX showed similar cytotoxic activity as MTX treatment alone on MCF-7 breast carcinoma cells.

Materials and Methods

1. Reagents

Methotrexate was obtained from Calbiochem (Darmstadt, Germany). For the cell culture, FBS and DMEM were purchased from Gibco Lab (Grand Island, NY, USA). XTT was acquired from Roche Diagnostics (Germany) for the cell viability assay. TRI-Reagent was purchased from Molecular Research Center Inc. (Cincinnati, OH, USA) to isolate the RNA, and a PCR kit was obtained from Solgent Co. (Daejeon, South Korea).

2. MPR extracts preparation

In this study, we prepared 5 different proportions of MPR by increasing the RMR ratio against 0.05mg/ml of WPG, in order to make an enhanced immunostimulant as compared to WPG and RMR alone. The herbs used in this study were collected and identified by the College of Oriental Medicine, Dongguk University (Gyeongju, South Korea) and Myco Company (Gyeongju, Korea), respectively. The method used for the preparation of WPG has been described previously. The RMR was extracted by mixing 50g of RMR with 300ml of 80% EtOH and heating the formula to 70°C under reflux for 3h. The extract was filtered with filter paper (Advantec No. 2, Toyo Roshi Kaisha, Japan), and the filtrate was concentrated to approximately 100ml using a rotary evaporator (Heidolph, Germany) at 70°C under vacuum, and then freeze-dried (Bondiro, Ilshin, Korea). The yield ratio of each dried extract was approximately 13.82% WPG and 1.48% RMR from the original weight, respectively. The combinations made by the lyophilized powders of each extract were dissolved in saline prior to use.

3. Cell culture

RAW264.7 cells (KCLB 40071), a mouse macrophage, were obtained from the Korean Cell Line Bank (Seoul, South Korea) and MCF-7 cells (HTB-22), a human breast carcinoma, were acquired from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified incubator (5% CO₂ in air at 37°C).

4. Cell viability assay

Cells (1 × 10^5 cells/ml) were seeded into a 96-well plate. After 18 h, the cells were cultured with serum free media for a further 18 h, and then treated with the different concentrations of WPG, RMR, or MPR, in the presence and absence of MTX for 24 h. At the end of the assay time period, 50µl of sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT)
was added to each well to measure the cell viability. After 4 h of incubation, the absorbance was measured on an ELISA reader (Molecular Devices, Toronto, Canada) at a wavelength of 490 nm. The background control wells containing the same volume of complete culture medium without cells were also analyzed. The percentage cell survival was defined as the relative absorbance of the untreated cells divided by that of the treated cells.

5. Determination of nitric oxide level (Griess reaction)

After 24 h treatment with MPR in the presence or absence of 1 μM LPS (2 hrs pretreatment), 50 μl of supernatant from each well of the cell culture plates was transferred into 96-well microplates, and equal volume of Griess reagent (1% sulfanilamide, 0.1% N- (1-naphthyl)-ethyline diamine hydrochloride, 2.5% H₃PO₄) was then added to the supernatant. The absorbance at 540 nm was determined in a UV-Vis microplate reader (SpectraMax Plus, Molecular Devices) after 15 min at room temperature. The concentrations of nitrite were derived from regression analysis using serial dilutions of sodium nitrite as a standard.

6. RT-PCR

Total RNA was isolated from the treated and untreated cells (5×10⁵ cells/ml in 6-well plate) using a Tri-Reagent, which is an RNA/DNA/protein isolation reagent. Reverse transcription-polymerase chain reaction amplification was conducted as described by Jun et al. (Briefly, the cDNA was synthesized by reverse transcriptase (RT) with 0.5 μl of Random 9 Primer, 20 units of AMV reverse transcriptase, 0.5 μg of the total cellular RNA, 4 μl of 5 X RT buffer, 0.5 mM each dNTP, and 2 μl of DEPC. The 20 μl reaction mixtures underwent the following treatments: incubation at 30°C for 10 min, followed by 42°C for 1 h, heating to 95°C for 5 min, and rapid chilling on ice.

<table>
<thead>
<tr>
<th>Table 1. Primer Sequence Used for Detection of Cytokine Gene Expressions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oligonucleotide sequence</strong></td>
</tr>
<tr>
<td><strong>IL-1α</strong></td>
</tr>
<tr>
<td><strong>IL-1β</strong></td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
</tr>
<tr>
<td><strong>IL-10</strong></td>
</tr>
<tr>
<td><strong>IL-4</strong></td>
</tr>
<tr>
<td><strong>TGF-β</strong></td>
</tr>
<tr>
<td><strong>INOS</strong></td>
</tr>
<tr>
<td><strong>COX-2</strong></td>
</tr>
</tbody>
</table>
The presence of cytokine expression was determined by the polymerase chain reaction (PCR) (MJ Research, Reno, NV, USA) using the sequence specific primers. β-actin was used as the internal control in order to ensure that equal amounts of RNA had been used. The PCR was carried out in a 1X reaction buffer containing 0.5 mM dNTP, 10 pmole of the sense and antisense primers, 0.5 μl cDNA, and 5 units of Taq DNA polymerase in a 25 μl reaction mixture. The following primers sequences were used: (Table 1)

The number of amplification cycles was empirically determined for each primer pair to identify the logarithmic phase. Each primer was designed using Primer 3 software (Whitehead Institute for Biomedical Research) to have a GC content of ~55%; BLAST searches were used to confirm the specificity of the selected nucleotide sequences. Band intensities of the amplified DNAs were compared after visualization on an UV transilluminator.

7. Statistics

The data are expressed as a mean ± S.E. The statistical comparisons were made using a sigma plot. The significant differences (P < 0.05, 0.005, 0.0005) between the means of the control and the treated cells were analyzed using a Student’s t-test.

### Table 2. Effects of WPG, RMR, and MPR on the Cell Viability of Raw 264.7 Macrophage Cells.

<table>
<thead>
<tr>
<th>WPG (mg/ml)</th>
<th>0.05</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of control</td>
<td>121.9±4.4***</td>
<td>129.0±4.9***</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RMR (mg/ml)</th>
<th>0.05</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of control</td>
<td>114.5</td>
<td>107.9</td>
</tr>
<tr>
<td>±5.8***</td>
<td>±3.7***</td>
<td>±2 **</td>
</tr>
</tbody>
</table>

| MPR | 135.5 | 127.8 | 136.0 | 129.6 | 124.8 |
| % of control | 1 | 2 | 3 | 4 | 5 |
| ±13.9** | ±8.8*** | ±9.5*** | ±7.4*** | ±7.8*** |

We prepared 5 different proportions of MPR by increasing RMR ratio against 0.05mg/ml of WPG concentrations. Values indicate the relative cell viability (relative level in untreated control=100%) of WPG, RMR, and MPR, respectively. Cell viability was determined by XTT assay as described in Materials and Methods. The data was obtained from six independent triplicate experiments. The values are reported as a mean ± S.E., **p<0.05, ***p<0.005, ****p<0.0001 compared with the control group. The number of 1–5 means the ratio of RMR against 0.05mg/ml of WPG concentration.

1. Effects of MPR and its components on the proliferation of RAW264.7 macrophage cells

RAW264.7 cells were stimulated with 5 different proportions of MPR for 24h, and then the mitogenic activity of the MPR was examined by XTT assay. According to the different proportions of MPR, cell proliferation increased to 136, 128, 136, 130 and 125% of the control, respectively (Table 2). Additionally, we estimated the effects of WPG and RMR, on RAW264.7 cell proliferation. WPG treatment (0.05-0.1mg/ml) increased cell proliferation dose-dependently to 122% and 129% of the control, respectively. In contrast, the addition of RMR showed enhanced cell proliferation at low doses (0.05-0.15 mg/ml), but decreased proliferation significantly at higher doses (0.2-0.25mg/ml).

2. Effects of MPR on MTX-induced cell toxicity in RAW264.7 macrophage cells

In this study, the effect of MTX on cell proliferation was evaluated using XTT assay. MTX treatment caused a dose-dependent decrease in cell viability, and the maximum effect was observed with 2mg/ml MTX, showing 59.7% cell viability of the control (Fig. 1A). We next examined whether MPR could
inhibit the suppression of cell proliferation by MTX. Here, 0.5mg/ml of MTX decreased the cell viability by 65.7% of the control, and this toxicity was significantly recovered to the level of the control group by co-treatment with MPR (Fig 1B). These results imply that MPR, even though the RMR ratio increase, can potentiate the proliferation of RAW264.7 macrophage cells in the presence or absence of MTX.

3. Effects of MPR on NO and COX-2 expression in RAW264.7 macrophage cells

To determine the effects of MPR on NO and COX-2 expression, RAW264.7 cells were treated with 5 different proportions of MPR with or without LPS (1µg/ml) for 24h, and then the levels of NO production, iNOS, and COX-2 mRNA expression were monitored (Fig 2). MPR alone slightly increased NO production; however, it was markedly increased in LPS-stimulated RAW264.7 macrophage cells.

The amount of NO released by the cells was measured by the Griess reaction (A). The levels of iNOS and COX-2 mRNA expression were assessed by RT-PCR (B). The results were confirmed from at least three independent experiments. The values are expressed as mean ± S.E. * p<0.05, ** p<0.005, *** p<0.0001 as compared to the control group. Numbers 1–5 correspond to the ratio of RMR against 0.05mg/ml of the WPG concentration.
expression, but it did not affect the expression of COX-2 mRNA. MPR reduced the NO accumulation caused by LPS in the order of RMR ratio (Fig 2A). Quantitative RT-PCR showed that MPR slightly induced iNOS mRNA expression; whereas, exposure to LPS clearly enhanced the expression of iNOS mRNA, but this enhancement was gradually decreased in a dose-dependent manner by the co-treatment with MPR. In addition, MPR also slightly induced COX-2 mRNA expression, but it did not affect the expression of COX-2 mRNA with LPS treatment (Fig 2B).

4. Effects of MPR on the cytokine expression of MTX–treated RAW264.7 macrophage cells

We examined that the influence of MPR on the cytokine mRNA expressions of the MTX-treated RAW264.7 cells. Cells were incubated with 5 different proportions of MPR with or without MTX (500μg/ml) for 24h, and then IL-1α, IL-1β, IL-6, IL-10, TNF-α and TGF-β mRNA expressions were examined (Fig 3). The RT-PCR analysis showed that there were significant inductions of IL-α, IL-1β and IL-6

![Fig. 3. Modulation of cytokine mRNA expressions in MTX–treated RAW264.7 macrophage cells by MPR.](image)

Cells were treated with MPR in the presence and absence of MTX (500µg/ml) for 24h. The level of each cytokine mRNA was assessed by RT-PCR as described in Materials and Methods. Results were confirmed by three independent experiments. The number of 1–5 means the ratio of RMR against 50µg/ml of the WPG concentration.

![Fig. 4. Effects of MPR on the cell viability in MTX–treated MCF–7 human breast carcinoma cell lines.](image)

Cells (1 × 10^6 cells/ml) were incubated with MPR in the presence and absence of MTX (500µg/ml) for 24h. Cell viability was measured by XTT assay and the data were obtained from six independent experiments. Data are mean ± S.E. The mark “a” indicates no significant relation against “a”. Numbers 1–5 correspond to the ratio of RMR against 0.05mg/ml of the WPG concentration.
Mixture of Wild Panax Ginseng and Red-Mold Rice Extracts Activates Macrophages through Protection of Cell Regression and Cytokine Expression in Methotrexate-Treated RAW264.7 Cells

mRNA expression by MPR, regardless of MTX co-treatment. In addition, TNF-α was induced by MPR independently of MTX treatment. However, neither MPR nor MTX changed IL-10 and TGF-β expression.

5. Effect of MPR on the proliferation of MTX–treated MCF–7 breast carcinoma cells

We extended our research to determine whether MPR could be used together with MTX in adjuvant chemotherapy. MCF-7 breast carcinoma cells were treated with 5 different proportions of MPR in the presence or absence of MTX (500μg/ml) for 48h, and then the effects of MPR on the cell viability were evaluated by XTT assay (Fig 4). The co-treatment of MPR with MTX decreased cell proliferation to the level of MTX treatment alone. However, MPR alone did not show any cytotoxic effect on MCF-7 cell proliferation. These results strongly suggest that MPR could be used with MTX in anti-cancer therapy, because it did not prevent cytotoxic action of MTX against human breast carcinoma cells.

Discussion

Almost all chemotherapeutic regimens, which affect malignant and other fast-dividing cells such as those responsible for hair growth and host defense, are physically exhausting for patients due to their side effects such as nausea, vomiting, and diarrhea, in addition to infection and sepsis by immunosuppression. Therefore, patients with advanced-stage diseases usually consider using herbal immune stimulants in an attempt to overcome immunosuppression or to counteract infections.

Mixtures of herbs can have greater beneficial effects as compared to a single plant extract, because of synergistic effects or prevention of side effects. MPR consists of wild Panax ginseng and red-mold rice, the solid-state fermentation product of Monascus, which is usually prescribed for increase and protection of host immunity.

Panax ginseng has been studied for its several immunomodulatory properties. Juzen-taiho-to, including Panax ginseng, enhanced host defense against an infectious agent via macrophage activation. Also, the systemic administration of Panax ginseng stimulated basal levels and the recovery of NK cell function in immunosuppressed mice caused by cyclophosphamide treatment. It is widely accepted in both Korea and China that wild Panax ginseng is more active than cultivated in stimulating host defense system, and contains higher levels of ginsenosides.

RMR is manufactured based on a traditional herbal remedy, and it is now used for disease prevention in the United States and many Asian countries. Recently, it has been studied for its beneficial compounds such as lovastatin, flavonoids, polyunsaturated fats, phytosterols, and pyrrolinic compounds, etc. Several other compounds, which have a variety of beneficial activities such as chemopreventive potential, antitumor activity, and macrophage stimulating activity, have been isolated from RMR. These findings indicate that each herb plays a role in cell proliferation and immune response. On the basis of the known function of each herb as described above, we examined whether MPR could potentiate the function of macrophages in MTX-treated RAW264.7 macrophage cells.

MTX, originally used as part of combination chemotherapy regimens to treat many kinds of cancers by inhibiting the metabolism of folic acid, acts mainly during DNA and RNA synthesis, and thus, inhibits the growth and proliferation of non-cancerous cells, which include rapidly dividing cells such as bone marrow and gastrointestinal mucosa cells, and also causes side effects such as myelosuppression and mucositis. In addition to its inhibitory effect on B cell function, MTX also suppresses macrophage function, modulates IL-1 and superoxide anion production, and inhibits neutrophil chemotaxis. It has been proposed that MTX may cause these effects on cellular and humoral immunity as well as on cytokine secretion, due to its ability which can...
modulate the purine and pyrimidine synthesis that is required for cell division, resulting in cytotoxicity on cells generate cytokines or incite other cells to generate cytokines\(^23\).

Macrophages are known to produce a variety of immuno-modulatory factors, including cytokines, leukocyte adhesion, and nitric oxide (NO). Our results showed that MPR not only significantly enhanced macrophage cell proliferation as compared to WPG alone, but also protected from the cell toxicity by RMR alone at high dosage (0.2~0.25mg/ml) as well as MTX (500\(\mu\)g/ml) treatment. In the immune system, NO is one of the primary effector molecules of the activated macrophage, is a double-edged sword that mediates both beneficial (microbicidal, tumoricidal) and detrimental effects (host cell death and immunosuppression on B and T cell function\(^24,25,26\)). COX-2 is responsible for PGE\(_2\) synthesis, which in turn triggers NO release and subsequent macrophage activation\(^27\). As shown in Fig. 2, MPR alone slightly increased NO production in RAW264.7 macrophage cells. Its production was predominantly elevated by LPS treatment; however, this accumulation gradually decreased when co-treated with MPR, in the order of the RMR concentration. MPR also mildly induced iNOS mRNA expression, whereas its induction was markedly up-regulated by LPS. In addition, the co-addition with LPS and MPR decreased this enhancement, dependent on the RMR ratio. These data were consistent with those of NO production. This study suggests that MPR could enhance the immune activity of macrophages via the induction of NO release within physiological range. Moreover, it may reduce the excessive NO production implicated in the pathogenesis such as chronic inflammation.

It has been reported that LPS induces the expression of an inducible cyclooxygenase-2 (COX-2), which is responsible for prostaglandin E2 (PGE\(_2\)) synthesis and subsequent macrophage activation\(^28\). PGE\(_2\) is involved in NO-mediated antileishmanial immunity by triggering NO release, and perhaps in the protective immune response against other intracellular pathogens\(^27,29,30\). In the present study, MPR alone moderately induced COX-2 mRNA expression, whereas it did not affect enhanced COX-2 mRNA expression in LPS stimulated RAW264.7 cells. The difference between the COX-2 mRNA expression and that of iNOS by MPR in LPS stimulated RAW264.7 cells might be due to other enzymes that may affect iNOS mRNA expression, and were inhibited by co-treatment with MPR.

In the search for new immunomodulating agents over the past few years, many traditional herbal medicines have been evaluated and their abilities reported, showing the enhancement of various types of immune response\(^31,32,33\). Recently, there was a report that cycloartane- and oleanan-type triterpenes from these species possess prominent IL-2 inducing activity, and might have a contributory role in the immunostimulating and anticancer effects of Astragalus species\(^34\). In addition, IL-1\(\alpha\) and IL-12p40 mRNA were induced by Astragalus radix regardless of MTX co-treatment in RAW264.7 macrophage cells\(^35\).

Our results demonstrate that MPR significantly induced IL-1\(\alpha\), IL-1\(\beta\) and IL-6 mRNA expression, as well as TNF-\(\alpha\), regardless of MTX co-treatment, which was dependent on the RMR ratio of the MPR. On the other hand, MPR or MPR plus MTX did not modulate IL-10 and TGF-\(\beta\) mRNA expression. Based on the previous report that IL-1\(\alpha\), IL-1\(\beta\), IL-6, and TNF-\(\alpha\) are generated in response to immunological reaction, inflammation, and microbial invasion in macrophages, leading to secondary immune response such as the proliferation of T and B cells, activation of macrophages for phagocytosis, and the killing of microorganisms\(^36\), therefore, the macrophage cell proliferation and induction of IL-1\(\alpha\), IL-1\(\beta\), IL-6, and TNF-\(\alpha\) at the transcription level by MPR suggest that MPR has a potential effect on macrophage activation, resulting in immune activation. In addition, the co-treatment with MTX and MPR caused a significant decrease in MCF-7 human breast carcinoma
Mixture of Wild Panax Ginseng and Red-Mold Rice Extracts Activates Macrophages through Protection of Cell Regression and Cytokine Expression in Methotrexate-Treated RAW264.7 Cells

cell viability. It was similar to MTX treatment alone, even if MPR alone did not show any cytotoxic effect on MCF-7 human breast carcinoma cells. These results propose that MPR may have therapeutic potential as an adjuvant in chemotherapy, as it did not disturb MTX cytotoxicity against the MCF-7 human breast carcinoma cells. In conclusion, we suggest that MPR could protect against the suppression of immune response by MTX via activation of macrophage cells.

Acknowledgments

This work was supported by the Dongguk University Research Fund of 2009.

References

15. Akagawa G, Abe S, Tansho S, Uchida K, Yama...


Mixture of *Wild Panax Ginseng* and *Red-Mold Rice* Extracts Activates Macrophages through Protection of Cell Regression and Cytokine Expression in Methotrexate-Treated RAW264.7 Cells


