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Citrus Peel Extract Inhibits LPS-induced Cytokines Secretion in Macrophage

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Abstract

Citrus peel is a useful research resource containing important physiological function materials. In this study, anti-inflammatory effects of Citrus peel extracts were identified in LPS-induced macrophage cells. After the pretreatment of lipopolysarcharide (LPS), inhibitory effects of nitric oxide (NO), inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2) and pro-inflammatory cytokines, tumor necrotic factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1 (IL-1), production were measured according to the treatment of citrus peel extracts. Study results showed approximately 40% inhibition of NO production but no concentration-dependent effects when citrus peel extracts were treated. According to the results of inhibitory effects of pro-inflammatory cytokines production, IL-6 had about 44% inhibition of production, TNF-α had about 13% inhibition of production, and IL-1 had no inhibition of production. According to western blot results, iNOS protein's expression was slightly decreased. Also, COX-2 protein's expression was observed to be slightly decreased in cells treated with citrus peels as iNOS's result.

Keywords: *Citrus peel, NO, TNF-α, IL-6, iNOS, COX-2*

1. Introduction

When any damage is caused to the cells and tissues of the body, it will immediately respond by localizing the damages and furthermore will cause a serious of localized reaction in order to restore the damaged areas to the original condition. Such initial tissue response to the damage is called an inflammation [1]. Appropriate inflammatory response is indispensable reaction that protects the body from external and internal factors, however excessive and inappropriate inflammatory response becomes a main reason for the cause of various chronic diseases, with the death of cells and tissue necrosis and septicemia.

Periodontal disease is infectious diseases caused by pathogens within dental plaque, causing periodontal tissue destruction due to inflammation and especially cause of tooth loss due to destruction of alveolar bone [2]. Progression of periodontal disease is achieved by the mutual interplay of bacteria and host defense reaction. In the inflammatory response, macrophagocytes and monocytes are activated by recognizing various pathogen-associated molecular patterns including LPS, and form pro-inflammatory cytokines such as TNF-, IL-6 and IL-1, and also form NO and prostaglandin E2 (PGE2) synthesized by iNOS and COX-2 [3].

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Physiologically, NO plays a variety of roles, such as removing bacteria and tumor or blood pressure control and mediating neurotransmission. However, when inflammatory reaction occurs, it increases the expression of iNOS in the related cells generating large amounts of NO and excessively generated NO causes tissue damage, generic mutation and nerve damage, and increases vascular permeability edema expediting the inflammatory response such as edema [4].

The treatment of the periodontal disease can be divided into antibiotics, antibacterial and anti-inflammatory, but antibiotics have side effects such as causing expression of resistant bacteria, hypersensitivity reactions and gastrointestinal disorders [4]. Anti-inflammatory drugs which represent the Non-steroidal anti-inflammatory drugs (NSAIDs) suppress inflammation by inhibiting the production of PGE2, and antimicrobial agents such as phenolic compound, quaternary ammonium compound and bisbiguanide that shows plaque inhibition and antibacterial effect but causes problems such as surface staining of the teeth, gingivitis causing dissection of the mucous membranes and hypersensitivity. In order to overcome these side effects, herbal formulation researches are being actively conducted [5].

Citrus fruits are # 1 fruit in Korea which accounts for 30% of the domestic fruit production. Citrus peel belonging to *Rutaceae* is the dried state of the pericarp called citripericarpium which is a mature fruit of *Citrus unshiu Markovich*. From the past, it was used as the herbal medicine for eliminating nausea, indigestion, sputum and for having diuretic effect. Citrus peel not only remove hazardous substances, such as antioxidant activity, but prevent circulatory diseases, anti-allergic, anti-bacterial, anti-viral, and lipid-lowering effect, immune functioning, and capillaries mineralization have been reported. Chi *et al.* [6], for antibacterial effects of extracts, Park *et al.* [7], for the fermentation extract's antioxidant effects and increased enzyme activity of the citrus peel, Eun *et al.* [8], for the effects in immune cell regulation to the mouse using the citrus peel and Jung *et al.* [9], for the anti-arthritic effects using the citrus peel have been reported. Also the citrus peel contains a large amount of functional substances such as flavonoid, alkolid, lignin, monoterpenoid, sesquiterpenoid and triterpenoid having a high research value.

As such, the citrus peel is clearly a useful resource containing important physiological functional substances having a variety of developing possibility. Although the citrus peel has been used as a treatment in the private sector and used for processed foods up to now, there has been almost no report relating to periodontal disease and inflammation.

In the inflammation response mediated by the Macrophagocytes, regulating the expression of inflammatory cytokines and COX-2 which is the speed control phase enzyme of the biosynthesis iNOS and prostaglandin enzyme generated by NO, for the expression of these genes, the control of key signaling molecules of the MAPK and NF-kB activation has been recognized as a key factor to control the inflammatory response. Research has been actively conducted to find a new candidate material to control the inflammatory response by regulating the activity of these factors. In this study, in order to determine the effects of citrus peel extract against the inflammatory disease, after the pretreatment of the citrus peel extract to the RAW 264.7 cells, the effects on LPS induced NO production, expression iNOS and COX-2, IL-1, IL-6 and TNF-α production were examined.

2. Materials and Methods

2.1. Manufacture of extracts

For the research material, 100% domestic (Jeju) citrus powder was purchased for use. Mixing 60 g of citrus power and 600 ml of ethanol and storing at room temperature for 24 hours then applied the ultrasound for 1 hour and passed the vacuum filter. The extract was filtered using a vacuum evaporator to get concentrated citrus extract. The extract was diluted in distilled water at a concentration of 1.25 g/ml, and stored in the refrigerator.

2.2. Cell culture

The RAW 264.7 cells (KCLB, Seoul, Korea) was incubated at DMEM medium containing 10% FBS and penicillin/streptomycin100 unit/mL at 37°C, 5% CO_2 incubator and were subcultured every 3 days. After dispensing Raw 264.7 cells in a 12 well plate and cultured for 24 hours, it was replaced with serum free medium. Then the LPS 100 ng/mL and citrus peel extract (0, 0.1, 0.5, 1, 2%) were incubated for 24 hours.

2.3. MTT cell viability assay

To measure the viability of RAW 264.7 cells, the cytotoxicity was measured by MTT analysis method. After processing the citrus peel extracts by concentration, and after the cells were cultured for 24 hours then processing the MTT solution having 5 mg/mL of concentration, Incubated for an additional 4 hours at 37°C incubator. After incubation removing the supernatant, and absorbance was measured at 570 nm using the ELISA microplatereader.

2.4. NO measuring generation inhibitory activity

The amount of NO generated from RAW 264.7 cells in the form of NO_2 present in the cell culture medium was measured using the Griess reagent. After mixing the cell culture supernatant with 50 μl of Griess reagent (1% sulfanilamide in 5% phosphoric acid, 1% α-naphthylamide in H_2O) 50 μl in a 96 well plate reacting for 15 minutes, absorbance was measured at 540 nm using a microplate reader.

2.5. Enzyme linked-immunosorbant assay (ELISA)

Inoculating the Raw 264.7 cells in a 6-well plate at a concentration of 3×105 cells/ml and incubating for 24 hours at 37℃, 5% CO_2 in the incubator, and processed the citrus peel extract with LPS and was cultured for 24 hours. The cell supernatant was collected and TNF-a, IL-1 and IL-6 were quantified by ELISA.

2.6. Immunoblot analysis

RAW 264.7 cells were cultured for 24 hours prior to the processing of citrus peel extract and LPS. After it has been cultured, the treated group and the control group were collected. The protein analysis was performed through SDS-PAGE method. The protein was quantified by the Bio-Rad protein reagent after electrophoresis on a 10% SDS-PAGE. PVDF membrane was used to blot the protein of the gel. After acting with 1st antibody of iNOS and COX-2, it was reacted with the secondary antibody. To

determine the degree of expression of the protein, ECL detection reagents (Thermo Scientific, Pierce Biotechnology, Waltham, MA, USA) was used.

2.7. Statistical analysis

Mean ± standard deviation was used to display the graph as the value of the experimental data and performed the Students t-test which were considered significant if P was less than 0.05 (P <005). For each data obtained by performing three independent experiments with n = 3, the value was indicated as mean ± standard error of the mean.

3. Results

3.1. Effect of citrus peels extracts on cellular cytotoxicity

To find if citrus peels extracts influence the cell's survival rate, the cell's survival rate was measured with MTT method. If 80% or higher survival rate show in MTT reaction, it is determined that there is no cytotoxicity. When the extract is processed with concentrations of 10, 20, 50, 100 and 200μg/mL, cytotoxicity did not appear on 10-50μg/mL concentrations (Figure 1).

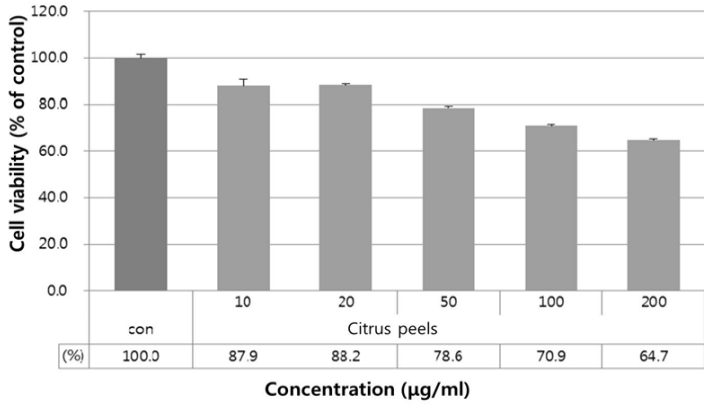

Figure 1. The cell viability of Raw 264.7 cells with citrus peels extracts. There was no difference between the control and the test group with citrus peels extracts at the concentration 10, 20, 50 μg /ml.

3.2. Inhibitory effect of citrus peels extracts on LPS-induced NO production

To observe NO creation inhibitory activities of citrus peels extracts in RAW 264.7 cells induced to LPS, citrus peels extracts is treated to cells in concentrations of 10 and 20 μg/ml, then the amount of NO was measured. In the group of cells treated with LPS, NO creation was found to be increased compared to the referenced group. And the experimental group of 10 μg/mL extract treatment was found to have about 40% of NO creation decreased (Figure 2).

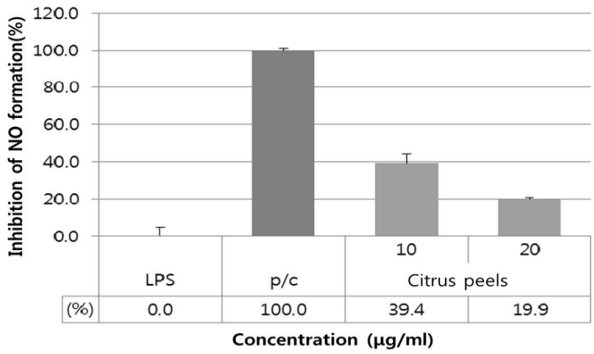

Figure 2. The inhibitory effect of citrus peels extracts at different concentrations for production of nitric oxide (NO). The level of nitrite in Raw 264.7 cells activated with LPS decreased as a dose-dependent pattern with citrus peels extracts

3.3. Inhibitory effect of citrus peels extracts on LPS-induced iNOS and COX-2

To investigate iNOS protein's relation on NO creation hindrance, immunoblot analysis was used to check iNOS protein's expression. According to the investigation, it was showed that LPS increased iNOS protein's expression, but with cells treated with citrus peels extracts, iNOS protein's expression was slightly decreased (Figure 3A). Also, COX-2 protein's expression was observed to be slightly decreased in cells treated with citrus peels extracts as iNOS's result (Figure 3B).

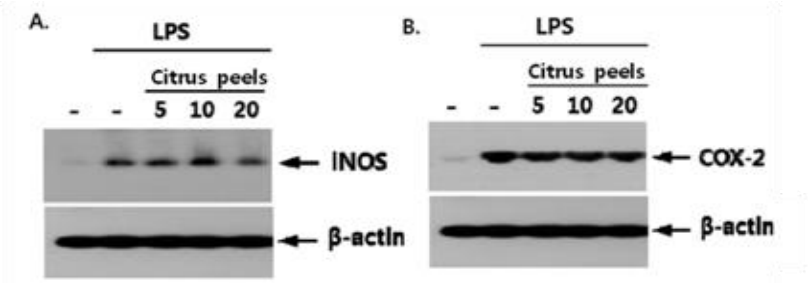

Figure 3. The expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in Raw 264.7 cells activated with LPS. The expression of iNOS was decreased in a dose-dependent pattern with citrus peels extracts. In accordance with western blot on COX-2, the expression was very slightly reduced

3.4. Inhibitory effect of citrus peels extracts on LPS-induced cytokine production

IL-1, IL-6, TNF-α and *etc.* are inflammatory cytokines and known to have interactions to each others. The inflammatory reaction occurred macrophage is a major forming cell. The experimentation was conducted by using ELISA for creations of IL-1, IL-6 and TNF-α, inflammatory cytokines from RAW 264.7 cell, the macrophage. The result showed that every cytokine creation was remarkably increased when LPS was treated (Figure 4). When 20 ug/ml citrus peels extracts were treated, IL-6 formation inhibitory efficacy showed to be about 44% and TNF-α formation inhibitory efficacy

showed to be about 13%. IL-1 formation inhibitory effect was not found in this experimentation.

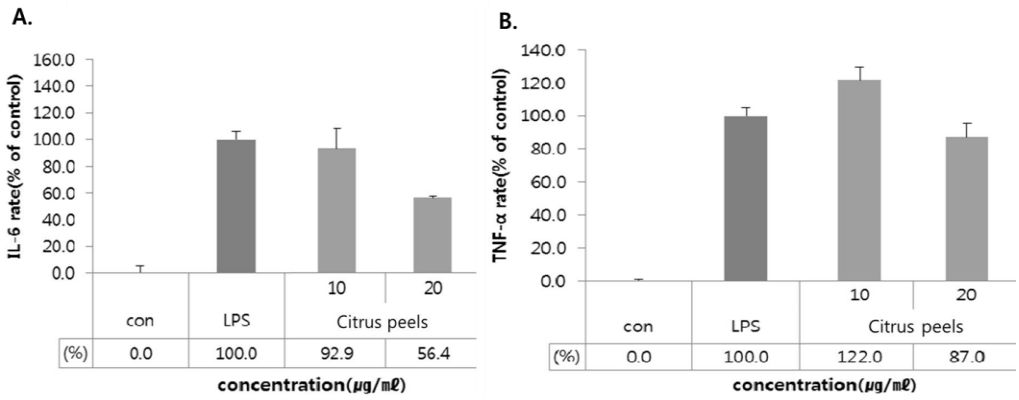

Figure 4. The anti-inflammatory effects of citrus peels extracts at different concentrations for production of interlukine-6 (IL-6) and tumor necrotic factor-α (TNF-α). The level of IL-6 and TNF-α in Raw 264.7 cells activated with LPS were decreased in a dose-dependent pattern with citrus peels extracts

4. Discussion

The drug research for periodontal diseases can be reviewed from two different perspectives as antimicrobials acting against bacteria and anti-inflammatory drugs controlling inflammatory progressions. The research regarding anti-inflammatory drugs are related to the periodontal disease progression and the critical factors for the tissue destruction such as cytokine, prostaglandin and collagenase.

NSAIDs are used as the main remedies for inflammatory diseases. However, since NSAIDs irritate the stomach wall causing the thickness reduction of the stomach wall as a complication, inflammations may be caused on not only the upper part, but, the lower part of gastrointestinal tract as well. Also, in case of chronic inflammatory disease, the drugs are mostly used for long terms with high doses so that the incident of a side-effect is higher than the acute inflammatory disease. Therefore, recently, as part of effort to develop substances to replace such medicines, trials to acquire effective substances from various natural materials are continuously conducted. And, as the importance of dietary treatments using natural food materials or health supplement foods increases relatively, the interest in natural materials is also growing [10].

The peel of tangerine orange, one of the most important fruits in Korea, is a useful research resource containing important physiological function materials. Yoo *et al.* [11] reported that the mineral content for each citrus peel 100 g includes potassium 3029±110 mg, calcium 705±20 mg and magnesium 495±10 mg in the order of potassium, calcium, magnesium, phosphorus, sodium, iron, and zinc. And Yang et al [12] reported that citrus peels extracts show high intestinal immune system activation caused by such polysaccharides in pectin system. Although the research for citrus peel has been conducted frequently in Pharmacology or with literatures, researches to find that with what mechanism, the citrus peel shows anti-inflammatory effects on macrophages haven't been conducted sufficiently. This study aims the confirmation of citrus peel extract's anti-inflammatory effect by using Raw 264.7 cell vitalized with LPS existing on the outside wall of Gram negative bacteria.

The inflammatory reaction is caused by issue damages or bacterial infections so to be a part of biological tissue's immune reaction to recover damaged part when damaged by various causes. Inflammatory symptoms include systemic symptoms such as fever, fatigue and loss of appetite and local symptoms such as seizures, fever, swelling, and pain, and are characterized pathologically with Cell infiltration including vascular hyperpermeability, granulocyte and macrophage. Especially, the macrophage plays very important role for immune reaction and controls various inflammatory mediators including NO, PGE2 and pro-inflammatory cytokines.

Among such mediators, NO is known as one of factors for controlling inflammatory reactors, and is easily interacting with other free radicals and heavy metals, engaging in bioactions such as the vasodilator, the neurotransmitter system, and the immune regulation and playing critical roles as the secondary signal deliverer in cells as the radical created via nitric oxide synthase (NOS) from L-arginine. NOS is categorized in large as two types, constitutive NOS (cNOS) and inducible NOS (iNOS). The creation of NO by cNOS is known to perform important roles for controlling homeostasis in the body [13]. iNOS is known to engage in immunotoxicity and create a large quantity of NO for a long period of time in macrophages, vascular smooth muscle cells, endothelial cells, liver cells, heart muscle cells and etc. by being vitalized with various stimuli including LPS, IL-1, IL-6 and TNF-α . Highly concentrated NO creation in the body may result tissue damages caused by occurrences of destruction of the host cell, vasodilatation caused by shock and/or inflammatory response induction [14]. Therefore, the material enabling the hindrance of NO creation has high possibilities to be developed as a drug to control septic shock, hemorrhagic shock, chronic disease, atherosclerosis and inflammatory response so that the research for such material is actively conducted.

To observe the citrus peel extract's NO creation hindrance level in RAW 264.7 cell, the amount of NO created by treating citrus peel extracts on the cell in 10 and 20 μ g/ml concentrations was measured. LPS increased NO creation amount, and the citrus peel extract reduced the NO creation increased by LPS by approx. 40% (Figure 2). To evaluate if the reduction of NO creation induced with LPS is caused by cytotoxicity, MTT assay was conducted. And, the result didn't show any cytotoxicity caused by the citrus peel extract in 10~50ug/ml (Figure 1).

iNOS creates NO by being induced by the endotoxin of germs such as LPS or various cytokines. And, the expression of iNOS is controlled by iNOS genes. To determine if NO creation suppression with citrus peels extracts was caused by iNOS expression control, the expression of iNOS was observed. And, the result showed that iNOS protein's expression was suppressed (Figure 3A). The above results suggest that citrus peels extracts may suppress iNOS expression through the suppression of signaling molecules and transcription factors in activated cells by LPS.

Currently, two types of COX, COX-1 and COX-2 are known. COX-1 is known to promote the synthesization of prostaglandin (PG). In the other hands, COX-2 is not expressed in normal physiological conditions, but is rapidly expressed by tumor promoters, growth factors and cytokines. COX-2 gene is controlled by macrophages related with inflammations, endothelial cells and fibroblasts. The transcriptional regulation of COX-2 is controlled through NF-κ B route. Controlling COX-2 gene expression is a critical mechanism for anti-inflammatory responses. According to COX-2's western blot result, COX-2 protein expression is slightly decreased by citrus peels extracts treatment (Figure 3B).

IL-1, IL-6 and TNF-α are pro-inflammatory cytokines and are reported to control the inflammatory response in body and having interactions among each others. IL-1 stimulates cell proliferation by secreting various lymphokines. IL-6 performs important roles for host defense, immune response and others. And, TNF-α is involved in functions such as the cell differentiation and growth for macrophages or mononuclear cells. In this study, LPS increased the creations of IL-1, IL-6 and TNF-α significantly, and the 20 μg/ml citrus peel extract reduced creations of IL-6 and TNF-α induced with LPS by approx. 13 – 44% (Figure 4). However, the formation inhibitory effect of IL-1 couldn't be confirmed. It is considered that additional research is required on the formation inhibitory effect of IL-1.

Through such study result, it was confirmed that citrus peel extracts hinders NO creation, expressions of iNOS and COX-2 protein and formation inhibitory effects of IL-6 and TNF-α .

Citrus peels extracts have higher economical efficacy so that the material is expected for easy large quantity products and income increase of the production farmers. iNOS, COX-2, IL-1, IL-6 and TNF-α observed in this study are representative inflammatory mediator genes commonly controlled by NF-κB and MAPK signal networks. Therefore, it is considered that further studies to find if the expression suppression of inflammatory mediator genes with citrus peel extracts is caused by the control of NF-κB and MAPK signal networks are necessary.

5. Conclusion

When RAW 264.7 cell is stimulated with LPS, citrus peel extracts' anti-inflammatory effect was investigated and the following results were acquired:

1. Citrus peels extracts suppressed NO creation in the LPS induced macrophages by approx. 40%.
2. Citrus peels extracts slightly suppressed protein expressions of the LPS induced NOS and COX-2.
3. Citrus peels extracts suppressed the expression of LPS induced Cytokine IL-6 by approx. 44%.
4. Citrus peels extracts suppressed the expression of LPS induced Cytokine TNF-α by approx. 13%.

According to such results, it was confirmed that citrus peel extracts are effective for anti-inflammatory by suppressing creations of NO, IL-6 and TNF-α and expressions of iNOS and COX-2 through affecting on macrophages.

References

[1] M. R. Kim, O. H. Kang, S. B. Kim, H. J. Kang, J. E. Kim, H. C. Hwang, I. W. Kim and D. Y. Kwon, Kor. J. Herbology, vol. 28, no. 1, **(2013)**, http://dx.doi.org/10.6116/kjh.2013.28.1.43.
[2] H. J. Moon, Journal of the Korea Academia-Industrial Cooperation Society, vol. 12, no. 12, **(2011),** http://dx.doi.org/10.5762/KAIS.2011.12.12.5648.
[3] M. Guha and N. Mackman, Cell Signal, vol. 13, **(2001).**
[4] H. H. Kim, J. H. Kwon, K. H. Park, M. H. Kim, M. H. Oh, K. I. Choe, S. H. Park, H. Y. Jin, S. S. Kim and M. W. Lee, Kor. J. Pharmacogn., vol. 43, no. 1, **(2012).**

[5] B. Chi, D. Jo, S. Cha, M. Chi, H. Jeong and K. Kang, Journal of the Korea Academia-Industrial Cooperation Society, vol. 12, no. 12, **(2011),** http://dx.doi.org/10.5762/KAIS.2011.12.12.5773.
[6] B. Choi, J. Kang and K. Kang, The journal of digital policy & management, vol. 10, no. 11, **(2012).**
[7] S. Y. Park , H. N. Yoo, J. M. Im, H. J. Kwon, Y. S. Cho, Y. H. Choi, E. M. Kim and T. Y. Kim, The Korean Society of Food Preservation, vol. 307, **(2010).**
[8] J. S. Eun and J. Y. Yum, Kor J Pharmacogn., vol. 29, no. 3, **(1998).**
[9] J. K. Jung, K. H. Son, Y. S. Kim and Y. K. Park, Kor J Herbology, vol. 26, no. 3, **(2011).**
[10] P. Kubes, Gut, vol. 47**, (2000).**
[11] Y. M. Yoo, C. E. Kim, D. I. Kim, D. Huh and I. Hwang, Korean J Food Cookery Sci., vol. 21, no. 3, **(2005).**
[12] H. S. Yang, K. W. Yu and Y. M. Choi, J. Korean Soc. Food Sci. Nutr., vol. 33, no. 9, **(2004).**
[13] C. Nathan, FASEB J., vol. 6, **(1992).**
[14] S. Chae, J. S. Kim, K. A. Kang, H. D. Bu, Y. Lee, J. W. Hyun and S. S. Kang, Biol. Pharm. Bull., vol. 27, no. 10, **(2004).**

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