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Full Length Article

Anti-Inflammatory Mechanism of Raddeanin A on RAW 264.7 Macrophage Inflammation Model Induced by LPS

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Abstract

Inflammation is a complex defensive response of organisms with vascular system to injury factors. Nonsteroidal anti-inflammatory drugs currently used in clinic have a series of adverse reactions. Thus searching for non-steroidal anti-inflammatory drugs with good anti-inflammatory activity and low adverse reactions has always been very important. The Rhizoma Anemones Raddeanae (RAR) is a kind of medicine for rheumatism treatment and applied clinically in various fields such as wind and cold symptoms, hand-paw and spasm, joint pain and ulcer pain. The proliferation effect of RDA worked on the RAW 264.7 cells was detected by MTT assay. Griess assay was used to detect the content of Nitric Oxide (NO). And the ELISA assay was used to detect the secretion of TNF- alpha, IL-6 and IL-1 beta. The changes of the protein contents of NF-kappa B p65, P-NF- kappa B p65, Akt, P-Akt, JNK, P-JNK in RAW 264.7 cells induced by LPS were detected by western blot assay. The RT-PCR assay was used to detect the changes of TNF-α, IL-6, IL-1beta, iNOS and COX-2 genes expression in the RAW 264.7 cells induced by LPS. RDA could play an anti-inflammatory effect by inhibiting NF- kappa B/MAPK and iNOS/COX-2 signal pathway. RDA could inhibit the proliferation of RAW 264.7 induced by LPS, effectively reduce the release of NO, and the anti-inflammatory activity of RDA was better. It could inhibit the secretion of TNF- alpha, IL-1 beta and IL-6, and showed a dose-dependent relationship. We found that RDA could inhibit the proliferation of RAW 264.7 induced by LPS, effectively reduce the release of NO, and the anti-inflammatory activity of RDA was better. It could inhibit the secretion of TNF- alpha, IL-1 beta and IL-6.

Keywords: Anti-inflammatory; LPS; RAW 264.7 Cells; RDA

Introduction

Inflammation is a defensive response when the body is stimulated by endotoxin and macrophages release a large number of inflammatory cytokines against various inflammatory factors and their induced injuries (Zheng *et al*., 2012). LPS is a major pathogenic substance of Gram-negative bacteria and a direct cause of severe inflammatory infection, which can stimulate the macrophages to produce a variety of inflammatory mediators and cytokines (Zhang *et al*., 2016). And the TNF-alpha is an important inflammatory factor, which has a strong inflammatory damage and can regulates the secretion of IL-6, IL-1 beta and the release of NO (Li *et al.,* 2015).

NF- kappa B is used for regulating the expression of cytokines and cell adhesion molecules (Gil *et al.*, 2012). It can play an important role in the process of inflammation. The main activation form of NF-kappa B is the heteromeric two dimer formed by the combination of p65 and p50. Under normal physiological condition, NF-kappa B (p65/P50) are combined with NF-kappa B inhibitory protein I kappa B to be an inactive compound in the cytoplasm (Hauek *et al*., 2007). When cells are stimulated by cytokines or oxidative stress, I kappa B degrades and separates from NF-kappa B (p65/P50). The NF-kappa B (p65/P50) transcription factor activates and moves into the nucleus (Roman-Blas and Jimenez, 2006). The activation of transcription factor NF-kappa B leads to the overexpression of inflammatory mediators by binding to the corresponding kappa B loci in the corresponding promoter region of inflammatory mediators (Qin *et al.*, 2011). TNF-alpha could induce nuclear shift of NF-kappa B in cells, resulting in activation of by NF-kappa B signal pathway (Zhu *et al.*, 2013).

The Rhizoma Anemones Raddeanae (RAR) is the dry rhizome of anemone raddeana RegeL (Anemone raddeana Regel), which belongs to Ranunculaceae. It is a kind of medicines for rheumatism treatment and applied clinically in various fields such as wind and cold symptoms, hand-paw and spasm, joint pain and ulcer pain (Kyriakis and Avruch, 2001). RDA, as its main component, has the anti-inflammatory effects (Chen *et al.*, 2016). In this article, we make use of the RAW 264.7 induced by LPS to establish a cellular inflammatory model and to study the anti-inflammatory effects and mechanisms of RDA.

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Materials and Methods

Cell Culture

RAW 264.7 cells are purchased from the Shanghai Institute of cell biology, Chinese Academy of Sciences (China, Shanghai). Cells were cultured in DMEM (Gibco) medium containing 10% fetal Bovine Serum (FBS) (Gibco), 100 units·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin at 37°C in 5% CO_2-95% air.

The studies were approved by the Animal Ethics Committee of Changchun University of Chinese Medicine.

The Relations of Time-effect and Dosage-effect of the RAW 264.7 Cells Induced by LPS

Study on the optional drug concentration: The logarithmic growth phase of the RAW 264.7 cells were inoculated on 96-well plate with the cell density of 1 × 10^5 ·mL^{-1}, 100 μL per well. The 96-well plate was placed in the carbon dioxide incubator (37°C, 5% CO_2) for 24 h. The culture medium was discarded and added different concentrations of LPS (0, 0.1, 1, 10 and 100 μM·mL^{-1}) in it. At the same time, the blank control group was set up and cultivated for 24 h. And then the culture medium was discarded and added 20 μL 5 mg·mL^{-1} MTT solution, cultivated for 4 h. We discarded the culture medium, washed the plate with PBS for 3 times, added 150 μL DMSO solution to each well, shaked the plate, put it in the dark for 15 min and determined the OD value at 490 nm with enzyme labelling instrument and calculated the cells proliferation rate (RGR).

Study on the optimal drug concentration of the RAW 264.7 cells induced by LPS: The RAW 264.7 cells were seeded in 96-well plate the same as above and cultured the cells for 24 h and then discarded the culture medium. Except the blank control group and the LPS model group, other groups were given indomethacin with a concentration of 10 μM·mL^{-1} and cultured the cells for 4 h. Except the blank control group, other groups were added LPS 1 μg·mL^{-1} cells to induce cells. The cells were cultured at 37°C and 5% CO_2 in 12 h, 24 h, 36 h and 48 h, respectively. The culture medium was discarded and determined the OD values at 490 nm wavelength by MTT assay and then calculated the relative inhibitory rate (IR) of the cells.

The Proliferation Effect on the RAW 264.7 Cells of RDA

When the cell density of RAW 264.7 cells was in the logarithmic growth period, adjusted the cell density to 1 × 10^5·mL^{-1} and seeded the cells in the 96-well plate. The 96-well plate was placed at 37°C and 5% CO_2 for 24 h. After that discarded the culture medium and set up the positive control group (10 μM·mL^{-1} indomethacin), the negative control group (1% DMSO) and the RDA administration group (20 μM·mL^{-1}, 10 μM·mL^{-1}, 5 μM·mL^{-1} and 2.5 μM·mL^{-1}) and the blank control group at the same time. Each group had 5 compound wells. After cultured for 20 h, the supernatant was discarded and added 20 μL 5 mg·mL^{-1} MTT solution, cultured the cells for 4 h and then discarded the supernatant, added 150 μL DMSO solution to each well, shaked the 96-well plate and placed it in the dark for 15 min. And determined the OD value at 490 nm with enzyme labelling instrument. Calculated the relative cell proliferation rate (RGR), and the RGR%= 1-OD test group / OD negative control group was 100%.

Effect of RDA on Proliferation of the RAW 264.7 Cells Induced by LPS

When the density of the RAW 264.7 cells was in the logarithmic growth period, adjusted the cell density to 1 × 10^5·mL^{-1} and seeded the cells in the 96-well plate. Cultured the cells for 4 h and added the LPS solution with a final concentration of 1 μg·mL^{-1} 50 μL respectively. After 20 h cultured, added 20 μL MTT solution (5 mg·mL^{-1}) to each well and cultured the cells for 4h, discarded the supernatant and added 150 μL DMSO to each well. Shaked the 96-well plate and placed it at room temperature in the dark. Calculated the relative inhibition rate of the cells at the wavelength 490 nm.

Effect of RDA on NO Content in Supernatant of the RAW 264.7 Cells Induced by LPS

The RAW 264.7 cells collected during the logarithmic growth period were seeded in the 24-well plate and adjusted the cell density to 2 × 10^5 mL^{-1}. 10 μM·mL^{-1} RDA and 10 μM·mL^{-1} indomethacin were added to the plate and held for 4 h, after that added 1 μg·mL^{-1} 250 μL LPS held for 20 h. At the same time, the LPS group and the blank group were set up. The cell supernatant was transferred to another 96-well plate. 100 μL Griess A and Griess B mixed reagent (1:1) were added to each well and placed the plate for 10 min in the dark. The values were measured at 540 nm and the content of NO was calculated.

The Effect of RDA on TNF-alpha, IL-1 beta and IL-6 Secretion in the RAW 264.7 Cells Induced by LPS

The same as above term, removed the cell supernatant solution and continued to operate according to the ELISA kits of tumor necrosis factor-alpha (TNF-alpha), interleukin 1 beta (IL-1 beta), interleukin 6 (IL-6). The content of TNF-alpha, IL-1 beta and IL-6 in all the supernatants was calculated according to the standard curve.

Western Blot Analysis of Protein Expression and Phosphorylation Level

Collected the logarithmic growth phase of RAW 264.7 cells and seeded the cells in 6 well-plate at 1 × 10^6·mL^{-1} cell density. These cells were divided into negative control group (1% DMSO), model group (LPS),

administration group (10 μM·mL^{-1} RDA) and positive drug group (10 μM·mL^{-1} indomethacin). After 4 h cultured, LPS (1 μg·mL^{-1}) was used to induce the RAW 264.7 cells to set up the inflammation model. After 20 h cultured, the medium was discarded and the RAW 264.7 cells were washed for 3 times with the precooled PBS buffer. Added 1 mL PBS buffer to each well and gently scraped the RAW 264.7 cells with cell scraper. Transferred the RAW 264.7 cells into the tube, centrifuged it at 1000 rpm at 4°C for 5 min and discarded the PBS. Each EP tube was added 100 μL RIPA lysate, vortex oscillation and put it in ice bath for 30 min to make RAW 264.7 cells lysate fully. And then centrifuged it at 4°C for 10 min. The protein concentration was measured by BCA assay. Statistical treatment: the Western-blot strip was analyzed with image J software and the result was expressed as the relative expression of the target protein. (The formula is as follows).

$$\text{Relative protein expression} = \frac{\text{Integral optical density of target protein (}IOD\text{)}}{\text{Internal reference integrated optical density (}IOD\text{)}}$$

The Effect of RT-QPCR on Related Genes after RDA Treatment

RAW 264.7 cell suspension was seeded in the 6 well-plate. The cell density was 5×10^5·mL^{-1} and 2 mL per well. Added the RDA (10 μM·mL^{-1}) and cultured for 4 h. The RAW 264.7 cells were set to zero well group, normal group, LPS group, LPS + RDA group and then added LPS 1 μg·mL^{-1} in each group. According to AxyPrep total RNA small quantity preparation kit, all the RNA was extracted, reversely transcribed to cDNA and then amplified. As shown in Table 1, the Primer sequence has been established.

Statistics Processing

Using the S.P.S.S. 20.0 software, the experimental group was expressed by $\bar{x}$ ±s, paired T test was used before and after comparison, and factorial analysis of variance was used for comparison between these groups. The statistical differences were determined at $P < 0.05$.

Results

Study on Dose-effect Relationship of the RAW 264.7 Cells Induced by LPS

Study on the best drug concentration of the RAW 264.7 cells induced by LPS: As is shown in Table 2, the proliferation rate of the RAW 264.7 cells induced by different concentration of LPS has significantly increased. When the concentration of LPS was 1 g·mL^{-1}, the proliferation rate of the blank group was significantly different from that of the LPS (0.1 μg·mL^{-1}) group (P<0.01), but proliferation rate of the blank group was not significantly different from that of LPS (10 μg·mL^{-1}) group and LPS (100 μg·mL^{-1}) group. In summary, the optimal concentration of LPS induced RAW 264.7 cells is 1 μg·mL^{-1}.

The Optional Administration time of the RAW 264.7 Cells Induced by LPS

As is shown in Fig. 1, it shows that with the increasing time of drug delivery, the cell absorbency has a tendency to decrease, indicating that the anti-inflammatory effect is enhanced with prolongation time of administration. When the time reaches 24 h, the curve tends to be stable. When the time reaches 48 h, the absorbance is the lowest. However, cultured cells for 8 h, some cells died because of the limited space of the 96-well plate, the concentration of cells was too dense, and the culture medium was not enough to cultivate excess cells. In summary, the optimal time for the RAW 264.7 cells induced by LPS is 24 h.

The Proliferation Effect of RDA on the RAW 264.7 Cells

As shown in Table 3, when the concentration was at 10 μM·mL^{-1}, the cell increment rate was low, and no subsequent experiments could be done. Compared with negative control group, when the concentration was 10 or 5 μM·mL^{-1}, the cell viability of RDA was significantly different. When the concentration was 2.5 μM·mL^{-1}, the RDA was significantly different. But when the concentration was 1.25 μM·mL^{-1}, there was no significant difference. Compared with the positive control group, the activity of the RDA in the concentration was 10 or 5 μM·mL^{-1}, which has the extremely significant difference. But when RDA concentration was 2.5 or 1.25 μM·mL^{-1}, there was no significant difference, so the safety dose of RDA was 5 μM·mL^{-1}.

Proliferation Effect on the RAW 264.7 Cells Induced By LPS

As is shown in Table 4, when the RAW 264.7 cells were induced by LPS, the cell viability of RDA was significantly different when the concentration was 5 μM·mL^{-1}compared with the negative control group. Compared with the positive control group, the cell viability of the RDA group was significantly different.

The Inhibition of NO Secretion from RAW 264.7 Cells Induced by LPS

RAW 264.7 cells released NO after induced by LPS. The release of NO could be significantly inhibited after administration. As is shown in Table 5, the RDA group and the positive control group could significantly inhibit the release of NO when compared with the negative control group. Compared with the positive control group, the content of NO was least when the concentration was 5 μM·mL^{-1}, which indicated that RDA could reduce the

Table 1: The primer sequence

TNF-α	upstream	downstream
	TCCCAGGTTCTCTTCAAGGGA	GGTGAGGAGCACGTAGTCGG
IL-6	upstream	downstream
	ACAAAGCCAGAGTCCTTCAGAG	GGTCCTTAGCCACTCCTTCTG
Cox-2	upstream	downstream
	TCAGCCAGGCAGCAAATCCT	TAGTCTCTCCTATGAGTATG
iNOS	upstream	downstream
	ATGTCCGAAGCAAACATCAC	TAATGTCCAGGAAGTAGGTG
IL-1β	upstream	downstream
	ATGGCAACTGTTCCTGAACTCAACT	TTTCCTTTCTTAGATATGGACAGGAC
GADPH	upstream	downstream
	TGGCAAAGTGGAGATTGTTGC	AAGATGGTGATGGGCTTCCCG

Table 2: The effects of LPS of different concentrations on the proliferation of RAW 264.7 cells (n=6)

Group	Dose ($\mu g \cdot mL^{-1}$)	Absorbance	Proliferation rate (%)
The blank group		0.608 ± 0.022	——
The LPS group	0.1	0.683 ± 0.023**	112.28
	1	0.831 ± 0.017**	136.73
	10	0.852 ± 0.028**	140.16
	100	0.841 ± 0.021**	138.35

Note: compared with the normal group, * * P< 0.01

Table 3: the effect of RDA on the proliferation of RAW 264.7 cells ($\bar{x}$ + s, n=6)

Group	Dose ($\mu M \cdot mL^{-1}$)	OD value	RGR (%)
The Negative control group	0	0.606 ± 0.022	96.95
The Positive control group (indomethacin)	5	0.563 ± 0.031	90.07
RDA	10	0.061 ± 0.002**/##	9.18
RDA	5	0.211 ± 0.034**/##	33.87
RDA	2.5	0.464 ± 0.046*	74.32
RDA	1.25	0.599 ± 0.132	95.93

Compared with the negative control group, *P < 0.05, **P < 0.01; compared with the positive control group, P < 0.05, P < 0.01

Table 4: The proliferation Effect on RAW 264.7 cells induced by LPS ($\bar{x}$ ± s, n=6)

Group	Dose (μg/mL)	OD value	Proliferation rate (%)
The blank group	0	0.668 ± 0.199	0
The Negative control +LPS group	0	0.665 ± 0.115	0.48
The Positive control +LPS group	5	0.647 ± 0.068	3.26
The RDA+LPS group	5	0.215 ± 0.039**/##	67.81

Compared with the negative control group, *P < 0.05, **P < 0.01; compared with the positive control group, P < 0.05, P < 0.01

Table 5: The effect of RDA on the secretion of NO from LPS induced RAW 264.7 cells ($\bar{x}$ + s, n=6)

Group	Dose (μM/mL)	[NO^{2-}] content	Proliferation rate (%)
The blank group	0	16.519 ± 0.493	
The Negative control +LPS group	0	19.667 ± 0.095	
The Positive control +LPS group	5	15.417 ± 0.238**	87.26
The RDA+LPS group	5	14.724 ± 0.131**/#	83.35

Compared with the negative control group, *P < 0.05, **P < 0.01; compared with the positive control group, P < 0.05, P < 0.01

synthesis and the release of NO in the body and achieve the anti-inflammatory effect.

The Inhibition of the TNF-alpha, IL-1 beta and IL-6 Secretion from the RAW 264.7 Cells

After stimulation of the RAW 264.7 cells with LPS, the levels of TNF-alpha, IL-1 beta and IL-6 in the supernatants of model group increased. Compared with group LPS, RDA (5 $\mu M \cdot mL^{-1}$) could significantly inhibit the secretion of TNF- alpha and IL-1 beta and significantly inhibit IL-6 secretion (Fig. 2).

Compared with the normal group, the increase of inflammatory factors was nearly two times more, indicating that the model was successful. Compared with the model group, the values of IL-1 beta, IL-6 and TNF- alpha were significantly reduced, and the levels of pro-inflammatory factors could be effectively reduced to the anti-inflammatory effect.

Effect of Western Bolt on Related Proteins after RDA Treatment

According to the results of Western blot, the expression of

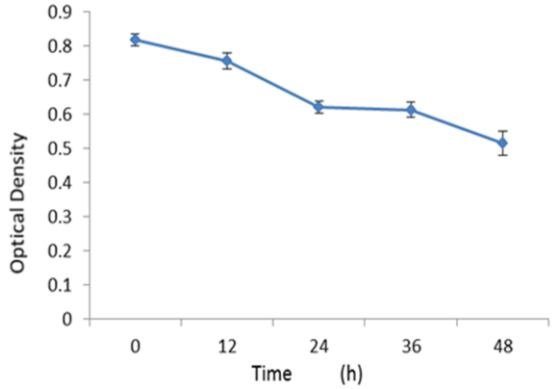

Fig. 1: OD values of RAW 264.7 cells induced by LPS at different times

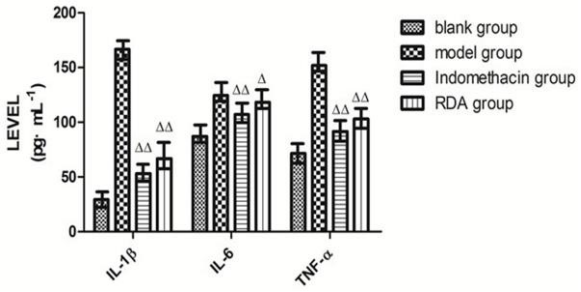

Fig. 2: The contents of IL-1β, IL-6 and TNF- alpha in cell supernatants of each group

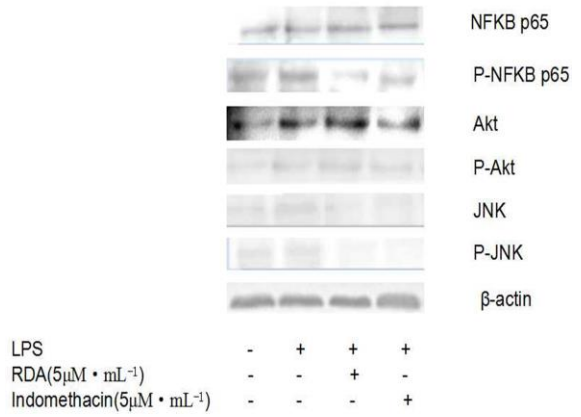

Fig. 3: Western bolt detection of NF- kappa B p65 and P-p65, Akt and P-Akt, JNK and P-JNK proteins after RDA detection

NF- kappa B p65 and P-p65, JNK and P-JNK protein were increased compared with the blank control group and the Akt and P-Akt did not change much after the cells were stimulated by LPS. Compared with the model group, the bands of P-p65 and P-p65, JNK and P-JNK were obviously shallower, and the expression of protein and phosphorylated protein decreased, indicating that the activity of NF- kappa B p65 and JNK protein was inhibited by drugs in the RDA group. The results were shown in Fig. 3.

Effect of RT-QPCR Method on Detection of COX-2 and iNOS Genes after RDA Treatment

The real-time fluorescent quantitative PCR method was used to detect the mRNA expression level of TNF- alpha, IL-6, IL-1 beta, COX-2 and iNOS in macrophages. Selected GAPDH to correct the expression and used a relative quantitative method to study the expression of the target gene. After repeating the three groups, the Ct mean of the internal reference gene of the Ct value box was used. The negative logarithm of the difference was taken as an index, and 2 was calculated as the base number. The relative expression of the target gene relative to the internal reference gene was counted and analyzed as the result. The formula was: the higher the value, the lower the expression quantity. The results were shown in Table 5. Compared with the blank group, the expression of cytokines TNF-α, IL-6, IL-1 beta, COX-2 and iNOS in the LPS induced model group showed a very significant difference ($P < 0.01$). Compared with the model group, mRNA expression of RDA TNF-α IL-6, IL-1 beta, COX-2 and iNOS was significantly reduced (Table 6).

Discussion

In this study, the results showed that RDA could inhibit the proliferation of RAW 264.7 induced by LPS, effectively reduce the release of NO, and the anti-inflammatory activity of RDA was better. It could inhibit the secretion of TNF-alpha, IL-1 beta and IL-6, and showed a dose-dependent relationship.

Some studies have shown that TNF- alpha could increase the expression of I NOS and promote the formation of NO (Nie *et al.*, 2019). It is presumed that RDA may inhibit the secretion of TNF- A and inhibit the expression of I NOS and reduce the formation of NO (Paulino *et al.*, 2016; Park and Son, 2017; Huo *et al.*, 2019).

When the cells were stimulated by LPS, the expression of NF- kappa B p65 and P-p65, JNK and P-JNK increased, and Akt and P-Akt did not change too much (Chao and Qing, 2019). But the RDA group decreased the expression of P-p65, P-p65, protein and phosphorylated protein (Neranjan Tharuka *et al.*, 2019). The phosphorylation activity of related proteins in the road could achieve anti-inflammatory effect (Neranjan Tharuka *et al.*, 2019). At the gene level, the expression of mRNA in TNF- alpha, IL-6, IL-1 beta, COX-2 and iNOS in the cells was significantly down regulated by RDA, indicating that RDA effectively inhibited the expression of TNF-alpha, IL-6 and beta of the downstream factor of NF- kappa B to eliminate inflammation, while the inducible nitric oxide synthase was a catalyst for the synthesis of arginine (Sun *et al.*, 2018). The key enzyme of nitrogen oxide, as a new second messenger neurotransmitter, is a new second messenger neurotransmitter and a highly active free radical (Murthuza and Manjunatha, 2018). It is widely involved in various

Table 6: Effects of RDA on TNF- alpha, IL-6, IL-1 beta, COX-2 and iNOS genes ($\bar{X}$ + s, n=3)

Group	TNF-α/GAPDH	IL-6/GAPDH	IL-1β/GAPDH	COX-2/GAPDH	iNOS/GAPDH
The blank group	5.48 ± 1.00	20.40 ± 0.91	15.76 ± 0.99	11.79 ± 1.00	16.38 ± 0.99
The Model group	1.93 ± 0.35	6.71 ± 0.29	2.03 ± 0.33	7.94 ± 0.39	6.58 ± 0.34
The RDA group	3.81 ± 0.88**	15.09 ± 0.37**	6.57 ± 0.54**	10.46 ± 0.66**	12.91 ± 0.60**
The Positive control group	4.33 ± 0.86**	19.47 ± 0.61**	10.22 ± 0.63**	7.84 ± 0.59**	14.26 ± 0.67**

Compared with the model group: *$P < 0.05$; **$P < 0.01$

pathophysiological processes *in vivo*, mediating inflammatory reaction and immune response (Tarasuntisuk *et al.*, 2018). Cyclooxygenase-2 (COX-2) is the speed limit of the peanut prostaglandin (PGs) synthesis of Xie Guocheng (Wang *et al.*, 2018). Enzyme, a membrane binding protein, exists in the nuclear membrane and microsome membrane, and also participates in the immune inflammatory response, ischemia, injury, and the formation of the tumor (Kim *et al.*, 2017).

After RDA acts on the cells, it could effectively reduce the expression of mRNA in the iNOS and COX-2, indicating that RDA could restrict the existence of iNOS and COX-2 by inhibiting the iNOS /COX-2 signaling pathway. The "cross-talk" effect reduces inflammation and achieves an indirect anti-inflammatory effect.

Conclusion

The anti-inflammatory mechanism of RDA was explored through an *in vitro* inflammatory model and it was found that RDA could inhibit the proliferation of RAW 264.7 induced by LPS, effectively reduce the release of NO and the anti-inflammatory activity of RDA was better, and it could inhibit the secretion of TNF- alpha, IL-1 beta and IL-6.

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