Effects of flavonoids from *Allium mongolicum* Regel on cell apoptosis, cell cycle and STAT1 mRNA expression in peripheral blood lymphocytes of sheep

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**ABSTRACT**

Flavonoids from *Allium mongolicum* Regel (AMR) have immunoregulatory and anti-inflammatory properties. However, the effect of flavonoids from *A. mongolicum* Regel on sheep peripheral blood lymphocytes is still unknown. In this study, we found that total flavonoids from *A. mongolicum* Regel reduced the apoptosis of peripheral blood lymphocytes in sheep, increased the cell cycle progression to the G2/M phase, and promoted mitosis. These molecules inhibited mRNA expression of the apoptosis-related gene STAT1, indicating that the flavonoids alter JAK-STAT signalling and through this classical pathway inhibit cell apoptosis. The bioactive effects are predominantly related to the structure of flavonoids from *A. mongolicum* Regel, and the 55% elution group, which contained rutin, quercetin, and mignonette element -5-0-glucose-4-hydroxybenzoic acid, compounds found in plants, was the most effective. The effects were the strongest at a flavonoid concentration of 100 μg/ml. In conclusion, the present study showed that flavonoids from *A. mongolicum* Regel inhibited the expression of STAT1 mRNA, reduced the apoptosis of sheep peripheral blood lymphocytes and promoted cell cycle progression. They also increased the proportion of cells in the G2/M phase. Our findings confirmed a novel role of flavonoids from *A. mongolicum* Regel in regulating immune function.

1. **Introduction**

*Allium mongolicum* Regel (Shacong in Chinese), a member of the Liliaceae family and the genus *Allium*, shows strong drought and cold resistance (Wu et al. 1980). It is widely distributed in northwest China and Inner Mongolia (Bao 1999). The leaf and flower of AMR are edible, have medicinal purposes and contain high levels of steroids, polysaccharides and flavonoids. Recently, researchers have investigated the non-volatile compounds, such as steroids and flavonoids, of Allium family members (Li et al. 2010, Mnayer D et al. 2015). In the 1980s, researchers analysed the steroid saponins in Allium plants and showed that AMR extracts had various bioactivities (Smoczkiwicz et al. 1982). Additionally, feeding sheep AMR increased their weight (Sechenbater 2002). In 2005, Li Hua Zhao and Chang Jin Ao demonstrated that AMR can improve the quality of mutton (Lu 2002). AMR flavonoids enhanced the antioxidant content and activity in the serum and liver and decreased lipid peroxidation in mice (Zhao 2005). Moreover, AMR polysaccharides improved the Ca^{2+} and NO levels of sheep peripheral blood lymphocytes in vitro and altered the abundance of cAMP and cGMP, which affect immune signalling. Thus, AMR polysaccharides have an immunomodulatory role (Zhao 2008). AMR polysaccharides also had anti-tumour activity by promoting the mRNA expressions of IFN-γ and STAT1 in sheep peripheral blood lymphocytes (Zhao 2013a).

Flavonoids are found in various vegetables, fruits, and wild and medicinal plants. They are secondary metabolites that have been subjected to long-term natural selection during the evolution of plants. Flavonoids have two benzene rings linked by three carbon atoms. They not only have chemical value but also, importantly, pharmacological value to humans, as they have various bioactive properties, such as enzyme inhibitory activity and anti-cancer, anti-microbial (Alencar et al. 2007), and anti-viral (Wang et al. 2010) effects. Adding flavonoids from *A. mongolicum* Regel to the diet can promote the transformation of peripheral blood lymphocytes of sheep, and transform the cells into the DNA synthesis phase, promote cell division, and significantly promote the apoptosis (Mu et al. 2017).

Signal transducer and activator of transcription (STAT1) are involved in cell differentiation and cell apoptosis. STAT1 was the first member of the STAT family to be identified. It controls tumour cell proliferation and promotes tumour cell apoptosis; thus, STAT1 is a potential tumour suppressor. Previous reports indicated that STAT1 plays an important role in the development of hepatocellular carcinoma (Zhao et al. 2013b). STAT1-deficient rats had reduced interferon signal transduction, which may result in infection and tumour progression (Zhou et al. 2004).

With the development of feed science, many feed additives have been investigated. In this study, we extracted the flavonoids from AMR using an alcohol extraction protocol, which yielded a water extract, 35% ethanol extract, 55% ethanol extract and 75% ethanol extract. Sheep blood lymphocytes...
were stimulated with the different extracts, and the cell cycle, cell apoptosis and immune responses were analysed. Currently, flavonoids are widely used in the chemical and medical fields but not in feed science research.

2. Materials and methods

2.1. Experimental materials

2.1.1. Experimental reagents

Flavonoid extracts from AMR, including crude flavonoids, water extract, the 35% elution group, the 55% elution group and the 75% elution group, were derived in the laboratory. Sheep peripheral blood lymphocyte separation medium was obtained from TBD. RPMI-1640 medium and foetal calf sera were from Gibco. Microbiological reagents, β-mercaptoethanol, concanavalin A, DPBS, an AMV First Strand cDNA Synthesis Kit, RNAisoPlus (D9108B), SYBR Premix Ex Taq™ II (TliRNaseH Plus) (DRR820A), DL 2000 DNA Marker (D501A), DL500 DNA Marker (D525A), RNA enzymes, propidium iodide (PI), and Triton X-100 were also purchased.

2.1.2. Experimental apparatus

A HEPA (High-Efficiency Particulate Air) class 100 CO₂ incubator (Thermo), AC2-4S1 biohazard safety equipment (ESCO), an IX71 inverted phase-contrast microscope (Japan, Olympus), (Thermo), AC2-4S1 biohazard safety equipment (ESCO), an IX71 inverted phase-contrast microscope (Japan, Olympus), iQ5 Multicolor Real-time PCR Detection System (Bio-Rad), T-GRADIENT Gene Amp PCR system (Biometra), a 3–30 K centrifugal machine (Sigma), and Champ Gel 5000 Digital gel image analysis system were used to perform the experiments.

2.2. Experimental treatment

A previously reported method (Wang et al. 2010) was used to obtain crude flavonoids from the AMR (the optimum temperature was 40°C, the optimum time was 15 min, 75% ethanol was used, and the ratio of solid to liquid was 1:30). Purification was performed with polyamide column chromatography, and crude flavonoids were eluted using water, 35% ethanol, 55% ethanol and 75% ethanol. For the experiments, 10 mg crude flavonoids were solubilized in 1 ml ultrapure water to obtain a 10 mg/ml crude flavonoid solution. Then, 100 μl of this solution was added to 900 μl ultrapure water for a 1 mg/ml crude flavonoid solution. Water extract, the 35% elution group, the 55% elution group and the 75% elution group were diluted by RPMI-1640 medium using the same method. The flavonoids from A. mongolicum Regeli were yellow in colour and insoluble in water. According to early research regarding structure identification (Sa, 2014), the structure contains saccharides, naphthenic hydrocarbon, 3-0, 4-0-epoxy group-7-0-5-methoxy flavonols, 7-0-5, 4-0-dimethoxy-3-0-hydroxide radical flavones, rutin, quercitrin, saccharides, mignonette element 5-0-glucose-4-hydroxybenzoic acid, and acacia.

2.3. Separation and collection of peripheral blood lymphocytes

A total of 10 six-month-old healthy meat sheep with an initial body weight of 39.9 ± 3.2 kg were selected. Experimental animals were provided by Fuchuan Inner Mongolia farming Ploytron Technologies Inc. The farming condition is well controlled. And all blood collection from animals (sheep) comply with the Inner Mongolia agricultural university animal ethical committee guidelines (NND2021040) and were carried out in accordance with the Chinese Institutes of Health guide for the care and use of Laboratory animals and Animal law. Five millilitre sterile blood from the jugular vein of a sheep was collected in heparin anticoagulation tubes and stored at a temperature of 22–26°C, a total of 10 sheep. Followed by centrifugation at 1500 r/min in a vertical centrifuge for 10 min. The upper plasma layer of the blood was discarded, and the remaining blood sample was added to a 50 ml centrifuge tube and diluted with isometric DPBS to 50 ml followed by gentle mixing. Then, 4 ml of peripheral blood lymphocyte separation medium was added to a centrifuge tube, followed by 5 ml of diluted blood. The mixture was centrifuged at 2300 r/min in a vertical centrifuge for 40 min. After centrifugation, the blood was divided into four layers, with the second layer containing the lymphocytes. Then, 3 ml of lymphocytes was added to a 15 ml centrifuge tube, and 5 times the volume of DPBS was added and gently mixed. The mixture was centrifuged at 1500 r/min in a vertical centrifuge for 10 min, and the supernatant was collected. This step was repeated to obtain purified lymphocytes. Then, 5 ml culture medium was added to the lymphocytes by gentle mixing. Lymphocytes solution was added to six-well plates at 2 ml, followed by 100, 200, or 400 μl of the flavonoid solution. The final concentrations were 5, 100, or 200 μg/ml, and the concentrations were followed by Saruli (Sa 2014), respectively, and the plates were cultured at 37°C in 5% CO₂ for 12 h. Then, cellular morphology was assessed in the next step.

2.4. Flow cytometry analysis of cell apoptosis

After incubation, the cells were centrifuged and collected. Then, the cells were resuspended in the precooled DPBS twice, and 100 μl precooled 1 × Annexin V Binding Buffer, 5 μl Annexin V-FITC and 5 μl PI were added. The samples were incubated in the dark at 20–25°C for 15 min. After the 15 min reaction, the cell solution was mixed with 400 μl precooled 1 × Annexin V Binding Buffer by gentle blending, and then, cell apoptosis was immediately assessed with flow cytometry analysis.

2.5. Flow cytometry analysis of the cell cycle

After a 24 h incubation, cells were centrifuged, the medium was discarded, and cells were resuspended in DPBS. This step was repeated twice. Then, the cells were stained for 15 min with 1 ml staining solution in the dark, and the cell cycle was immediately assessed with flow cytometry.

2.6. RNA isolation

After the cell incubation, total RNA was isolated from the peripheral blood lymphocytes using an RNA Prep Pure Tissue kit (Tiangen Biotech Co., Ltd., Beijing, China). The purity of the RNA was determined by measuring the 260/280 ratios using a
2.7. Reverse transcription and quantitative reverse transcription–PCR

The cDNA was synthesized using 200 ng of RNA in 2 μl, which was mixed with 2 μl of 5 × Prime Script RT Master Mix and 6 μl of DNase/RNase-free water (TaKaRa, Tokyo, Japan). The reaction was performed using the following protocol: 37°C for 15 min and 85°C for 5 s. Quantitative reverse transcription–PCR was performed using the Prime Script RT reagent kit (TaKaRa) in a 20 μl reaction volume with 10 μl of 2 × SYBR premix Ex TaqII (TaKaRa), 0.4 μl of each of 10 μM forward and reverse primers, and 7.2 μl of DNase/RNase-free water in 96-well micro-well plates with a Bio-Rad IQ5 Multicolor Real-time PCR Detection System (Hercules, CA). The quantitative RT–PCR was conducted under the following conditions: 40 cycles of pre-denaturation for 30 s at 95°C, denaturation for 5 s at 95°C, annealing for 30 s at 60°C, and extension for 3 s at 70°C for 51 cycles. The RT–PCR analysis was performed using the 2ΔΔCT method (Livak et al. 2001), and β-actin was used as the reference gene. The primer sequences and annealing temperature are displayed in Table 1. The primers were synthesized by TaKaRa Biotechnology Co., Ltd. (Dalian, China).

2.8. Statistical analysis

Statistical analyses were performed with the GLM method of one-way ANOVA using SAS9.0. The values are shown as the mean ± standard deviation. A significance level of 0.05 was used.

3. Results

3.1. Lymphocyte apoptosis of meat sheep

Flavonoids could be acting on lymphocytes, it depended on stimulating the signal transduction between cells and enhancing free radical scavenging and anti-oxidation function. Flow cytometry was used to detect the apoptotic rate in each treatment group. Apoptotic cells were stained with PI, shown in Figure 1. Thus, the DNA of cells with damaged membranes was stained and showed red fluorescence; however, the DNA of cells with intact membranes was not stained. Figure 2 shows that the addition of 50 μg/ml flavonoids from AMR to peripheral blood lymphocytes of meat sheep reduced the apoptosis rate compared to that of the control group (P < 0.05). Peripheral blood lymphocytes were then treated with 100 μg/ml flavonoids from AMR. The apoptosis of treated lymphocytes was lower than that of the control group (P < 0.05), and the 200 μg/ml flavonoid test group had a higher apoptotic rate than that of the 50 and 100 μg/ml flavonoid group (P > 0.05). Therefore, flavonoids from AMR acted on peripheral blood lymphocytes in vitro. We confirmed that different concentrations of flavonoids could inhibit the apoptosis of lymphocytes; this may be related to the structure of flavonoids from AMR, the bioactivity of flavonoids from AMR and the effects on genes related to apoptosis, which promoted the immunomodulatory activity.

3.2. Cell cycle of lymphocytes from meat sheep

Flavonoids from AMR enhanced peripheral blood cell proliferation, and the optimal cell culture time was 12 h. After 12 h, time had no significant effect on the cell proliferation. We also found that the intermediate concentration of flavonoids, 100 μg/ml flavonoids from AMR, had the strongest effect on lymphocyte proliferation (Sa 2014). Based on the results of the lymphocyte proliferation analyses, we selected different concentrations of flavonoids from AMR, stimulated the peripheral blood lymphocytes cell for 12 h, and assessed the changes in the cell cycle. The results of this study showed (Figure 3) that the control group (0 μg/ml) and the treatment groups had no significant changes in G0/G1 phase (P > 0.05), but the cell proportion in the treatment groups had an increasing trend. The proportion of cells in the S phase was slightly lower than that of cells in the G0/G1 phase and G2/M phase, but the treatment groups had a higher percentage of cells in S phase than that of the control group; cells treated with the flavonoids from AMR (50 and 200 μg/ml) had a significant change in S phase than that of the control group (P < 0.05). The proportion of cells in the G2/M phase was higher than that of cells in the S phase (P < 0.05); cells treated with the intermediate concentration of flavonoids from AMR (100 μg/ml) had the highest percentage in the G2/M phase (P < 0.05). Thus, total flavonoids from AMR had an impact on the peripheral blood lymphocytes of sheep, significantly increased cell cycle progression to the G2/M phase and promoted cell mitosis. The results indicated that flavonoids from AMR had an active effect on DNA repair and could effectively maintain the cell cycle progression, decreasing the apoptosis of lymphocytes.

3.3. STAT1 mRNA in sheep peripheral blood lymphocytes

This study explored the effect of different flavonoids from AMR on the expression of genes related to the cell cycle and apoptosis and showed that AMR extracts contain bioactive substances that alter the apoptosis of sheep peripheral blood lymphocytes.

Based on a preliminary analysis of the structure of flavonoids from AMR, we separated and purified the total flavonoids from AMR using polyamide column chromatography with elution by distilled water, 35% ethanol, 55% ethanol, and 75% ethanol. Separate elution fractions with different components were obtained. We used liquid chromatography-tandem mass spectrometry, infrared spectrum analysis and ultraviolet spectrum analysis to identify the compounds in the different elution groups. The results showed that the water elution group contained polysaccharides, and the 35% elution group contained...
3′,4′-epoxy-7-O-5-methoxyflavonols and 7-O-5,4′-dimethoxy-3′-hydroxyl radical flavone. The 55% ethanol elution group contained rutin, quercetin, mignonette element -5-0-glucose-4-hydroxybenzoic acid, compounds found in plants. The 75% ethanol elution group contained polysaccharides found in acacia. Figure 4(A) shows that in sheep peripheral blood lymphocytes stimulated with 100 and 200 μg/ml total flavonoids from AMR, expression of the apoptosis-related gene STAT1 was lower than that of the control group (P < .05). Cells treated with 50 μg/ml had a trend of decreased STAT1 expression relative to that of the control group, but this was not significant (P > .05).

**Figure 1.** Flavonoids from *A. mongolicum* Regel sensitizes sheep peripheral blood lymphocytes to apoptosis.
Figure 4(B) shows that the different elution groups of flavonoids from AMR had inhibitory effects on the expression of STAT1 in sheep peripheral blood lymphocytes. STAT1 expression was decreased in a concentration-dependent manner, and when the cells were stimulated with 100 and 200 μg/ml of flavonoids, the inhibitory effect was the greatest in the 55% elution group. When the cells were stimulated with 50 μg/ml, all elution groups had no obvious changes in the expression of STAT1 mRNA (P > .05). At a concentration of 100 μg/ml, the STAT1 expression in the 35% and 55% elution groups was lower than that of the control group and the 75% elution group (P < .05). STAT1 mRNA expression in the 35% elution group was not significantly different from that of the 55% elution group (P > .05). When the flavonoid concentration was 200 μg/ml, STAT1 mRNA expression in all elution groups was lower than that of the control group (P < .05). The lymphocytes stimulated with 100 μg/ml of all elution groups showed decreased STAT1 mRNA expression compared with that of the 50 and 200 μg/ml groups. Among the elution groups, the 55% elution group had the strongest effect, and the water elution group, 35% elution group and 75% elution group had a decreasing trend (P < .05). These results indicated that all elution groups of flavonoids from AMR inhibited the expression of STAT1 mRNA, and the 55% elution group had the strongest effect. This may be related to the components of the 55% elution group; the immunoregulatory activity of the flavonoids is due to the iroes-trogen-like structure and C2=C3 double bond structure.

4. Discussion

Cell apoptosis, a normal physiological phenomenon known as programmed cell death, is the foundation of development and breeding of all organisms. Cell apoptosis differs from cell death in that it is an active process of cell self-destruction determined by the gene. Cell apoptosis plays an important role in the development of tissues. Senescent cells are removed by the mechanism of cell apoptosis in the process of body metabolism (Hong et al. 2009, Hu 2010). Many researches about flavonoids on cell apoptosis prove resveratrol could induce malignant NK cell apoptosis by inhibiting JAK2/STAT3 pathway and blocking the cell cycle (Trung et al. 2013). Excessive apoptosis of lymphocytes may have a detrimental effect on lymphocyte-mediated immunity. Lymphocyte apoptosis was predominantly mediated by the blockage of the cell cycle, an increase in apoptosis-related gene expression and a decrease in anti-apoptotic gene expression. We wanted to examine whether flavonoids from AMR played an anti-inflammatory role in cell apoptosis inhibition. To our surprise, our result found that flavonoids from AMR could inhibit the lymphocytes in meat sheep, and the 200 μg/ml flavonoid act the best effect. Cell cycle and cell apoptosis have a mutual support role in the body. The cell cycle is divided into mitosis and inter phase, and the normal cell cycle has four periods, G1 phase, S phase, G2 phase and M phase. G1 phase is the DNA pre-synthesis phase, and RNA and ribosomes are synthesized in preparation for DNA
replication. Then, cells enter the S phase to begin to synthesize DNA, and histones and enzymes required for DNA replication are produced. After DNA replication, cells enter G2 phase, the latest age of DNA synthesis, and principally prepare for mitosis. Mitosis occurs in the M phase, and DNA synthesis is terminated (Tang 2014). The result showed that AMR had an active effect on the cell cycle and enhanced cell cycle progression to the G2/M phase and promoted cell mitosis. These two results are related to the STAT1 mRNA inhibition.

STATs have kinase activity and have been shown to be involved in tumour cell signalling in Gao et al. (2013); Zhu et al. (2011); Chin et al. (1996); Ilkovitch et al. (2008). Researchers have identified seven members of the STAT family, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Calò et al. 2003). IFN-γ binds to specific receptors on the surface of target cells in vivo, the receptors dimerize, and the tyrosine kinase JAK binds to the receptor. Then, STAT1 is recruited and activated, and activated STAT1 initiates the JAK-STAT signal transduction pathway to induce gene expression (Fowler et al. 2012). JAK-STAT signalling is an important cellular pathway, and it is involved in various physiological and pathological processes, such as cell proliferation, differentiation, apoptosis, inflammation, and tumour genesis (Xu et al. 2012). Scarabelli (Scarabelli et al. 2001) and Chung (Chung et al. 1997) found that STAT1 and STAT3 played opposing roles in myocardial cell survival, and STAT1 activation is related to myocardial cell apoptosis induced by ischaemia. Several studies have suggested that altering the JAK-STAT signal transduction pathway to inhibit STAT1 activation could have anti-inflammatory effects; for example, EGCG, a polyphenol found in green tea, inhibited the activation of STAT1 to exert its anti-inflammatory effects (Wu et al. 2014). This study found that sheep peripheral blood lymphocytes stimulated with total flavonoids from AMR and different elution groups showed decreased STAT1 mRNA, and it confirmed that flavonoids from AMR inhibit STAT1 and decrease apoptosis of lymphocytes. The effects of these compounds are related to their structure, and the 55% elution group had the best activity. The 55% elution groups contained rutin, quercetin, mignonette element-5-O-glucose-4-hydroxybenzoic acid, compounds found in plants, which had the strongest anti-inflammatory and antioxidant properties.

5. Conclusion

Flavonoids from AMR inhibited the apoptosis of sheep peripheral blood lymphocytes, promoted progression to the G2/M phase, and enhanced cell mitosis. Total flavonoids from AMR and all elution groups inhibited the expression of STAT1 mRNA; thus, we hypothesized that flavonoids from AMR had immunoregulatory effects. The results showed that the immunoregulatory effects of the 55% elution group were significantly higher than those of the other groups due to the composition of the 55% elution group; it contained many synergistic components that may have increased the anti-inflammatory and antioxidant properties. Additionally, flavonoids from AMR had oestrogenic activity.

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