

# Effects of rutin on osteoblast MC3T3-E1 differentiation, ALP activity and Runx2 protein expression

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## ABSTRACT

As a flavonoid, rutin has been found to have a wide range of biological functions, such as resisting inflammation and oxidation, and preventing cerebral hemorrhage and hypertension. It has been found to play an important role in osteoporosis and other orthopedic diseases in recent years. MC3T3-E1 cells were randomly divided into a control group, a rutin-1 group (0.01 mmol/L), a rutin-2 group (0.05 mmol/L) and a rutin-3 group (0.1 mmol/L). Osteogenic differentiation of cells was induced by osteogenic induction fluid. The control group was treated with the maximum dose of drug solvent. 2~3 days later, the solvent was replaced with fresh osteogenic induction fluid containing rutin. After a certain period of routine culture, the cells were collected for subsequent experiments. The expression of *Runx2* gene in cells in all groups was detected by Real-time PCR; the expression of Runx2 protein was detected by Western blot and immunocytochemistry (IHC); the activity of ALP was detected by reagent kit method; osteogenic differentiation was analyzed by alizarin red staining. The results of Real-time PCR showed that, compared with the control group, the treatment of cells with rutin can significantly increase the expression of *Runx2* gene ( $p < 0.05$ ); the higher the concentration, the higher the expression of *Runx2* gene, and significant differences were found among groups in which different concentrations were used ( $p < 0.05$ ); the results of Western blot and IHC showed that the expression trend of Runx2 protein in each group was consistent with PCR results. In drug treatment groups, the activity of ALP was significantly higher than that in the control group ( $p < 0.05$ ); there were significant differences among groups in which different concentrations were used ( $p < 0.05$ ). The results of alizarin red staining showed that calcified nodules were formed in all groups and that the area of calcified nodules formed in groups treated with rutin was greater than that in the control group; the greater the concentration, the larger the area. Rutin can promote osteoblastic differentiation; and the greater the concentration, the more effective it is.

**Key words:** xRutin; Runx2; ALP; osteogenic differentiation.

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**Contributions:** XWL, BM, experiments concept and design; YZ, LBX, experiments performing; TYH, analysis and interpretation of experiments results. All the authors have read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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**Informed consent:** Not applicable.

## Introduction

Rutin is a kind of flavonoid mostly from rutin leaves, tobacco leaves and *Sophora japonica* buds, etc. It is widely used and has such functions as resisting inflammation and oxidation and maintaining vascular resistance.<sup>1,2</sup> It is clinically often used to treat hypertension, prevent vascular sclerosis, cerebral hemorrhage and other diseases.<sup>3</sup> In this study, mouse osteoblast precursor cells, MC3T3-E1 cells, were used as study subjects to explore the effects of different concentrations of rutin on the expression of Runx2 protein and the activity of ALP during MC3T3-E1 differentiation. MC3T3-E1 cells biological functions are close to those of primary cultured osteoblasts. Therefore, they are extensively used to study the differentiation, proliferation and molecular mechanism of osteoblasts.<sup>4</sup>

During bone development, the expression of Runt family associated transcription factor 2 (Runx2) is the beginning of osteoblast differentiation. Runx2 can initiate and activate the differentiation of bone marrow stromal stem cells into osteoblasts. Meanwhile, it can upregulate the expression of many osteogenic genes. It is a key marker in bone formation.<sup>5-7</sup> In addition to playing a biological role in osteoblasts, Runx2 is highly expressed in hypertrophic chondrocytes and promotes chondrocyte hypertrophy and endochondral ossification. Runx2 also promotes osteoclast differentiation by inducing the nuclear factor kB receptor activator ligand (RANKL) and inhibiting the osteopontin (OPN) to play corresponding roles<sup>8</sup>. Besides, Runx2 can also promote the synthesis of related extracellular matrix proteins, including type 1 collagen, osteocalcin (OCN) and osteialin (BSP).<sup>9</sup>

Alkaline phosphatase (ALP) is an isoenzyme widely existing in liver, bone, placenta and other tissues. It is clinically used for the diagnosis and differentiation of rickets, malignant tumor bone metastasis, hepatobiliary diseases and other diseases.<sup>10,11</sup> During bone formation, ALP can hydrolyze phosphate and pyrophosphate to eliminate their inhibitory effect on bone formation, which is beneficial to further bone formation. The activity of ALP can indicate the degree of differentiation of osteoblasts and is used as one of the indicators to evaluate the mineralization ability of osteoblasts.

## Materials and Methods

### Cell culture

Mouse embryonic osteoblast precursor cells, MC3T3-E1 cells, were purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Routine culture was performed with the  $\alpha$ -MEM medium containing 10% FBS and 1% P/S (PM150421, Procell, Wuhan, China) at 37°C and 5% CO<sub>2</sub>. The state of MC3T3-E1 cells was adjusted. MC3T3-E1 cells in logarithmic phase were randomly divided into a control group, a rutin-1 group (0.01 mmol/L), a rutin-2 group (0.05 mmol/L) and a rutin-3 group (0.1 mmol/L). Osteogenic differentiation of cells was induced by osteogenic induction fluid. The control group was treated with the maximum dose of drug solvent; 2~3 days later, the solvent was replaced with

fresh osteogenic induction fluid containing rutin. After a certain period of routine culture, the cells were collected for subsequent experiments. Rutin was purchased from Solarbio Technology Co., Ltd. (SR8250; Beijing, China).

### Real-time PCR

Cells were collected from all groups after 3 days of treatment with rutin. The total RNA in cell samples from each group was extracted according to the instructions for TRIpure extraction kit (RP1001; BioTek, Beijing, China). The ultraviolet spectrophotometer NanoDrop-2000 (ThermoFisher Scientific, Waltham, MA, USA) was used to determine the RNA concentration in each sample. Real-time PCR (Exicycler 96, BIONEER, Republic of Korea) was used; and super M-MLV reverse transcriptase (PR6502; BioTeke, Beijing, China) was used for reverse transcription. Real-time fluorescence quantitative PCR reaction was carried out by adding 2×Power Taq PCR MasterMix (PR1702; BioTeke), SYBR Green I (SY1020; Solarbio), cDNA template and upstream and downstream primers. Upon the completion of the reaction, *B-actin* was used as a reference. The formula  $2^{-\Delta\Delta Ct}$  was used to calculate the relative expression level of mRNA of Runx2 gene. See Table 1 for primer sequence. It is synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

### Western blot

The cells in all groups were treated with rutin for 3 days. The treated cells were split with the RIPA lysis buffer (P0100; Solarbio) on the icebox for 5 min; then the cells were centrifuged at 12,000 rpm and 4°C for 10 min. The supernatant was separated to obtain protein extract. BCA protein assay kit (WLA004, Wanleibio, Shenyang, China) was used for quantitative analysis of the total protein (TP) extracted to get protein concentration. The electrophoresis apparatus was assembled and polyacrylamide gel prepared. 8% SDS-PAGE was used for electrophoresis. After electrophoresis, the protein bands separated were transferred to the PVDF membrane (IPVH00010; Millipore, Burlington, MA, USA). The PVDF membrane was sealed with 5% skim milk; then Runx2 primary antibody (diluted by 1:500; WL03358; Wanleibio) was added. The PVDF membrane was incubated at 4°C overnight. Upon the completion of incubation, the PVDF membrane was washed with TBST. The horseradish peroxidase (HRP)-labeled sheep anti-rabbit IgG (diluted by 1:5000; SE134; Solarbio) was added for incubation. The reference  $\beta$ -actin was incubated by the same experimental procedures. Finally, ECL luminous fluid (PE0010; Solarbio) was added for exposure in a darkroom. The film was scanned. The gel image processing system (the software Gel-Pro-Analyzer) was used to analyze the optical density of the target band.

### Immunocytochemistry (IHC)

After 3 days of treatment with rutin, the cells treated in each group were made into cell slides. Four percent paraformaldehyde solution was used for fixation for 15 min. Next, 0.1% TritonX-100 was added for incubation at ambient temperature for 20 min and then discarded. 3% H<sub>2</sub>O<sub>2</sub> was added for incubation. The serum was added for sealing for 15 min. The serum was discarded, the Runx2 primary antibody diluted with PBS (diluted by 1:500; WL03358;

**Table 1. Primer sequence.**

Name of primer	Former primer (5'–3')	Back primer (5'–3')
Runx2	GCAGACTCCATATCTCTACT	TTCCGTCAGCGTCAACAC
$\beta$ -actin	CTGTGCCCATCTACGAGGGCTAT	TTTGATGTCACGCACGATTTC

Wanleibio) was added. The mixture was placed at 4°C overnight. The primary antibody was discarded. PBS was used to dilute the HRP-labeled secondary antibody by 1000 times (diluted by 1:1000; #31460, ThermoFisher). The mixture was added to completely cover the cells. The cells were incubated at 37°C for 1h. The secondary antibody was removed. Hematoxylin was used for redyeing (G1004-100; Servicebio, Wuhan, China). Sealing was performed. The immunopositivity was observed and photographed under the microscope at 400x magnification. The software Image-Pro Plus was used for optical density analysis.

### Determination of ALP activity

After the treatment of cells with rutin, the activity of ALP in cells in each group was detected according to the instructions for ALP activity detection kit (WLA064; Wanleibio).

### Alizarin red staining

After 14 days of rutin intervention, alizarin red staining was performed to analyze the osteogenic differentiation of cells in different treatment groups. The medium in each group was removed. 4% paraformaldehyde was used for fixation at ambient temperature for 15 min. The fixative was discarded. Cells were washed with distilled water for 3 times. Alizarin red S (G1450; Solarbio) was added for staining for 30 min. The staining solution was removed. Cells were washed with distilled water for 3 times again. The magnification of the microscope was adjusted to 100 times for photographing and analyzing.

### Statistical analysis

The data were analyzed with SPSS 20.0. All results were expressed as means  $\pm$  SD. The counting data were tested by chi-square test; the difference was deemed statistically significant if  $p < 0.05$  (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). The software GraphPad Prism 8.0 was used to draw charts.

## Results

### Effects of rutin on expression of *Runx2* gene in cells

After 3 days of treatment of MC3T3-E1 cells with different concentrations of rutin, the expression of *Runx2* gene was detected by PCR. The results showed that, compared with the control group, 3 concentrations of rutin could significantly increase the expression quantity of *Runx2* gene, and there were significant differences ( $p < 0.05$ ). The relation between different concentrations of rutin was compared; and it was found that the greater the concentration, the higher the expression quantity of *Runx2* gene. There were highly significant differences among the 3 concentrations ( $p < 0.05$ ) (Figure 1).

### Effects of rutin on expression of *Runx2* protein in cells

After the treatment of cells in all groups with rutin for 3 days, the expression of *Runx2* protein was detected by Western blot (WB) and IHC, respectively. WB results showed that rutin can significantly increase the expression level of *Runx2* protein, and the difference was statistically significant; with the increase of rutin concentration. The expression level of *Runx2* protein also got higher and higher, and there were significant differences ( $p < 0.05$ ). The IHC results showed that, there was no significant difference between the low concentration group and the control group. The comparative results among other groups were consistent with those of WB (Figure 2 A,B).

### Effects of rutin on ALP activity in cells

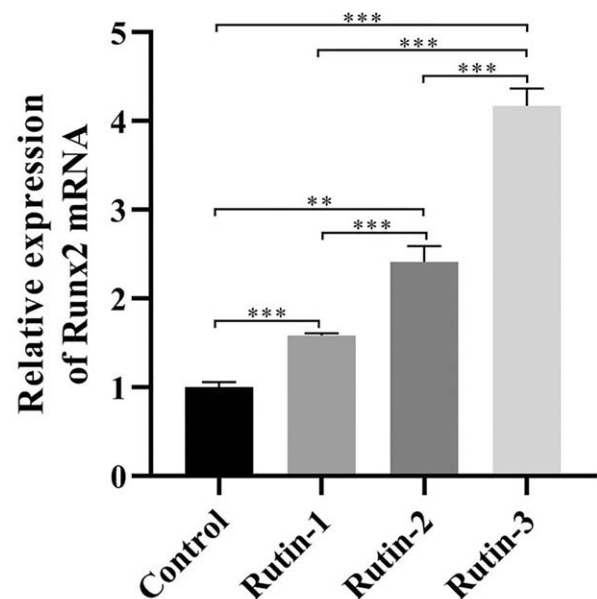
The ALP activity was detected with ALP activity detection kit after the cells in all groups were treated with rutin for 5 days. It was found that, compared with the control group, the ALP activity of all treatment groups was significantly improved, and the greater the concentration, the higher the ALP activity ( $p < 0.05$ ); and there were significant differences in the ALP activity among different concentration groups ( $p < 0.05$ ) (Figure 3).

### Detection of osteogenic differentiation of cells by alizarin red staining

The differentiation in cells was analyzed by alizarin red staining after 14 days of treatment with rutin. The results showed that calcified nodules were formed in all groups and that the area of calcified nodules formed in groups treated with rutin was greater than that in the control group; the greater the concentration of rutin, the larger the more obvious the nodules (Figure 4).

## Discussion

Rutin is a flavonoid found in many plants, has a wide range of biological activities, and can be used to resist oxidation and inflammation, protect the nerve and liver, and prevent cerebral hemorrhage.<sup>12</sup> In addition to some diseases that have been known and clinically treated with rutin, the role of rutin in other diseases has also been gradually taken seriously by people. Previous studies showed that rutin has important potential in the prevention and treatment of diabetes.<sup>13,14</sup> The effects of rutin in orthopedic diseases have also been gradually known by people. Osteoporosis is a chronic disease; the main cause is that the bone has lost its mineralization quality and mechanical flexibility. Since the currently used anti-osteoporosis agents have some efficacy but also some side effects, including an increased risk of blood clots and cancer, phytochemicals may be a safer and more effective option. The

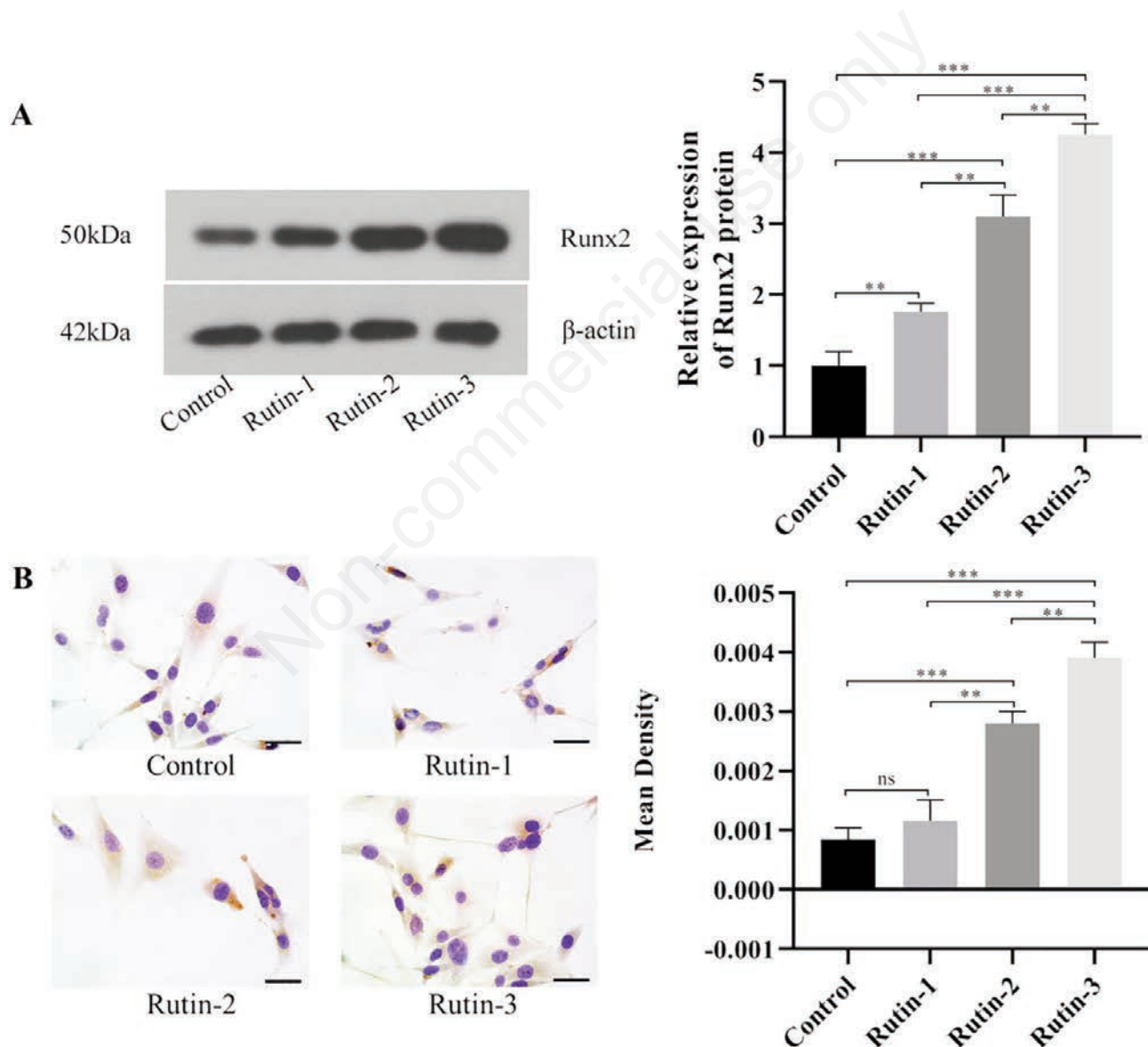


**Figure 1.** Expression of *Runx2* gene after treatment of MC3T3-E1 cells with different concentrations of rutin.

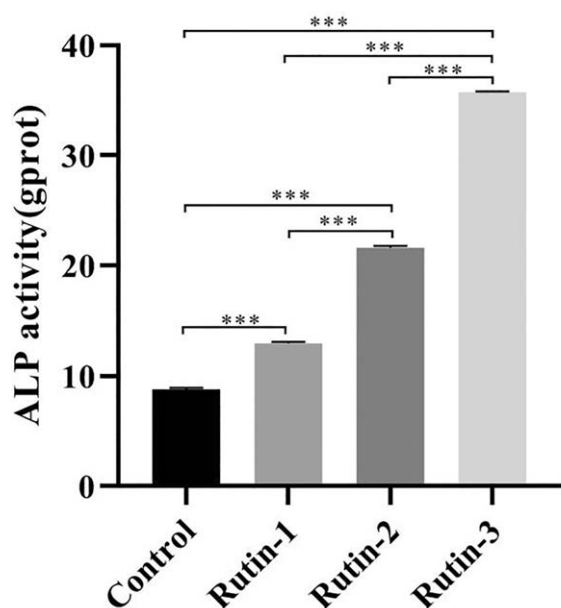
study on human osteosarcoma cells, SAOS-2 cells, showed that rutin can increase the expression of osteocyte and osteoblast-related genes and decrease the expression of Runx inhibitor and osteoclast gene. The study on the effects of rutin on ALP activity demonstrated that rutin can improve ALP activity and decrease the activity of acid phosphatase, a marker for osteoporosis. Therefore, rutin can promote the proliferation of osteocyte and act as an ossification marker.<sup>15</sup> The results of this study are consistent with those of our study, proving that rutin can be used as an effective drug for future treatment of orthopedic diseases. Xiao *et al.* explored the mechanism of action of rutin in osteoporosis and found by analyzing by multiple experimental methods, including Micro-CT, Western-Blotting, Real-time PCR, transmission electron microscope and alizarin red staining, that rutin can regulate FNCD1 level and autophagy through the Akt/mTOR signaling pathway, which provides a new strategy for the treatment of osteoporosis.<sup>16</sup> Rutin can also protect human periodontal ligament stem cells (HPDLSCs) from the TNF- $\alpha$ -induced osteogenic differentiation

damage in an inflammatory environment, and is expected to be a new candidate drug for the treatment of periodontitis bone defects.<sup>17</sup>

The formation and reconstruction of bone is a complicated process, including osteoblasts forming new bone and osteoclasts absorbing old bone to maintain a dynamic balance. *Runx2*, also known as core-binding factor  $\alpha 1$ , is one of the important members of the Runt family. The other two members are *Runt1* and *Runx3*. Some studies have shown that, in the early stage of osteoblast differentiation, *Runx2* gene can trigger the synthesis of bone matrix protein and provide a great number of immature osteoblasts.<sup>18</sup> It can be used as a specific marker gene to indicate osteoblast differentiation and bone formation. Moreover, *Runx2* gene activates the transcription and expression of bone sialoprotein, osteopontin and type I collagen gene by binding to cis-acting elements of osteoblasts.<sup>19</sup> The molecular osteoprotegerin (OPG) produced by osteoblasts plays an important role in the formation of osteoclasts; OPG can not only inhibit the formation of osteoclast-like cells *in*



**Figure 2.** Expression of Runx2 protein in cells after treatment with rutin. A) Western blot; B) Immunohistochemistry.

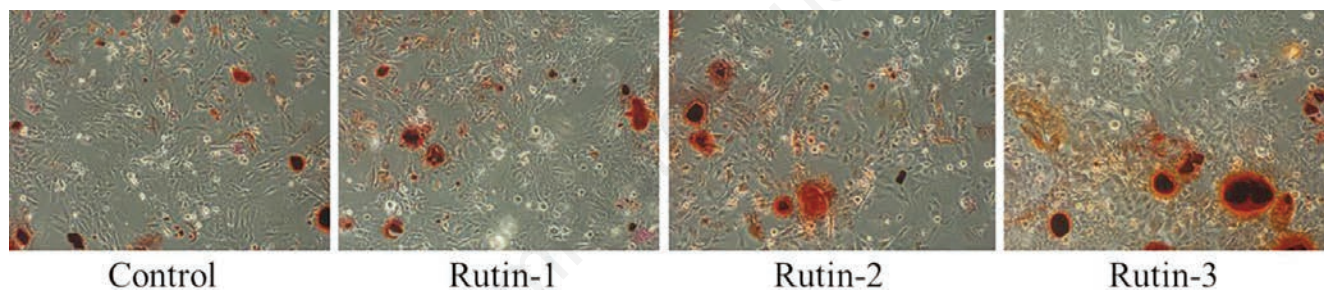


**Figure 3.** ALP activity after treatment of cells with rutin.

*in vivo* and *in vitro* but also absorb bone. The sequence cloning of human OPG showed that there is a binding site for OPG in *Runx2* gene sequence, indicating that *Runx2* can regulate OPG expression.<sup>9,20</sup> *Runx2* gene is highly expressed in multiple myeloma cells and is one of the major drivers of myeloma progression. Lowering the abundance of *Runx2* by regulating the upstream genes of *Runx2* can slow down the progression multiple myeloma.<sup>21</sup> The results are consistent with ours. With the deepening of study and the understanding of the richer functions and mechanism of action of *Runx2* gene, it will certainly provide a detailed theoretical basis and practical significance for the treatment of bone-related diseases.

ALP is a specific enzyme secreted by osteoblasts. Its activity directly reflects the degree of the differentiation of osteoblasts. Zhang *et al.* confirmed that ALP was highly expressed in both *in vitro* osteogenic differentiation and *in vivo* bone formation.<sup>22</sup> Similar to rutin, astragaloside is also a bioactive flavonoid mostly from medicinal plant. After the treatment of MC3T3-E1 cells with astragaloside, high expression of ALP was also detected and significantly increased; besides, the expression level of *Runx2* was also higher than that in the control group. The results are consistent with those of this study.<sup>23</sup>

It is believed that, with the deepening of study, rutin will play a better role in the treatment of orthopedic diseases, with great significance for their cure.



**Figure 4.** Analysis of osteogenic differentiation of cells by alizarin red staining.

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