—Full Paper—

Effects of resveratrol and its analogues on the cell cycle of equine mesenchymal stem/stromal cells

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Resveratrol (RSV; trans-3,5,4'-trihydroxystilbene) strongly activates sirtuin 1, and it and its analogue V29 enhance the proliferation of mesenchymal stem/stromal cells (MSCs). Although culture medium containing 5-azacytydine and RSV inhibits senescence of adipose tissue–derived MSCs isolated from horses with metabolic syndrome, few studies have reported the effects of RSV on equine bone marrow–derived MSCs (eBMMSCs) isolated from horses without metabolic syndrome. The aim of this study was to investigate the effects of RSV and V29 on the cell cycle of eBMMSCs. Following treatment with 5 μM RSV or 10 μM V29, the cell proliferation capacity of eBMMSCs derived from seven horses was evaluated by EdU (5-ethynyl-2'-deoxyuridine) and Ki-67 antibody assays. Brightfield images of cells and immunofluorescent images of EdU, Ki-67, and DAPI staining were recorded by fluorescence microscopy, and the number of cells positive for each was quantified and compared by Friedman's test at $P<0.05$. The growth fraction of eBMMSCs was significantly increased by RSV and V29 as measured by the EdU assay (control 28.1% ± 13.8%, V29 31.8% ± 14.6%, RSV 32.0% ± 10.8%; mean ± SD; $P<0.05$) but not as measured by the Ki-67 antibody assay (control 27.0% ± 11.2%, V29 27.4% ± 10.8%, RSV 27.7% ± 6.8%). RSV and V29 promoted progression of the cell cycle of eBMMSCs into the S phase and may be useful for eBMMSC expansion.

Key words: *cell cycle, mesenchymal stem cell, resveralogue, resveratrol*

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Resveratrol (RSV; *trans*-3,5,4′-trihydroxystilbene) is a plant polyphenol and is found at high concentrations in grape skin and wine [7]. It has beneficial effects on disorders such as osteoarthritis [31], metabolic diseases [13], and inflammatory bowel disease [22]. RSV has a strong effect on the inflammatory phenotype of senescent cells and can reverse cell senescence [18]. Investigations of its effects on mesenchymal stem/stromal cells (MSCs) are increasing in the field of human regenerative medicine [14] because of its strong activation of sirtuin 1 (SIRT1), which is associated with biological aging [5, 26, 34]. Long-term culture of human MSCs decreases SIRT1 [39, 40], but RSV activates SIRT1 to promote cell cycling, survival, and proliferation of MSCs [8, 23, 36]. Studies suggest that RSV could improve the therapeutic efficacy of human regenerative medicine [27, 29] through its actions via multiple pathways [10] in addition to its canonical SIRT1 activity [30]. Analogues and prodrugs of RSV have recently been developed that can act via SIRT1-dependent or SIRT1-independent activity [2, 3, 18, 37].

Equine MSCs are used in equine regenerative medicine [11, 24]. Equine MSC therapies were effective in treating musculoskeletal diseases of tendons, ligaments, and joints [9, 25, 32]. One study [35] showed promising results in the treatment of tendon injury with a local administration of 2×10^7 MSCs into lesions, much higher than the 1×10^6 MSCs reported in a previous study [4]. The use of MSC-derived extracellular vesicles prepared from culture media of highly culture-expanded MSCs is also increasing in equine regenerative medicine [12, 24]. These approaches

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require significant scale-up in the manufacture of MSCs, which is a major challenge for efficient methods of sustained cell proliferation and maintenance of the quality of equine MSCs, to realize the clinical use of these therapies [11].

Several studies have reported the effects of RSV on equine MSCs. Culture media containing 5-azacytydine and RSV inhibited senescence of adipose tissue–derived MSCs isolated from horses with metabolic syndrome and promoted cell proliferation, osteogenesis, and chondrogenesis [16, 19, 20]. However, few studies have reported the effects of RSV on equine bone marrow–derived MSCs (eBMMSCs) isolated from horses without metabolic syndrome, and to the best of our knowledge, no studies have reported the effects of novel RSV analogues (resveralogues) on eBMMSCs.

The aim of this *in vitro* study was to investigate the effects of RSV and V29, a novel analogue of RSV [3], on the cell cycle of eBMMSCs.

Materials and Methods

EBMMSC culture

The study was approved by the Ethics and Welfare Committee of the Royal Veterinary College (ref. URN 2022 2127-2). The authors have no competing interests.

Standard culture medium (D10) contained Dulbecco's Modified Eagle's Medium (DMEM) with 1 g/*l* of glucose (31885023, Gibco, Thermo Fisher Scientific, London, UK) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin (all Gibco, Thermo Fisher Scientific).

Equine MSCs were derived from bone marrow aspirates of horses (n=7) for isolation, expansion, and osteogenic, adipogenic, and chondrogenic differentiation as described previously [32]. The eBMMSCs were stored in liquid nitrogen with 1 m*l* aliquots of cell freezing medium (Cellbanker 2, AMS Biotechnology, London, UK) in accordance with the manufacturer's instructions. The passage number of the eBMMSCs at freezing was 2.1 ± 1.1 (2.0).

eBMMSCs were rapidly thawed in a 37°C water bath, immediately diluted in 10 m*l* of D10, and then centrifuged at 300 × *g* for 5 min at 21°C (Rotina 380 R, Andreas Hettich GmbH, Germany). The cell pellet was suspended in 15 m*l* D10. The MSCs were seeded in a 75-cm^2 flask and incubated at 37°C in humidified 5% CO_2 (CB 170, Binder GmbH, Tuttlingen, Germany). The medium was replaced after 24 hr to remove non-adherent cells and then replaced every 2 to 3 days until the cells reached 80% confluence. Cell cultures were passaged for experimental treatment as describe below.

Preparation of resveratrol and V29

Resveratrol was purchased (ab120726, Abcam, London, UK) and V29 was synthesized in house [3]. Stock solutions of both were prepared in DMSO (Invitrogen, Thermo Fisher Scientific) and diluted into working solutions with D10. The working solutions were used at final concentrations of 5 and 10 μM, respectively, at which both enhance proliferation of human fibroblasts [3] and are not toxic.

Treatment with resveratrol and V29

Cells were detached from flasks with TrypLE Express (Gibco, Thermo Fisher Scientific, UK) and centrifuged at 300 × *g* for 5 min at 21°C. The cell pellet was suspended in 1 m*l* of culture medium, and the cell concentration was assessed with an automated cell counter (Countess II FL, Thermo Fisher Scientific).

For experiments, eBMMSCs were seeded into 12-well plates at 8 × 10^3 cells/well in D10 and allowed to adhere for 24 hr. Non-adherent cells were carefully removed by washing with phosphate-buffered saline (PBS; Gibco, Thermo Fisher Scientific). Cells were then treated with 2 m*l* of D10 (control group), 10 μM V29 in D10 (V29 group), or 5 μM RSV solution in D10 (RSV group) and incubated for 24 hr. The medium was then discarded, and the cells washed with PBS before culture in D10 for a further 72 hr.

The population doubling level (PDL) of eBMMSCs was calculated as

$$PDL = 3.322\,(\log N_{end} - \log N_{ini}) + PDL_{ini},$$

where N_{end} was the cell number at the end of the growth period, N_{ini} was that at the beginning of the growth period, and PDL_{ini} was the initial population doubling level, which was assumed to be 11 for all eBMMSCs.

Cell proliferation assay

The cell proliferation rate was evaluated with an EdU (5-ethynyl-2′-deoxyuridine) labelling kit (Click-iT Plus EdU Cell Proliferation Kit for Imaging Alexa Fluor 594, Gibco) and Ki-67 antibody (Recombinant Alexa Fluor 488 Anti-Ki-67, Abcam) in accordance with the manufacturers' instructions. Cells were labelled with EdU in D10 for 3 hr, washed with PBS, fixed in 3.7% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature, incubated overnight in blocking buffer (1% bovine serum albumin, 0.1% Triton X-100 in PBS), and then incubated with EdU reaction solution for 30 min at room temperature in the dark.

After washing with PBS, cells were incubated with Ki-67 antibody diluted 1:50 with 3% bovine serum albumin in PBS for 3 hr at room temperature in the dark. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific).

Image analysis

Brightfield images of cells and immunofluorescent images of EdU (red), Ki-67 (green), and DAPI (blue)

staining in the same region of interest were recorded by fluorescence microscopy (EVOS FL, Thermo Fisher Scientific; Fig. 1).

The numbers of cells positive for EdU, Ki-67, and DAPI staining were quantified in the ImageJ v. 1.53t software (National Institutes of Health, Bethesda, MD, USA) [28]. To evaluate the proliferating fraction, at least 1,000 total nuclei were counted in random regions in each well, and the percentage of EdU- or Ki-67-positive cells was calculated.

Statistical analysis

Data are expressed as the mean ± standard deviation (median in parentheses). Friedman's test with Dunn's *post hoc* test was used to determine the significance of differences between treatment groups at $P<0.05$ in the GraphPad Prism v. 7.04 software (GraphPad Software, San Diego, CA, USA).

Results

Microscopic observation confirmed good growth and proliferation of all eBMMSCs. The passage number and PDL of eBMMSCs at the time of treatment were 3.4 ± 1.2 (3.0) and 16.2 ± 3.9 (15.5), respectively.

The percentages of EdU-positive cells were 28.1% ± 13.8% (31.3%) in the control group, 31.8% ± 14.6% (37.0%) in the V29 group, and 32.0% ± 10.8% (36.5%) in the RSV group, with those of the V29 and RSV groups being significantly higher than that of the control group (Fig. 2).

The percentages of Ki-67-positive cells were 27.0% ± 11.2% (28.5%) in the control group, 27.4% ± 10.8% (25.1%) in the V29 group, and 27.7% ± 6.8% (26.5%) in the RSV group, with no significant difference among the groups (Fig. 3).

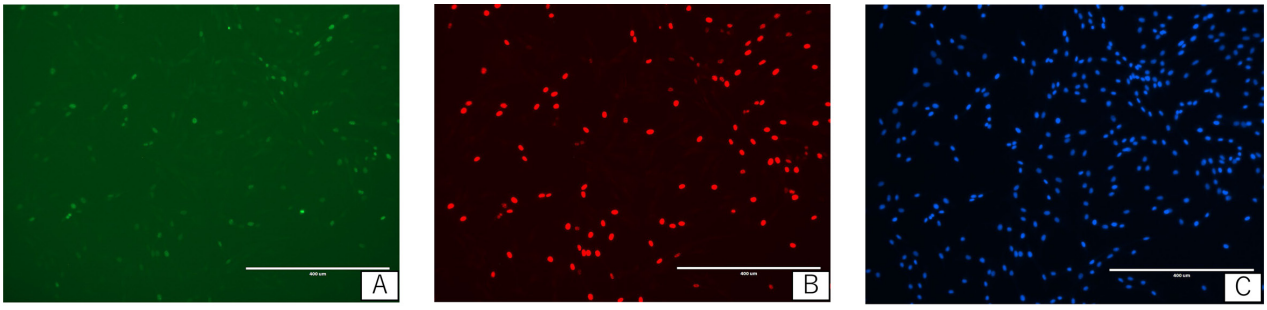

Fig. 1. Representative immunofluorescent images of the same field: (A) Ki-67, green; (B) EdU, red; and (C) DAPI, blue. Scale bars; 400 μm.

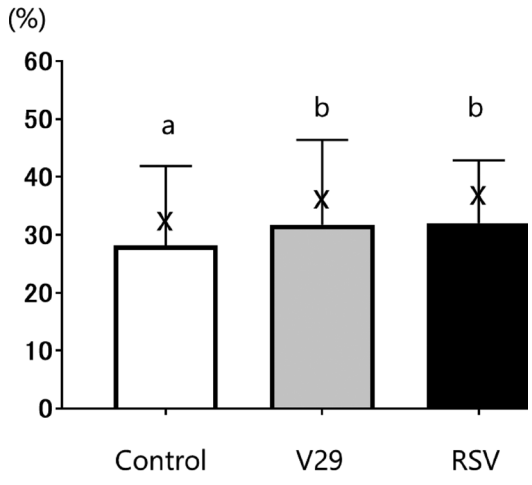

Fig. 2. Proportions of EdU-labelled cells following treatment with V29 or RSV. Values are indicated as the mean ± standard deviation (error bars), and median values are indicated by an x. Different letters above groups indicates statistical significance ($P<0.05$ by Friedman's test).

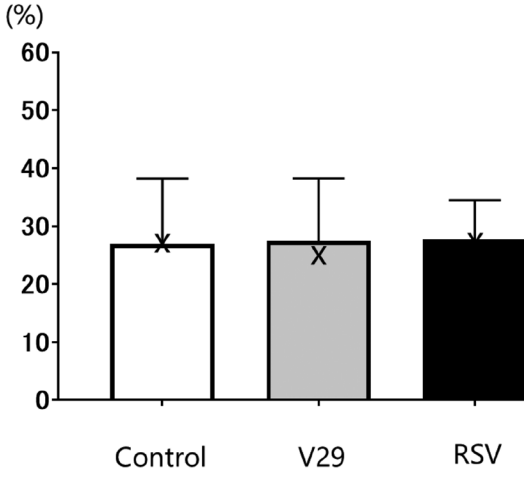

Fig. 3. Proportions of Ki-67-labelled cells following treatment with V29 or RSV. Values are indicated as the mean ± standard deviation (error bars), and median values are indicated by an x.

Discussion

The purpose of this study was to evaluate the effects of RSV and V29, a novel resveralogue, on the cell cycle of eBMMSCs. The Ki-67 assay is generally used to evaluate the whole cell cycle, because Ki-67 is a nuclear protein antigen that is expressed in cells during all phases of the cell cycle but is strongly downregulated during the G0 phase [15]. In this study, it revealed no significant differences among the three groups. Although some antioxidants show cytotoxic effects on MSCs [14], neither of the RSV and V29 concentrations used in this study adversely affected the eBMMSCs. EdU staining is an excellent indicator of cell proliferation [6] owing to its incorporation into newly synthesized DNA by cells during the S phase. The proportion of EdU-labelled cells during the same incubation period was dependent on the cell type because of their differences in cell cycle acceleration [21]. The percentages of EdU-positive cells were significantly higher in both the RSV and V29 groups than in the control group. Our results indicate that both RSV and V29 promoted progression of the cell cycle of eBMMSCs to the S phase and cell proliferation without affecting the whole cell cycle.

Our results support previous reports that RSV can increase the proportion of human MSCs in the S phase *in vitro* [23, 36]. They are also consistent with the report that both 10 μM RSV and 5 μM V29 (and other resveralogues) accelerated the cell cycle of human fibroblasts [3].

RSV promotes proliferation of mouse [33] and human MSCs [36, 38]. Our results also suggest that both RSV and V29 would be useful to promote the proliferation of autologous eBMMSCs. Autologous eBMMSCs are widely used to treat musculoskeletal diseases, including injuries of the superficial digital flexor tendon [25, 32], arthritis [9], and laminitis [1]. A longer culture period, however, is required to increase eBMMSCs to sufficient numbers for regenerative therapies in equine clinical use [24]. The use of RSV and V29 to promote progression of the cell cycle to the S phase will contribute to equine regenerative medicine by shortening the period of eBMMSC preparation.

Synthetic analogues and prodrugs of RSV have been developed to enhance pharmacological activity [2, 3, 37]. There are, however, few reports of the effects of these RSV derivatives on MSCs [14]. This study shows the effects of V29 on eBMMSCs for the first time. Our study also revealed that a novel synthesized resveralogue showed similar effects to conventional RSV, because there was no difference between the effects of RSV and those of V29. Several structurally related resveralogues induce higher SIRT1 activation of human fibroblasts and are less toxic to them than RSV [3]. Although further research is needed, our results suggest that other resveralogues may be candidates for enhancing the proliferation of eBMMSCs and motivate the development of new RSV analogues for use in regenerative medicine.

The effects of RSV on cells have been shown to be different depending on the concentration [8, 23, 36]. Although dose-dependent cell proliferation of human MSCs was observed in the range of 0.01 μM to 10 μM of RSV [8], 60 μM RSV caused irreversible cell cycle arrest, DNA damage, and premature senescence in human MSCs [17]. Our previous study also showed that resveralogues promoted cell proliferation at 10 μM but caused increasing cell death at 100 μM due to their toxicity [3]. Several studies of RSV have shown that these concentration-dependent effects vary by animal species, cell origin, and culture duration [8, 14, 33]. Although studies of RSV in equine cells are limited, culture medium containing both 0.5 μM 5-azacytydine, which was incorporated into DNA and inhibited the methylation pattern of specific gene regions, and 5 μM RSV induced the proliferation of eMSCs isolated from equine metabolic syndrome cases [16]. Our preliminary experiments performed prior to this study also resulted in increased proliferation of eBMMSCs and equine tendon cells with 10 μM RSV and 10 μM V29 (data not shown). Although the concentrations of RSV and V29 used here promoted cell proliferation in the short period examined, further experiments are necessary to evaluate the effects in long-term cultures or on cells of different ages because it takes approximately one month to culture eBMMSCs for clinical use.

In conclusion, RSV and V29 promoted progression of the cell cycle of eBMMSCs into the S phase. They may be useful for eBMMSC expansion for autologous use but will be especially useful for allogeneic applications where extensive cell expansion is necessary.

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