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# Methylsulfonylmethane inhibits cortisol-induced stress through p53-mediated *SDHA*/*HPRT1* expression in racehorse skeletal muscle cells: A primary step against exercise stress

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**Abstract.** Cortisol is a hormone involved in stress during exercise. The application of natural compounds is a new potential approach for controlling cortisol-induced stress. Tumour suppressor protein p53 is activated during cellular stress. Succinate dehydrogenase complex subunit A (*SDHA*) and hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) are considered to be two of the most stable reference genes when measuring stress during exercise in horses. In the present study cells were considered to be in a 'stressed state' if the levels of these stable genes and the highly stress responsive gene p53 were altered. It was hypothesized that a natural organic sulphur-containing compound, methylsulfonylmethane (MSM), could inhibit cortisol-induced stress in racing horse skeletal muscle cells by regulating *SDHA*, *HPRT1* and *p53* expression. After assessing cell viability using MTT assays, 20 $\mu$g/ml cortisol and 50 mM MSM were applied to horse skeletal muscle cell cultures. Reverse transcription-quantitative PCR and western blot analysis demonstrated increases in SDHA, HPRT1 and p53 expression in cells in response to cortisol treatment, which was inhibited or normalized by MSM treatment. To determine the relationship between *p53* and *SDHA*/*HPRT1* expression at a transcriptional level, horse gene sequences of *SDHA* and *HPRT1* were probed to identify novel binding sites for p53 in the gene promoters, which were confirmed using a chromatin immunoprecipitation assay. The relationship between p53 and SDHA/HPRT1 expression was confirmed using western blot analysis following the application of pifithrin-α, a p53 inhibitor. These results suggested that MSM is a potential candidate drug for the inhibition of cortisol-induced stress in racehorse skeletal muscle cells.

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## Introduction

Exercise is pivotal for the maintenance of physical and mental wellbeing. Although essential, exercise often results in stress, particularly in animals. The degree of stress is dependent on the conditions of the exercise. During exercise, a racehorses may experience different types of stress, including oxidative (1), heat (2), hypoxic (3), hormonal (4) and glucose stress (5). As a result of these aforementioned stress responses, levels of hormones become altered, including cortisol (6), adrenaline (7) and noradrenaline (8). Among these hormones, the steroidal hormone cortisol is the primary stress hormone produced by the adrenal gland. During the onset of stress, the pituitary-adrenal axis secretes corticotrophin-releasing hormone, which stimulates the secretion of adrenocorticotropic hormone (ACTH). ACTH then in turn stimulates the adrenal gland to secrete cortisol (9). Cortisol increases blood glucose levels and suppresses the digestive system (10,11). In addition, it affects the brain regions that control fear, motivation and mood (12). Prolonged secretion of cortisol may lead to physical and psychological effects (13). Indeed, serious mental issues associated with cortisol secretion include exaggerated negative cognitions, increased feelings of anxiety and helplessness in response to stress (14).

Prolonged secretion of cortisol occurs with prolonged stress, and is associated with disease, including heart disease, weight gain and depression (15). To reduce stress, the level of

cortisol should be controlled or optimized externally, such as through the use of pharmacological agents that control cortisol levels. The administration of natural compounds to optimize cortisol levels in thoroughbred racehorses during stressful conditions is a convenient approach, as natural compounds may have fewer off-target effects than other pharmacological agents (16). Among these natural compounds, methylsulfonylmethane (MSM) is one such natural organic sulphur-containing compound that is present in fruit, vegetables and some beverages (17). MSM has properties that make it a suitable drug candidate to alleviate stress, as it has been previously shown to exhibit anti-cancer (18), anti-inflammatory and antioxidant activity (19). It has also been demonstrated to inhibit ketosis *in vitro* by regulating the STAT5B signalling cascade (20).

Several genes undergo changes in expression when a racehorse encounters stress. The most stable reference genes for the assessment of exercise-induced stress are succinate dehydrogenase (SDH) complex subunit A (*SDHA*) and hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) (21). SDHA is a flavo-protein located in the mitochondria, which is involved in the citric acid cycle and the respiratory chain. It is closely associated with other subunits in the SDH complex (SDHB, SDHAC, and SDHD). SDHA contains a flavin adenine dinucleotide (FAD) cofactor-binding site and catalyzes the transelimination of two hydrogen molecules from succinate to form fumarate. In this reaction FAD accepts the two hydrogen molecules and FAD is converted to $FADH_2$ (22). In a previous study that implemented knockdown of SDH complexes, SDHB knockdown resulted in increased cytosolic oxidative stress, whereas SDHA knockdown did not, indicating that SDHA is a stable reference gene under stress conditions (23).

The HPRT1 enzyme converts hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate. It serves a vital role in the production of purine nucleotides via the purine salvage pathway. HPRT deficiency can lead to replication stress, which may result in pathological consequences as a result of genome instability due to inappropriate repair of chromosomal DNA double-strand breaks (24) and diseases such as Lesch-Nyhan disease in humans (25,26). Although these two genes are conventionally considered as housekeeping or reference genes for stress, the potential regulation of their expression in racehorse skeletal muscle cells remains unknown and has not been studied in the presence of cortisol.

p53 is a tumour suppressor in multicellular organisms (27). p53 expression is upregulated in response to oxidative stress, as a result of an increase in its half-life and stability (28). Oxidative stress activates p53 to regulate the transcription of genes associated with stress (29). Although p53 has been found to transcriptionally regulate the expression of *SDHA* and *HPRT1* in mice (30,31), the relationship between p53 and the expression of *SDHA* and *HPRT1* in racehorse skeletal muscle cells remains unknown.

In the present study, it was hypothesized that MSM may inhibit cortisol-induced stress in the skeletal muscle cells of thoroughbred racehorses by regulating the expression levels of *SDHA* and *HPRT1*, by acting as a transcription factor for these two genes.

## Materials and methods

*Antibodies and cell culture reagents*. Medium 199 was purchased from Gibco; Thermo Fisher Scientific, Inc. Penicillin-streptomycin solution and FBS were purchased from HyClone; GE Healthcare Life Sciences. Trypsin-EDTA (0.05%) was obtained from Gibco; Thermo Fisher Scientific, Inc. Antibodies specific for β-actin (cat. no. sc-47778) and horseradish peroxidase-conjugated secondary antibodies [goat anti-mouse (cat. no. sc-2005) and anti-rabbit (cat. no. sc-2004)] were obtained from Santa Cruz Biotechnology, Inc. Anti-SDHA antibody (cat. no. ab66484) was purchased from Abcam. The primary antibody against HPRT1 (cat. no. LS-C81245) was purchased from LifeSpan BioSciences, Inc. and the anti-p53 primary antibody (cat. no. ARP37163_T100) was obtained from Aviva Systems Biology Corporation. Pifithrin-α (PFT-α; P4359) was purchased from Sigma-Aldrich; Merck KGaA (32).

*Isolation of racehorse skeletal muscle cells*. A skeletal muscle tissue biopsy was performed on the leg of a male neonatal thoroughbred racehorse to cultivate primary horse skeletal muscle cells. The obtained muscle tissue was first chopped into 1x1 mm sections, washed twice using PBS and subsequently transferred to 15 ml tubes containing 2 ml trypsin/EDTA for 18 h at 4˚C. The trypsin was then discarded and the tissue pieces were further incubated with residual trypsin at 37˚C for 30 min, followed by the addition of 5 ml media containing 10% FBS. The resulting suspension was centrifuged for 3 min at 670 x g at room temperature to collect the cell pellet. After centrifugation, a filter was used (200 $\mu$M; Cell Strainers; cat. no. 08-771-1; Falcon™; Thermo Fisher Scientific, Inc.) to completely disperse any remaining tissues and to collect single cells. The resulting supernatant was centrifuged further (3 min; 670 x g at room temperature) before the cell pellet was collected and cultured in media. This cell isolation protocol was as previously described (33). The Pusan National University-Institutional Animal Care and Use Committee approved the study design (approval no. PNU-2015-0864).

*Cell culture and treatment*. Racehorse skeletal muscle cells were cultured in Medium 199 supplemented with 10% FBS and 1% antibiotic-antimycotic (ABAM; Invitrogen; Thermo Fisher Scientific, Inc.). Horse skeletal muscle cell cultures were incubated in a humidified atmosphere with 5% $CO_2$ at 37˚C. For each experiment, at between 70 and 80% confluence, cells were gently washed twice with PBS and then treated by adding MSM with fresh media. Unless specified otherwise, cells were treated with 50 mM MSM for 24 h at 37˚C.

*Cell viability assay*. Cell viability was assessed using an MTT assay (Sigma Aldrich; Merck KGaA). Briefly, cells were resuspended in Medium 199 and seeded into 24-well culture plates at a density of $1x10^4$ cells/well, 1 day prior to drug treatment. The next day, culture medium was replaced with fresh Medium 199 (vehicle control) or different concentrations of MSM (5-400 mM), and the cells were incubated for a further 24 h at 37˚C. MTT (5 mg/ml) was subsequently added into each well, and the culture dishes were incubated at 37˚C for 4 h. Formazan crystals in each well were then dissolved using DMSO (Sigma Aldrich; Merck KGaA), and the absorbance at 550 nm was

measured using an Ultra multifunctional microplate reader (Tecan Group, Ltd.). Cell viability was determined from these readings using the calculation % Viability=(fluorescence value of MSM/fluorescence value of non-treated control) x100. All measurements were performed in triplicate, and experiments were repeated at least three times.

*Western blotting*. Whole cell lysates were prepared from untreated or MSM-treated racehorse skeletal muscle cells by incubation with radioimmunoprecipitation lysis buffer (EMD Millipore) containing phosphatase and protease inhibitors on ice. Cells were disrupted by aspiration through a 23-gauge needle with the resultant lysate centrifuged at 18,300 x g for 10 min at 4˚C to remove cellular debris. Protein concentrations were measured using the Bradford assay (Thermo Fisher Scientific, Inc.). Equal amounts of protein (100 $\mu$g/lane) were separated on a 10% SDS-PAGE gel, followed by transferal onto nitrocellulose membranes. The blots were then blocked for 1 h at room temperature with 5% skim milk dissolved in TBS buffer supplemented with 0.1X Tween-20 (TBS-T). The membranes were then probed overnight at 4˚C with the relevant primary antibodies [anti-SDHA (1:500), anti-HPRT1 (1:500), anti-p53 (1:500) and anti-β-actin (1:1,000)] diluted in 5% bovine serum albumin (BSA; EMD Millipore) or skim milk (Difco™ skim milk; BD Biosciences). Membranes were then washed with TBS-T and incubated for 1 h at room temperature with HRP-conjugated secondary antibodies (1:2,000). Detection was performed using an Enhanced Chemiluminescence Plus detection kit (Amersham; GE Healthcare) and imaged on an ImageQuant™ LAS 4000 imaging device (Fujifilm Corporation). Blots were stripped using Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific, Inc.). Densitometry values were determined using FUJI FILM Multi Gauge version 3.1 (Fuji Photo Film Co., Ltd.).

*Reverse transcription-semiquantitative polymerase chain reaction (RT-sqPCR)*. Total RNA was extracted with the RNeasy Mini kit (Qiagen GmbH) according to the manufacturer's protocol. RNA was quantified spectrophotometrically at 260 nm. Subsequently, RT-sqPCR analyses were performed to detect *SDHA*, *HPRT1* and *GAPDH* RNA expression. Briefly, cDNA was synthesized from total RNA at 42˚C for 1 h, and at 95˚C for 5 min using first-strand cDNA synthesis kit (AccuPower® RT PreMix; cat. no. K-2041; Bioneer Corporation) and oligo d(T) primers. The RT-PCR Premix kit (AccuPower® PCR PreMix; cat. no. K-2016; Bioneer Corporation) was used to amplify *SDHA*, *HPRT1* and *GAPDH* with primers synthesized by Bioneer Corporation. To generate a 200-bp *SDHA* fragment, the following primers were used: *SDHA* forward, 5'-CTACAAGGGGCAGGTTCTGA-3' and reverse, 5'-TCTGCAATACTCAGGGCACA-3'. To generate a 290-bp *HPRT1* fragment, the following primers were used: *HPRT1* forward, 5'-TCTTTGCTGACCTGCTGGAT-3' and reverse, 5'-GGGTCCTTTTCACCAGCAAG-3'. To generate a 211-bp *p53* fragment, the following primer pair was used: *p53* forward, 5'-AGGTTGGCTCTGACTGTACC-3' and reverse, 5'-TCCTCCTTCTTGCGGAAGTT-3'. Finally, a 320-bp *GAPDH* mRNA fragment was generated using the following primer pair: *GAPDH* forward, 5'-AAGGCCATCACCATCTTCCA-3' and reverse, 5'-ACGATGCCAAAGTGGTCATG-3' and an *18S* mRNA fragment was generated using the following primer pair: *18S* forward, 5'-AGCCTTCGGCTGACTGGCTGG-3' and reverse, 5'-CTGCCCATCATCATGACCTGG-3'. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 31 cycles at 95˚C for 45 sec, 58˚C for 60 sec and 72˚C for 60 sec, followed by final extension at 72˚C for 10 min. The PCR products were resolved by electrophoresis on a 2% agarose gel, and were visualized using ethidium bromide (cat. no. E7637; Sigma-Aldrich; Merck KGaA) staining. Quantification was performed using FUJI FILM Multi Gauge version 3.1 (Fuji Photo Film Co., Ltd.).

*p53 binding motif analysis*. The p53 binding motif was identified using Geneious Prime software (Geneious; version R6.1; https://www.geneious.com). The sequences of *SDHA* and *HPRT1* were screened for the p53 binding motif (AGACAT). The results obtained showed 4 binding motifs for p53 in the *SDHA* sequence and 6 binding motifs for p53 in the *HPRT1* sequence. Primers were designed on the basis of these sequences.

*Chromatin immunoprecipitation (ChIP) assay*. The ChIP assay was performed using the Imprint® chromatin immunoprecipitation kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. Briefly, racehorse skeletal muscle cells were fixed using 1% formaldehyde at room temperature for 10 min and quenched using 1.25 M glycine at room temperature. Samples were then mixed and washed with ice-cold PBS by centrifugation at room temperature for 5 min at 180 x g. After washing, the cells were suspended in nuclei preparation buffer and shearing buffer prior to their sonication (30% amplitude for 30 sec followed by 30 Sec rest for 20 cycles) on ice. The sheared DNA was subsequently centrifuged at 4˚C for 5 min at 180 x g and the cleared supernatant was used for protein/DNA immunoprecipitation. The clarified supernatant was diluted with buffer at a 1:1 ratio and 5-$\mu$l aliquots of the diluted samples were used as internal controls. The diluted supernatant was then incubated in 96 well plates pre-coated with 4 $\mu$g/$\mu$l anti-p53 antibody for 90 min at room temperature. The negative and positive controls were incubated with 1 $\mu$g normal goat IgG and 1 $\mu$g anti-RNA polymerase II (Sigma-Aldrich; Merck KGaA), respectively. The unbound DNA was washed using immunoprecipitation wash buffer, and the bound DNA was collected by applying the crosslink reversal method, using DNA release buffer containing proteinase K. The released DNA and DNA from the internal control were subsequently purified using a GenElute™ Binding Column G (Sigma-Aldrich; Merck KGaA), following which they were quantified using conventional PCR. The thermocycling conditions for PCR were as follows: Initial denaturation at 95˚C for 10 min, followed by 35 cycles at 95˚C for 40 sec, 58˚C for 50 sec and 72˚C for 50 sec, followed by final extension at 72˚C for 10 min. The PCR products were resolved by electrophoresis on a 1.5% agarose gel, and were visualized using ethidium bromide (cat. no. E7637; Sigma-Aldrich; Merck KGaA) staining. Quantification was performed using FUJI FILM Multi Gauge version 3.1 (Fuji Photo Film Co., Ltd.).

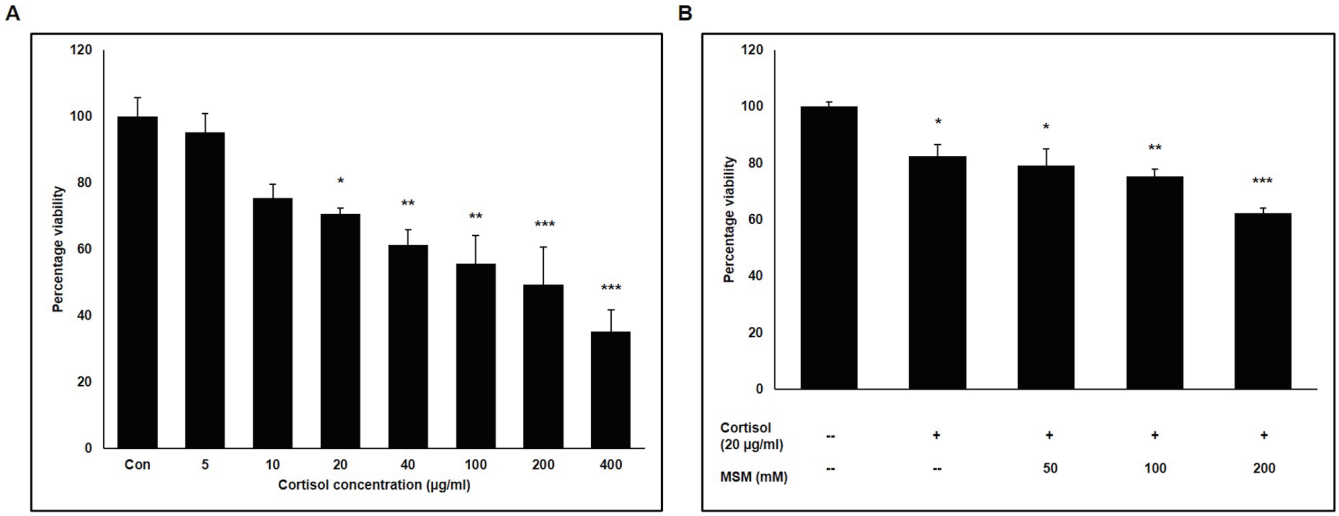


Figure 1. Cortisol and MSM reduces cell viability in racehorse skeletal muscle cells. (A) Changes in horse muscle cell viability after 24-h treatments with increasing concentrations of cortisol, according to the MTT assay. Statistical analysis was performed using ANOVA. (B) The MTT assay results showed changes in racehorse skeletal muscle cell viability after 24 h combined treatment with increasing concentrations of MSM in the presence of 20 $\mu$g/ml cortisol. Statistical analysis was performed using ANOVA. $^{*}$P<0.05, $^{**}$P<0.01 and $^{***}$P<0.001 vs. respective control. MSM, methylsulfonylmethane; Con, control.

*Statistical analyses*. All experiments were performed at least three times. Data are presented as the mean ± SEM. Statistical analyses were conducted with one-way ANOVA and Student's t-test. They were performed with Duncan's multiple range test as a post hoc test. Analyses were performed using the SAS 9.3 program (SAS Institute, Inc.). P<0.05 was considered to indicate a statistically significant difference.

## Results

*Effect of cortisol and MSM on horse skeletal muscle cell viability*. A candidate drug for the treatment of stress should be designed in such a way that it causes little or no toxicity in racehorse skeletal muscle cells. Therefore, the effect of increasing concentrations of cortisol and MSM on thoroughbred racehorse skeletal muscle cells was analysed using an MTT assay. It was found that ~70% of the cells remained viable following treatment with 20 $\mu$g/ml cortisol (Fig. 1A). Subsequently, the effect of three ascending concentrations of MSM on horse skeletal muscle cell viability was checked in the presence of 20 $\mu$g/ml cortisol. Combined with 20 $\mu$g/ml cortisol, little difference was observed in cell viability between 50 mM MSM treatment and no MSM treatment (Fig. 1B). Therefore, doses of 20 $\mu$g/ml cortisol and 50 mM MSM were selected for subsequent experiments.

*Cortisol-induced expression of SDHA and HPRT1 is inhibited by MSM*. It was hypothesized that the expression pattern of *SDHA* and *HPRT1* is a significant factor in cortisol-induced stress. Therefore, in the present study, the expression of *SDHA* and *HPRT1* following treatment with increasing concentrations of cortisol was analysed. The expression levels of SDHA and HPRT1 were elevated in response to 20 $\mu$g/ml cortisol for 24 h, at the mRNA and protein levels (Figs. 2A, B and S1). From this, it was hypothesized that cortisol treatment induced stress in horse skeletal muscle cells. To examine whether the impact of cortisol-induced stress, could be minimized using MSM, increasing concentrations of MSM were applied in conjunction with cortisol. Treatment of cells with 50 mM MSM or higher reduced the expression of *SDHA* and *HPRT1* (Figs. 2C, D and S1). MSM at 100 and 200 mM reduced the expression of *GAPDH,* which may have been due to the increased cytotoxicity caused by the synergistic effect of cortisol and MSM (data not shown). These results suggested that MSM inhibited cortisol-induced stress.

*MSM inhibits cortisol-induced stress by regulating SDHA/HPRT1 and p53 expression*. RT-PCR was performed to assess the expression patterns of *SDHA*, *HPRT1* and *p53* in cortisol-treated and untreated (control) horse skeletal muscle cells (Fig. 3). MSM treatment appeared to reverse cortisol-induced increases in *SDHA*, *HPRT1* and *p53* expression significantly (Fig. 3A and B), an observation that was replicated at the protein level (Fig. 3C and D). These results suggested that p53 may serve an important role in MSM treatment of cortisol-induced stress.

*MSM inhibits the binding of p53 to the SDHA and HPRT1 gene promoter regions*. It was hypothesized that p53 serves an important role in cortisol-induced stress through its interactions with SDHA and HPRT1 at a post-translational level. Therefore, the ability of p53 and MSM to transcriptionally regulate *SDHA* and *HPRT1* genes in horse skeletal muscle cells was investigated. p53 binding motifs were discovered at four sites (labelled a-d) in the *HPRT1* gene promoter (Fig. 4A) and at six sites (labelled a-f) in the *SDHA* gene promoter region using Geneious prime software (Geneious; Version R6.1) (Fig. 4A and C). These binding sites were confirmed using a ChIP assay. Positive p53 binding was found at sites a, b and d in the *HPRT1* sequence, whereas no binding was observed at site c (Fig. 4B). MSM treatment acted on site b, where it inhibited p53-induced *HPRT1* expression (Fig. 4B). Strong positive p53 binding was also found in the *SDHA* promoter sequence at sites a, b, c, d and e, but a negative result

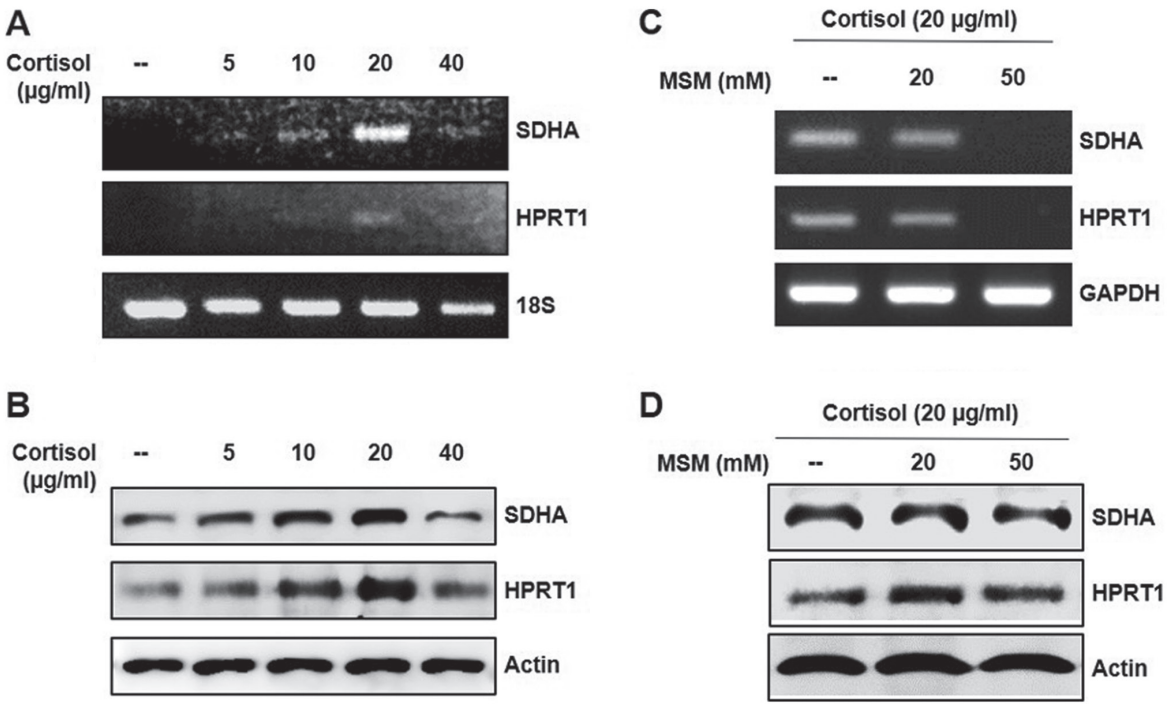


Figure 2. Cortisol induces the expression of SDHA and HPRT1 in racehorse skeletal muscle cells, which were reversed by concomitant MSM treatment. (A) RT-PCR analysis of *SDHA* and *HPRT1* gene expression in horse skeletal muscle cells following 24 h treatments with increasing concentrations of cortisol. (B) Western blot analysis showing the expression pattern of SDHA and HPRT1 proteins after 24 h treatment with increasing concentrations of cortisol. (C) RT-PCR analysis of *SDHA* and *HPRT1* gene expression after 24 h treatments with increasing concentrations of MSM in the presence of 20 $\mu$g/ml cortisol. (D) Western blot analysis of horse skeletal muscle cells showing the expression of SDHA and HPRT1 proteins after 24 h treatment of MSM in the presence of 20 $\mu$g/ml cortisol. SDHA, succinate dehydrogenase complex subunit A; HPRT1, hypoxanthine phosphoribosyl transferase 1; RT-PCR, reverse transcription-PCR; MSM, methylsulfonylmethane.

was observed at site f (Fig. 4D). Here, it was also found that MSM reversed cortisol-induced p53 binding to sites a and e in the *SDHA* promoter. These results suggest that p53 serves a vital role in the cortisol-induced expression of *SDHA* and *HPRT1,* which is inhibited by MSM driven regulation of p53 expression.

*Role of p53 in the cortisol-induced expression of SDHA and HPRT1.* To confirm the relationship between p53 and SDHA/HPRT1 expression in the presence of cortisol, western blotting analysis was performed using, PFT-α, a p53 inhibitor. Significant increases in SDHA and HPRT1 expression were observed in racehorse skeletal muscle cells in response to 20 $\mu$g/ml cortisol, which was significantly reversed with the concomitant addition of PFT-α (Fig. 5A). Significantly elevated p53 expression was also observed in cortisol-treated cells, which was also reversed by PFT-α treatment (Fig. 5B). These results further support the role of p53 in cortisol-induced increases in SDHA and HPRT1 expression.

## Discussion

When a thoroughbred racehorse participates in a race, cortisol levels are elevated, as a result of a variety of factors. To control stress, cortisol levels should be optimized. Treating the racehorses with drugs may affect performance, since these drugs typically produce side effects. Therefore, treating racehorses with natural remedies may provide a better alternative for overcoming cortisol-induced stress in racehorses. In particular, MSM is a natural organic sulphur-containing compound, which has been previously reported to increase growth hormone receptor expression (34) and bone growth by regulating bone morphogenetic protein-2 expression (35). In the present study, it was demonstrated that 20 $\mu$g/ml cortisol induced ~30% cell death according to the MTT assay, whilst enhancing the expression of SDHA and HPRT in racehorse muscle cells. By contrast, 40 $\mu$g/ml cortisol reduced the expression of 18S due to ~40% cell death. Therefore, 20 $\mu$g/ml cortisol was used for further studies. In the presence of 20 $\mu$g/ml cortisol, 50 mM MSM did not aggravate cell death, suggesting that 50 mM MSM may be non-toxic to racehorse muscle cells. Based on the findings in the present study, MSM may make a promising candidate drug for controlling cortisol-induced stress.

The present study was, to the best of our knowledge, the first to analyse stress in racehorses by culturing racehorse skeletal muscle cells and inducing stress *in vitro* using cortisol. To confirm stress induction, the expression levels of the two most stable reference genes, *SDHA* and *HPRT*, were analysed (21). These two genes exhibited altered expression following cortisol treatment. Indeed, SDHA is a key factor in oxidative stress (36), but, to the best of our knowledge, no evidence exists that identifies a role for SDHA in cortisol-induced stress conditions. Additional studies have reported that HPRT is involved in cellular stress responses (37,38), HPRT expression in response to stress in the presence of cortisol remains unclear. Recently, Morgan *et al* (39) used *SDHA* and *HPRT* as housekeeping genes when studying cortisol metabolism. In the present study it was hypothesized that if treatment with

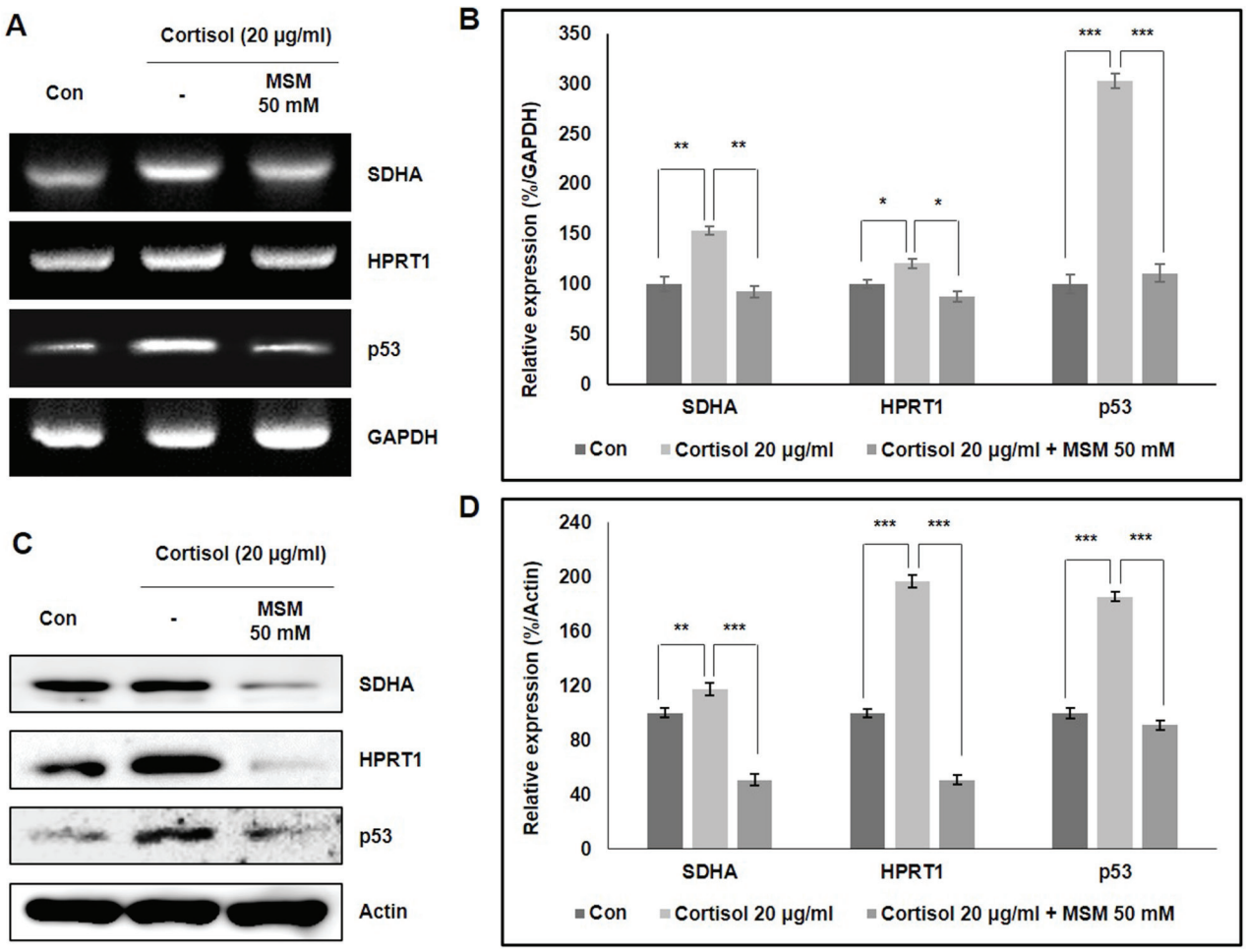


Figure 3. MSM reverses cortisol-induced increases in SDHA, HPRT1 and p53 expression levels. (A) RT-PCR analysis of *SDHA, HPRT1* and *p53* gene expression in horse skeletal muscle cells following 24 h treatment with 20 $\mu$g/ml cortisol and 50 mM MSM. (B) Quantified densitometry data, with comparisons performed using Student's t-test. (C) Western blot analysis of horse skeletal muscle cells showing the expression levels of SDHA, HPRT1 and p53 proteins after 24 h treatment with 20 $\mu$g/ml cortisol and 50 mM MSM, with respect to β-actin expression. (D) Quantified densitometry data, with comparisons was performed using Student's t-test. $^{*}$P.0.05, $^{**}$P<0.01 and $^{***}$P<0.001 vs. respective control. $^{*}$P.0.05, $^{**}$P<0.01 and $^{***}$P<0.001 between cortisol group and cortisol + MSM group. SDHA, succinate dehydrogenase complex subunit A; HPRT1, hypoxanthine phosphoribosyl transferase 1; RT-PCR, reverse transcription-PCR; MSM, methylsulfonylmethane.

cortisol was able to alter the expression of *SDHA* and *HPRT*, then it could be concluded that the cells were in a 'stressed state', since these are often considered to be the most stable reference genes (25). Results from the present study showed that the expression levels of both SDHA and HPRT1 were elevated in 20 $\mu$g/ml cortisol-treated cells. These findings suggested that cortisol treatment induced stress in thoroughbred racehorse skeletal muscle cells. It was also hypothesized in the present study that MSM may reduce stress in the same cell type, and the data showed that 50 mM MSM could reverse the cortisol-induced elevation of SDHA and HPRT1 expression.

Many studies have provided evidence that p53 serves an integral part in stress. As a result of cellular stress such as oxidative stress, p53 becomes activated, which then takes part in cell cycle arrest and apoptosis induction (28). The amount of stress is directly proportional to the degree of p53 activation, which becomes saturated in response to prolonged stress (40). Data from the present study showed that the addition of cortisol induced the expression of p53, which was inhibited or normalized to levels comparable to those of control cells following additive MSM treatment. In addition, p53 expression was found to be directly proportional to the expression of SDHA and HPRT1. Previous evidence has demonstrated that p53 acts as a transcription factor for *SDHA* and *HPRT* in mice (30). Therefore, to determine the relationship between p53 and *SDHA*/*HPRT* in the horse genome, the sequences of horse *SDHA* and *HPRT1* genes were analysed. A number of novel binding sites for p53 were found in the promoter regions of the *SDHA* and *HPRT1* genes. Since these binding sites were new in the horse genome, to the best of our knowledge, their function was validated by performing ChIP assays to detect p53/SDHA and p53/HPRT1 binding complexes. Once p53 binds to the promoter region of these genes, it may promote the transcription of the respective genes.

MSM can induce p53 independent apoptosis in cancer (41). In the present study, it was observed that MSM could inhibit or reverse the cortisol-induced formation of p53/SDHA and p53/HPRT1 complexes. These results suggested that MSM could be a candidate drug for treating cortisol-induced stress in thoroughbred racehorse skeletal muscle cells. In tumour cells, MSM induces p53 independent apoptosis, and therefore

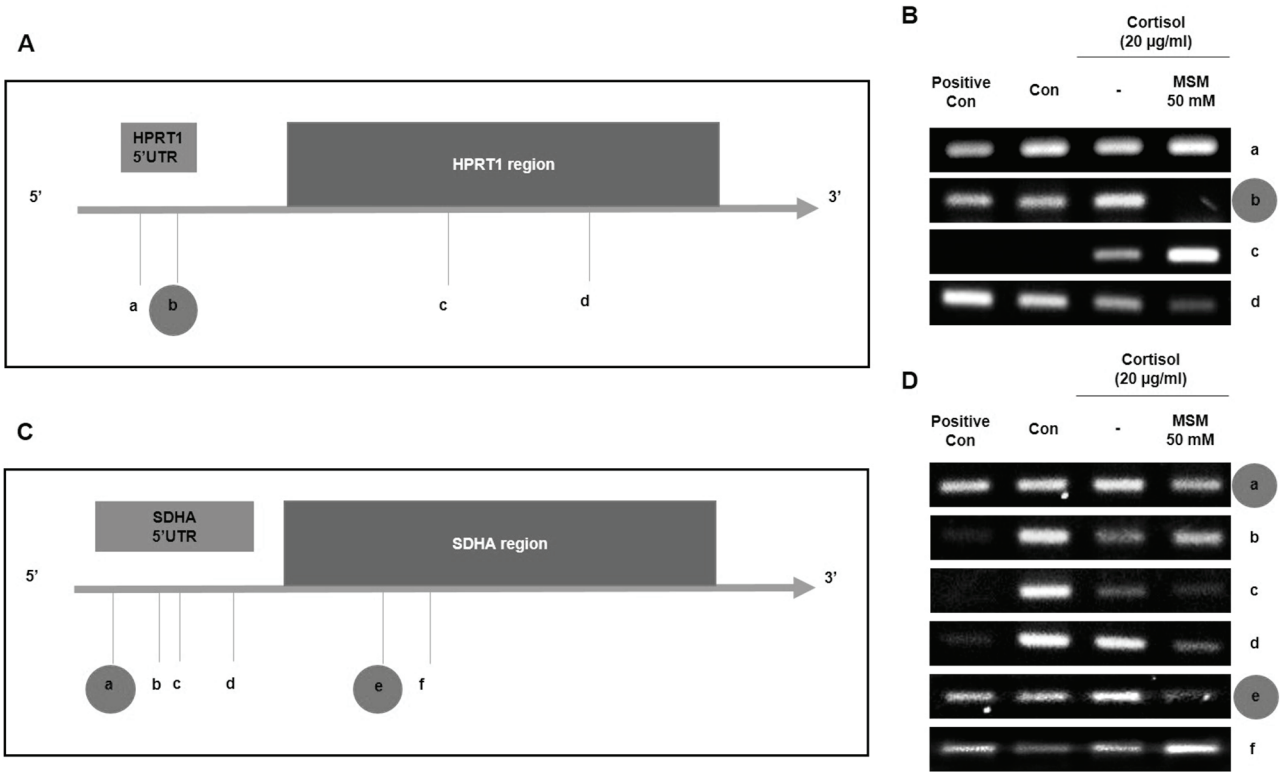


Figure 4. MSM inhibits p53 binding to the promoter regions of the *SDHA* and *HPRT1* genes. (A) Schematic representation of p53 binding sites in the promoter of the *HPRT1* gene. (B) ChIP assay results showing p53/HPRT1 complex formation and its inhibition by MSM at site b. (C) Schematic representation of p53 binding sites in the promoter of the *SDHA* gene. (D) ChIP assay results showing p53/SDHA complex formation and its inhibition by MSM at sites a and e. SDHA, succinate dehydrogenase complex subunit A; HPRT1, hypoxanthine phosphoribosyl transferase 1; RT-PCR, reverse transcription-PCR; MSM, methylsulfonylmethane; ChIP, chromatin immunoprecipitation.

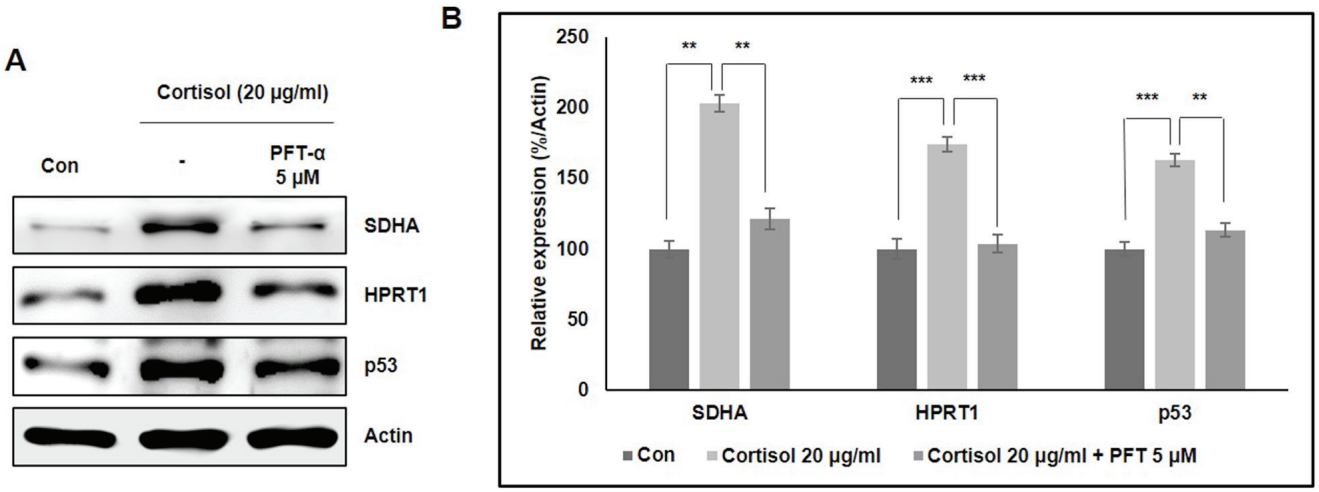


Figure 5. Role of p53 in cortisol-induced changes in SDHA and HPRT1 expression. (A) Western blot analysis of horse skeletal muscle cells following 24 h treatment with 20 $\mu$g/ml cortisol and 5 $\mu$M p53 inhibitor PFT-α. (B) Quantified densitometric analysis of SDHA, HPRT1 and p53 protein expression with respect to β-actin expression. Statistical analysis was performed using Student's t-test. $^{**}$P<0.01 and $^{***}$P<0.001 vs. respective control. $^{**}$P<0.01 and $^{***}$P<0.001 between cortisol group and cortisol + PFT-α group. SDHA, succinate dehydrogenase complex subunit A; HPRT1, hypoxanthine phosphoribosyl transferase 1; PFT-α, pifithrin-α; MSM, methylsulfonylmethane, Con, control.

controls tumour growth (41). However, in normal cells, p53 is activated in response to cellular stress (42). Therefore, the potential anti-stress activity of MSM was analysed by comparing cellular responses to MSM treatment with that of a p53 inhibitor, PFT-α. PFT-α inhibited the cortisol-induced regulation of *SDHA* and *HPRT1* expression in a pattern similar to that observed with MSM, suggesting that MSM acted as a p53 inhibitor in stressful conditions. Taken together, these findings suggested that MSM may serve as a candidate anti-stress drug for treating cortisol-induced stress conditions.

In conclusion, the present study demonstrated that MSM inhibited cortisol-induced stress in thoroughbred

horse skeletal muscle cells by regulating p53-mediated SDHA/HPRT1 expression. Novel binding sites for p53 in the *SDHA* and *HPRT1* gene promoter regions were also found in this cell type. Therefore, MSM may be a candidate anti-stress drug for treating stress in racing horses.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

NS and DYK conceived and designed the experiments, performed the experiments and wrote the paper. YMY and KJJ contributed in designing the experiments and data analysis. DHK, HGL, YMP, IHK, HKL and BWC analyzed experiments and data along with KJJ and YMY. All authors contributed to revising the manuscript and approved the final version for publication.

**Ethics approval and consent to participate**

The Pusan National University-Institutional Animal Care and Use Committee approved the study design (approval no. PNU-2015-0864).

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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