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Protective effect of p53 in vascular smooth muscle cells against nitric oxide-induced apoptosis is mediated by up-regulation of heme oxygenase-2

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The tumor suppressor gene p53 regulates apoptotic cell death and the cell cycle. In this study, we investigated the role of p53 in nitric oxide (NO)-induced apoptosis in vascular smooth muscle cells (VSMCs). We found that the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) increased apoptotic cell death in p53-deficient VSMCs compared with wild-type cells. The heme oxygenase (HO) inhibitor tin protoporphyrin IX reduced the resistance of wild-type VSMCs to SNAP-induced cell death. SNAP promoted HO-1 expression in both cell types. HO-2 protein was increased only in wild-type VSMCs following SNAP treatment; however, similar levels of HO-2 mRNA were detected in both cell types. SNAP significantly increased the levels of non-heme-iron and dinitrosyl iron-sulfur clusters in wild-type VSMCs compared with p53-deficient VSMCs. Moreover, pretreatment with FeSO4 and the carbon monoxide donor CORM-2, but not biliverdin, significantly protected p53-deficient cells from SNAP-induced cell death compared with normal cells. These results suggest that wild-type VSMCs are more resistant to NO-mediated apoptosis than p53-deficient VSMCs through p53-dependent up-regulation of HO-2. [BMB reports 2008; 41(2): 164-169]

INTRODUCTION

Nitric oxide (NO), synthesized from L-arginine by nitric oxide synthase (NOS), plays an important role in the regulation of vascular function and apoptosis (1). Low levels of NO are constitutively produced by endothelial nitric oxide synthase (NOS) in the vasculature and a large amount of NO is generated by the induction of inducible NOS following endothelium injury as well as in atherosclerotic plaques (1, 2). Although a small amount of NO plays an important role in vascular tone, endothelial survival, and vascular inflammation (3), high levels of NO has been shown to induce VSMC apoptosis (4). Regulation of VSMC apoptosis or proliferation has recently been found to be involved in the physiological remodeling of the vasculature as well as in the pathogenesis of vascular diseases such as atherosclerosis, hypertension, and restenosis after angioplasty (5, 6).

Heme oxygenases (HOs) are the rate-limiting enzymes in heme degradation, catalyzing the cleavage of the heme ring to form ferrous iron, carbon monoxide (CO), and biliverdin. Three distinct variants of HOs have been cloned (7). HO-1 is an inducible enzyme, whose synthesis is elicited by inflammatory stimulants or stress, and HO-2 which is constitutively expressed is concentrated mostly in the brain and testes, accounting for most HO activity in brain (7). HO-3 is also largely localized in the brain, although its enzymatic activity is lower than that of the other isoforms (8). Both HO-1 and -2 identically metabolize heme to iron, CO, and biliverdin, which have been described as cytoprotective factors (9). Morita et al. demonstrated that VSMCs expressed both HO-1 and -2 (10), indicating that both isotypes may be involved in the protection of VSMCs from apoptotic cell death.

The p53 tumor-suppressor protein functions as a key regulator of growth and apoptosis of stressed or abnormal cells. It has been demonstrated that p53-dependent apoptosis can contribute to the inhibition of cancer development at several stages during tumorigenesis (11). Although the apoptotic function of p53 depends on its ability to induce apoptotic genes, p53-null cells have been shown to be more sensitive to the drug induced mitotic catastrophe (12). Evidence has been presented which suggests that depending on the cell type and situation,

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p53 can also prevent apoptosis; this is especially true in cells that harbor mutations of genes affecting the apoptosis pathway (13). Moreover, some p53 mutants fail selectively in their ability to express the apoptotic target genes while retaining anti-proliferative activity, indicating that the apoptotic and anti-proliferative functions of p53 can be dissociated in certain mutants (14). Although p53 is involved in regulating VSMC apoptosis (15), the functional role of p53 in NO-induced VSMC apoptosis remains largely unknown.

In this study, we examined the role of p53 in NO-induced VSMC cell death. We found that p53 deficiency renders VSMCs more susceptible to NO-induced cell death than p53 wild-type cells. The functional role of p53 in the resistance to NO-induced cell death appears to be conferred by up-regulation of HO-2 expression.

RESULTS

p53 protects against NO-induced apoptotic cell death in VSMCs

To analyze p53 function against NO-induced cell death in VSMCs, we first examined NO-induced cell death in wild-type and p53-deficient VSMCs. The chemical NO donor, SNAP, induced a significantly greater level of cell death in p53-deficient VSMCs as compared with normal cells in both time and dose-dependent manners (Fig. 1A and 1B). For the subsequent studies, we used 1 mM SNAP as the source of NO to simplify our approach. Cell death was effectively inhibited by the addition of the pan-caspase inhibitor Ac-ZVAD-fmk (data not shown), indicating that SNAP induces apoptotic cell death in both cell types. To investigate whether the sensitivity of p53-deficient VSMCs to NO-induced cell death was specific, we examined the effect of known apoptotic agents on cell viability in wild-type and p53-deficient VSMCs. Apoptotic cell death induced by etoposide (10 μM) and staurosporin (1 μM) was not significantly different in both cell types (Fig. 1C and 1D). Furthermore, a similar sensitivity to NO-induced cell death was observed in tumor cells with mutant p53 (PANC-1) or cells lacking p53 (Hep3B) versus a tumor cell line with wild-type p53 (MCF-7) (Fig. 1E). These results indicate that p53 plays an important role in the sensitivity of NO-induced apoptotic cell death.

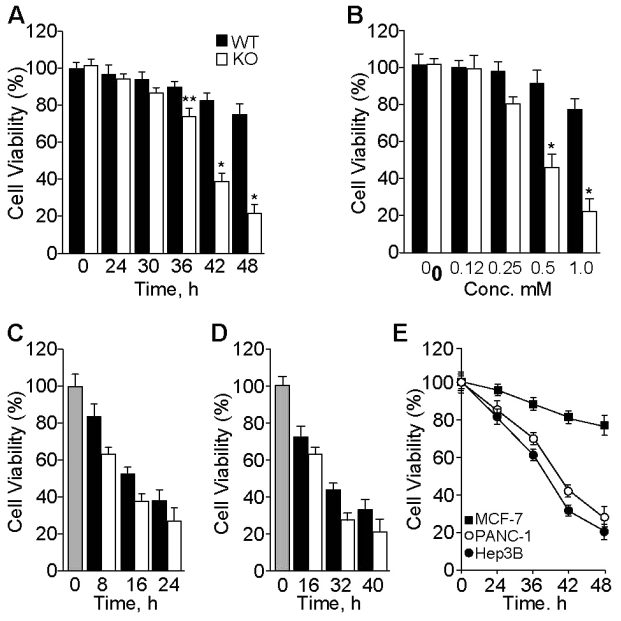

Fig. 1. Protective effect of p53 on NO-induced apoptotic cell death in VSMCs. (A) wild-type and p53-deficient VSMCs were treated with 1 mM SNAP for various time periods. (B) VSMCs were treated with various concentrations of SNAP for 48 h. VSMCs were treated with 1 μM staurosporin (C) and 10 μM etoposide (D). (E) Tumor cells with wild-type p53 (MCF-7) and mutant p53 (PANC-1) or without p53 (Hep3B) were treated with 1 mM SNAP for various time periods. Cell viability was measured by crystal violet staining. All data are the mean ± SD (n=4). **, $P < 0.05$ and *, $P < 0.01$ versus wild-type cells.

Differential HO-2 expression in wild-type and p53-deficient VSMCs exposed to SNAP

To distinguish the difference between wild-type and p53-deficient VSMCs, we analyzed the expression levels of HO-1 and -2 in cells treated with SNAP. SNAP treatment markedly increased HO-1 protein expression, but HO-1 protein levels were similar in both cell types (Fig. 2A). When treated with SNAP for 24 h, HO-2 protein levels were significantly increased in a time-dependent manner in wild-type VSMCs, but not detectable in p53-deficient VSMCs (Fig. 2A). Northern blot analysis was next performed to examine the steady-state levels of HO-2 mRNA in VSMCs after exposure to SNAP. HO-2 mRNA levels

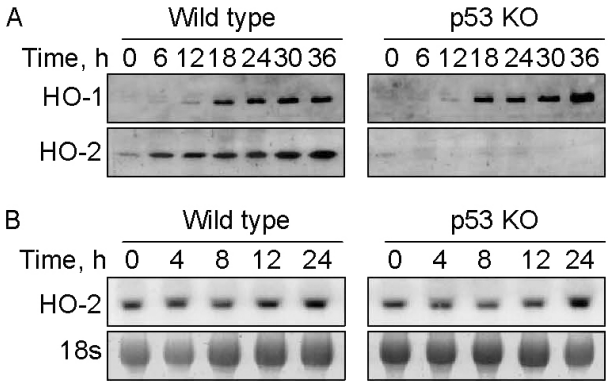

Fig. 2. Expression of HO-1 and -2 in wild-type and 53-deficient VSMCs. (A) VSMCs were treated with 1 mM SNAP for various time periods, and the protein levels of HO-1 and -2 were determined by Western blot analysis. (B) The levels of HO-2 mRNA were determined by Northern blot analysis, and 18S rRNA was used as an internal control.

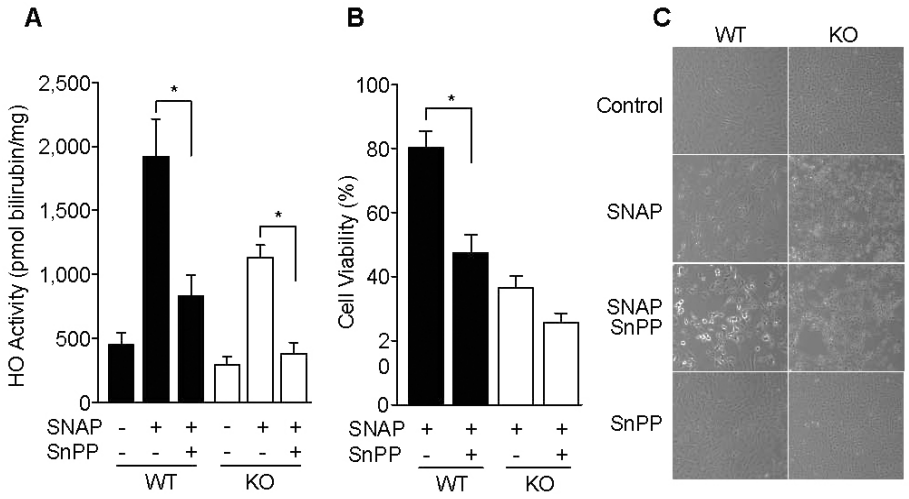

Fig. 3. The inhibition of HO activity increases SNAP-induced smooth muscle cell death. (A) VSMCs were treated with 1 mM SNAP in the presence or absence of 25 μM SnPP IX for 24 h. HO enzyme activity was determined by measuring bilirubin formation. (B) Under the same experimental condition, cell viability was determined after 42 h incubation. (C) Morphological changes were determined after 42 h incubation using a light microscope. Data shown in (A) and (B) are the mean ± SD (n=3). *, $P < 0.01$.

were nearly identical in both cell types during the entire period of SNAP treatment (Fig. 2B). These results indicate that p53 may be involved in the up-regulation of HO-2 protein in NO-treated VSMCs at the translational or post-translational step.

Resistance of wild-type VSMCs to NO-induced cell death was attenuated by inhibition of HO activity

To investigate the role of HO in the resistance of VSMCs to NO-induced apoptosis, we examined the effect of the HO inhibitor tin protoporphyrin IX (SnPP IX) on HO activity and cell death in both cell types. SNAP treatment increased HO activity in both cell types, but higher enzyme activity was observed in wild-type VSMCs than p53-deficient cells (Fig. 3A). The enzyme activity was effectively inhibited by treatment with SnPP IX. Under the same experimental condition, SnPP IX significantly reduced the resistance of wild-type VSMCs to NO-induced cell death, but partially decreased cell viability in p53-deficient VSMCs (Fig. 3B), suggesting that HO-2 may be important for the resistance of wild-type cells to NO-induced cell death. Fig. 3C shows typical morphological changes with markedly decreased survival of wild-type VSMCs following co-treatment with SNAP and SnPP IX compared with SNAP treatment alone.

Effects of heme metabolites on the resistance of NO-induced cell death

HO metabolizes heme to free iron, biliverdin, and CO, which play an important role in cytoprotection (7). We examined iron contents in SNAP-treated VSMCs. The total cellular iron contents were similar in both cell types, and the levels of heme iron was significantly lower in wild-type VSMCs compared with p53-deficient VSMCs, resulting in an increase in non-heme iron levels in wild-type VSMCs (Fig. 4A). Iron released from heme metabolism interacts with intracellular thiols and forms iron-sulfur clusters (16). It has been shown that the nitroslyated form of the iron-sulfur cluster, dinitrosyl iron-sulfur complex (DNIC), protects cells from NO-induced toxicity by either scavenging NO or converting NO to a potent S-nitrosylating species (16). We next determined the cellular levels of DNIC in wild-type and p53-deficient VSMCs by EPR spectroscopy. The formation of DNIC was higher in wild-type VSMCs than p53-deficient cells at 16 h following SNAP treatment, whereas this signal was not apparent in untreated control cells (Fig. 4B). Furthermore, pretreatment with FeSO4 (10 μM) for 12 h to restore cellular iron levels prevented NO-induced cell death in p53-deficient VSMCs, but not in wild-type VSMCs (Fig. 4C). Treatment with the chemical CO donor CORM-2, but not biliverdin, also slightly protected p53-deficient VSMCs, but not wild-type cells (Fig. 4C). These results indicate that iron and CO produced from heme by HO may contribute to the resistance of wild-type VSMCs to NO-mediated apoptotic cell death.

DISCUSSION

It has been postulated that vascular integrity and lesion formation are determined in part by a balance between proliferation and apoptotic cell death of vascular cells including endothelia cells and VSMCs. VSMC apoptosis has recently been identified as an important process in various human vascular diseases, including coronary atherosclerosis and restenosis (5, 6, 15). Among the various vasoactive substances that modulate vascular function, NO has been known to be one of the key molecules in terms of the control of vascular tone and vascular cell survival (1). Recently, NO has emerged as a bifunctional regulator of apoptosis. Although NO exerts anti-apoptotic activity in vascular endothelial cells (1), this molecule induces apoptosis in VSMCs (4). Regulation of apoptosis by NO largely depends on the capability of cells to express numerous genes, such as HO and p53 (9, 13). We examined the

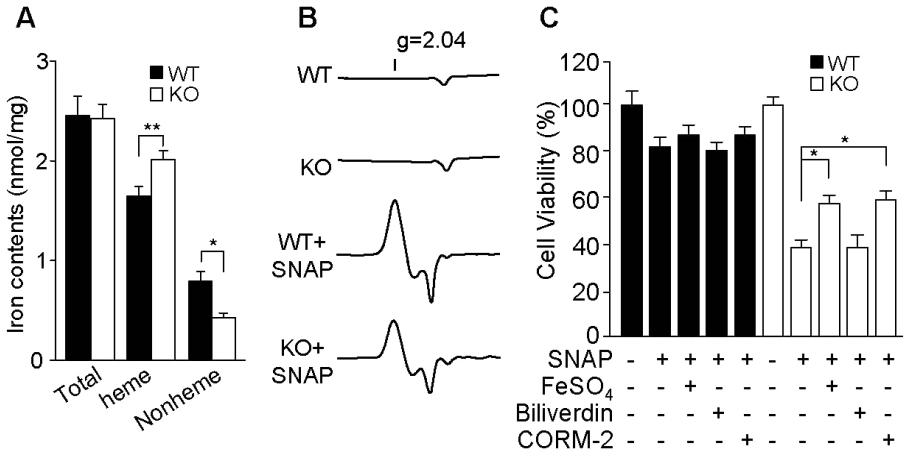

Fig. 4. Iron and carbon monoxide protects p53-deficient VSMCs from SNAP-induced cell death. (A) VSMCs were treated with 1 mM SNAP for 16 h, and total, heme, and non-heme iron contents were determined as described in "Materials and Methods". (B) VSMCs were treated with 1 mM SNAP for 16 h, washed with phosphate buffer, and then exposed to 400 μM SNAP for 3 h. The same amount of cells were transferred into a quartz tube and placed in an EPR device. Formation of dinitrosyl iron-sulfur complex was determined at g=2.04. (C) VSMCs pretreated with 10 μM FeSO4 for 24 h or 20 μM bilirubin for 2 h were exposed to 1mM SNAP. Cells were simultaneously treated with 1 mM SNAP and 25 μM CORM-2, and CORM-2 (25 μM) was added to the culture media every 12 h. Cell viability was determined 42 h after SNAP treatment. Data shown in (A) and (C) are the mean ± SD (n=4). **, $P < 0.05$ and *, $P < 0.01$ versus wild-type.

role of these gene products in NO-mediated apoptosis of VSMCs. We here found that normal VSMCs were more resistant to NO-mediated cytotoxicity than p53-deficient cells. This resistance was attenuated by treatment with the HO inhibitor SnPP IX, suggesting that the catalytic activity of HO may be important for protecting normal VSMCs from NO-induced cell death. Moreover, there was no difference in NO-mediated HO-1 expression in both cell types. Interestingly, NO significantly elevated HO-2 protein levels in wild-type cells, but not in p53-deficient cells, without altering HO-2 mRNA levels in both cell types. These results suggest that NO significantly increased HO-2 expression, probably by translational or post-translational regulation of HO-2 expression in a p53-depenednt manner, and subsequently prevented apoptotic cell death in wild-type VSMCs.

We have first showed that pretreatment with a low level of NO induced HO-1 in hepatocytes and protected cells from apoptotic cell death induced by subsequent treatment with cytotoxic levels of NO and H_2O_2 (17). This protective effect was blocked by the addition of SnPP IX, indicating that the catalytic activity of HO-1 is critically involved in NO-mediated hepatoprotection. A constitutive form, HO-2, is also important in the protection of vascular endothelium against apoptotic changes induced by oxidative stress and cytokine-mediated inflammation (18). These observations indicate that both isoforms of HO play an important role in the protection of various cells from apoptotic cell death. VSMCs expressed both HO-1 and -2 (10), which contributed to the cytoprotection of VSMCs from stress-induced cell death (19). We here also found that both isotypes of HO are expressed in NO-treated VSMCs isolated from wild-type mice. However, HO-2 mRNA was detected in both cell types, but HO-2 protein was expressed only in wild-type VSMCs, suggesting that p53 may play a role in the translational and/or post-transcriptional regulation of HO-2. Although p53 has been shown to function as a transcription factor, the regulation of p53-dependent HO-2 expression is entirely new. This regulatory mechanism will be further investigated. Our data also showed that the catalytic activity of HO is critically involved in the protective effect of p53 on NO-induced cell death, indicating that HO-mediated heme metabolites such as iron, CO, and biliverdin are important for the cytoprotection of VSMCs. Indeed, the addition of iron and CO, but not biliverdin, to the culture media prevented against NO-induced cell death of p53-deficient VSMCs.

p53 induces apoptosis in cell-type and cell-context specific manners (20). Some cell types derived from p53 knockout animals are more sensitive to genotoxic stresses, such as UV and ionizing irradiation, and certain chemotherapeutic agents including Taxol (21, 22), whereas other cell types from the same animals are resistant to these same agents (20, 22). Therefore, the role of p53 in determining cell fate is complex and multifaceted. Our data showed that wild-type VSMCs were more resistant to NO-induced apoptotic cell death than p53-deficinet VSMCs via the p53-dependent up-regulation of HO-2. This resistance was highly correlated with the protein level and catalytic activity of HO-2 in wild-type VSMCs, but not in p53-deficient cells, indicating that p53 protects VSMCs from NO-mediated apoptosis by up-regulating HO-2 protein levels.

Although the regulatory mechanism of p53-dependent HO-2 expression has not been clearly identified, p53 can increase HO-2 gene expression in NO-treated smooth muscle cells at the translational and/or post-translational step.

In conclusion, we demonstrated that NO effectively induced apoptotic cell death of p53-deficient VSMCs compared with wild-type cells. The resistance of wild-type VSMCs to NO-induced apoptosis depended on HO-2 expression and its catalytic activity. These results indicate that the p53-mediated protective effect on NO-induced cell death is associated with HO-2 expression, probably via the translational or post-translational regulation.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium, penicillin, and streptomycin were purchased from Life Technology Inc. (Rockville, MD). HO-1 and HO-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fetal bovine serum was obtained from Hyclone (Logan, Utah). CORM-2 was purchased from Sigma-Aldrich (St. Louis, MO), and SnPP IX, an inhibitor of heme oxygenase activity, was from Porphyrin Products (Logan, UT). Unless indicated otherwise, all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

Aortic VSMC were cultured from explanted thoracic aortas of wild-type and p53-deficient C57BL/6 mice (Taconic Laboratories, Germantown, NY) using the explant method. Cultured cells had the characteristic appearance of hills and valleys and were routinely more than 95% pure by α-smooth muscle actin staining. Cells were grown in Dulbecco modified Eagle medium (low glucose)/Ham's F12 (1:1 vol:vol), supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 4 nmol/l L-glutamine; and maintained in a 37°C , 95% air/5% CO_2 incubator.

Cell viability

Cell viability was determined by the crystal violet staining method, as described previously (16). In brief, cells were stained with 0.5% crystal violet in 30% ethanol and 3% formaldehyde for 10 min at room temperature. Plates were washed four times with tap water. After drying, cells were lysed with 1% SDS solution, and dye uptake was measured at 550 nm using a 96-well plate reader. Cell viability was calculated from relative dye intensity compared with untreated cells.

Western blot analysis

Cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and resuspended in 20 mM Tric-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1 μg/ml chymostatin). Protein concentration was determined with the BCA assay (Pierce). Samples (40 μg) were subjected to electrophoresis in a 12% SDS-polyacrylamide gel and then transferred to nitrocellulose. The membranes were blocked with 5% nonfat-dried milk and hybridized with primary and secondary antibodies against HO-1 and HO-2 as previously described (23). Protein bands were visualized by incubating membranes with chemiluminescent solution for 2 min and exposure to X-ray film.

Northern blot analysis

Total mRNAs were isolated from VSMCs using the Trizol reagent kit (Life Technology Inc., USA). Twenty μg of total mRNA was heat denatured and subjected to 1.0% agarose gel electrophoresis and transferred to nitrocellulose membrane. The membrane was hybridized overnight in a solution containing ^{32}P-labelled cDNA probe to human HO-2 and exposed to X-ray film.

Measurement of HO activity

VSMCs were lysed with ice-cold PBS. Cytosolic HO activity was assayed utilizing a previously described method (23). Briefly, cell lysates were added to the reaction mixture containing NADPH, rat liver cytosol as a source of biliverdin reductase, and heme albumin. The reaction was carried out in the dark for 1 h at 37°C, terminated by the addition of 1 ml chloroform, and bilirubin concentrations were calculated by the difference in absorbance between 464 and 530 nm.

Determination of iron contents and DNIC

Total iron was determined using a colorimetric micromethod after acid-permaganate treatment (23). Heme iron was analyzed by the pyridine-chromogen method (23). Non-heme iron was expressed as the difference between total iron and heme iron. VSMCs were treated with 1 mM SNAP for 16 h, washed with phosphate buffer, and then exposed to 400 μM SNAP for another 3 h. Cells were harvested, washed twice with ice-cold PBS, and resuspended in a small volume of PBS. Cell concentration was adjusted by measuring total protein using a Lowry protein assay kit (P5626, Sigma). An equal volume of cell suspension was placed in a sample tube and frozen in liquid nitrogen for EPR spectroscopy. The formation of cellular DNIC was determined by the intensity of the EPR spectrum at g = 2.04. EPR examination was performed at 77 K as described previously (23).

Statistical analysis

Data are presented as means ± SD of at least three separate experiments except where results of blots are shown, in which case a representative experiment is depicted in the figure. Comparisons between two values were analyzed using Student's *t* test. Differences were considered significant at P values ≤ 0.05.

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