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Expression of Thyroid Stimulating Hormone Receptor mRNA in Mouse C2C12 Skeletal Muscle Cells

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Background: We analyzed whether thyroid stimulating hormone receptor (TSH-R) is expressed in a skeletal muscle cell line and if TSH has influence on the differentiation of muscle cells or on the determination of muscle fiber types.
Methods: TSH-R gene expression was detected with nested real-time polymerase chain reaction (RT-PCR) in C2C12, a mouse skeletal muscle cell line. The effect of TSH on myotube differentiation was assessed by microscopic examination of myotube formation and through the measurement of expression of muscle differentiation markers, i.e., myogenin and myoD, and muscle type-specific genes, i.e., MyHC1, MyHC2a, and MyHC2b, with quantitative RT-PCR before and after incubation of C2C12 myotube with TSH.
Results: TSH-R was expressed in the mouse skeletal muscle cell line. However, treatment with TSH had little effect on the differentiation of muscle cells, although the expression of the muscle differention marker myogenin was significantly increased after TSH treatment. Treatment of TSH did not affect the expression of muscle type-specific genes.
Conclusion: TSH-R is expressed in a mouse skeletal muscle cell line, but the role of TSH receptor signaling in skeletal muscle needs further investigation.

Keywords: Receptors, thyrotropin; Muscle, skeletal; Muscle differentiation

INTRODUCTION

Thyrotropin or thyroid stimulating hormone (TSH) regulates thyroid hormone biosynthesis and secretion in thyroid follicular cells through TSH receptor (TSH-R) [1]. TSH-R is a G-protein-coupled receptor with seven membrane spanning segments [2].

Hyperthyroid or hypothyroid states affect multiple body systems, including muscle function and mass, and it has been thought that those changes were from alternations of serum thyroid hormone levels. However, serum TSH levels are also decreased or increased, respectively, in these states; thus, it might be possible that TSH-R has a role in muscle tissues, independent of serum thyroid hormone. Recently, extrathyroidal expression and the role of TSH-R, independent of thyroid hormonal status, have been the subject of intense research. It has been reported that TSH-R is expressed in tissues like lymphocytes, pituitary gland, thymus, testes, kidney, adipose tissue, and osteoblasts [3-5]. TSH-R in preadipocytes is thought to induce lipolysis and thermogenesis [6,7]. TSH-R in osteoblasts or osteoclast precursors is known to be involved in skeletal remodeling, bone formation, and bone resorption [8].

TSH-R has also been reported to be expressed in extraocular muscles and cardiomyocytes, although contradictory results exist [9,10]. Clinically, muscle function is affected by altered

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thyroid function. Hyperthyroid patients often experience skeletal muscle wasting and weakness, while hypothyroid patients suffer from myalgia, muscle weakness, and exercise intolerance. Therefore, it is necessary to explore whether TSH-R is present in skeletal muscle and is involved in the pathogenesis of muscle disorders.

In the present study, we demonstrate the expression of TSH-R mRNA in the C2C12 mouse muscle cell line by the nested real-time polymerase chain reaction (RT-PCR) method and direct sequencing and investigate whether TSH affects myogenic differentiation and fiber type determination through quantitation of muscle differentiation markers and myosin heavy chain (MHC) isoforms before and after incubation of C2C12 myotubes with TSH.

METHODS

Materials

Triiodothyronine (T3, thyroid hormone, 3,5,3'-triiodo-L-thyronine) and bovine TSH were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture

C2C12 myoblasts were maintained at 37ºC in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum in an atmosphere of 10% CO_2. At 80% confluence, differentiation was induced by treatment with DMEM supplemented with 2% horse serum at 37ºC in 10% CO_2, and cells were maintained in this medium postdifferentiation. Fresh media was changed every other day. Myotubes were used for experiments 5 days after differentiation.

Mice

C57BL/6J mice were used. The mice were housed in groups of four or five in plastic microisolator cages at 22ºC with a 12-hour light/12-hour dark cycle and fed a laboratory chow diet (Purina irradiated laboratory chow 38057, Purina Korea, Seoul, Korea) and water *ad libitum*. Animals were sacrificed after fasting for 6 hours from 6:00 AM. Mice were anesthetized by intraperitoneal injection of zoletil (Virvac, Carros, France). The thyroid, skeletal muscle (gastrocnemius), heart, hypothalamus, and small intestine were quickly removed, frozen in liquid nitrogen and used for RNA extraction. All procedures followed the guidelines of the Seoul National University Bundang Hospital Animal Policy and Welfare Committee.

RNA extraction

Total RNAs of C2C12 myotubes, myoblasts, and mouse hypothalamus were isolated with Trizol (Invitrogen, Carlsbad, CA, USA) and purified with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. cDNA was generated with reverse transcription, which was performed with 1 μg of total RNA and random primers by SuperScript II reverse transcriptase (Invitrogen).

RT-PCR, nested RT-PCR, and sequencing

RT-PCR was conducted using a 40-cycle, two step PCR in an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master Mix (ABI) and 200 nM primers. Reactions were performed in duplicate. Primer sequences for RT-PCR are available upon request.

TSH-R cDNA was amplified by nested PCR using primers designed according to the NCBI reference sequence NC 000078. The first round of amplification was done with a forward primer (peptide position 41 to 47) spanning exon 1 to exon 6, 5′-CAAGGAGCTCCACCGAATCC-3′, and a reverse primer (peptide position 168 to 173), 5′-GCATAGGCCCTGGAATGCGT-3′. PCR was performed in 25 μL of reaction mixture using 1 μL of the cDNA and Platinum Taq DNA polymerase (Invitrogen). The PCR was performed as follows: incubation at 95ºC for 5 minutes, followed by 46 amplification cycles of 95ºC for 40 seconds, 58ºC for 40 seconds, and 72ºC for 40 seconds, and a final extension at 72ºC for 10 minutes. The second round of PCR was performed with 2 μL of the first-round PCR product, nested primer sets, and the Platinum Taq DNA polymerase. The nested primer set for TSH-R spanned exons 3 to 6 with the forward primer (peptide position 89 to 95), 5′-GCAGCGGCTGGAACCACATT-3′ and the reverse primer (peptide position 171 to 177), 5′-GGAATGCGTTTTCAGGGACC-3′. This PCR, which produced one possible product (215 bp), was performed as follows: incubation at 95ºC for 5 minutes, followed by 30 amplification cycles of 95ºC for 40 seconds, 58ºC for 40 seconds, and 72ºC for 40 seconds, and a final extension at 72ºC for 10 minutes. The amplified PCR products were recovered from the agarose gel using a gel extraction kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and directly sequenced with Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems).

Statistical analysis

The means were compared by the nonparametric Wilcoxon rank-sum test, and data with P values less than 0.05 were de-

noted as statistically significant. All analyses were performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The expression of TSH-R in C2C12 skeletal muscle cells

TSH-R expression was analyzed with RT-PCR in thyroid and

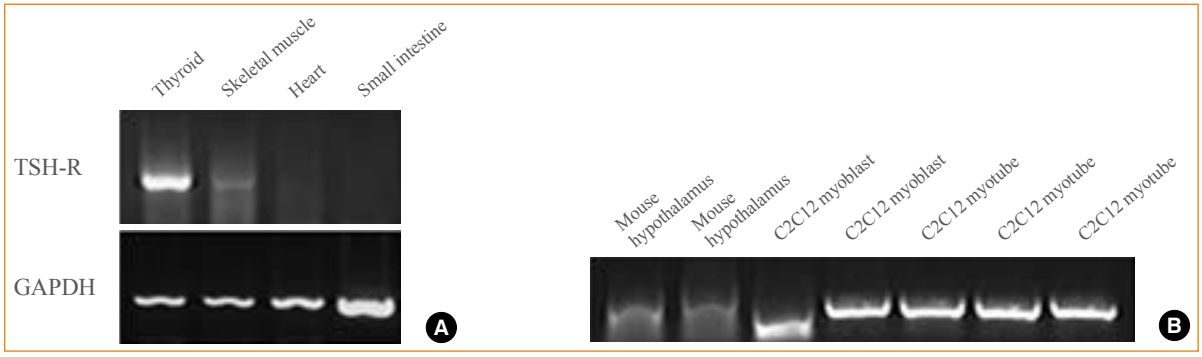

Fig. 1. (A) Expression of thyroid stimulating hormone receptor (TSH-R) in mouse thyroid (positive control), skeletal muscle (gastrocnemius), heart, and small intestine (negative control) using real-time polymerase chain reaction (RT-PCR) detected with shorter primer sets used in nested RT-PCR (see Methods). TSH-R is expressed in skeletal muscle (gastrocnemius). (B) Expression of TSH-R in mouse hypothalamus (positive control) and C2C12 myoblast and myotube, detected with nested RT-PCR. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

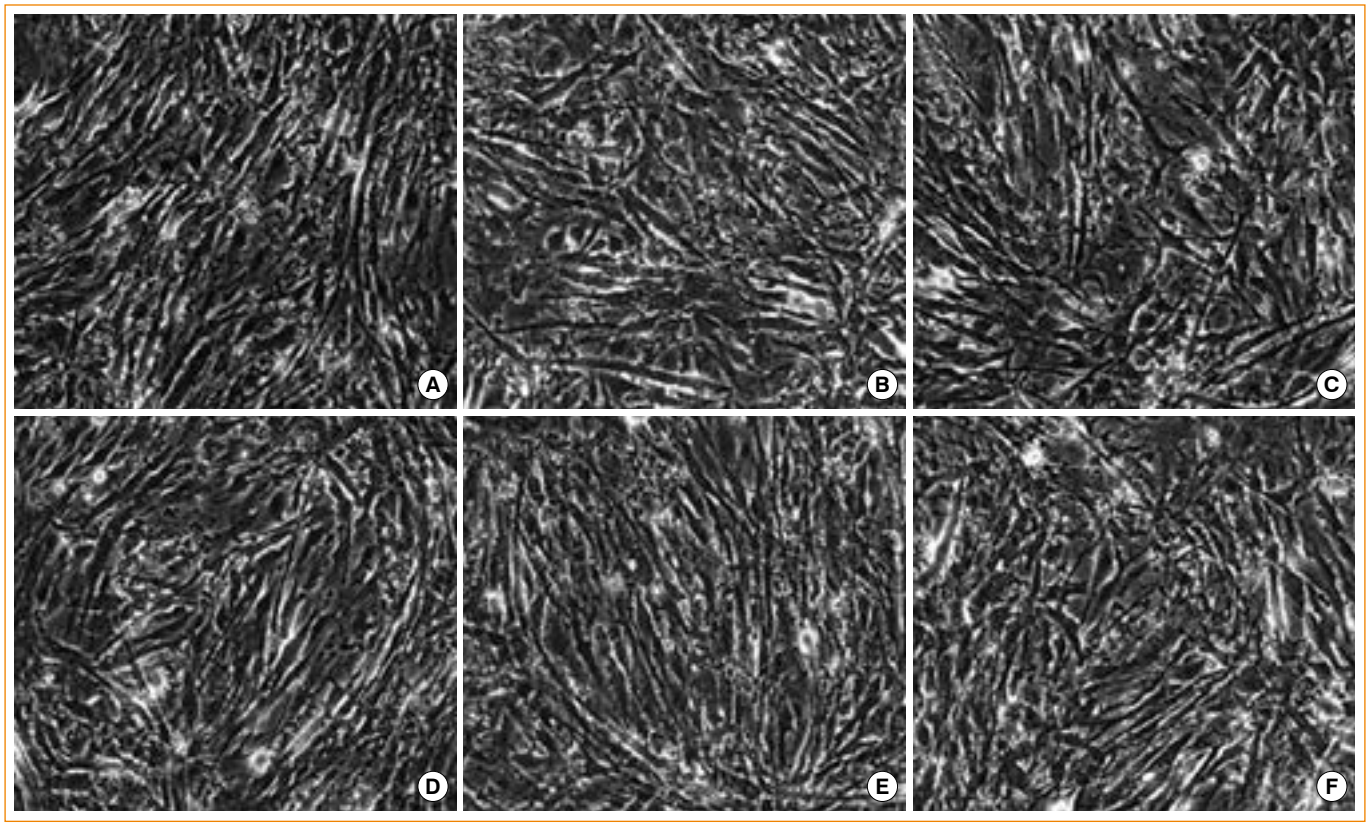

Fig. 2. Light microscopic images of C2C12 myotube after induction of differentiation with 2% horse serum without thyroid stimulating hormone (TSH) (A) or with TSH at concentrations of (B) 0.001, (C) 0.01, (D) 0.1, (E) 1, and (F) 10 mU/mL (×200).

hypothalamus tissues as positive controls and in the intestine as a negative control (Fig. 1A). The expression of TSH-R in thyroid tissue was very strong, while that in the heart and small intestine was almost negligible. However, expression was observed in skeletal muscle, although it was very weak. We also verified the expression of TSH-R in the mouse muscle cell line, C2C12. To minimize false-positive results of RT-PCR and to exclude nonspecific binding of primers, nested RT-PCR was performed using two sets of primers specific for the TSH-R cDNA (see Methods). Primers were designed to span exons to exclude nonspecific genomic amplification. This method showed amplification of PCR fragments of the expected size in C2C12 myoblasts and myotubes (Fig. 1B). PCR fragments were extracted from the agarose gel and sequenced to confirm the expected PCR product of TSH-R. TSH-R was expressed as abundantly in C2C12 myoblasts and myotubes as in the positive control from the hypothalamic tissue of C57BL/6J mice (Fig. 1B).

The effects of TSH on the differentiation of muscle cells

After induction of C2C12 myotube differentiation with 2% horse serum, we incubated C2C12 myotubes with or without TSH at different concentrations (0.001, 0.01, 0.1, 1, and 10 mU/mL) and observed the cells daily for 5 days. However, under light microscopic examination, we could not find any significant differences in myotube formation at various concentrations at once daily time points for 5 days (Fig. 2).

The expression of master regulators of muscle differentiation like myogenin and myoD was determined by RT-PCR before and after treatment with TSH (10 mU/mL) and T3 at a

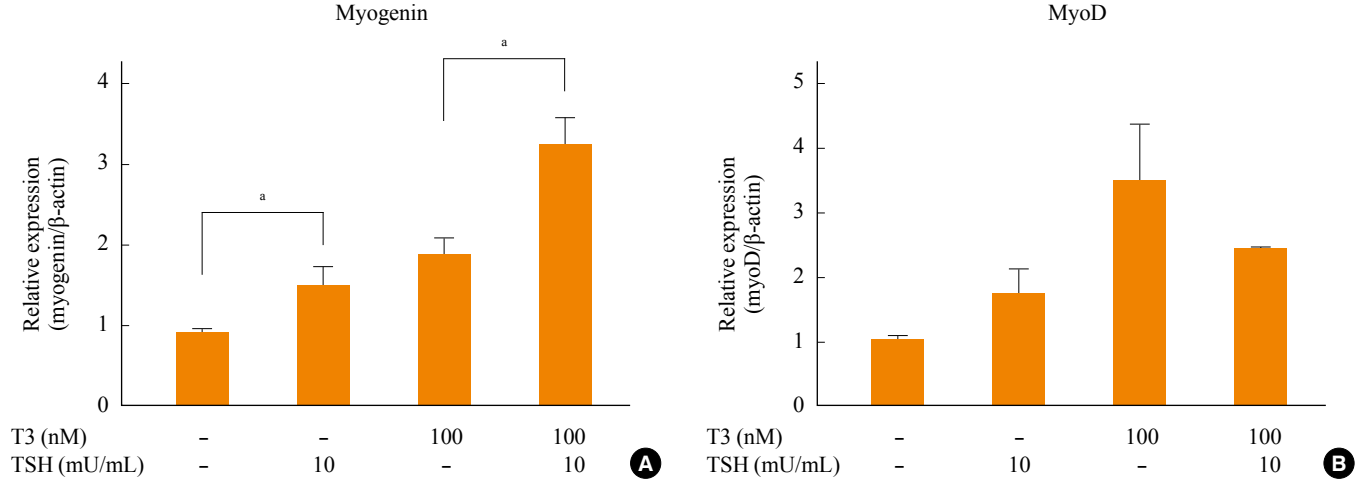

Fig. 3. Expression of muscle differentiation markers, (A) myogenin and (B) myoD, in C2C12 myotube before and after treatment with thyroid stimulating hormone (TSH) and T3 for 24 hours at postdifferentiation day 5. The results are from three independent experiments. TSH and T3 significantly increase the expression of myogenin. [a]$P<0.05$.

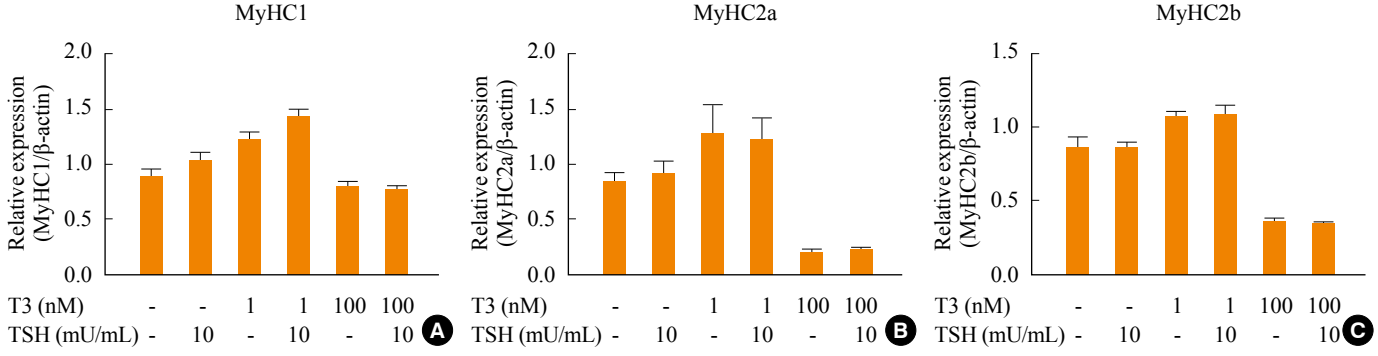

Fig. 4. Expression of myosin heavy chain isoforms, (A) MyHC1, (B) MyHC2a, and (C) MyHC2b, in C2C12 myotube before and after treatment with thyroid stimulating hormone (TSH) and T3 for 24 hours at post-differentiation day 5. The result is from three independent experiments.

concentration of 100 μM for 24 hours at postdifferentiation day 5 (Fig. 3). At this physiologic condition, TSH and T3 concentrations are around 0.4 to 4 mU/mL and 1.0 to 2.0 nM, respectively. Since horse serum, which is essential for myogenic differentiation, contains physiologic concentrations of TSH and T3, we incubated with higher than physiologic doses of TSH and T3 at the respective concentrations of 10 mU/mL and 100 nM. Incubation with TSH increased the expression of myogenin and myoD by 50% (Fig. 3). As previously reported, T3 significantly induced the expression of myogenin and myoD by 2- to 3-fold [9]. TSH further significantly increased the expression of myogenin induced by T3 (Fig. 3).

The effects of TSH on the myofiber type determination

We further investigated the expression of myofiber-type specific genes (MyHC1, MyHC2a, and MyHC2b) with RT-PCR before and after treatment with TSH (10 mU/mL) and T3 at concentrations of 1 and 100 nM respectively for 24 hours at postdifferentiation day 5. TSH at 10 mU/mL did not induce or repress the expression of MyHC1, MyHC2a, or MyHC2b (Fig. 4). While 1 nM of T3 marginally (nonsignificantly) induced the expression of all MHC isoforms, 100 nM of T3 reduced the expression of MyHC2a and MyHC2b but not MyHC1. This pattern of T3 effect on MHC isoforms was not modified by TSH cotreatment.

DISCUSSION

Hyperthyroid patients frequently suffer from muscle wasting and weakness. In these patients TSH is suppressed and thyroid hormones are increased, which led us to investigate the role of TSH in skeletal muscle tissue. In the present study, we demonstrate the expression of TSH-R in C2C12 myoblast and myotube with nested RT-PCR and sequencing. TSH treatment did not affect muscle differentiation, but the expression of genes related to muscle differentiation, myogenin, and myoD, were slightly increased after TSH treatments. The expression of fiber type determination-related genes in C2C12 myotube did not change after TSH treatment.

We provide the first evidence that TSH-R is expressed in a skeletal muscle cell line. As for cardiomyocytes, contradictory results exist regarding the presence of TSH-R in cardiomyocytes from tissue specimens [10,11], which is thought to result from fibroblast-like cells between myocytes that express TSH-R [10]. Since the cell line and muscle tissues used in our study could have also been contaminated by fibroblasts, we excluded the possibility of contamination by cells other than myocytes that express TSH-R. Unlike a study of Busuttil and Frauman [10] that failed to detect mRNA in mouse abdominal skeletal muscle tissue, we applied the nested RT-PCR technique, which is more sensitive and specific than conventional RT-PCR.

The inherent problem with PCR is its sensitivity, allowing the amplification of target mRNA present at very low levels. However, TSH-R was expressed in a similar amount compared to the expression level in mouse hypothalamic tissue, where TSH-R is reported to be present [12]. Also, we confirmed that the PCR bands were the true TSH-R using a direct sequencing method after DNA extraction from the expected bands. Of course, the presence of a transcript product does not necessarily mean functional protein, and we need to further confirm the presence of TSH-R protein in C2C12 myotube.

In the present study, we failed to show the functional significance of TSH-R signaling in skeletal muscle cell differentiation by morphologic analysis. However, the finding that TSH mildly elevated the expression of myogenin at 10 mU/mL suggests that TSH could contribute to the myogenic differentiation process. At 100 nM of T3, which is 50- to 100-fold higher than the physiologic concentration of T3, myogenin was significantly elevated by 2- to 3-fold, and this increase in myogenin expression is further enhanced by TSH. However, we could only conclude that overall TSH does not have a major role in myogenic differentiation, although the possibility of some minor role still remains.

As muscular weakness in a thyrotoxicosis state could result from muscle fiber-type switching [13], and suppressed TSH could play a role in this process, we investigated muscle fiber type-related gene expression at high T3 concentrations. T3 treatment at 1 nM concentration increased the expression of all MHC isoforms, and 100 nM T3 repressed the expression of MyHC2a and MyHC2b. However, incubation with TSH alone or in combination with T3 did not significantly affect the pattern of the T3 effect on muscle fiber type-related genes. We conclude that TSH receptor signaling does not affect the muscle type determination process.

From the above results, we could not identify a significant functional role of TSH-R in mouse skeletal muscle cells, and further studies are necessary to reveal the biological significance of TSH-R expression in these cells. However, there have been reports on several important functional roles of TSH-R in other types of muscle cells, including extraocular muscle cells and cardiomyocytes. Since Graves ophthalmopathy presents about the same time as Graves disease, the two diseases have

been proposed to share a common autoimmune pathogenesis, and the presence of a common antigen has been suggested. Stringent PCR of extraocular muscle tissue from normal subjects revealed the expression of TSH-R in extraocular muscles, and TSH-R is hypothesized to function as a shared antigen with the thyroid and contribute to the occurrence of Graves ophthalmopathy [10]. Although contradictory data exists as to the expression of TSH-R in cardiomyocytes, a case of a 25-year-old man with Graves disease who suffered from severe cardiomyopathy reported expression of TSH-R in a myocardial biopsy [14]. This also suggests the existence of a common antigen between the thyroid and heart, contributing to the occurrence of cardiomyopathy in Graves disease. These hypotheses on the pathogenesis of extrathyroidal manifestations in Graves disease propose that skeletal muscle may also be the subject of autoimmune attack, given the skeletal muscle weakness and atrophy observed in patients with Graves disease. Future studies should focus on the autoimmune destruction of skeletal muscle in Graves disease.

In conclusion, we confirmed the expression of TSH-R in a mouse skeletal muscle cell line using nested RT-PCR. Overall, TSH-R seems to have little effect on skeletal muscle differentiation or fiber type determination, warranting further study to investigate its functional significance in skeletal muscle.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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