Expression of basigin in reproductive tissues of estrogen receptor-α or β null mice

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Abstract

Basigin plays important roles in both male and female reproduction because basigin (Bsg) null male and female mice are infertile. The aim of the present study was to determine whether basigin expression in reproductive organs requires estrogen receptor-α (ESR1, ERα) or -β (ESR2, ERβ). Expression of basigin protein in the testis, ovary, and male and female reproductive tracts was studied in adult wild-type (WT), Esr1-null (αERKO), and Esr2-null (βERKO) mice by immunohistochemistry and immunoblotting. Basigin mRNA levels in ovary and uterus were examined by quantitative RT-PCR. In females, basigin protein expression was observed mainly in granulosa and interstitial cells of the ovary and epithelial cells of the proximal oviduct in all genotypes. Basigin protein was also expressed in the uterine epithelium at proestrus and estrus in WT and βERKO mice but not in αERKO mice. However, a higher level of basigin mRNA was observed in uteri of αERKO mice compared with WT and βERKO mice. In males, basigin was expressed in Leydig cells and all germ cells except spermatogonia in all genotypes. Basigin was present in epithelial cells lining the efferent ductules in WT and βERKO mice, but expression was greatly reduced in αERKO mice. In epididymal ducts, basigin expression was observed in epithelial cells in the caput and cauda in all genotypes. These data suggest that expression of basigin protein requires ESR1, but not ESR2, in the uterus and efferent ductules, but is independent of estrogen receptor in the ovary, oviduct, testis, and epididymis.


Introduction


Basigin is abundant in the reproductive organs, including the testis, ovary, and uterus (Igakura et al. 1998, Kuno et al. 1998, Chen et al. 2001, Noguchi et al. 2003, Chang et al. 2004), and plays essential roles in both male and female reproduction. Targeted disruption of the Bsg gene in mice results in the failure of spermatogenesis and defects in fertilization and implantation (Igakura et al. 1998, Kuno et al. 1998). The steroid hormone, estradiol, acting via the nuclear receptors, estrogen receptor-α (ESR1, ERα) and -β (ESR2, ERβ), is fundamental to the regulation of numerous genes in reproductive tissues. Basigin expression in the mouse uterine epithelium is reportedly upregulated by estrogen (Xiao et al. 2002), but the role of ESR1 and ESR2 in the regulation of basigin expression remains unknown.
The goal of the present study was to compare the expression of basigin in the reproductive tissues of wild-type (WT), $Esr1$-null ($\alpha$ERKO), and $Esr2$-null ($\beta$ERKO) mice to determine the contribution of each estrogen receptor isoform to basigin expression. The results showed that ESR1 not ESR2 was required for the proper expression of basigin protein in the uterus and efferent ductules, while basigin protein expression in the ovary, oviduct, testis, and epididymis was estrogen receptor-independent.

Results

Antibody specificity

Basigin immunoreactivity was identified on the apical and bilateral surface of uterine epithelial cells in endometrium of WT mice (Fig. 1A), consistent with a previous report (Xiao et al. 2002). We also observed basigin immunoreactivity on the surface of erythrocytes in the blood vessels and myometrium in the uteri (Fig. 1A). Furthermore, no basigin immunoreactivity was observed among uterine sections from the $Bsg$-null mice (Fig. 1B), confirming the specificity of the antibody for mouse basigin.

Basigin protein expression in the reproductive tissues of WT, $\alpha$ERKO, and $\beta$ERKO females

Basigin immunoreactivity in WT ovaries was intense on the surface of granulosa cells and interstitial cells but weak or absent in theca cells and corpus luteum cells (Fig. 2A–C and Table 1). In the corpora lutea of WT ovaries, strong basigin immunoreactivity was identified on the surface of erythrocytes in the blood vessels, not in corpus luteum cells (Fig. 2C). Basigin immunoreactivity was also detected on the surface of cumulus oophorus cells surrounding the ovulated oocytes that were retained in the oviduct of WT mice at estrus (Fig. 3A). Similarly, strong immunoreactivity for basigin was observed in granulosa and interstitial cells in $\alpha$ERKO and $\beta$ERKO ovaries (Fig. 2D and E; Table 1). As expected, ovaries from $\alpha$ERKO mice exhibited large, hemorrhagic, and cystic follicles as well as lacked corpora lutea (Couse & Korach 1999), but no aberrant basigin expression was associated with these pathologies.

Strong basigin immunoreactivity was observed on the basolateral surface of ciliated and non-ciliated epithelial cells of the proximal oviducts from WT mice (Fig. 3A and Table 1). This expression pattern was not...
affected by the stage of estrous cycle (data not shown) or the loss of either estrogen receptor isoform (Fig. 3B and C; Table 1). On the other hand, basigin immunoreactivity clearly fluctuated throughout the estrous cycle in the uteri of WT females. Basigin immunoreactivity was high on the apical and lateral surfaces of the luminal and glandular epithelia at proestrus and estrus (Fig. 4A and B; Table 1), while that in endometrium was weak or absent at metestrus (Fig. 4C) and diestrus (Fig. 4D). Fibroblasts in the uterine stroma were consistently negative for basigin, while basigin immunoreactivity was seen on the surface of erythrocytes and myometrium in the uteri at all stages of estrous cycle (Fig. 4A–D). The expression pattern of basigin in uteri of βERKO mice was comparable to that in WT mice (Fig. 4E and Table 1). In the αERKO uteri, basigin was undetectable in the luminal and glandular epithelia (Fig. 4E, G and H; Table 1), although it was still detectable in the erythrocytes and myometrium (Fig. 4E, G and H).

### Table 1

Summary of basigin expression in ovary, testis, and reproductive tracts of wild-type (WT), αERKO, and βERKO mice. Three or more mice of each different genotype were used for immunohistochemistry analysis. Three slides per tissue and at least five fields within a slide were evaluated by two observers.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Cells</th>
<th>WT</th>
<th>αERKO</th>
<th>βERKO</th>
</tr>
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<tbody>
<tr>
<td>Ovary</td>
<td>Granulosa cells</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Interstitial cells</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Theca cells</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>Corpus luteum</td>
<td>−</td>
<td>NA</td>
<td>−</td>
</tr>
<tr>
<td>Oviduct</td>
<td>Epithelial cells</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Uterus</td>
<td>Epithelial cells</td>
<td>++ (proest, est)</td>
<td>−</td>
<td>++ (proest, est)</td>
</tr>
<tr>
<td></td>
<td>Stroma cells</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Testis</td>
<td>Sertoli cells</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>Leydig cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Spermatogonia</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>Spermatocytes</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Spermatid</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Sperm</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Efferent ductule</td>
<td>Ciliated epithelial cells</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>Non-ciliated epithelial cells</td>
<td>+ +</td>
<td>+ +</td>
<td></td>
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<tr>
<td>Epididymis</td>
<td>Initial segment</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>Caput</td>
<td>+</td>
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<td></td>
<td>Corpus</td>
<td>±</td>
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<tr>
<td></td>
<td>Cauda</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+++, strongly positive; +, positive; ±, weakly positive; −, negative; proest, proestrus; est, estrus; NA, not applicable.

**Figure 3** Basigin immunoreactivity in oviducts of WT (A), αERKO (B), and βERKO (C) mice. Sections were stained with goat anti-mouse basigin antibody (A–C) or goat non-specific IgG (D). Basigin expression is evident on the surface of ciliated and non-ciliated epithelial cells of WT, αERKO, and βERKO mice. Basigin expression is observed on the surface of cumulus oophorus cells (A, arrow) surrounding the ovulated oocyte (A, arrow head) that were retained in the oviduct of WT mice at estrus. Scale bar = 50 μm for A–C and 100 μm for D.
Basigin protein expression in the reproductive tissues of WT, αERKO, and βERKO males

Basigin immunoreactivity in WT testes was seen on the cell surface of Leydig cells, spermatocytes, spermatids, and sperm tails, while undetectable in spermatogonia (Fig. 5A and A′; Table 1). Weak immunoreactivity was detected on the surface of Sertoli cells (Fig. 5A and A′; Table 1). Basigin immunoreactivity in αERKO and βERKO testes was comparable to that in WT males (Fig. 5B and C; Table 1), despite the flattened epithelia and diluted lumen that are characteristic abnormalities of the testes of αERKO mice.

The efferent ducts of WT and βERKO mice exhibited strong basigin immunoreactivity on the basolateral surface of ciliated and non-ciliated epithelial cells (Fig. 6A and C; Table 1), while this immunoreactivity was reduced or absent in the efferent ductules of αERKO males, all of which were dilated and lined with cuboidal epithelia (Fig. 6B and Table 1).

Basigin expression varied along the epididymal duct in WT mice. Moderate basigin immunoreactivity was localized on the lateral surface of epithelial cells in the caput (Fig. 7B) and apical surface in the cauda (Fig. 7D), while weak immunoreactivity was seen on lateral surfaces of epithelial cells in the initial segment (Fig. 7A) and apical surface in the corpus (Fig. 7C). Basigin expression in the αERKO and βERKO epididymis was comparable to that in WT males (Table 1).

Basigin protein and mRNA levels in uteri of WT, αERKO, and βERKO mice

Our immunostaining clearly shows the absence of basigin protein expression in the uterine luminal and glandular epithelia of αERKO mice (Fig. 4E and G). We further quantified basigin protein levels in αERKO uteri by immunoblotting. Basigin protein levels of αERKO uteri were greatly reduced to around 40% of those of WT uteri (Fig. 8A). These data underestimate the down-regulation of basigin protein in uterine epithelium of αERKO mice, because basigin expression by erythrocytes and myometrium (data not shown) in the WT uteri at all stages of estrous cycle. In the αERKO uterus, basigin is not observed in the luminal or glandular epithelium, whereas basigin immunoreactivity is detectable in erythrocytes (arrows in G) and myometrium (arrow heads in H).

The estrogen–ESR1 complex directly regulates the transcription of some genes including lactoferrin and progesterone receptor. Although our immunohistochemistry and immunoblotting results suggest that ESR1 is required for proper basigin protein expression in uteri, especially in uterine epithelium, it is not clear whether the signaling mediated by ESR1 regulates basigin mRNA levels in uteri. To answer this question, we examined basigin mRNA levels in uteri of αERKO and βERKO mice. Since lactoferrin transcript is regulated by the action of ESR1, we used lactoferrin as a control gene. As expected, lactoferrin mRNA levels in the uteri of αERKO mice were only 20% of those of WT mice (Fig. 8B). In contrast to the absence of basigin protein expression in uterine epithelium of αERKO mice, basigin mRNA levels in uteri of αERKO mice were threefold increased.
compared with those of WT mice (Fig. 8B). As expected, basigin mRNA levels in uteri of βERKO mice and ovaries of αERKO and βERKO mice were comparable to those of WT mice (Fig. 8B).

Discussion
Basigin is a glycosylated transmembrane protein that is expressed in multiple reproductive organs and plays critical roles in male and female fertility, as evidenced by the infertility in Bsg-null mice (Igakura et al. 1998, Kuno et al. 1998). The present study was conducted to determine whether basigin expression in male and female reproductive organs requires the actions of ESR1 or ESR2. By comparing the level and pattern of basigin expression in the reproductive tissues of WT, αERKO, and βERKO mice, we identified the uterine epithelium in females and the efferent ductule epithelium in males as tissues in which basigin expression requires functional ESR1, while expression in the gonads, oviduct, and epididymis is estrogen receptor-independent.

Our finding of markedly high basigin levels in ovarian granulosa cells, interstitial cells, and ovarian surface epithelial cells but the absence of expression in luteal cells is consistent with the report of Kuno et al. (1998). In contrast, basigin mRNA and protein were detectable in both new corpora lutea and corpora lutea from previous cycles in the rat ovary (Smedts & Curry 2005), suggesting that basigin expression in corpus lutea is species-specific. Still, the ovaries of Bsg-null mutant mice exhibit normal folliculogenesis and corpus lutea (Kuno et al. 1998), as well as normal progesterone levels on day 8 of pseudopregnancy (L Chen, unpublished observations). However, the reduced rate of fertilization in vivo among Bsg-null oocytes (Kuno et al. 1998) suggests a function of basigin in granulosa cells that is critical to oocyte maturation and function. Our demonstration that basigin expression in mouse ovaries was not altered by the absence of either ESR1 or ESR2 indicates that estrogen signaling is not required for basigin expression in the ovary. Instead, basigin expression in granulosa cells may depend on epidermal growth factor or amphiregulin (Yoshino et al. 2006), both of which are produced by granulosa cells and have been shown to induce basigin expression in NS2T2A1 human breast tumor cells (Menashi et al. 2003).

Basigin is reportedly overexpressed in all malignant ovarian tumors including serous adenocarcinoma, mucinous adenocarcinoma, yolk sac tumor, clear cell carcinoma, and granulose cell tumors (Jin et al. 2006). The level of basigin expressed by ovarian cancer cells is positively related with cancer cell invasive ability (Millimaggi et al. 2007). Several ovarian epithelial tumor cell lines have been demonstrated to release basigin protein by microvesicle shedding. Microvesicles shed from ovarian carcinoma cells stimulate proangiogenic activities of human umbilical vein endothelial cells in a basigin-dependent manner, indicating the significant role of basigin in ovarian tumor invasion and metastasis (Millimaggi et al. 2007).

Basigin expression in the uterine endometrium was localized to the luminal and glandular epithelia and clearly fluctuated during the cycle such that the expression was markedly high at proestrus and estrus.
Further evidence of estrogen regulation of basigin protein expression in the uterine epithelium, and the importance of ESR1 in mediating this effect, was the total lack of basigin protein in the uterine epithelium of Esr1-null females. Paradoxically, we observed a significant increase, instead of a decrease, in basigin mRNA levels in the uteri of Esr1-null females. These apparently contradictory observations suggest that the signaling mediated by ESR1 must be necessary for basigin protein translation, instead of mRNA transcription. Knockout of Esr1 results in a lower basigin protein expression in the uteri, which may lead to a compensatory response with increases in basigin mRNA levels.

MicroRNAs (miRNAs), a group of ~22 nucleotide-long non-coding RNAs, powerfully negatively regulate gene expression by targeting mRNAs at the post-transcriptional level. miRNAs inhibit the translation of or degrade their target mRNAs by binding to the 3′-UTR of target mRNAs (Ambros 2004). Each miRNA targets ~200 transcripts directly or indirectly (Zhang et al. 2006), and ~30% of human genes are potential targets of miRNA (Yu et al. 2006). Interestingly, some miRNAs are regulated by steroid hormones. For example, estrogen downregulates miR-21 expression, which leads to increased expression of miR-21-target genes at the protein levels (Wickramasinghe et al. 2009). It is possible that some miRNAs that are downregulated by estrogen suppress basigin mRNA translation in uteri. Blocking estrogen signaling by knocking out Esr1 could cause an increase in some miRNAs, which in turn inhibits translation of basigin mRNA.

In contrast, and despite the substantial level of ESR1 expression in the oviduct epithelium, basigin expression in the oviduct was not affected by the loss of functional ESR1 nor did it overtly fluctuate during the estrous cycle in WT females. These data illustrate very nicely the differential regulation of basigin expression between epithelial cell types of the uterus and oviduct in mice.

In males, the intense basigin expression observed in Leydig cells and all stages of germ cells except spermatogonia is in agreement with previous reports (Igakura et al. 1998, Maekawa et al. 1998). Not surprisingly, Bsg-null mice exhibit a complete arrest of spermatogenesis at the metaphase of the first meiosis (Igakura et al. 1998). ESR1 and ESR2 are expressed in various somatic and germ cell types of the testis (Zhou et al. 2002). However, deletion of either estrogen receptor isoform does not affect basigin expression in the testis, as expression was not altered in aERKO or bERKO mice.

The epithelial cells of the efferent ductules are reported to have a markedly high concentration of ESR1 (Schleicher et al. 1984, Cooke et al. 1991, Zhou et al. 2002). However, deletion of either estrogen receptor isoform does not affect basigin expression in the testis, as expression was not altered in aERKO or bERKO mice.

The epithelial cells of the efferent ductules are reported to have a markedly high concentration of ESR1 (Schleicher et al. 1984, Cooke et al. 1991, Zhou et al. 2002). Furthermore, there is convincing evidence that ESR1-mediated estrogen actions are required to maintain the fluid resorption of the efferent ducts (Hess et al. 1997). Herein, we found that basigin expression is localized to the basolateral surface of efferent ductule epithelial cells, similar to the reported site of the Na⁺,K⁺-ATPase that is thought to be important in fluid absorption (Lee et al. 2001). Still, Bsg-null mice possess efferent ducts that exhibit normal morphology and no overt functional deficits (Nakai et al. 2006). Bsg-null mice do, however, exhibit impaired expression of monocarboxylate transporter 1 (MCT1; Nakai et al. 2006), a transmembrane protein responsible for the transport of monocarboxylates across the plasma membr

Figure 6 Basigin immunoreactivity in efferent ductules of WT (A), aERKO (B), and bERKO (C) mice. Sections were stained with goat anti-mouse basigin antibody (A–C) or goat non-specific IgG (D). Intense basigin immunoreactivity is observed in the ciliated and non-ciliated epithelial cells of WT (A) and bERKO (C) mice. Basigin immunoreactivity is greatly diminished or absent in epithelial cells of aERKO efferent ductules (B, arrow). Scale bar = 50 μm for A–C and 200 μm for D.
membrane. Deletion of \( \text{Esr}1 \) may lead to impaired MCT1 expression as well as \( \text{Na}^+,\text{K}^+\)-ATPase, which may in turn contribute to the invariable phenotype of poor fluid resorption in \( \text{Esr}1 \)-null males.

In summary, we have employed estrogen receptor-null mice to demonstrate that basigin expression is estrogen- and ESR1-dependent in some reproductive tissues (e.g. uterine and efferent ductile epithelia), but not in all reproductive tissues (e.g. gonads, oviduct, and epididymis) in both sexes. Future studies are required to identify the factors that regulate basigin expression in those reproductive tissues in which estrogen receptor-mediated estrogen actions appear to have little influence.

**Materials and Methods**

**Animals**

All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were pre-approved by the NIEHS IACUC. The generation of mice null for the \( \text{Esr}1 \) (ER\( \alpha \)) or \( \text{Esr}2 \) (ER\( \beta \)) genes was in a C57BL6/J background and has been described previously (Lubahn et al. 1993, Krege et al. 1998). Estrous cycle phase for WT and \( \text{Esr}2 \)-null females was determined by vaginal smear over a minimum period of 1 week. Three or more mice for each genotype at different estrous cycles were used for this study.

**Tissue collection**

All mice were killed by carbon dioxide asphyxiation. For immunohistochemistry uteri, ovaries and oviducts were fixed in 10% formalin, whereas testes and efferent ductules were fixed in Bouin’s solution overnight. All tissues were then processed for paraffin embedding. For immunoblotting and quantitative RT-PCR analyses, uteri and ovaries were snap-frozen with nitrogen and kept at \(-80^\circ \text{C} \) for future use.

**Immunohistochemistry**

Tissues were sectioned at 5 \( \mu \text{m} \) and mounted on poly-l-lysine-coated slides. Sections were boiled in 10 mM citrate buffer (pH 6.0) for 10 min to promote antigen retrieval. Endogenous peroxidase activity was blocked in methanol containing 0.3% hydrogen peroxide for 15 min. After blocking with 5% normal rabbit serum in PBS for 20 min, sections were incubated with 2 \( \mu \text{g/ml} \) goat polyclonal antibody against mouse basigin (R&D, Minneapolis, MN, USA) at 4 \( ^\circ \text{C} \) overnight. Normal goat IgG (2 \( \mu \text{g/ml} \)) was applied as a negative control. After washing in PBS, sections were incubated with biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA, USA) diluted at 1:100 with PBS for 1 h at room temperature. Sections were then incubated in ABC solution (Vector Labs) for 45 min, reacted with metal-3,3’-diaminobenzidine (Sigma) for 3 min, and counterstained with hematoxylin.

**Immunoblotting**

Frozen whole uteri isolated from WT and \( \alpha \text{ERKO} \) mice were ground in liquid nitrogen and solubilized in modified RIPA buffer in the presence of protease inhibitors. Protein concentrations were measured by BCA protein assay (Pierce, Rockford, IL, USA). Twenty micrograms of total proteins were resolved by 10% SDS-PAGE. The gels were transferred to nitrocellulose membranes, blocked in 5% non-fat dry milk, and probed with 0.2 \( \mu \text{g/ml} \) goat polyclonal antibody against mouse basigin (R&D) for overnight at 4 \( ^\circ \text{C} \). The membranes were washed and incubated with the HRP-conjugated donkey anti-goat IgG.
antibody (Santa Cruz Company, Santa Cruz, CA, USA) at 1:3000 dilution for 45 min at room temperature. The bound secondary antibody was detected using chemiluminescent reagents (Pierce). The same membranes were stripped and reprobed with anti-GAPDH antibody (Cell Signaling, Boston, MA, USA) as a loading control.

Densitometric analysis of immunoblotting
Films developed after exposure to chemiluminescence were scanned, and images were analyzed using the ImageJ software from NIH (available at http://rsbweb.nih.gov/ij/download.html). Measurements of basigin bands were normalized for loading differences using measurements of GAPDH. Graphs represent means and S.E.M.

RNA isolation and quantitative RT-PCR
Total RNA was extracted from mouse uteri and ovaries using TRIzol according to the manufacturer's instructions. Two micrograms of total RNA were reverse transcribed using the RETROscript First Strand cDNA Synthesis Kit, and quantitative RT-PCR analysis was performed using TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, Atlanta, GA, USA). Primer and probe set for basigin were designed using Primer Express software (Perkin-Elmer, Waltham, MA, USA). For basigin cDNA amplification, 200 nM forward primer (5'-TGGACTCCTGTTGATCCT-3'), 200 nM reverse primer (5'-ACACCTGAGATGCGGAAGT-3'), and 200 nM probe (5'-ATCCCTTGGCTGAGGTGAGC-3') were used. Lactoferrin was amplified using the 20X Assays-on-Demand Gene Expression Assays purchased from Applied Biosystems. Relative mRNA levels were calculated using the relative standard curve method. Standard curves were generated from dilution series constructed from a 'reference sample', which was cDNA from a WT mouse uterus or ovary. Quantitative PCR was performed on both experimental samples and reference standards. Relative values for basigin, lactoferrin, and ribosomal 18S abundance in all samples were extrapolated from the standard curves from the reference standards. Relative mRNA levels were normalized to 18S. To show changes in gene expression relative to control levels (WT mouse tissue, set as 1), each of the samples was normalized to the average of the control groups.

Statistical analysis
Results are presented as the mean ± S.E.M. of at least three samples. Statistical analyses were performed by ANOVA. Values of $P<0.05$ were considered significant.
Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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