Life Sciences 228 (2019) 112–120

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Trichostatin A inhibits uterine histomorphology alterations induced by cigarette smoke exposure in mice

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ARTICLE INFO

Keywords:
Cigarette smoking
Uterus
Macro-autophagy
TSA

ABSTRACT

Aims: Cigarette smoking results in well-known negative reproductive consequences. However, the role of histone deacetylase 1 and 2 (HDAC1/2) in the structural changes of uterine tissues induced by cigarette smoke (CS) exposure and the therapeutic potential of trichostatin A (TSA), a HDAC inhibitor, have not been investigated.
Main methods: Female mice were exposed to CS twice daily for 30 days and TSA was injected intraperitoneally into CS-exposed mice on alternate days in the TSA-treated group. Uteri in the estrus phase were weighed and uterine histomorphology and HDAC1 cell distribution were examined by HE and immunohistochemistry. Markers associated with macro-autophagy (Beclin-1), autophagic flux (increased LC3-II and a lack of p62 accumulation), autophagy inhibiting factor (mTOR, phosphorylated mTOR and its upstream IRS, phosphorylated IRS), HDAC1/2, FOXO1 and FOXO3 were assessed by Western blot.
Key findings: CS exposure decreased body weight and triggered uterine histomorphologic alterations, including a thinner myometrium and a reduced number of glandular and interstitial cells. HDAC1/2 were activated in uterine tissues after CS exposure and TSA effectively inhibited HDAC1/2 activation and attenuated the loss of body weight and uterine wet weight induced by CS exposure. TSA effectively restored the thickness of the myometrium and number of glandular and interstitial cells. TSA also restored the expression of markers of macro-autophagy (LC3-II and Beclin-1) and reduced phosphorylated mTOR, phosphorylated IRS, FOXO1 and FOXO3 activation.
Significance: TSA inhibited uterine histomorphologic alterations induced by CS exposure. The TSA effect might be associated with resumption of macro-autophagy via HDAC1/2 inhibition.

1. Introduction

Smoking in women has nocuous effects on some stages of the reproductive process, including folliculogenesis, embryo transport, endometrial angiogenesis and uterine blood flow [1–3]. According to the National Bureau of Statistics of China, there are 380 million women 15–49 y of age and an estimated 100 million pregnant women in China in 2010 [4]. Of note, 65.1% of non-smoking women of childbearing age (15–49 y) are exposed to secondhand tobacco smoke at home and 52.6% are exposed in the workplace [5]. Cigarette smoking is associated with lower fecundity rates, adverse reproductive outcomes and a higher risk of in vitro fertilization (IVF) failure [6,7]. Autophagy appears to play an important role in the normal development and maintenance of homeostasis in the female reproductive tract [8]. Exposure to cigarette smoke (CS) activates the Atg pathway, which ultimately leads to ovarian follicle loss. Previous studies have suggested that exposure to CS induces dysfunction of mitochondrial repair mechanisms and leads to autophagy-mediated follicle death [9,10]. The effect of smoking on ovarian function can partly explain the effect of smoking on female reproductive ability. Furthermore, the uterus is an important hormone-responsive reproductive organ in mammals. Histologically, the uterus is composed of three major cell types (epithelial, stromal, and myometrial cells). CS contains several thousand components, such as nicotine, polycyclic aromatic hydrocarbons and cadmium. Each endometrial cell type (epithelial and stromal cells, as well as endothelial and smooth muscle cells) might be targets for CS

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https://doi.org/10.1016/j.lfs.2019.04.069
Received 20 February 2019; Received in revised form 28 April 2019; Accepted 30 April 2019
Available online 01 May 2019
0024-3205/

components. Endometrial thickness is significantly lower in patients who smoke cigarettes than non-smoking patients, which may help to further explain the negative effect of CS on implantation and pregnancy rates during assisted reproduction [11]. Oral nicotine results in height variation in the surface and glandular epithelium in rats [12] and CS exposure adversely affects reproductive organs of diabetic female rats. In the uterus, the expression of matrix metalloproteinase (MMP)-9, C-X-C chemokine receptor type 4 (CXCR4) and estrogen receptor-alpha (ER-α) were increased by CS exposure in the uterus and ovary [13]. However, macro-autophagy underlying the structural changes in uterine tissues induced by CS exposure has not been established.

The expression of histone deacetylases (HDACs) are related to the change in autophagy level. HDACs are required for stress-induced cardiomyocyte autophagy, especially the class I HDACs (HDAC1/2) and HDAC2 overexpression significantly promotes autophagy [14]. HDAC1/2 regulate skeletal muscle autophagy by mediating the induction of autophagic gene expression and the formation of autophagosomes [15]. HDAC inhibitors, such as trichostatin A (TSA), display inconsistent autophagy activation in different cell types. Both inhibition and genetic knock-down of HDAC1 in Hela cells induces autophagic vacuole formation [16]. HDAC inhibitors attenuate cardiac hypertrophy by suppressing autophagy [14]. Owing to the crucial importance of HDAC activity in physiologic and pathologic processes, HDAC inhibitors have been developed as therapeutic options for a variety of diseases, including cancer, mood disorders and AIDS [17]. Studies involving HDAC 1/2 and TSA in the uterus are limited. Nuclear expression of HDAC1 protein has been detected in all endometrial cell types and HDAC1 mRNA and protein are expressed without cyclic changes in the human endometrium [18]. The levels of gene and protein expression of HDAC1/2 are significantly increased in endometriotic lesions [18,19]. Impaired HDAC1 protein expression has been reported in human endometrial adenocarcinomas and uterine cancer in mice caused by neonatal exposure to diethylstilbestrol [20,21]. TSA induces apoptosis in human endometriotic stromal cells via induction of NAG-1 expression [22]. Treatment with TSA significantly reduces lesion growth and relieves pain symptoms in women with endometriosis and TSA suppresses IL-1beta-induced COX-2 gene and protein expression in endometrial stromal cells, which suggests that HDACs are a promising class of compounds that have therapeutic potential for endometriosis [23–25]. The important roles of HDAC1/2 in autophagy and the structural changes in uterine tissues induced by CS exposure and therapeutic potential of TSA in this process, however, have not been determined. Thus, in this work the structural changes in uterine tissues and macro-autophagic activity induced by CS exposure and therapeutic potential of TSA in this process were studied.

2. Materials and methods

2.1. Ethics

Female C57BL/6 mice (8 weeks old at the start of CS exposure) were obtained from the Animal Center of Shengjing Hospital (China Medical University). All studies were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee of the China Medical University (No. 2013PS06K). All surgical procedures were performed under anesthesia and all efforts were made to minimize animal suffering.

2.2. Antibodies and other reagents

Antibodies and reagents were purchased from the following sources: GAPDH (KangChen Bio-tech, Shanghai, China); LC3B (CST, Danvers, MA, USA); Beclin1 (Abcam, Cambridge, UK); P62 (Sigma-Aldrich, St. Gallen, Switzerland); IRS1 (CST, Danvers, MA, USA); phosphorylated IRS1-Ser636/639 (CST, Danvers, MA, USA); MTOR (CST, Danvers, MA, USA); phosphorylated MTOR-Ser2448 (CST, Danvers, MA, USA); HDAC2 (CST, Danvers, MA, USA); HDAC3 (Wanleibio, Shen Yang, China); HDAC6 (CST, Danvers, MA, USA); FOXO1 (Millipore, MA, USA); FOXO3 (CST, Danvers, MA, USA); caspase-9 (CST, Danvers, MA, USA); TSA (Sigma-Aldrich, St. Louis, MO, USA); RIPA protein lysis buffer (Beyotime, Shanghai, China); PMSF (Millipore, Basel, Switzerland); BCA (Thermo Fisher, Rockford, IL, USA); Roche Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland); SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA); Trizol reagent (Takara Bio Inc., Dalian, China); PrimeScriptTM RT Reagent Kit with gDNA Eraser (Takara Bio Inc., Dalian, China); SYBR Green (Takara Bio Inc., Dalian, China); SuperScript II (Takara Bio Inc., Dalian, China); ABC Elite Reagent (Maxim, Fuzhou, China).

2.3. CS exposure

XiongShi cigarettes (China Tobacco Zhejiang Industrial Co., LTD, Zhejiang, China) with 0.7 mg of nicotine and 8 mg of tar were used in this study. The methods of CS exposure and dose of TSA used in this study were based on previous studies [26–28]. Mice were exposed to CS twice daily for 30 days using a whole body, mainstream smoke exposure system. Briefly, CS from 6 cigarettes was mixed with room air and delivered into the exposure chamber over a 120-min period twice daily. Mice were acclimated to the exposure chamber over a 3-day period before the experiment. During the acclimatization period, mice were exposed to CS from 1 cigarette for 20 min twice daily. Control animals were placed in the restrainer for 120 min twice daily and exposed to room air only. TSA (0.6 mg/kg of body weight on alternate days for 30 days) was injected intraperitoneally into CS-exposed mice in the TSA-treated group. Mice were weighed every day. After CS exposure for 30 days, vaginal exfoliative cells from each mouse were stained and examined to confirm the estrous cycle. Female mice in the estrous phase were euthanized and at least five uteri in each group were collected for further studies.

2.4. Hematoxylin and eosin (HE) staining

Following 30 days of CS exposure, at least 5 uteri were harvested and weighed. One horn of the uterus from each animal was then fixed in 4% paraformaldehyde, embedded with paraffin, and cut into cross-sections (5 μm thick) and stained with HE. One horn of the uterus was quickly frozen in liquid nitrogen for the subsequent detection of protein. Uterine histomorphologic examinations were performed under a Nikon Ni upright microscope using bright field microscopy with 4×, 10×, 20× and 40× objective lenses and photographed using Nis-Elements software.

2.5. Western blot analysis

Total protein was extracted from frozen uteri (n = 4–5/group) using RIPA Protein Lysis Buffer containing Roche Complete Protease Inhibitor Cocktail and 1 mM PMSF. Protein concentrations were determined using the BCA method. Protein extracts were subjected to SDS-PAGE electrophoresis and visualized with SuperSignal West Pico Chemiluminescent Substrate. The primary antibodies used in immunoblots were as follows: GAPDH (1:10000); LC3B (1:1000); Beclin1 (1:500); P62 (1:500); IRS1 (1:1000); phosphorylated IRS1-Ser636/639 (1:1000); MTOR (1:500); phosphorylated MTOR-Ser2448 (1:500); HDAC1 (1:500); HDAC2 (1:1000); HDAC3 (1:500); HDAC6 (1:1000); FOXO1(1:500); FOXO3(1:500); Caspase-9 (1:500).

2.6. Immunohistochemistry

Uteri were dissected, then fixed in 4% paraformaldehyde, embedded with paraffin, and cut into cross-sections (5 μm thick). Sections were deparaffinized in xylene, hydrated and incubated with 3% H_2O_2 in methanol for 30 min at room temperature to block endogenous

peroxidase, then washed twice in PBS (2 × 5 min) and incubated in normal fetal bovine serum for 30 min at room temperature to block non-specific sites. Sections were incubated overnight at 4 °C with the primary antibody against HDAC1 (1:150) or Caspase-9 (1:200), washed 3 times with PBS (3 × 10 min), then transferred to biotinylated IgG anti-rabbit serum in PBS for 90 min at room temperature, washed 3 times with PBS (3 × 5 min), incubated again in ABC Elite Reagent in PBS for 30 min at room temperature, and washed 3 times with PBS (3 × 5 min). Negative controls were obtained by omitting the primary antibody. The sections were viewed under a Nikon Ni upright microscope using bright field microscopy with 4×, 10×, 20× and 40× objective lenses and photographed using Nis-Elements software.

2.7. Quantification of mRNA expression

Total RNA was isolated from uteri (n = 3–4/group) using Trizol reagent and RNA (1 mg) was reverse-transcribed with oligo (dT) primer and SuperScript II. A 1:5 dilution of the complementary DNA (cDNA) mixture served as a template for subsequent PCR. Real-time PCR was performed on a Roche 480 PCR system with SYBR Green. *Gapdh* was used as an endogenous control and the relative levels of gene expression for each sample were calculated using the $2^{-\Delta\Delta ct}$ method. The primer sequences of genes, including *Gapdh*, *Mtor*, *Becn1*, *Foxo1*, *Foxo3,* and *caspase-9* were shown in Table 1.

2.8. Statistical analyses

All graphs represent the mean ± standard error of the mean (SEM). A two-tailed two sample Student's *t*-test of unequal variance was performed using GraphPad software to determine statistical significance.

3. Results

3.1. Uterine histomorphologic alterations

CS exposure decreased body weight and uterine wet weight compared with non-exposed controls (Fig. 1A, B). CS exposure triggered uterine histomorphologic alterations, as shown in Fig. 1B. Myometrium (both the circular and longitudinal muscle layers) and endometrium from mice exposed to CS were thinner than mice exposed to room air. Furthermore, glandular tissue and interstitial cells were reduced after CS exposure (Fig. 1C).

3.2. Exposure to CS resulted in macro-autophagy inhibition and HDAC1/2 activation

Markers associated with macro-autophagy (Beclin-1) and autophagic flux (increased LC3-II and a lack of p62 accumulation) were investigated and the results showed that Beclin-1 and LC3-II were decreased in the uteri after CS exposure. At the same time, p62 accumulation was clearly evident (Fig. 1D). The results indicated that macro-autophagy was inactivated in CS-treated tissues. CS exposure triggered mTOR, the important autophagy inhibiting factor, to increase in the uteri (Fig. 1D). HDAC1, HDAC2, HDAC3 and HDAC6 expression were also detected. The results showed that HDAC1/2 were increased, HDAC3 was decreased, and HDAC6 was not changed in CS-treated uteri (Fig. 1E). The results indicated that HDAC1/2 might participate in the uterine histomorphologic alterations induced by CS exposure.

Table 1
Primers for genes used in Q-PCR.

Gene	Forward	Reverse	Length
Gapdh	ATGTTTGTGATGGGTGTGAA	ATGCCAAAGTTGTCATGGAT	122 bp
Mtor	TGTGCCGAGACCTTGAG	ATTGCTGCCCATCAGAGT	140 bp
Becn1	CTGTAGCCAGCCTCTGAAA	CCTCTTCCTCCTGGGTCT	125 bp
Foxo1	GTGGATGGTGAAGAGCGT	AACTTGCTGTGAAGGGACA	105 bp
Foxo3	AAATGGGCAAAGCAGACC	CTGTTCCACGGGTAAGGG	189 bp
Casp9	CAGATGCTGTCCCCTATCA	GGAGCCACTTTTCTTGTCC	143 bp

3.3. TSA inhibited uterine histomorphologic alterations and HDAC1/2 activation

HDACs came into focus as a therapeutic target and the effects of TSA (a HDAC inhibitor) on uterine histomorphologic alterations induced by CS exposure have not been demonstrated. HDAC1 up-regulation was shown in CS-treated tissues. However, the effect of TSA inhibition on HDAC1 expression in specific endometrial cells was not clear. Our results showed that nuclear HDAC1 protein was activated in smooth muscle, glandular endothelial, and luminal endothelial cells after CS exposure (Fig. 2). In addition, TSA effectively inhibited HDAC1 activation induced by CS exposure in the above-mentioned cells (Fig. 2). Furthermore, our results showed that TSA attenuated the loss of body weight and uterine wet weight induced by CS exposure (Fig. 3A, B). TSA also effectively restored the thickness of the myometrium and the number of glandular and interstitial cells (Fig. 3C). TSA also inhibited the expression of HDAC1/2 and aggravated the suppression of HDAC3 caused by CS exposed, but had no significant effect on HDAC6 activity (Fig. 3D).

3.4. TSA reactivated macro-autophagy and inhibited mTOR activation

TSA effectively restored markers of macro-autophagy (Beclin-1) and autophagic flux (increased LC3-II and a lack of p62 accumulation; Fig. 4A). These results indicated that TSA inhibited uterine histomorphologic alterations induced by CS exposure and the effect was associated with reactivation of macro-autophagy. CS exposure triggered mTOR, an important autophagy inhibiting factor, and increased mTOR in the uterus (Fig. 1D). TSA reactivated macro-autophagy. However, the association between this effect and mTOR activation was not clearly illustrated. Our results showed that TSA limited phosphorylated mTOR and IRS activation after CS exposure (Fig. 4B). The results indicated that TSA inhibited uterine histomorphologic alterations induced by CS exposure and the effect was associated with inhibited mTOR activation and reactivation of macro-autophagy. Furthermore, TSA can inhibit the activation of FOXO1 and FOXO3 in the uterus induced by CS exposure (Fig. 4C). Caspase-9 activation was found in stromal cells and TSA inhibited its activation (Fig. 4C, D), which suggested that FOXO signaling and apoptosis might also play important roles in inhibiting uterine morphologic changes induced by CS exposure to TSA.

3.5. TSA inhibited the transcriptional activity of Mtor and Foxo1

The effects of smoking and TSA exposure on the transcriptional activities of *Mtor, Becn1, Foxo1, Foxo3,* and *Casp9* were investigated by real-time quantitative RT-PCR. The results showed that the level of *Mtor*, *Foxo1,* and *Casp9* mRNA were increased and *Becn1* was decreased in the uteri after CS exposure and TSA significantly inhibited the transcriptional activity of *Foxo1* and *Mtor*. Treatment with smoking and TSA had no effect on the transcriptional activity of *Foxo3* (Fig. 5).

4. Discussion

Cigarette smoking results in well-known negative reproductive consequences [29]. However, the molecular mechanism underlying the structural changes in uterine tissues induced by CS exposure has not been established. The uterus is an important hormone-responsive reproductive organ in mammals and all cell types might be targets for CS components. In this study structural changes in the uterus induced by CS exposure were investigated and the molecular mechanism underlying this process was explored. Whole body exposure of mice to CS closely mimicking human exposure was used in this study, as described

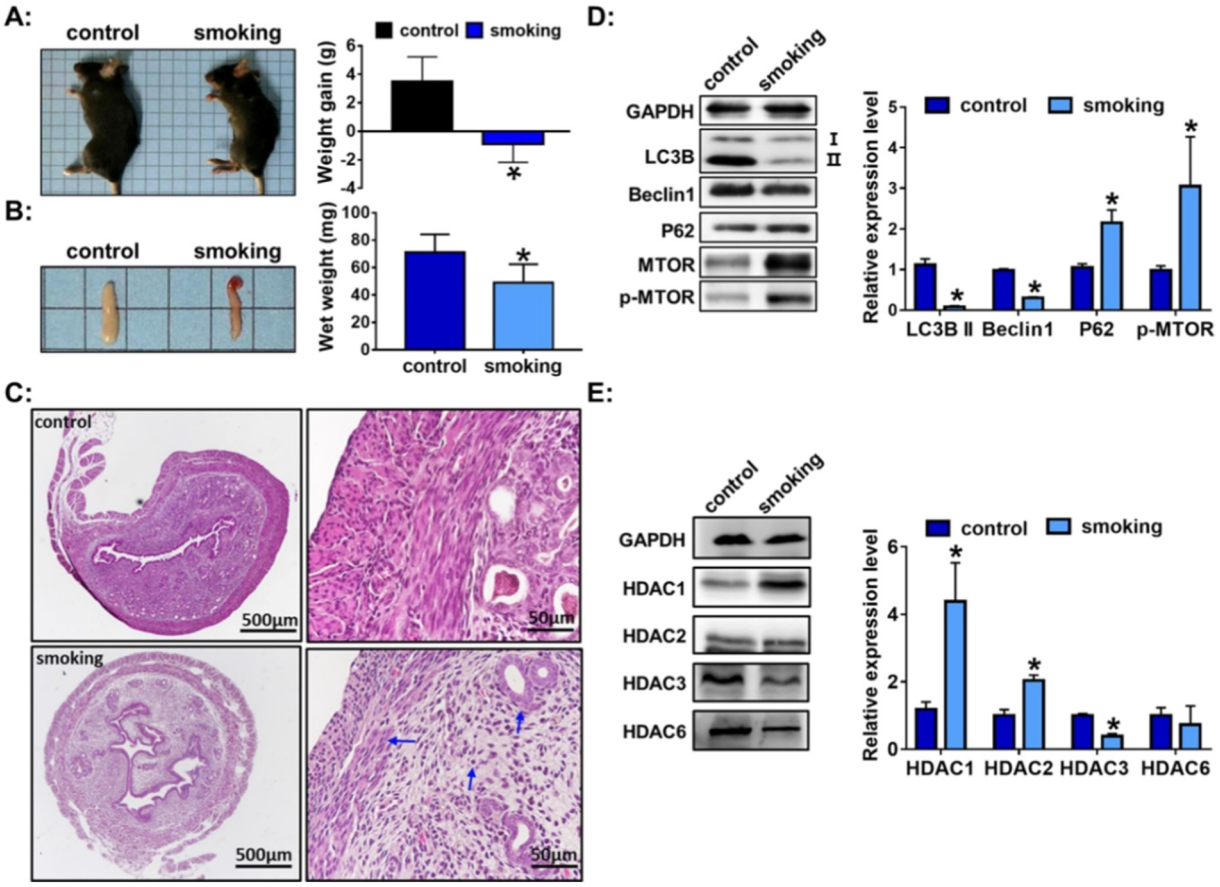

Fig. 1. Exposure to cigarette smoke triggered uterine histomorphologic alterations and resulted in macro-autophagy inhibition and HDAC1/2 activation
A: CS exposure decreased body weight compared with controls. B: CS exposure decreased the uterine wet weight compared with controls. C: Myometrium (both circular and longitudinal muscle layers) and endometrium from mice exposed to CS were thinner than mice exposed to room air. Furthermore, glandular tissue and interstitial cells were reduced after CS exposure, while the blue arrow points to thinner myometrium and reduced glandular tissue and interstitial cells. D: Western blot analysis of markers for macro-autophagy in controls and CS-treated uteri. Statistical analysis indicated that the LC3-II and Beclin1 (macro-autophagy markers) were significantly decreased in CS-treated uteri. There was also evidence of p62 accumulation, which indicated that macro-autophagy was depressed in CS-treated uteri. Furthermore, mTOR, an important autophagy inhibiting factor, was increased in CS-treated uteri. E: Western blot analysis of HDACs, including HDAC1, HDAC2, HDAC3, and HDAC6, in controls and CS-treated uteri. Statistical analysis indicated that HDAC1/2 increased, HDAC3 was decreased, and HDAC6 was not changed in CS-treated tissues. Data are shown as the mean ± SEM. $^{*}P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in a previous study [30]. Our results showed that CS exposure twice daily for 30 days triggered a loss in uterine wet weight and uterine histomorphologic alterations. Myometrium and endometrium from mice exposed to CS were thinner than mice exposed to room air. Furthermore, glandular tissue and interstitial cells were reduced after CS exposure. Card and Mitchell [31] reported lower uterine weight after nicotine administration. Our uterine weight results were consistent with the Card and Mitchell study [31]. We did not observe height variation in surface and glandular epithelium in rats induced by oral nicotine [12]. The myometrium was thinner after CS exposure and this alteration in murine uteri was similar to the uteri of humans who smoked cigarettes [32]. Moreover, after CS exposure the contractile sensitivity of the myometrium increased in response to oxytocin by up-regulating the expression of oxytocin receptor mRNA, which might increase the risk of premature delivery in smokers [33–35]. Khorram et al. [36] reported a significant dose-dependent (aqueous cigarette smoke solution) decrease in human endometrial epithelial cell proliferation in nitric oxide (NO)-mediated pathways. Thus, we reasoned that a decrease in proliferation might partially cause a reduction in the number of glands and interstitial cells after CS exposure.

Autophagy is a major cellular catabolic pathway that is tightly associated with cell survival. Park et al. [37] reported that autophagy is increased in BEAS-2B cells, a normal human bronchial epithelial cell line, which exposure to whole CS condensate. Autophagy suppression and phosphorylation of mTOR activation are associated with hormone effects in the uterus during pregnancy [38,39]. Moreover, mTOR signaling is involved in endometriosis, adenomyosis, leiomyoma, decidualization, and hormone effects in the uterus [38,43]. However, the role of mTOR in CS-treated uteri has not been defined. Our results showed that mTOR was activated in CS-treated tissues. The results indicated that macro-autophagy suppression mediated by activation of mTOR phosphorylation played a vital role in uterine histomorphologic alterations induced by CS exposure. Furthermore, our results showed that total HDAC1/2 protein was up-regulated in uterine tissues and nuclear HDAC1 protein was activated in smooth muscle, glandular endothelial, and luminal endothelial cells after CS exposure. HDAC1 is expressed in three major cell types (epithelial, stromal, and myometrial cells) [10]. In undifferentiated endometrial sarcoma, the HDAC1/2/4/6/7/8 series exhibited strong immunoreactivity and can be considered potential therapeutic targets [44]. Sakai et al. [45] showed that HDAC inhibitors enhance the progesterone-induced decidualization of human endometrial fibroblasts cultured in vitro. In mice, HDAC inhibitors, like TSA, enhance estradiol (E2)-induced proliferation as well as estrogen (ER) and progesterone receptor (PR) expression of uterine cell populations [46]. Our results showed that HDAC1/2 were activated in CS-treated tissues, therefore HDAC1/2 came into focus as a therapeutic

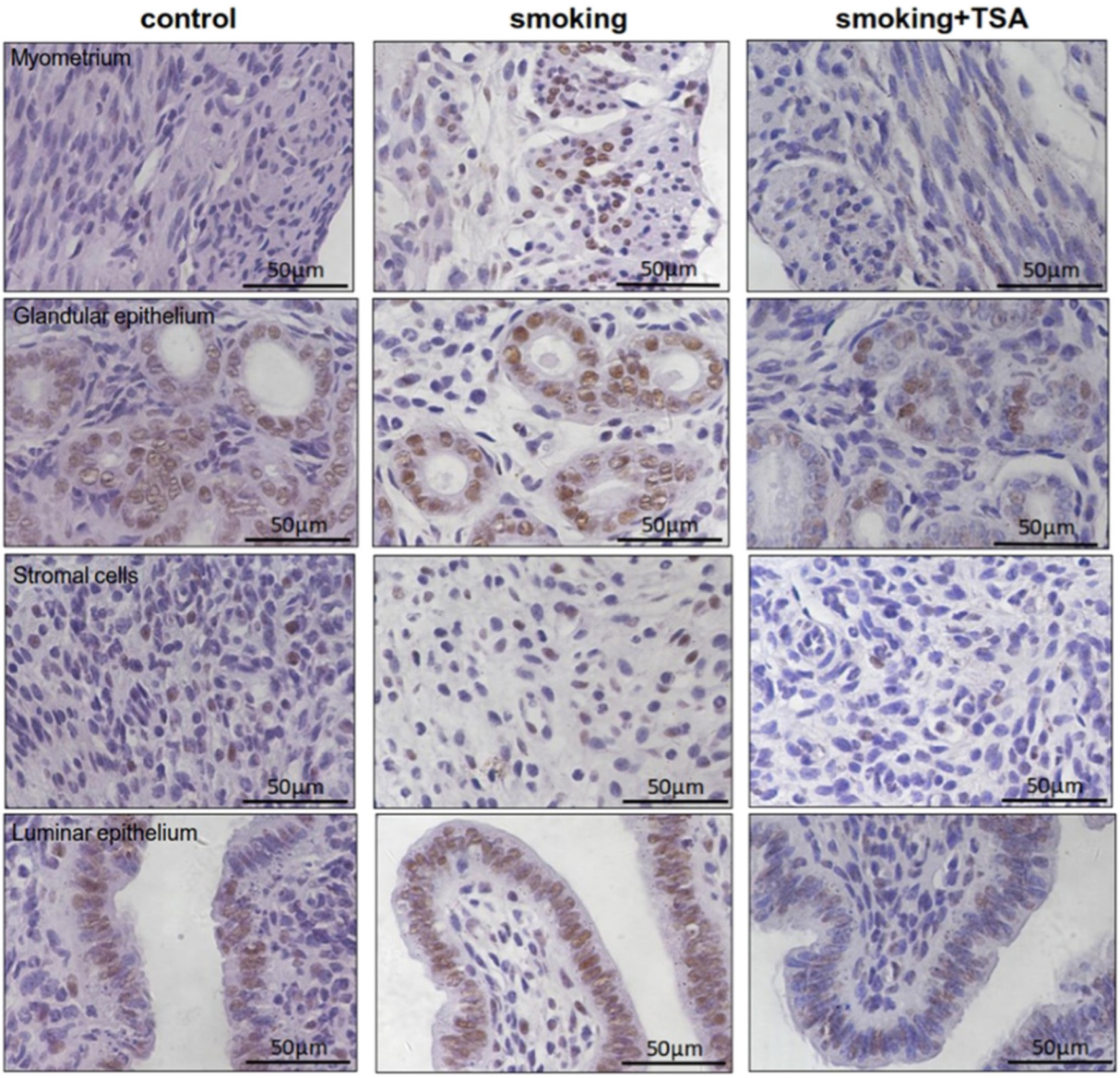

Fig. 2. Nuclear HDAC1 protein distribution in the uteri.
Nuclear HDAC1 protein was expressed in endometrial cell types and smooth muscle cells in control uterus. After CS exposure nuclear HDAC1 was activated in smooth muscle, glandular endothelial, and luminal endothelial cells and TSA could effectively inhibit HDAC1 activation in the above-mentioned cells.

target and the effects of TSA, a HDAC inhibitor, on uterine histomorphologic alterations induced by CS exposure and mTOR signaling in this process were further investigated.

TSA effectively inhibited HDAC1/2 activation and attenuated the loss of body weight and uterine wet weight induced by CS exposure. TSA effectively restored myometrium thickness and the number of glandular and interstitial cells. TSA also restored the expression of markers of macro-autophagy (Beclin-1) and autophagic flux (increased LC3-II and a lack of p62 accumulation). Furthermore, our results showed TSA limited phosphorylated mTOR activation and IRS after CS exposure. The PI3K/mTOR signaling pathway is activated by E2 in uterine myocytes, suggesting that this activation is responsible for the induction of myometrial hyperplasia during early gestation [38]. The PI3K/Akt/p-mTOR signaling pathway is also involved in endometrial cell proliferation, migration, and angiogenesis, which provided an underlying theoretical target for endometriosis and adenomyosis [40]. Activation of mTOR is followed by loss of negative feedback to IRS-1 during the initial stages of development of endometrial hyperplasia and carcinoma [41]. Activated mTOR signaling is associated with leiomyoma etiology [42]. In the rat uterus, it has been shown that phosphorylation of mTOR is increased under the influence of E2 and uterine autophagy is suppressed by E2 and PR treatment in mice [38,39]. TSA availably limited phosphorylated mTOR activation and IRS after CS exposure, which indicated that TSA inhibited uterine histomorphologic alterations induced by CS exposure. The TSA effect might be associated with resumption of macro-autophagy via HDAC1/2 inhibition.

Furthermore, TSA inhibited the activation of FOXO1 and FOXO3 in uteri induced by CS exposure. FOXO1 and FOXO3 are transcriptional regulators of decidualization in human endometrial stromal cells [47,48]. Aberrant expression or perturbed activity of FOXO transcription factors have been increasingly linked to prevalent reproductive disorders, such as endometriosis, endometrial cancer, primary ovarian insufficiency, and pregnancy failure [49]. Our results indicated that abnormal decidualization of endometrium may be involved in decreased fertility caused by CS exposure. Caspase-9 activation was demonstrated in stromal cells and TSA inhibits caspase-9 activation, which suggested that apoptosis in stromal cells might also play a role in

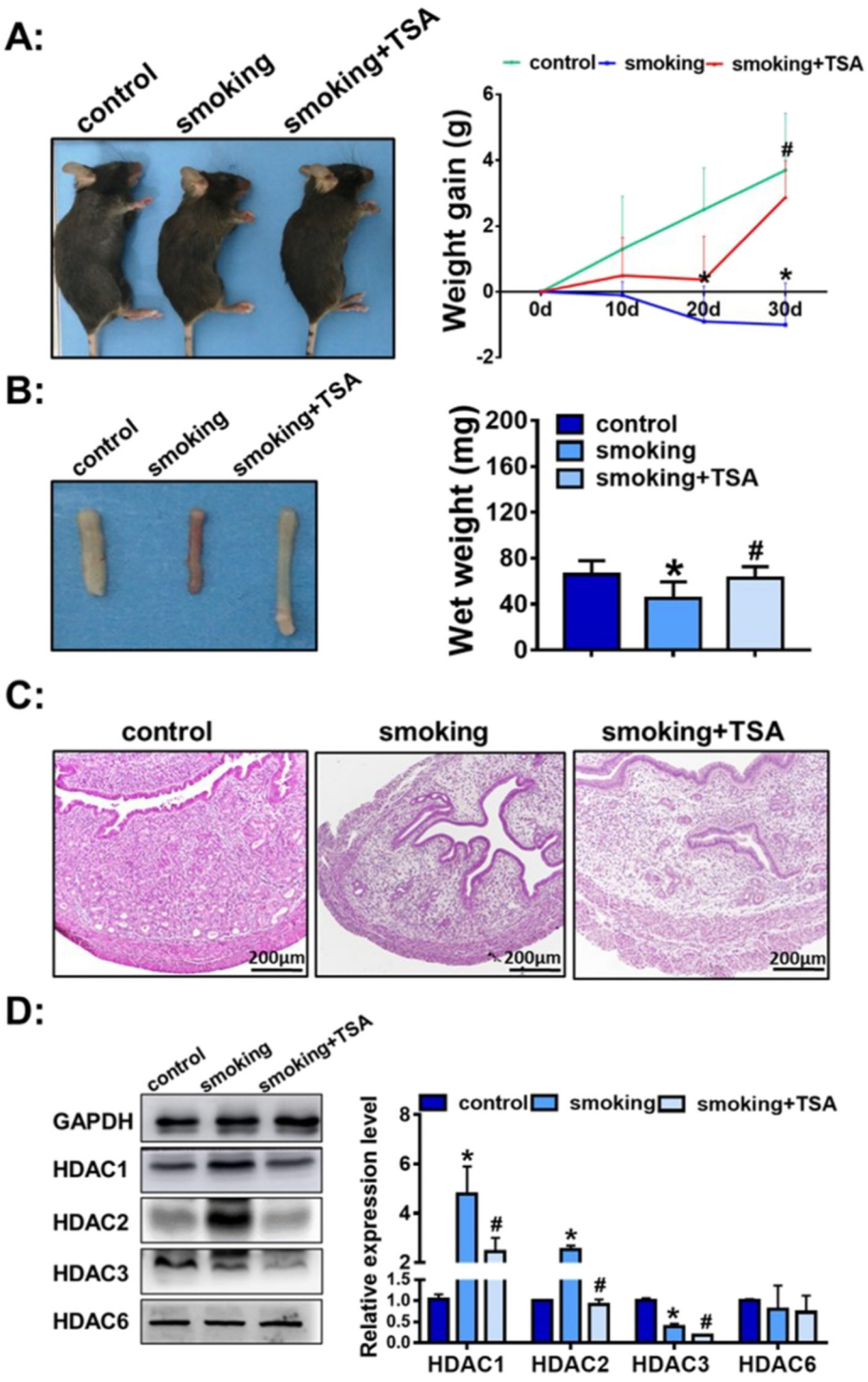

Fig. 3. TSA inhibited uterine histomorphologic alterations and HDAC1/2 activation.
A: TSA attenuated the loss of body weight induced by CS exposure. Data are shown as the mean ± SEM. $^{*}P < 0.05$ (control vs. smoking), $^{\#}P < 0.05$ (smoking vs. smoking + TSA). B: TSA attenuated the loss of wet weight of the uterus induced by CS exposure. Data are shown as the mean ± SEM. $^{*}P < 0.05$ (control vs. smoking), $^{\#}P < 0.05$ (smoking vs. smoking + TSA). C: TSA effectively restored the thickness of the myometrium and number of glandular and interstitial cells. D: TSA inhibited the expression of HDAC1/2 and aggravated the suppression of HDAC3 caused by CS exposed, but had no significant effect on HDAC6 activity. Data are shown as the mean ± SEM. $^{*}P < 0.05$ (control vs. smoking), $^{\#}P < 0.05$ (smoking vs. smoking + TSA).

inhibiting uterine morphologic changes induced by CS exposure by TSA.

Histone modification, including deacetylation of lysine residues by HDACs, is a key mechanism of gene expression regulation [50]. In this work, we also investigated the transcriptional activities of genes, including *Mtor*, *Becn1*, *Foxo1*, *Foxo3,* and *Casp9,* after smoking and TSA exposure. Our results showed TSA inhibited the transcriptional activity of *Mtor* and *Foxo1* induced by CS exposure, which were consistent with the results of Western blot. However, our results did not demonstrate whether the transcriptional activity of *Mtor* and *Foxo1* induced by CS exposure were directly caused by HDAC1/2.

5. Conclusion

This study demonstrated structural changes and HDAC1/2 protein activation in the uterus after CS exposure. TSA might inhibit uterine histomorphologic alterations induced by CS exposure by reactivating macro-autophagy via inhibition of HDAC1/2 and mTOR activation. The results provided a better understanding of CS compounds on the reproductive system and help reduce the prevalence of cigarette smoking. Further studies are necessary to explore whether TSA directly affects macro-autophagy by regulating HDAC1/2 in specific cells, such as smooth muscle, endothelial, or interstitial cells after CS exposure.

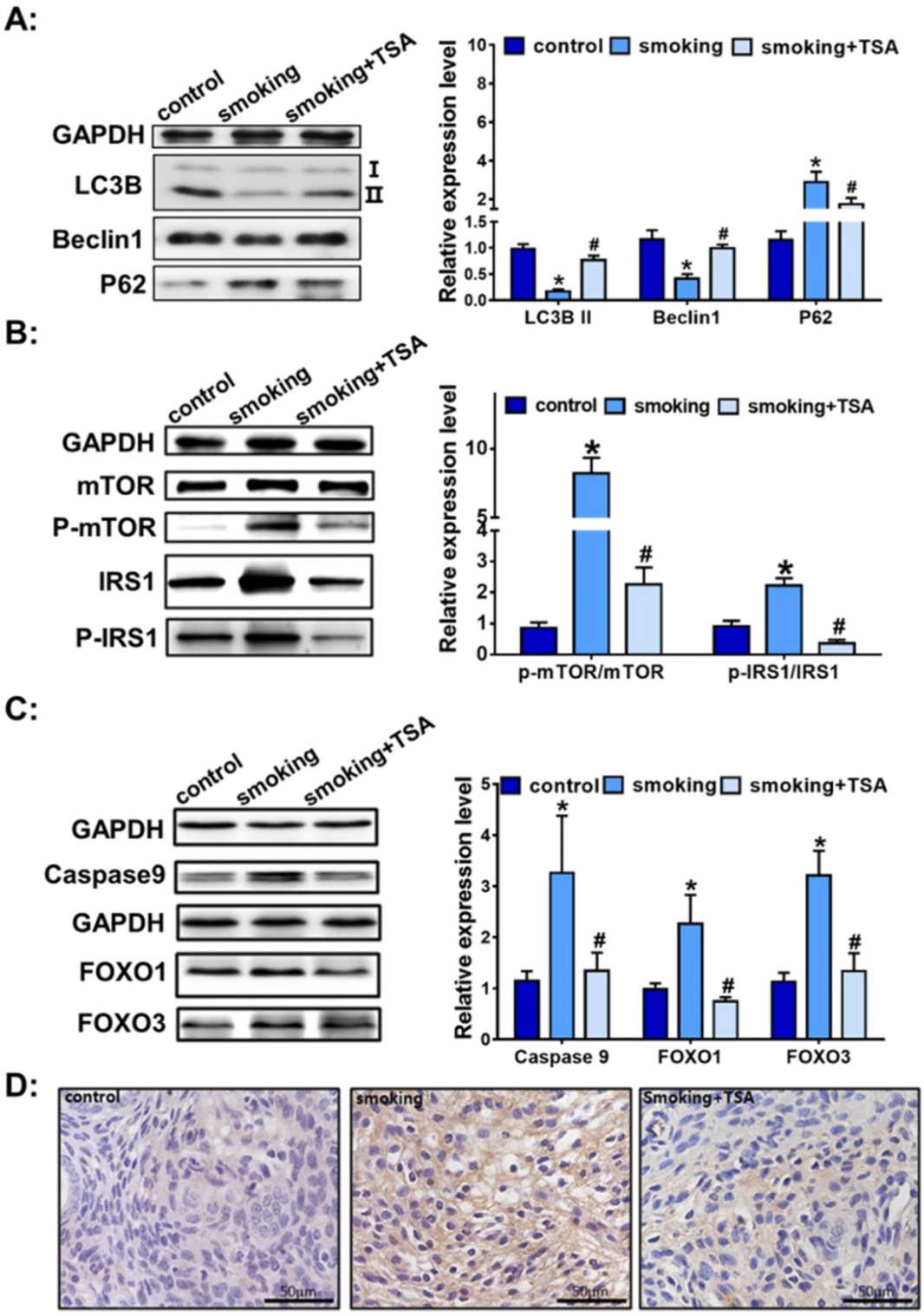

Fig. 4. TSA inhibited the activation of markers of macro-autophagy, mTOR, IRS, and FOXO.
A: TSA resumed the expression of markers of macro-autophagy (LC3-II and Beclin-1) and decreased p62 accumulation. Data are shown as the mean ± SEM. $^{*}P < 0.05$ (control vs. smoking), $^{\#}P < 0.05$ (smoking vs. smoking + TSA). B: TSA limited phosphorylated mTOR and IRS activation induced by CS exposure. Data are shown as the mean ± SEM. $^{*}P < 0.05$ (control vs. smoking), $^{\#}P < 0.05$ (smoking vs. smoking + TSA). C: TSA inhibited FOXO1, FOXO3, and caspase-9 expression activated by CS exposure. Data are shown as the mean ± SEM. $^{*}P < 0.05$ (control vs. smoking), $^{\#}P < 0.05$ (smoking vs. smoking + TSA). D: TSA inhibited caspase-9 expression in stromal cells activated by CS exposure.

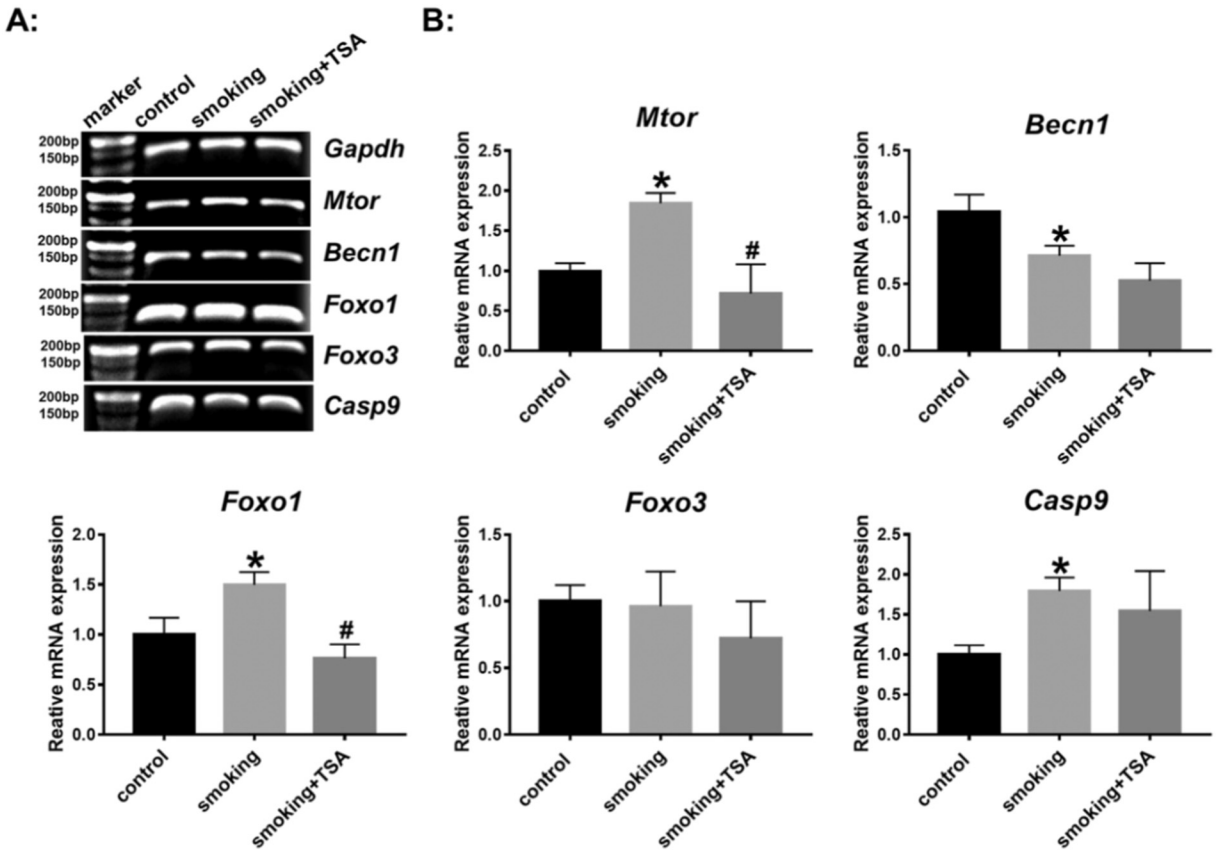

Fig. 5. TSA inhibited the transcriptional activity of *Mtor* and *Foxo1*.
A: The electrophoresis result of PCR products of *Gapdh*, *Mtor*, *Becn1*, *Foxo1*, *Foxo3,* and *Casp9*. B: The relative mRNA expressions of *Mtor*, *Becn1*, *Foxo1*, *Foxo3,* and *Casp9*. The mRNA level of *Mtor*, *Foxo1,* and *Casp9* were increased and *Becn1* was decreased in the uterus after smoking exposure and TSA significantly inhibited the transcriptional activity of *Foxo1* and *Mtor*. Treatment with smoking and TSA had no effect on the transcriptional activity of *Foxo3*. Data are shown as the mean ± SEM. $^{*}P < 0.05$ (control vs. smoking), $^{\#}P < 0.05$ (smoking vs. smoking + TSA).

Acknowledgments

We would like to express our gratitude to all the subjects who participated in the study. This study was supported by the National Natural Science Foundation of China (81471466) and the Program for Liaoning Excellent Talents in University (LR2015066).

Declaration of interests

The authors declare that there are no conflicts of interest.

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