Medical Research Archives

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Published: November 30, 2022

Citation: Luo M, Cai X, et al., 2022. Tetramethylpyrazine Stalls Lesional Progression via Inducing Senescence in Mouse with Induced Deep Endometriosis, Medical Research Archives, [online] 10(11). https://doi.org/10.18103/mra.v10i11.3341

DOI
https://doi.org/10.18103/mra.v10i11.3341

ISSN: 2375-1924

RESEARCH ARTICLE

Tetramethylpyrazine Stalls Lesional Progression via Inducing Senescence in Mouse with Induced Deep Endometriosis

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ABSTRACT

So far, the pressing need for the development of non-hormonal therapeutics for endometriosis has been unfulfilled. In light of the evidence that platelets play important roles in the development of endometriosis and that tetramethylpyrazine has therapeutic potential, we tested the hypothesis that tetramethylpyrazine can induce cellular senescence in endometriotic lesions, hindering fibrogenesis in mice with induced deep endometriosis and that the combined use of tetramethylpyrazine and sodium tanshinone IIA sulfonate may have a synergistic effect. We induced deep endometriosis in 32 female Balb/C mice, and then randomly divided mice into equal-sized four groups: low- and high-dose tetramethylpyrazine, low-dose tetramethylpyrazine plus low-dose tanshinone IIA sulfonate and inert vehicle control. After two weeks of treatment, their lesion tissues were removed and procured. All lesions were weighed, and lesion fibrosis was quantitated by Masson trichrome staining. In addition, cellular senescence in lesions was evaluated by senescence-associated β-galactosidase, along with immunohistochemistry analyses of p53, Salvador 1, cellular communication network factor 1, hyaluronan synthase 2, surviving and granulocyte-macrophage colony stimulating factor and other markers. We found that tetramethylpyrazine treatment significantly decreased lesion weight, arrested lesional progression and ameliorated pain behaviour ostensibly via inducing senescence by p53 activation, and induction of Salvador 1 and cellular communication network factor 1 while suppressing hyaluronan synthase 2, survivin and granulocyte-macrophage colony stimulating factor, resulting in increased apoptosis and reduced lesional infiltration of alternatively activated macrophages. Tetramethylpyrazine treatment also significantly reduced the plasma concentration of P-selectin and hyaluronic acid, possibly leading to reduced lesional platelet aggregation. Thus, we conclude that tetramethylpyrazine holds the promise as therapeutics for treating endometriosis, but the tetramethylpyrazine plus tanshinone IIA sulfonate did not further enhance the therapeutic effect. These results further underscore the notion that induced senescence may play an antifibrotic role in endometriosis, and activating the senescence pathway, through treatment with tetramethylpyrazine or other similar drugs, may be a novel avenue for treating endometriosis.

Keywords: Endometriosis; mouse; platelet; senescence; tanshinone IIA; tetramethylpyrazine

INTRODUCTION

Featuring the deposition of endometrial epithelial and stromal cells outside of the uterine cavity, endometriosis is a common gynecologic disease affecting mostly women of reproductive age [1]. As of now, all medications for the treatment of endometriosis are hormonal drugs with varying degrees of side effects [1]. As such, there is a great deal of discontent over the current medications, as well as the demand for non-hormonal drugs [2]. Thus, the development of more efficacious and cost-effective non-hormonal medical treatment is an unmet need for endometriosis. Dishearteningly, the development of such drugs for endometriosis so far has been fraught with failure and disappointment [3,4], probably due to the fibrotic content in lesions [4] in conjunction with the well-documented diagnostic delay in endometriosis [5]. By the time when the patient is accurately diagnosed with endometriosis, her lesions may already become highly fibrotic, and, as such, become very difficult to treat by medication [6]. In addition, many perceived targets, such as angiogenesis and inflammation, are also needed for normal endometrial physiological function, leaving little room for manoeuvre.

In general, fibrotic diseases are very difficult to treat [7], and endometriosis is no exception. In particular, lesions become more fibrotic as endometriosis progresses, along with reduced vascularity and cellularity, and aberrant hormonal receptor expression and epigenetic changes [6,8]. To combat fibrosis, strategies to change the cell fates through degradation of the fibrotic extracellular matrix and/or elimination of fibrogenic myofibroblasts have been proposed, which include apoptosis, senescence, dedifferentiation and reprogramming [9]. In particular, induction of senescence following liver damage [10] and cutaneous wound [11] has been shown to prevent excessive fibrosis.

Capitalizing on an established mouse model of deep endometriosis [12], we have recently reported that sodium tanshinone IIA sulfonate (STS) treatment resulted in significantly smaller lesions, retarded lesional progression and improved pain behavior [13]. Like STS, which is derived from tanshinone IIA that is a pharmacologically active diterpenoid extracted from the roots of *Salvia miltiorriza Bunge*, tetramethylpyrazine (TMP) also is derived from an herb, called *Rhizoma Chuanxiong*, which has been used in traditional Chinese medicine (TCM) for thousands of years as a remedy for blood stasis (hypercoagulation). Recently, we have also shown through *in vitro* and *in vivo* experimentations that TMP stalls endometriosis progression by inhibiting epithelial-mesenchymal transition (EMT), fibroblast-to-myofibroblast transdifferentiation (FMT) and fibrogenesis [14].

Officially listed in the Chinese Pharmacopodia (2010, Expanded and revised edition) for the treatment of cerebrovascular disorders, TMP is a major component in Foshousan, a TCM formula, in treating endometriosis [15-17]. Commercially available in China, the injective formulation of TMP is a prescription drug and has been demonstrated to inhibit platelet activation [18], inflammation [19,20], and oxidative stress [21]. In addition, it also has been shown to be anti-fibrosis [22-24]. In endometriosis, it has been shown that, as one major ingredient in a concocted herbal formula, TMP suppresses lesional growth, inflammation, and EMT and induces apoptosis [25-27], suggesting that it may be an admissible compound for treating endometriosis.

Along this line, we hypothesized that TMP, like STS, may induce cellular senescence in endometriotic lesions through activation of p53 [28] or cysteine-rich protein 61 (CYR61/CCN1) [29,30], and activate Salvador 1 (Sav1) [31] but suppress hyaluronan synthase 2 (HAS2) [32], Survivin [33] and granulocyte-macrophage colony stimulating factor (GM-CSF) and thus inhibit lesional infiltration of CD163-positive alternatively activated or M2 macrophage [34,35], achieving its therapeutic effect through suppressing lesional progression and fibrogenesis. In addition, inspired by the report that a senolytic cocktail, dasatinib plus quercetin (D+Q), can induce senescence and then *remove* senescent fibroblasts to improve health and function in idiopathic pulmonary fibrosis [36], we wondered whether the joint treatment with STS and TMP would have any additive or synergistic effect in treating endometriosis similar to the D+Q cocktail. This study was designed to test these hypotheses in a recently established mouse model of deep endometriosis [12]. We chose this mouse model due to two reasons. First, deep endometriosis is the most challenging to manage among all subtypes of endometriosis [4]. Second, so far the animal model of deep endometriosis has been confined to baboon [37] and mouse [12] only, yet the baboon model is prohibitively expensive and is apparently out of reach by the majority of laboratories, especially when a moderate sample size is required.

METHODS AND MATERIALS

Animals

Seventy-two virgin female Balb/C mice, 6-week old and about 16-18 g in bodyweight, were purchased from the Lingchang Biotechnology Company (Shanghai, China) and used for this study.

All mice were housed under controlled conditions with a light/dark cycle of 12/12 hours, and had access to chows and water *ad libitum*. All experiments were carried out following the guidelines of the National Research Council's *Guide for the Care and Use of Laboratory Animals* [38] and were approved by the Experimental Animals Review Board of Shanghai OB/GYN Hospital, Fudan University on 17th January 2017.

Protocol for mouse experiment

Forty-eight recipient Balb/C mice were divided, at random, into 6 groups of eight animals; low-dose STS (5 mg/kg/d; #S832653, Macklin, Shanghai, China), high-dose STS (15 mg/kg/d), low-dose TMP (25 mg/kg/d; 183938, Sigma, MO, USA), high-dose TMP (50 mg/kg/d), low-dose STS + low-dose TMP, and control. The results of single drug treatment on STS have been previously reported [13]. In this study, only the results from the TMP-treated and TMP+STS-treated groups, along with the control group, are reported. To use the data more efficiently, we also used data from low- and high-dose STS groups that have reported in [13] when appropriate.

After 4 weeks of infusion with substance P (SP) (see below), all recipient mice received a surgery, and SP-containing Alzet pumps were taken out and replaced with new Alzet pumps (Model 2002, 0.5 µL/h, DURECT Corporation, Cupertino, CA, USA) which contained different concentrations of TMP or just vehicle. The choice of TMP dosages was based mainly on the recommended dosage of TMP for human adults, which is 50-100 mg/day via the intravenous route (National Drug Approval number: H20061205, Changchun Leiyunshang Pharmaceutical Co., Ltd). Therefore, according to the conversion formula, the mouse equivalent dose (mg/kg) = human dose (mg/kg) × 37 (human K_m factor)/3 (mouse K_m factor) [39], assuming an average body weight of 50 kg in female adults. In female Balb/C mice, the doses of 25 to 100 mg/kg/day TMP administrated intragastrically have been reported previously [40]. The ratio of the systemic bioavailability of intravenous administration vs. intranasal route is roughly 1.2, and the ratio between intranasal and intragastric routes is about 1.7 [41]. This led to the 25 and 50 mg/kg/day, respectively, for low- and high-doses as we used.

For controls, we used Alzet pumps containing vehicle only (sterile saline with 50% anhydrous ethanol), similar to the active groups. Fourteen days later, all mice were sacrificed and evaluated.

Induction of deep endometriosis in mouse

We used a recently established mouse model of deep endometriosis by intraperitoneal injection of endometrial fragments as described [42] with infusion of SP [12]. SP is a sensory nerve-derived neuropeptide and an inflammatory mediator involved in pain transmission [43-45], and it can accelerate the acute and chronic wound healing processes [46-48]. We have previously shown that SP induces EMT, fibroblast-to-myofibroblast transdifferentiation (FMT) and further turns stromal cells into smooth muscle cells (SMCs) in endometriotic lesions, resulting ultimately in fibrosis [49,50]. Here, SP was used to accelerate lesional progression and adhesion.

Among the 72 mice, 24 were randomly selected as donors that contributed uterine tissue fragments and the remaining 48 were recipients that received endometrial tissues from donor mice. Results from the first 24 of those recipient mice have been reported (19), and the results from the remaining 24 are reported here.

Briefly, after 2 weeks of acclimatization, donor mice were injected intramuscularly with 100 µg/kg estradiol benzoate (XinYi Chemistry, Shanghai, China) to stimulate the growth of endometrium. One week later the donor mice were sacrificed and their uteri were removed and harvested. The harvested uterine tissues were then seeded in a Petri dish containing warm sterile saline, and, with a pair of scissors, split longitudinally [51]. The uterine tissues were cut into small fragments, ensuring that the maximal diameter was consistently ≤1 mm. The fragments were injected intraperitoneally into recipient mice. To minimize individual variations, the fragments from 3 donor mice were mixed together and then divided into 6 parts, each injected intraperitoneally to one mouse each from one of 6 groups.

As previously reported [12,52], one day before the induction procedure, all recipient mice were anaesthetized with 40 mg/kg pentobarbital (China National Medicines Corporation, Ltd., Shanghai, China), and a transverse incision about 0.5 cm in length on the nape was made. The skin was separated from muscles towards the back, and an Alzet pump (Model 1004, 0.11 µL/h, DURECT Corporation) was placed into the subcutaneous pocket and closed. Each Alzet osmotic pump was filled with SP (100 µg/kg/day; #ab120170, Abcam, Cambridge, UK) [53], and was ensured consistent and controlled release of its contents with a constant speed.

Lesion measurement and pain evaluation

The bodyweight and hotplate latency of all mice were evaluated and recorded on day 1, 12, 26 and 40 after the induction of deep endometriosis. All mice were sacrificed after collecting blood samples from eyeballs. Briefly, about 1mL of peripheral blood was taken from the inner canthus vein of the right eye by eyeball enucleation, which was used to measure plasma HA and P-selectin levels. Immediately after sacrifice, the abdominal cavity was then opened up and thoroughly examined and evaluated. All visible ectopic endometrial tissues were carefully harvested and weighed (dry weight) as previously reported [12]. Briefly, endometriotic lesions were meticulously excised from the surrounding tissues and washed several times in sterile normal saline. Then the cyst liquid was aspirated, and dry lesion weight was evaluated 24 hours after the harvest of lesions. Then the endometriotic lesions were fixed immediately and embedded in paraffin for pathologic examination, immunohistochemical analysis, in conjunction with Masson trichrome staining. The procedures for lesional measurement and pain evaluation using hotplate test are described in more detail in the Supplementary Information.

Immunohistochemistry analyses and Masson trichrome staining

For all mice, the harvested endometriotic lesion samples were fixed with 10% formalin (w/v) and paraffin-embedded, and serial 4-μm sections were performed for each block. The first resultant slide was stained for haematoxylin and eosin (H&E) for confirmation purpose, while the remaining slides were stained for senescence-associated markers using the following respective antibodies; senescence-associated β-galactosidase (SA-β-gal) (1:200, #sc-377257, Santa Cruz, CA, USA), p53 (1:100, #ab31333, Abcam), CCN1 (1:100, #ab10760, Abcam), HAS2 (1:50, #sc-365263, Santa Cruz), CD163 (1:500,#ab182422, Abcam), GM-CSF (1:100, #ab9741, Abcam), Survivin (1:500, #ab469, Abcam), and Sav1 (1:150, #NBP2-13282, Novusbio, Littleton, CO, USA). Since the report of our STS results [13] which used phosphate buffered saline (PBS) for negative controls, we redid all the staining for negative controls using non-immune serum in lieu of primary antibodies, and found that the results were exactly what we expected. A more detailed description of the IHC and Masson staining procedures is provided in the Supplemental Information, along with representative figures for positive and negative controls (Supplementary Figure S1).

Quantification of plasma hyaluronic acid and P-selectin concentrations

We used a sandwich enzyme-linked immunosorbent assay (ELISA) to measure the plasma P-selectin (Abcam, Cambridge, UK) and hyaluronic acid (HA) (R&D systems, Minneapolis, MN, USA) concentrations. Following the manufacturer's instructions, the harvested blood samples were collected in sterile tubes containing heparin, and were kept cold on ice. The samples were centrifuged at 2,000 g for 10 min at 4°C and then the supernatant liquid (plasma) was harvested and stored at -20°C until use. The absorbance was measured at 450 nm and read immediately on a microplate reader (Biotek, Winooski, VT, USA). Then the mean optional density was converted into concentration. Each sample was evaluated in triplicate.

Statistical analysis

Wilcoxon and Kruskal tests were used, respectively, to compare distributions of continuous variables between or among two or more groups and the paired Wilcoxon test was used when the before-after comparison was made for the same group of subjects. Pearson's correlation coefficient was used when evaluating correlations between two variables when both variables were continuous. Linear regression analysis was used to ascertain whether there was a linear increasing or decreasing trend in lesion weight, hotplate latency or immunoreactivity measure with respect to TMP dosage, after square-root or log-transformation to improve normality, or no transformation at all, whenever appropriate. Since in our data there was only one STS group (i.e. TMP+STS group), we also used the data on low- and high-dose STS data published in [13], and this permitted analysis of dose-dependent effect of STS as well as the interaction of TMP and STS.

Linear regression also was used to draw the regression line in scatter plots that show linear trends. *P*-values of less than 0.05 were considered statistically significant. All computations were made with R 4.1.3 [54] (www.r-project.org).

RESULTS

As with STS [13], TMP also was well-tolerated and all mice survived the entire experimental period until sacrifice. Consistent with previously reported [12], deep endometriosis was successfully induced in all mice per histological confirmation. Multiple linear

regression analysis incorporating time since induction (in days), TMP and STS dosages indicated that the bodyweight of all groups of mice increased gradually and significantly as with time after induction of endometriosis ($p<2.2x10^{-16}$; Figure 1A). However, there was no statistically significant difference in bodyweight between mice taking TMP or STS ($p=0.23$ and $p=0.13$, respectively; $R^2=0.77$; Figure 1A).

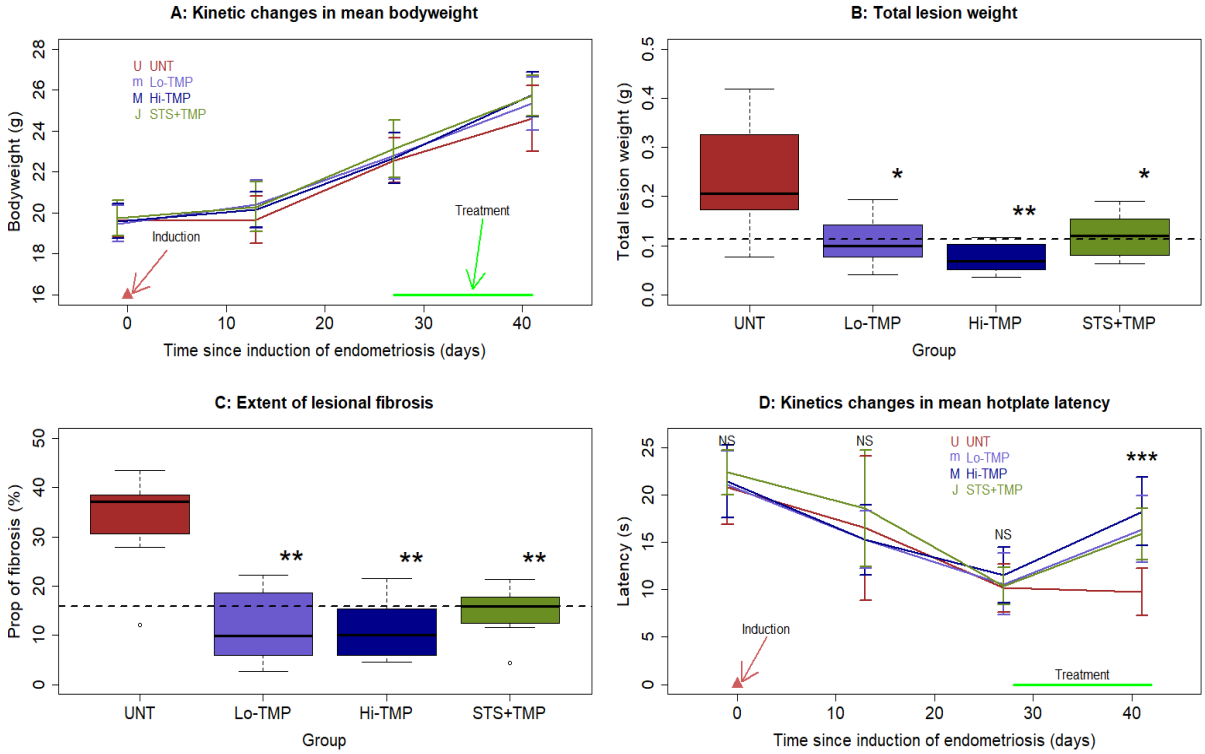

Figure 1. (A) Kinetic changes in the mean body weight in different treatment groups of mice; (B) Boxplot summarizing the lesion weight among different groups of mice. (C) Boxplot of the lesional fibrotic content among different groups of mice. A single dot outside of the whisker denotes an outlier. (D) Kinetic changes in the mean hotplate latency in different treatment groups of mice. Group labels: Control: control group; Lo-TMP, low-dose TMP; Hi-TMP, high-dose TMP; STS+TMP, low-dose STS+ low-dose TMP. In (A) and (D), Kruskal's test was used. Numbers shown above each time point are p values. Symbols of statistical significance levels: NS: $p>0.05$; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

TMP decreases lesion weight as well as fibrotic content, and prolongs hotplate latency

Using TMP dosage as a co-variable, the linear regression analysis indicates that TMP treatment yielded significantly reduced lesion weight than that of the control mice in dose-dependent manner ($p=2.8x10^{-5}$, $R^2=0.45$; Figure 1B). The average weight in Lo-TMP and Hi-TMP groups was decreased by 54.9% and 68.5%, respectively, as compared with that of the controls (Figure 1B), suggesting that the TMP treatment hinders lesional outgrowth. Mice that received the joint treatment with TMP and STS had their lesion weight reduced by an average of 49.4% ($p=0.010$; Figure 1B), suggesting no further reduction in lesion weight as compared with treatment with TMP (or STS) alone.

In addition, the content of lesional fibrosis also was reduced in a dose-dependent fashion ($p=1.1x10^{-5}$, $R^2=0.50$, per linear regression, using TMP dosage as a co-variable; Figure 1C and Figure 2). Joint treatment with TMP and STS also decreased the lesional fibrosis ($p=0.005$; Figure 1C). Since the lesional weight and the extent of fibrosis were highly correlated ($r=0.64$, $p=9.4x10^{-5}$), these findings suggest that TMP and STS retarded lesional progression and fibrogenesis dose-dependently. TMP+STS joint treatment also stalled lesional progression as well.

Prior to the induction of endometriosis or before the TMP treatment, there was no difference in hotplate latency among the 4 groups ($p=0.86$, $p=0.56$, and $p=0.75$, respectively; Figure 1D), and there was a progressive and significant shortening in latency 2 and 4 weeks after the induction of endometriosis ($p=0.0001$, and $p=4.7x10^{-10}$, respectively; Figure 1D), as we reported previously [55,56]. However, 2

weeks after treatment with TMP or TMP+STS, the difference in hotplate latency among the 4 treatment groups became highly significant (p=0.0007; Figure 1D). Treatment with TMP led to significantly and dose-dependently prolonged latency than the untreated mice ($p=4.8x10^{-6}$, R^2=0.51, per linear regression, using TMP dosage as a co-variable; Figure 1D). Mice treated with TMP+STS also had significant improvement in latency (p=0.0006; Figure 1D).

TMP induces senescence in endometriotic lesions

We also carried out SA-β-gal staining and immunostaining of p53, Sav1, CCN1, HAS2, GM-CSF, and Survivin for endometriotic lesions. Moreover, we stained CD163-positive macrophages in lesions. We found that SA-β-gal was seen mostly in nuclear and perinuclear regions in both epithelial and stromal components in endometriotic lesions (Figure 2). The immunostaining of p53, CCN1, Sav1, HAS2, and GM-CSF was seen in both glandular epithelium and stroma and localized in the cytoplasm, but predominantly in the epithelial component (Figure 2). In contrast, CD163 staining was seen primarily in membranes and cytoplasm in macrophages, which were located chiefly in lesional stroma (Figure 2).

Using TMP dosage as a co-variable, we found via multiple linear regression that, similar to STS [13], TMP treatment significantly increased, in a dose-dependent fashion, SA-β-gal staining in both epithelium and stroma of lesions (p=0.0015, R^2=0.24, and p=0.011, R^2=0.17, respectively; Figures 2, 3A and 4A), indicating the induction of senescence in lesions. However, joint treatment with TMP and STS attenuated this increase (p=0.046 and p=0.091, respectively). Similarly, immunostaining of p53, Sav1, and CCN1 in lesions was significantly increased, in a dose-dependent manner (p=0.024, R^2=0.23, p=0.0096, R^2=0.21, p=0.0003, R^2=0.32, p=0.0024, R^2=0.22, p=0.0081, R^2=0.19, p=0.0013, R^2=0.24, respectively, for p53, Sav1, and CCN1 in the epithelial and stromal components; all based on linear regression using TMP dosage as a co-variable; Figures 2, 3B-D and 4B-D), similar to STS [13]. The staining levels of HAS2 and GM-CSF were also significantly decreased, in a dose-dependent fashion, after TMP treatment (p=0.0067, R^2=0.23, p=0.0073, R^2=0.18, $p=2.8x10^{-5}$, R^2=0.39, p=0.0032, R^2=0.25, for epithelial and stromal staining of HAS2 and GM-CSF, respectively; all based on linear regression using TMP dosage as a co-variable; Figures 2, 3E,F and 4E,F), similar to STS [13]. Again, the joint treatment had no further effect, except that it also reduced the HAS2 and GM-CSF staining levels (all 4 p values <0.05; Figures 2, 3E,F and 4E,F). Moreover, the reduction of Survivin staining in lesional epithelium and stroma did not achieve statistical significance (both p's>0.072).

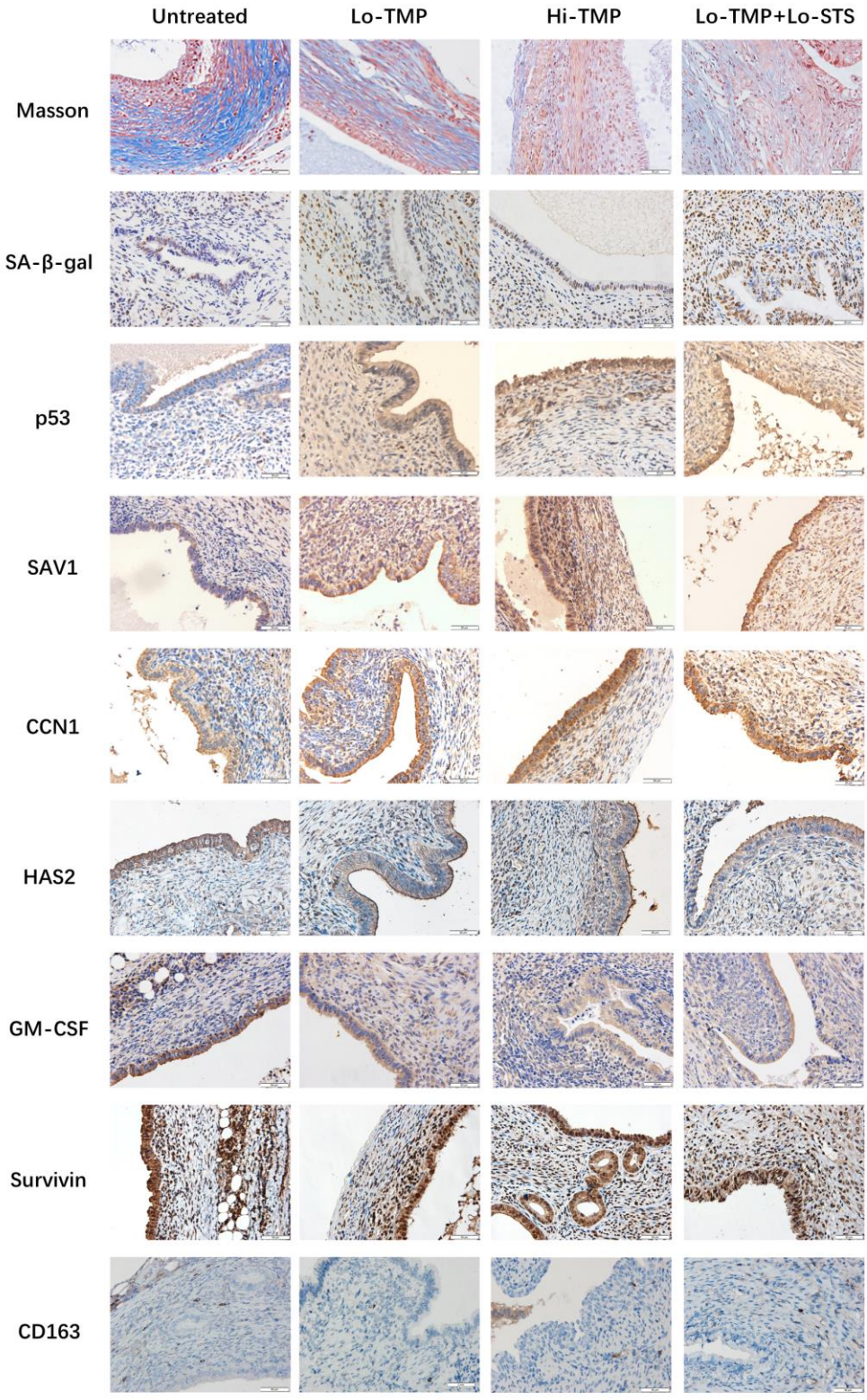

Figure 2. Representative photomicrographs of immunohistochemistry and histochemistry (Masson's trichrome staining) analyses of SA-β-gal, p53, Sav1, Ccn1, Has2, GM-Csf, Survivin and CD163, along with the extent of fibrosis in endometriotic lesions from different groups of mice. The four columns are for the Untreated (control), Lo-TMP, Hi-TMP and TMP+STS groups, respectively, while different rows show different immunostaining markers as indicated. Magnification: ×400. Scale bar = 50 μm.

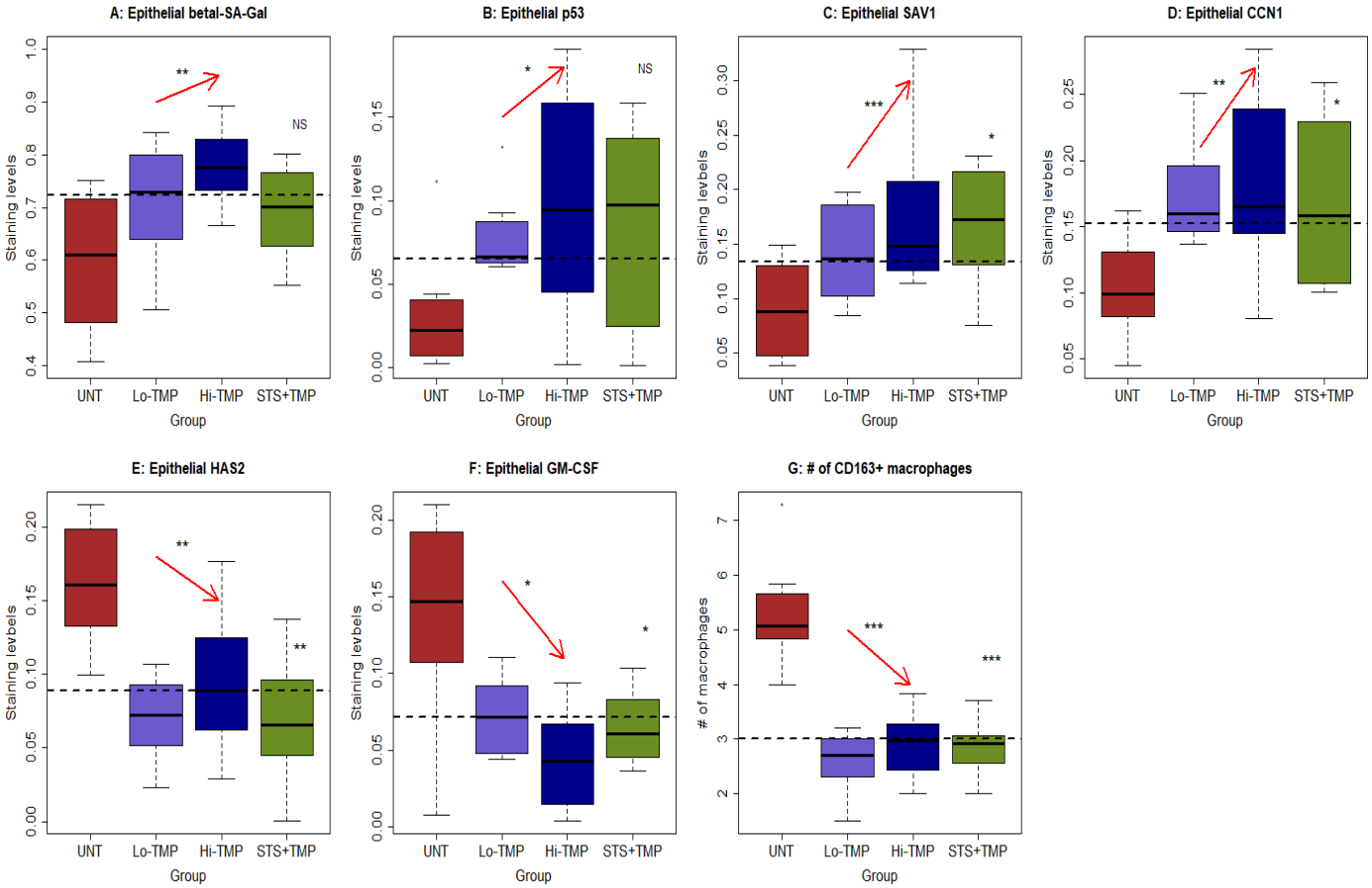

Figure 3. Boxplot summarization of the histochemistry and immunohistochemistry staining results for SA-β-gal(A), p53 (B), Sav1 (C), CCN1 (D), HAS2 (E), GM-CSF (F), and the density of CD163+ macrophages, i.e. the number of CD163+ macrophages per high-power field (HPF), (G) in the epithelial component of endometriotic lesions (except for the density of CD163+ macrophages, which were counted in the entire lesions). Group labels: UNT: untreated control group; Lo-TMP, low-dose TMP; Hi-TMP, high-dose TMP; STS+TMP, low-dose STS+ low-dose TMP. In all plots, the dashed line represents the median value of all three groups combined, and the linear regression results incorporating the TMP dosage as a covariable are shown. The results include a p value depicting the statistical significance level of the linear trend increasing or decreasing with the TMP dosage and the R^2 value of the regression. Symbols of statistical significance levels: NS: $p>0.05$; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. OD: Optical density.

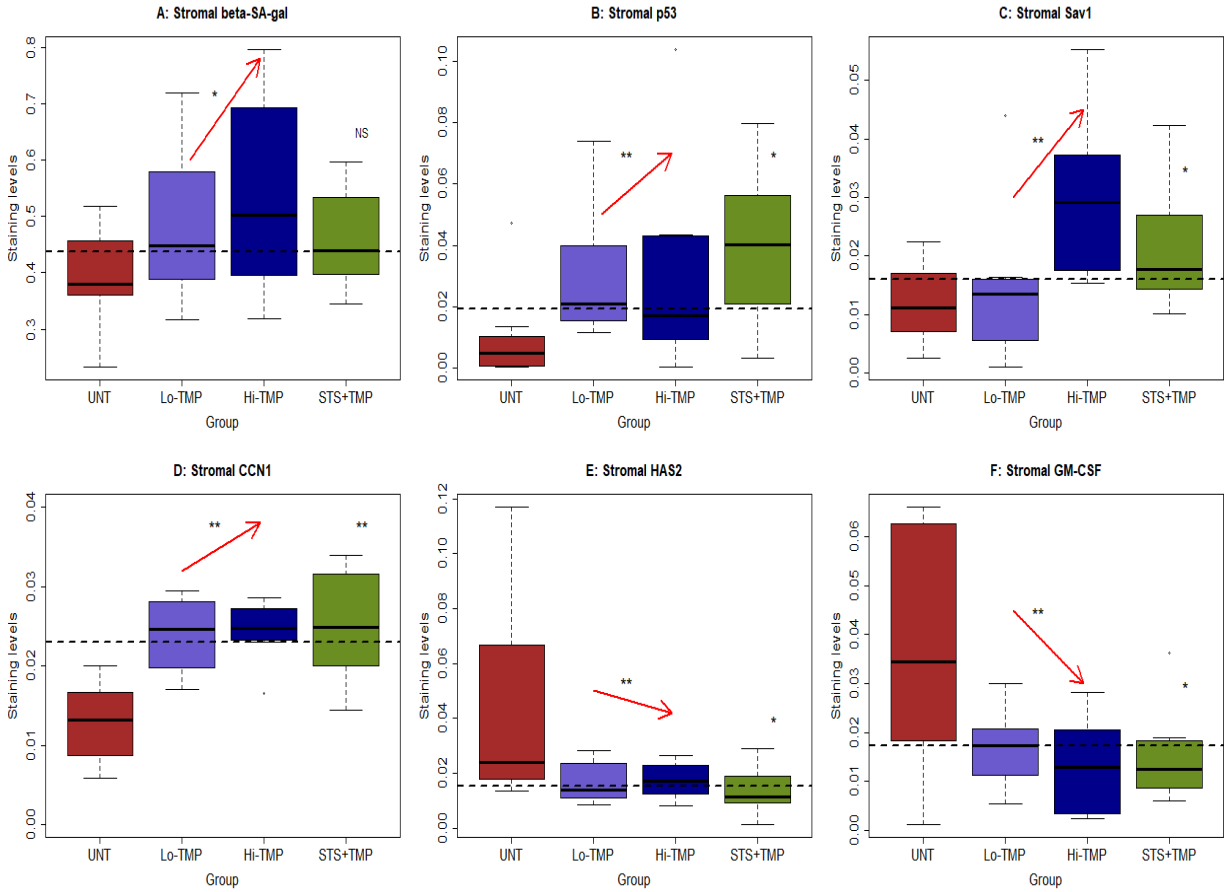

Figure 4. Summary of immunohistochemistry and histochemistry analyses. Boxplot summarization of the immunohistochemistry staining results for SA-β-gal (A), p53 (B), Sav1 (C), CCN1 (D), HAS2 (E), GM-CSF (F), and Survivin (G) in the stromal component of endometriotic lesions. Group labels: UNT: untreated control group; Lo-TMP, low-dose TMP; Hi-TMP, high-dose TMP; STS+TMP, low-dose STS+ low-dose TMP. In all plots, the dashed line represents the median value of all three groups combined, and the linear regression results incorporating the TMP dosage as the sole covariable are shown. The results include a p-value depicting the statistical significance level of the linear trend increasing or decreasing with the TMP dosage. Symbols of statistical significance levels: NS: $p>0.05$; *: $p<0.05$; **: $p<0.01$. OD: Optical density.

In agreement with the reduction, in a dose-dependent manner, in GM-CSF staining, the number of lesional infiltrated M2 macrophages in lesions was significantly and dose-dependently decreased ($p=1.5\times10^{-5}$, $R^2=0.38$; based on linear regression using TMP dosage as a co-variable; Figures 2 and 3G), similar to STS [13].

Except Survivin ($r=0.01$, $p=0.96$), the epithelial and stromal staining levels of SA-β-gal, p53, CCN1, Sav1, HAS2, and GM-CSF were closely and positively correlated ($r=0.49$, $p=0.0042$, $r=0.86$, $p=3.4\times10^{-10}$, $r=0.71$, $p=5.6\times10^{-6}$, $r=0.72$, $p=4.0\times10^{-6}$, $r=0.59$, $p=0.0004$, $r=0.73$, $p=5.7\times10^{-5}$, $r=0.92$, $p=8.6\times10^{-14}$, respectively).

TMP reduces systemic HA and P-selectin levels

Consistent with the anti-platelet property of TMP [18], mice treated with TMP had significantly decreased plasma HA and P-selectin levels, especially the latter (Figure 5A,B). While plasma HA concentrations correlated positively but marginally with the lesion weight ($r=0.31$, $p=0.090$; Figure 5C), P-selectin levels correlated positively and significantly with the lesion weight ($r=0.71$, $p=5.1\times10^{-6}$; Figure 5D). Both HA and P-selectin levels correlated positively with the lesional fibrotic content ($r=0.47$, $p=0.007$, and $r=0.67$, $p=2.6\times10^{-5}$, respectively; Figure 5E,F), but negatively with the hotplate latency after treatment ($r=-0.52$, $p=0.0023$, and $r=-0.54$, $p=0.0015$, respectively; Figure 5G, H).

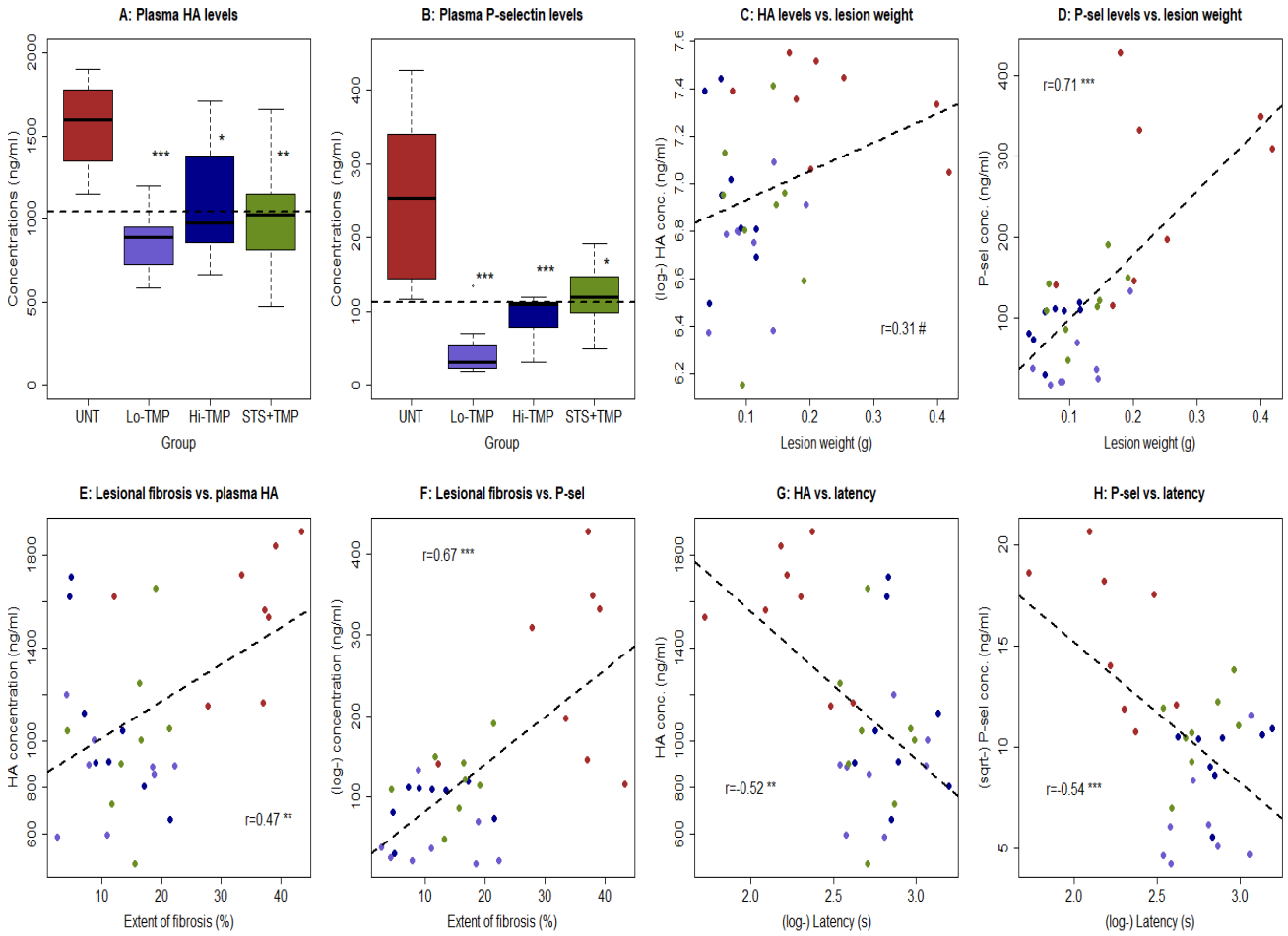

Figure 5. Summary results of plasma measurements and their correlation with lesion weight, extent of lesional fibrosis and hotplate latency. Boxplots of plasma P-selectin (A) and HA levels (B) in different treatment groups of mice. Scatter plots between lesion weight and plasma P-selectin levels (C) and HA levels (D), between the extent of lesional fibrosis and plasma P-selectin levels (E) and HA levels (F), and between hotplate latency and plasma P-selectin levels (G) and HA levels (H). In panels (A) and (B), Wilcoxon's test was used, with the untreated group as reference. The dashed line represents the median value of all groups combined. In panels of (C)-(F), each dot represents one data point (mouse), and the dashed line represents the regression line. The Pearson's correlation coefficient and its statistical significance level also are shown. Symbols of statistical significance levels: *: p<0.05; **: p<0.01; ***: p<0.001. P-sel: P-selectin.

Correlates of the extent of lesional fibrosis

The lesional fibrotic content correlated negatively with the senescence levels (r=-0.42, p=0.016) and the Sav1 staining levels (r=-0.55, p=0.0001) in the epithelial component, and CCN1 staining levels in both the epithelial (r=-0.54, p=0.0015) and stromal (r=-0.61, p=0.0002) components, but positively with the number of infiltrated M2 macrophages in lesions (r=0.77, $p=3.2x10^{-7}$).

DISCUSSION

In this study, we have shown that, similar to STS [13], TMP treatment hindered lesional progression and ameliorated the pain behaviour in mice with induced deep endometriosis ostensibly via inducting cellular senescence by the activation of p53, Sav1 and CCN1 while suppressing HAS2, Survivin and GM-CSF, leading to increased apoptosis and reduced lesional infiltration of pro-reparative M2 macrophages. Additionally, TMP treatment, similar to STS, significantly reduced the plasma concentration of P-selectin and HA, possibly leading to reduced lesional platelet aggregation. However, the joint use of TMP and STS did not yield any synergistic or even additive effect. In fact, the effect of joint treatment is apparently on par with that of the single drug treatment, suggesting that the TMP and STS likely have identical mechanisms of action. Regardless, our results complement our prervious report that TMP stalls progression and fibrogenesis in endometriosis [14] and support the notion that senescence may restrain lesional progression and fibrogenesis, and that targeting the senescence pathway may have desirable therapeutic potential.

TMP hydrochloride or phosphate injections have been used widely in China to treat cardiovascular and cerebrovascular diseases, due mainly to their low cost and good safety profile. Despite its excellent safety profile, a recent paper reported some uncommon side effects of TMP in hydrochloride injection formulation that have been reported in Chinese journals during 2000-2013 [57]. What is unclear, though, is the true causes for these side effects: is it due to the compound itself? Or because of the formulation? Or contamination problems in production? Notably, the adverse events arising from the injective formulations of TCM herbs/extracts have been well-documented in China. Future studies are warranted to clarify this issue and to evaluate the risk vs. benefit issue.

Given the documented anti-platelet property of TMP [58], and in light of the positive correlation between the lesional fibrotic content and plasma P-selectin levels as previously reported [59], our results are consistent with our previous findings that platelet depletion and anti-platelet treatment resulted in reduced lesion growth and improved pain behavior in mice with induced endometriosis [55,60,61]. In particular, TMP inhibits platelet activation seemingly through the phosphorylation of Akt [62], which is activated in endometriosis [63,64]. Moreover, our data are consistent with the increased lesional staining of GM-CSF as previously reported [65], the facilitative role of M2 macrophages in lesional progression [34,66]. Our data also are in line with the report that Survivin is overexpressed in endometriosis [67,68].

Data accumulated in the last few years indicate that endometriotic lesions are fundamentally wounds undergoing repeated tissue injury and repair (ReTIAR) since the ectopic endometrium experiences cyclic bleeding and thus tissue injury just as in eutopic endometrium [69-73]. Platelets are the first cells, though anucleated, to go to and aggregate at the wounding site to initiate hemostasis, inaugurating the tissue repair process of inflammation, proliferation and tissue remodelling [74]. Activated platelets secret a plethora of bioactive molecules, including various cytokines/chemokines and growth factors, including PDGF and TGF-β1 [75]. In addition, endometriotic stromal cells also produce potent platelet-activating molecules such as thrombin and thromboxane A2 [76] and collagens [71], which, coupled with increased angiogenesis and thus vascular permeability, may further lead to platelet aggregation. Therefore, endometriotic lesions and platelets engage active cross-talks to maintain lesion growth and facilitate lesional progression and fibrogenesis [60,71,72].

Our data are consistent with the report that the combination of TMP, ferulic acid, and ligustrazine attenuates epithelial-mesenchymal transformation via Wnt/β-catenin signaling pathway and inhibits invasion and metastasis through MMP/TIMP in endometriosis [16,17]. Aside from its anti-platelet capability, TMP also has been reported to be anti-fibrotic through suppressing Akt [62,77] and hedgehog signalling [24], interfering the PDGF-βR-mediated NLRP3/caspase-1 pathway [22], promoting autophagy [78], and suppressing YAP and activating p53 [79]. Remarkably, all these pathways have been implicated in endometriosis [63,80-83].

Cellular senescence is viewed as a terminal stress response triggered by a diverse range of stress-inducing stimuli, resulting in stable and essentially irreversible cell-cycle arrest [84]. Although non-proliferative, senescent cells are nonetheless viable and metabolically active, accumulating SA-β-gal and releasing a plethora of secreted proteins called the senescence-associated secretory phenotype (SASP), which include growth regulatory factors, proinflammatory cytokines, and factors involved in extracellular matrix (ECM) turnover [85].

Senescence also plays important roles in tissue repair, a point quite pertinent for endometriosis, which is fundamentally wounds undergoing ReTIAR [69-73]. In particular, induction of senescence following liver damage [10] and cutaneous wound [11] is shown to prevent excessive fibrosis. Increased production of senescence-linked fibrolytic enzymes and enhanced immune-regulated clearance of injury-expanded cell populations help to abrogate fibrosis [10]. Ectopic expression of CCN1, which induced cardiac senescence, decelerated fibrogenesis [29,30]. In contrast, genetic ablation of p53 and p16—two proteins known to promote senescence [28,85], promoted fibrogenesis [86,87].

Given the role of senescence in limiting fibrosis [10] and the synergistic effect of D+Q [36], our choice of joint treatment with STS and TMP was based on the thinking that perhaps STS might induce senescence in endometriotic cells, and the addition of TMP might help remove those senescent cells, thus facilitating the antifibrotic effect. Unfortunately, our results suggest that no such synergistic effect exists when STS and TMP were used jointly. Instead, our data suggest that the mode of action of the two compounds appears to be similar, hence the

additional compound did not yield further beneficial therapeutic effect. Future studies are warranted to see whether the addition of quercetin to either STS or TMP would have any synergistic effect.

CCN1 overexpression has been reported in endometriosis [88,89]. CCN1 is known to mediate fibroblast senescence as a wound healing response to injury to limit fibrosis during wound healing [11,30,90]. Our data suggest that TMP treatment increases CCN1 expression, which may induce senescence and thus restraining lesional fibrogenesis.

Our study has two notable strengths. First, we employed a recently established mouse model of deep endometriosis that recapitulates faithfully the most conspicuous features of the human condition [4]. The use of an animal model that resembles most closely with the real human condition should help improve the chance of success in later clinical trials [4]. Second, through testing the hypothesis that senescence may stall the progression of endometriotic lesions and the careful choice of select markers for immunohistochemistry, we have pieced together several bits of information from disparate sources, embedding them into the context of lesional progression and fibrogenesis.
Our study also has a few limitations. First, this study only employed immunohistochemistry and histochemistry analyses, and did not provide direct evidence that TMP treatment induces cellular senescence in endometriotic lesions, and whether it also activates the p53, CCN1, Sav1 or HAS2 signalling pathways. Hence the best we can say is that our data are highly consistent with the documented changes. Also due to the methods we used, only the senescence pathway was explored. Further research is warranted to clearly delineate the mechanism of action for TMP. Second, since we did not stain p16 and p21, whether the TMP treatment has any effect on the p16 or p21 pathway is unknown. Lastly, our data only provide highly suggestive, but certainly not conclusive, evidence that senescence plays a role in restricting lesional progression even though our data are coherently consistent with the hypothesis, and agree with many published studies. Further mechanistic research is warranted.

Conclusions

We have shown in this study that, similar to STS, TMP has therapeutic potentials in treating deep endometriosis as it stalls lesional progression via inducing the cellular senescence by the activation of p53, Sav1 and CCN1 while suppressing HAS2, Survivin and GM-CSF, leading to reduced cellular proliferation as well as decreased infiltration of M2 macrophages in lesions. The joint use of TMP and STS does not seem to further enhance the treatment effect. These results, taken together, provide evidence that induced cellular senescence may be antifibrotic in endometriotic lesions, and the activation of the senescence pathway, through treatment with either TMP or other compounds, may hold great potential in the treatment of endometriosis.

Conflict Of Interest

The authors declare no conflict of interest.

Funding

This research was supported in part by grants 82071623 (SWG) and 81871144 (XSL) from the National Natural Science Foundation of China, an Excellence in Centers of Clinical Medicine grant (2017ZZ01016) from the Science and Technology Commission of Shanghai Municipality, and grant SHDC2020CR2062B from Shanghai Shen Kang Hospital Development Center.

Ethics Statement

All experiments were performed under the guidelines of the U. S. National Research Council's Guide for the Care and Use of Laboratory Animals [91] and were approved by the Institutional Experimental Animals Review Board of Shanghai OB/GYN Hospital, Fudan University.

Author Contributions

S.W.G. conceived and designed the study, performed data analysis and data interpretation, and drafted the manuscript. M.L., X.C. and X.L. performed all the experiments and carried out initial data analysis. D.Y. was involved in data management, interpretation and figure preparation. All participated in the writing and approved the final version of the manuscript.

Acknowledgments

This research was supported in part by grants from the National Science Foundation of China (81771553 and 82071623 to SWG; and 81871144 to XSL), an Excellence in Centers of Clinical Medicine grant (2017ZZ01016) from the Science and Technology Commission of Shanghai Municipality, and grant SHDC2020CR2062B from Shanghai Shen Kang Hospital Development Center.

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