Original Paper

Suppression of STAT3 Signaling by Δ^9 -Tetrahydrocannabinol (THC) Induces Trophoblast Dysfunction

Xinwen Chang^a Yiding Bian^a Qizhi He^b Julei Yao^a Jingping Zhu^c Jinting Wu^a
Kai Wang^a Tao Duan^{ac}

^aClinical and Translational Research Center, ^bDepartment of Pathology, ^cDepartment of Obstetrics, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, China

Key Words

Marijuana/Cannabis • Δ^9 -tetrahydrocannabinol/THC • Perinatal outcomes • Trophoblast dysfunction • Signal Transducer and Activator of Transcription 3/STAT3

Abstract

Aims: Marijuana is a widely used illicit drug and its consumption during pregnancy has been associated with adverse reproductive outcomes. The purpose of this study was to determine the effects of chronic intake of Δ^9 -tetrahydrocannabinol (THC), the major component of marijuana, on trophoblast function, placental development, and birth outcomes. **Methods:** The pathological characteristics and distribution of cannabinoid receptors in placenta were observed by immunohistochemical (IHC) staining. Cell migration in response to THC was measured by transwell assays. The levels of cannabinoid receptors and Signal Transducer and Activator of Transcription 3 (STAT3) were detected by western blot. **Results:** We found the placenta expressed two main cannabinoid receptors, suggesting that THC induced biological responses in placental cells. Supporting this hypothesis, we observed dramatic alterations of placental morphology in marijuana users. Using THC and inhibitors of cannabinoid receptors, we demonstrated that THC impaired trophoblast cell migration and invasion partly via cannabinoid receptors. Additionally, pregnant mice injected with THC showed adverse reproductive events including reduced number of fetuses, lower maternal and placental weights. Mechanistically, STAT3 signaling pathway was involved in the THC-induced suppression of trophoblast cell motility and pregnancy outcomes. **Conclusion:** Our study indicates that the STAT3 signaling pathway plays a critical role in THC-induced trophoblast dysfunction.

Introduction

© 2017 The Author(s)
Published by S. Karger AG, Basel

Marijuana, one of the most frequently used illicit drugs around the world, is made from the whole plant (dried leaves, flowers, stems and seeds) of the hemp plant (*Cannabis sativa*) [1]. In 2013, 7.5% of the US population reported using marijuana within the last month of survey [2]. Currently, more than 60 types of Cannabinoids, the terpenophenolic constituents

X. Chang and Y. Bian contributed equally to this paper.

Kai Wang, M.D., Ph.D.
and Tao Duan, M.D., Ph.D.

Clinical and Translational Research Center and Department of Obstetrics, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, No. 536, Changle Road, Jing'an District, 200040, Shanghai, 200040 (China)
E-Mail kaiwangcn@yahoo.com / tduan@yahoo.com

of the Cannabis plant, have been identified. The major cannabinoid component, delta-9-tetrahydrocannabinol (Δ^9 -THC, often referred to just as THC), is primarily responsible for the psychoactive effects of marijuana [3]. THC also produces a variety of negative effects including tachycardia and impairment of memory and motor function [4]. These effects of THC are mainly mediated by binding to CB1R (cannabinoid receptor 1, brain-type) [5] and CB2R (cannabinoid receptor 2, spleen-type) [6], two major cannabinoid receptors. CB1R is abundant in the central nervous system also presents in peripheral tissues including the heart, uterus, embryo, placenta, and peripheral blood lymphocytes. CB2R is predominantly located in the spleen and immune cells where it may regulate inflammatory responses [7].

The global reported prevalence rates of marijuana use during pregnancy vary from 2% to 27%, depending on the different populations studied and methods of surveying marijuana usage [8, 9]. As more states have passed laws legalizing marijuana for recreational and medical use in United States, the rate of marijuana usage during pregnancy will likely grow. Although the placenta acts as a barrier to protect the fetus from toxic chemicals present in the maternal bloodstream [10], THC readily crosses the placenta and enters into the fetal bloodstream [11]. Consequently, THC may act to alter the normal function of placental trophoblasts, fetal development and influence the growth of newborns as it is also detected in breast milk [12]. Not surprisingly, marijuana use before and/or during pregnancy has been implicated in producing negative pregnancy outcomes such as abortion [13], preterm birth [14] and fetal growth restriction [15, 16].

Signal transducer and activator of transcription 3 (STAT3), a member of the STAT families, is activated mainly through STAT3 phosphorylation on Tyr705. STAT3 activation is involved in regulating essential cellular functions including cell motility, a key cellular activity required for proper placental development, in many cell types [17]. Placental development requires a precise coordination of cellular proliferation, differentiation and migration, and importantly the invasion of trophoblast cells into the endometrium to establish a fetal blood supply. It has been shown that STAT3 activation was essential for trophoblast cell invasion [18], in addition, THC suppressed STAT3 activation to regulate cell survival [19]. Given these observations, we hypothesized that exposure to marijuana during pregnancy may result in placental dysfunction. In this study, we provide new evidence that chronic THC exposure is associated with adverse reproductive events, which are likely facilitated by THC-induced inhibition of STAT3 signaling that leads to trophoblast cell dysfunction.

Materials and Methods

Patients and sample collections

Study participants were recruited at the Department of Obstetrics, Shanghai First Maternity and Infant Hospital from 2013 to 2016. The normal pregnant women (n=5) and marijuana users (n=3) occurred at the time of admission for elective cesarean delivery at term (38-40 weeks). Marijuana users were defined as pregnant women who smoking marijuana at least once a month without using alcohol or tobacco during pregnancy. The healthy women in first trimester (n=5) who underwent induced abortions were not marijuana users. Women with any adverse underlying medical condition (including hypertension, preeclampsia, gestational diabetes mellitus) were excluded. The demographic and clinical characteristics of this study were recorded (Table. 1). Gestational age was calculated using menstrual dates or ultrasound. Sample collection was approved by the Research and Ethics Committee of Shanghai First Maternity and Infant Hospital. Placental tissues were obtained from control pregnant subjects and marijuana users immediately (< 30min) after delivery by caesarean section. The chorionic villi samples in the first trimester of pregnancy were obtained and dissected out immediately after vacuum aspiration then washed in sterile PBS. Small pieces (~0.5cm³) were cut from the fetal part of the placenta or villi and washed briefly in sterile PBS to remove maternal blood contamination. All the samples were frozen within 20 min of delivery and stored in liquid nitrogen for Western blot and real-time PCR analysis. Additional placental tissues from first and third trimester pregnancies were fixed at 4°C in methanol and embedded in paraffin for hematoxylin & eosin (H&E) and immunohistochemical (IHC) staining.

Table 1 Characteristics of pregnant women and patients exposed to marijuana. All the data are expressed as mean $\pm$ S.E.M. * $p < 0.05$

	First Trimester (N = 5)	Third Trimester (N = 5)	Marijuana Users (N = 3)
Patient age (year)	27.6 $\pm$ 3.84	29.6 $\pm$ 2.04	26.00 $\pm$ 2.00
Gestation age (week)	6.6 $\pm$ 0.42	38.34 $\pm$ 0.45	38.55 $\pm$ 1.55
Fetal weight (g)	-	3267 $\pm$ 127.9	2673.50 $\pm$ 178.62*

Drugs

Δ^9 -tetrahydrocannabinol (THC, 10mg/mL in ethanol solution) was purchased from Cerilliant (Sigma-Aldrich, St. Louis, MO, USA) and stored at -20°C . SR141716 (Rimonabant) was from Selleckchem (Houston, TX, USA). SR144528 was purchased from SANOFI Research (Montpellier, France). Cryptotanshinone (CTP) was purchased from Selleckchem (Houston, TX, USA). They were initially dissolved in DMSO to a concentration of 10mM and stored at -80°C .

H&E and IHC

Placental tissues were immersion-fixed in 4% buffered formalin and then transferred to paraffin. Tissue sections of 3-5 μm in thickness were cut from the paraffin-embedded tissues, and mounted on poly-L-lysine-coated slides and then deparaffinized in xylene, dehydrated in alcohol then stained with the H&E. Some sections were stained for CB1R (1:100; Abcam, Cambridge, MA), CB2R (1:100; Abcam) and pSTAT3 (1:100; Cell Signaling Technology (CST) Danvers, MA), respectively using the streptavidin-biotin-horseradish peroxidase complex formation method.

RNA extraction and cDNA synthesis

Total tissue RNA was extracted by using the TRIzol reagent (Invitrogen™, Carlsbad, CA, USA) and the RNA simple Total RNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The concentration of RNA was quantified using the NanoDrop 2000c Spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The purity of the RNA extracts was high as indicated by the A260/280 nm ratios that ranged from 1.9–2.1. The RNA was treated with RNase-free DNase (Tiangen) before reverse transcription to eliminate amplification of residual amounts of genomic DNA in the RNA extracts. The RNA was converted to cDNA using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). The reaction conditions were performed at 37°C for 20 minutes followed by 85°C for 5 seconds on an Eppendorf thermocycler.

Quantitative RT-PCR analysis

Real-time quantitative reverse transcription–polymerase chain reaction (qPCR) was carried out to evaluate the levels of CB1, CB2 receptor mRNA expression using the SYBR Green Premix Ex Taq (Tiangen) on an ABI Prism 7000 Sequence Detection System (Life Technology). The primer sequences for real-time PCR analysis are as follows: human CB1 receptor – forward primer 5'-TTACAACAAGTCTCTCTCGTCCT-3', and reverse primer 5'-GGCTGCCGATGAAGTGGTA-3'; human CB2 receptor – forward primer 5'-GGGTGACAGAGATAGCCAATGG-3', and reverse primer 5'-TGAACAGGTATGAGGGCTTCC-3'; The relative levels were normalized to the control human GAPDH mRNA, the primer sequence was forward 5'-AGGGCTGCTTTTAACTCTGGT-3' and reverse 5'-CCCCACTTGATTTTGGAGGGA-3'. Mouse CB1 receptor – forward primer 5'-CTTAGACGGCCTTGCAGATAC -3', and reverse primer 5'-GGATGCCATGTCTCCTTTGATA -3'; Mouse CB2 receptor – forward primer 5'-GGGTCTCTCAGCATTGATTT-3', and reverse primer 5'-AGCCCAGTAGGTAGTCGTTAG-3'; The relative levels were normalized to control mouse GAPDH mRNA, the primer sequences were forward 5'-CAGTATGACTCCACTCACGGCAA-3' and reverse 5'-CTCGCTCTGGAAGATGGTGAT-3'. The real-time PCR reactions were carried out for 30s at 95°C and then 40 cycles of 15s at 95°C and 20s at 56°C . To confirm the amplification specificity, the PCR products were subjected to melting curve analyses. Relative mRNA levels were calculated using the $2^{-\Delta\Delta\text{CT}}$ method.

Western Blot analysis

For analysis of cannabinoid CB1R, CB2R, STAT3 and pSTAT3^{Y705} expression, placental tissues and HTR-8/SVneo and BeWo cells were lysed in RIPA Lysis buffer, then homogenized by vigorous mixing for 30 min on ice, and centrifuged at 13000 $\times$ g for 20min. Total protein concentration was measured using

the bicinchoninic acid assay (Pierce®, Thermo Fisher Scientific, Bonn, Germany). Proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gels. Following transfer to PVDF membranes and blocking with 5% non-fat milk powder, blots were probed with the following antibodies, CB1R (1:1000, Abcam), CB2R (1:1000, Abcam), STAT3 (1:2000, CST), p-STAT3^{Y705} (1:1000, CST), β -actin (1:5000, Abmart, Shanghai, China) and GAPDH (1:10000, Abmart) at 4°C overnight. Subsequently, membranes were washed and incubated with horseradish peroxidase-conjugated Fab-specific anti-mouse IgG (1:5000, CST) or anti-rabbit IgG (1:3000, CST). Ultimately, proteins were visualized using the enhanced chemiluminescence reagents (Thermo Scientific), and the relative expression of CB1R, CB2R, STAT3 and pSTAT3^{Y705} protein levels were analyzed by densitometry using the Image-J imaging analysis software (NIH, Bethesda, MD).

Cell culture

HTR-8/SVneo and BeWo human trophoblast cell lines obtained from ATCC (Rockville, MD, USA) were maintained (passages 10-20) at 37°C in a humidified atmosphere with 5% CO₂ and 95% air. Cells were cultured in 75-cm² flasks in DMEM/F-12(1:1) medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco) and 1% P/S.

Cell proliferation assay

To test the effect of THC on HTR-8/SVneo and BeWo cell proliferation, 3 x 10³ cells per well were plated in 96-well culture plates in 100 μ L of DMEM/F12 medium. The medium was changed to one that contained different doses of THC (0–30 μ M) for 48 and 96h with fresh medium replaced at 48h. After 48 and 96h, relative cell numbers were determined using the MTS (Promega) reagent in a 96-well plate reader at 490nm. Cell numbers were obtained by calibration against a standard curve of untreated HTR-8/SVneo and BeWo cell numbers grown in parallel. To make direct comparisons between cultures, we then converted the cell numbers to a percentage of the 1% methanol control cells.

Cell migration and invasion assay

Migration of HTR-8/SVneo and BeWo cells was determined using a modified system according to the manufacturer's instructions (BD Bioscience, Heidelberg, Germany). In this assay, cell migration towards a chemoattractant was monitored using a 24-well transwell plate with insert of 8 μ m in pore size. In brief, 5 x 10⁴ cells were seeded on the upper side of the transwell inserts in serum free medium. DMEM/F12 containing 10% FBS supplemented with test substances or vehicle alone were used as chemoattractant in the companion plate. Following incubation at 37°C with 5% CO₂ and 95% air for 16 hours, the fluorescent stain (calcein-AM) was added to each chamber and incubated for 30 minutes. The numbers of migrated cells was determined by fluorescence image analysis (Nikon, Japan).

The capacity of HTR-8/SVneo cells to invade Matrigel was determined using 24-well transwell plates (BD Bioscience, Becton Dickinson GmbH, Heidelberg, Germany). In this assay, the upper chamber of the insert was pre-coated with 60 μ L of a 1:10 dilution of Matrigel (BD Biosciences) in standard medium for 60 min at 37°C. After hydration by PBS for 60 min, 1.5 x 10⁵ cells were seeded into the upper side of the transwell inserts in serum free medium. DMEM/F12 containing 10% FBS was used as chemoattractant in the companion plate. Following incubation at 37°C with 5% CO₂ and 95% air for 24 hours, fluorescent stain was added to each chamber and incubated for 30 minutes. The numbers of invading cells were counted by fluorescence analysis (Nikon, Japan).

Animal studies

For a consistent and accurate assessment of the gestational age of mouse embryos, C57BL/6 males (n=20, 18-22g) and females (n=40, 18-22g) mice were pair housed (two females: one male) for one night and the next morning females were examined for the presence of a vaginal plug. The day when a vaginal plug was first noted was designated day 0.5 (GD0.5). Pregnant mice (n=14) were equally divided into two groups randomly and injected intraperitoneally with THC (5 mg/kg) or vehicle from GD5.5 to GD18.5 daily. Embryos were harvested on GD19.5, and placental tissues were processed as described previously. This project was approved by the Animal Investigation Ethics Committee of the East China Normal University, and mouse studies were performed in accordance with animal protocol procedures approved by the Department of Laboratory Animal Science, East China Normal University.

Statistics

All data are expressed as the mean $\pm$ the standard error of the mean (S.E.M) and analyzed using the SPSS 23.0 statistical analysis software (SPSS Inc., Chicago, IL, USA). Statistical significance was determined using a one-way analysis of variance (ANOVA) or two-way ANOVA. A p-value ≤ 0.05 was considered statistically significant.

Results

Histopathological changes and cannabinoid receptors expression in placental tissues of marijuana users

Few studies have examined the morphologic changes induced in the placenta by marijuana at the microscopic level. Therefore, we screened placental tissues for histopathologic changes in three marijuana users by H&E staining. Compared with normal controls (non-users) (Fig. 1a), marijuana user placentas showed increased syncytiotrophoblastic knots and fibrin exudation in the villous stroma. In addition, women who consumed marijuana had babies that weight less than non-users (Table. 1). The results illustrate that pregnant women who smoked marijuana at least once a month without reporting alcohol or tobacco use during pregnancy had an impaired placental development, which may lead to adverse pregnancy outcomes.

IHC analysis of human placental villous tissue obtained at about 7-week of gestation revealed that cannabinoid receptors are abundant in both syncytiotrophoblasts and

Fig. 1. Histopathological characteristics and expression of cannabinoid receptors in placentas. a H&E staining of placental tissues. Increased syncytial knots (arrowheads) and fibrin exudation in the villous stroma (arrows) of placental tissues of pregnant women taking marijuana. b Immunohistochemical localization of CB1R and CB2R in first trimester and term placentas of control pregnant women and placental tissue from marijuana users. Brown color indicates positive staining for the cannabinoid receptors. Arrows: syncytiotrophoblasts; arrowheads: cytotrophoblast. c,d qPCR and western blot analysis for CB1R and CB2R in human placental tissues from first trimester and third trimester pregnancies. Magnification 400X. Data are expressed as the mean $\pm$ S.E.M. fold of third trimester after normalization to GAPDH (One-way ANOVA, *p<0.05).

Fig. 2. Effect of THC on cell proliferation, migration and invasion. **a** Cell growth analysis was performed after treatment with THC for 48 and 96 hours in HTR-8/SVneo and BeWo cells. **b,c** Migration was analyzed in HTR-8/SVneo and BeWo cells with or without THC treatment. **d** Invasion was analyzed in HTR-8/SVneo cell treated with THC. The graph reports the mean percentage fold relative to methanol control values $\pm$ S.E.M of three independent experiments. (One-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

cytotrophoblasts (Fig. 1b). The positive brown staining of cannabinoid receptors in the placental tissues show that both CB1R and CB2R are expressed in the placenta tissues. IHC showed that CB1R and CB2R levels were decreased in the placenta of marijuana users compared with normal pregnant women. Next, qPCR and western blotting were used to determine the relative expression of CB1R and CB2R mRNA and protein levels in the placenta. No significant differences of CB1R were found between the first and third trimester placental tissues of non-users, but CB2R mRNA (Fig. 1c) and protein were (Fig. 1d) increased in the third trimester tissues, suggesting that CB2R expression is positively correlated with pregnancy progression.

Effect of THC on HTR-8/SVneo and BeWo cell proliferation, migration and invasion

Since appropriate trophoblast function is critical for normal placental development and pregnancy maintenance, we selected the most widely used trophoblast cell lines, HTR-8/SVneo and BeWo, to determine the effect of THC on cell proliferation, migration and invasion. The THC concentrations used in our studies ranged from 0.3 to 30 μ M, which was similar with previously study used *in vitro* [20]. Our data indicated that THC at low doses (0-15 μ M) did not affect cell proliferation whereas 30 μ M significantly induced cell death (Fig. 2a).

Furthermore, we performed trans-well assays to study the impact of THC on trophoblast cell migration and invasion. THC at a concentration of 3 μ M had no effects on either cell migration or invasion. However, 15 μ M of THC significantly impaired HTR-8/SVneo and BeWo cell migration (Fig. 2b-c), as well as HTR-8/SVneo cell invasion (Fig. 2d) compared to vehicle treated cells. In this assay, BeWo cells showed weak invasive potential on Matrigel, therefore the effects of THC on BeWo invasion was not tested. Taken together, these data suggest that THC disrupts placental development by impairing the migration and invasion capacity of trophoblasts.

Fig. 3. Involvement of cannabinoid receptors in THC-mediated inhibition of placental trophoblast cell migration and invasion. **a** Cannabinoid receptors expression levels in HTR-8/SVneo and BeWo cells after treatment with THC for 0, 12, 24, 48 hours. **b,c** Migration was analyzed in HTR-8/SVneo and BeWo cells pre-treated with SR141716, SR144528 or both for 1 hour and then treated with THC. **d** Invasion was analyzed in HTR-8/SVneo cell pre-treated with SR141716, SR144528 or both for 1 hour then treated with THC. The graph reports the mean percentage fold relative to vehicle control values $\pm$ S.E.M of three independent experiments. (Two-way ANOVA, *** $p < 0.001$ vs control, # $p < 0.05$ vs THC group).

Involvement of cannabinoid receptors in THC-induced changes in placental trophoblast cells

Although THC serves as an agonist of CB1 and CB2 receptors, the mechanism by which trophoblasts respond to THC upon receptor binding is not clear. To begin to answer this question, we first examined the protein levels of CB1R and CB2R before and after treatment of THC. We did not observe significant differences between HTR-8/SVneo and BeWo cells after the treating with THC (Fig. 3a), suggesting that changes in cannabinoid receptor expression are not required for trophoblasts to respond to THC. Next, we analyzed the effect of SR141716 (CB1 receptor antagonist) [21], and SR144528 (CB2 receptor antagonist) [22] on cell motility and invasion. Both of them had no obvious effect on trophoblast cell survival. In addition, migration and invasion responses to SR141716 or SR144528 alone or both were not different from that of vehicle treated cells. Although THC reduced the number of migrated cell number compared with the vehicle control, the effect of THC was reversed by pre-treating with the receptor antagonists (Fig. 3b-d). Hence, pre-treatment with cannabinoid receptor inhibitors can slightly attenuate the response to THC, which means that the effect of THC on cell migration and invasion is partly dependent on CB1R and CB2R.

The STAT3 signaling pathway is involved in THC-induced trophoblasts dysfunction

Due to dynamic changes in the phosphorylation status of proteins, we examined STAT3 phosphorylation at early time points (1, 2 and 4h) after THC stimulation of trophoblast cells. As shown in Fig. 4a-b, incubation of HTR-8/SVneo and BeWo cells with 15 μM of THC led to decreased phosphorylation of STAT3^{Y705}. To clarify the role of the STAT3 signaling pathway in THC-mediated impairment of trophoblast migration, trophoblast cell migration assays were conducted in the presence of cryptotanshinone, a selective STAT3^{Y705} inhibitor, which

Fig. 4 The STAT3 signaling pathway is involved in THC-induced trophoblast dysfunction. a,b Western blot analysis of pSTAT3^{Y705} levels in HTR-8/SVneo and BeWo cells after the treatment with THC for 0,1,2, and 4 hours. c,d Western blot analysis of pSTAT3^{Y705} levels in HTR-8/SVneo and BeWo cells after the treatment with cryptotanshinone for 0,1,2, and 4 hours. e Migration analysis of HTR-8/SVneo and BeWo cells treated with cryptotanshinone for 2 hours. (One-way ANOVA, ** $p < 0.01$, *** $p < 0.001$).

Table. 2 Perinatal outcomes of mice exposure to THC. All the data are expressed as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$

	Control (N=7)	THC (5mg/kg) (N=7)
Body weight (g) (GD 0.5)	20.08 $\pm$ 0.56	21.02 $\pm$ 0.29
Body weight (g) (GD 19.5)	36.28 $\pm$ 1.01	32.94 $\pm$ 0.82 *
Number of fetus (per litter)	10.75 $\pm$ 0.73	8.00 $\pm$ 0.76 *
Weight of fetus and placenta (g/litter)	11.56 $\pm$ 0.67	7.72 $\pm$ 0.93 **
Average weight of fetus and placenta	1.10 $\pm$ 0.03	0.92 $\pm$ 0.03 *

was isolated from *Salvia miltiorrhiza Bunge* (Danshen) [23]. Compared with the control DMSO treated cells, p-STAT3^{Y705} protein levels after treatment with cryptotanshinone was significantly reduced (Fig. 4c-d). Additionally, we observed a significant reduction in the migration potential of HTR-8/SVneo and BeWo cells treated with cryptotanshinone (Fig. 4e). Moreover, our previous researches have demonstrated that overexpression of STAT3 obviously improved the migration of HTR-8/SVneo trophoblast cells [24]. These results above suggest that STAT3, or signaling events upstream of STAT3, might be targeted by THC *in vitro*.

Effect of THC exposure on pregnancy outcomes in mice

Pregnant mice were randomly injected with a THC dose of 5mg/kg or diluent from GD 5.5 to 19.5. The training dose of 5mg/kg was chosen because the plasma THC concentration

Fig. 5. Effect of THC on mouse reproductive outcomes. Effect of THC exposure on body weight of pregnant mice on GD 0.5 and GD 19.5 (a), number of fetuses per litter (b), morphology of fetuses and placentas (c) and weights of fetuses and placentas (d). (One-way ANOVA, * $p < 0.05$).

produced by this dose is similar to that described for human marijuana smokers [25]. The characteristics of mice pregnancy outcomes are shown in Table 2. The body weight of pregnant mice on GD 19.5 (Fig. 5a) was significantly decreased in the THC treated group. This decrease in weight was most likely caused by the decreased numbers of fetuses per litter (Fig. 5b). In addition, the total weights of both the fetuses and placentas and the average weights were clearly reduced (Fig. 5c-d). The results are in agreement with previous epidemiological surveys and animal studies [2], indicating that exposure to marijuana during pregnancy may result in adverse reproductive outcomes including fetal loss and intrauterine growth restriction.

Effect of THC on placental histopathology and expression of cannabinoid receptors and phosphorylation of STAT3 *in vivo*

To explore the potential mechanism of adverse pregnancy events in marijuana users, H&E stained placental tissues obtained from THC treated and vehicle treated mice were histologically examined (Fig. 6a). Histologic differences in the placenta of THC treated mice were obvious relative to control mice. The placentas of THC treated animals showed clear narrowing of the maternal sinusoids (Fig. 6b, MS) with increased diameters of trophoblastic septa in the labyrinth zone. In addition, the spongiotrophoblasts were disordered in structure and there were significantly fewer glycogen cells in the basal zone (Fig. 6c, Sp, GC). Collectively these histologic changes lead to poor blood and nutrition supply to the developing fetuses in THC exposed animals.

Importantly, we found the activation of STAT3 is inhibited in response to THC *in vivo*, including the mice exposed to THC (Fig. 6d-e) and in women who used marijuana during pregnancy (Fig. 6g). Interestingly, we observed that CB1R and CB2R mRNA and protein levels were lower in the THC exposed group (Fig. 6d, f).

Discussion

Currently, recreational use of marijuana is common among people of reproductive age. However, most studies about chronic substance exposure are related to cigarette smoking [26], alcohol consumption [27] and cocaine addiction [28] while the hazards of chronic marijuana use especially the effects on trophoblast function and its potential mechanism of

Fig. 6. Effect of THC on placental histopathology and expression of cannabinoid receptors and phosphorylation of STAT3 *in vivo*. a-c H & E staining of mouse placentas (MS, maternal sinusoid; Arrowheads: trophoblastic septa; GC, glycogen cell; Sp, spongiotrophoblast). d,e Western blot analysis of STAT3 activity in mouse placentas. d,f mRNA and protein levels of cannabinoid receptors in mouse placental tissues. g IHC analysis of pSTAT3^{Y705} expression in human placental tissue. Magnification 40X (a), 200X (b,c) and 400X (g). (One-way ANOVA, * $p < 0.05$).

influencing pregnancy outcomes is not well researched. THC as one of the major bioactive cannabinoids, has been widely used to study the effects of marijuana on various physiology and pathology processes [29], thus we provided more insight into the effects of THC on the biological function of human trophoblasts and the potential mechanism underlying the influence of marijuana exposure on placental development during pregnancy.

In this study, we observed increased syncytiotrophoblastic knots and fibrin exudation in the villous stroma of marijuana users by H&E staining, suggesting that marijuana exposure impaired placental development and lead to the changes observed in placenta structure and morphology. These morphologic changes are most probably triggered by an inflammatory response and/or poor supply of nutrients and oxygen when the placental tissue is exposed to THC. In order to eliminate the effects of other potential confounding factors, we selected only three marijuana smokers without tobacco or alcohol addiction. Previously, studies focused on placental pathological characteristics reported that marijuana exposure was associated with larger placenta [30] and no significant differences in the placental villi between controls and marijuana using mothers [31], while potential effects of marijuana exposure on placental development were still largely unknown. Their findings were not consistent with our present results. Differences in the volume and frequency of marijuana intake and the way study subjects took marijuana in prior studies could explain the lack of agreement between

different studies. Costa et al. [32] showed that THC interferes with trophoblast turnover and impaired cytotrophoblasts (CTs) differentiation into syncytiotrophoblasts (STs) in women that consume marijuana during gestation.

In mouse models, we demonstrated that intake of THC during pregnancy resulted in adverse reproductive outcomes. As in human placental specimens from marijuana users, we observed histopathologic changes in the placentas obtained from THC treated mice. We found severe narrowing of the maternal sinusoids and increased diameters of trophoblastic septa in labyrinth zone, which indicated an increased diffusion distance between the fetal and maternal blood supplies that led poor nutrition to the developing fetus [33]. In addition, the disordered structure of spongiotrophoblasts and decreased number of glycogen cells in basal zone were likely responsible for adverse pregnancy outcomes.

It has been reported that cannabinoid receptors are expressed in many organs and tissues including the lung, heart, uterus, embryo and various types of cells particularly in cancer cells. A body of evidence indicates that cannabinoids mediate their biological effects through specific cannabinoid receptors. In this study, we demonstrated that CB1R and CB2R were highly expressed in human placental tissues of both the first and third trimesters. In addition, CB1R and CB2R expression levels appeared to increase at later stages of pregnancy, indicating that endocannabinoid signaling may play an important role in placental development. Indeed endocannabinoid signaling has been widely reported to play a crucial role in reproductive processes including fertilization, implantation, and embryonic growth [34, 35]. Interestingly, we observed that CB1R and CB2R levels in the placenta were significantly decreased in mice injected with THC. Contrary to this, we observed CB1R and CB2R levels were unaffected by THC in cultured trophoblast cells. Both the THC concentration differences and duration of THC exposure to trophoblast cells could explain the discrepancies in CB1R and CB2R expression between *in vitro* and *in vivo* experiments upon THC treatment. The lower cannabinoid receptor levels observed were probably related to desensitization of cannabinoid receptors in placentas following the chronic THC treatment. This rationale is consistent with a previous study conducted on the mouse hippocampus [36].

It was well known that extravillous cytotrophoblast (EVT) invasion is a complex process including attachment to the extracellular matrix (ECM), releasing proteolytic enzymes to break down the ECM then moving through to invade into the decidua. Neither excessive or shallow invasion are implicated in placental dysfunction, which is responsible for pregnancy-related disorders [37]. Manjiri et al. [20] found that concentrations of Δ^9 -THC equivalent to those found in the serum of cannabis users (i.e. $\sim 20 \mu\text{M}$) inhibited proliferation of BeWo trophoblast cell line. Our present data indicated that THC ($15 \mu\text{M}$) greatly impaired the biological activity of human trophoblasts, specifically trophoblast migration and invasion. Furtherly, we also confirmed that THC-impaired trophoblast functions were partly mediated by CB1R and CB2R, two main cannabinoid receptors. However, other receptors such as the transient receptor potential cation channel subfamily V member 1 (TRPV1) and the orphan G protein-coupled receptor GPR55 have been proposed to act as endocannabinoid receptors. Current knowledge about GPR55 expression and function in human placenta is very limited, gene expression analysis revealed low GPR55 levels in human placenta, when compared to spleen and lung. Moreover, expression analysis showed increased placental GPR55 expression at term compared to first trimester [38]. Therefore, the effect of THC on trophoblast functions may be mediated by other cannabinoid receptors. This finding was in agreement with previous reports showing that several cannabinoids, including THC and synthetic cannabinoid agonists, were shown to inhibit tumor growth and progression of several types of cancers, such as breast and liver cancers [39]. Studies also showed that CB1R and CB2R were novel targets for inhibition of non-small-cell lung cancer growth and metastasis [40]. The studies of trophoblast function have revealed the striking similarities in molecular circuits between trophoblasts and cancer cells, which may be primarily related to the inherent invasive nature of both cell types.

We shed light on the potential mechanism by which THC modulated human trophoblast biological functions. The STAT3 signaling pathway is involved in the process of cell division,

migration and invasion of various cell types. Moreover, STAT3 is detected in primary trophoblast cells isolated from first-trimester placenta and the activity is essential for implantation and pregnancy progression as STAT3^{-/-} knockout embryos degenerate and die [41]. Our data showed that STAT3 activation in trophoblasts was remarkably inhibited after exposure to THC. Therefore, we suggested that THC reduced the migration and invasion of human trophoblast cells probably via suppression of STAT3 activation. This was also supported by researches that interleukin-6 (IL-6) greatly increased trophoblast cell migration and invasion via activation of STAT3 signaling pathway [42]. Importantly, we also observed that the STAT3 phosphorylation levels in the placenta were significantly decreased in mice injected with THC and women exposed to marijuana, which was consistent with our *in vitro* results.

We revealed that THC impaired trophoblast cell migration and invasion through regulating the activation of STAT3 without affecting cell proliferation. Moreover, our study demonstrated that THC caused adverse reproductive events in pregnant mice. In conclusion, marijuana exposure during pregnancy does harm placental and fetal development as THC can impair trophoblast cell migration and invasion through the STAT3 signaling pathway.

The limitations of our study are inherent in research associated with toxicology exposure in pregnancy. First, although most medical histories of marijuana use during pregnancy are collected, but multiple confounding factors (including predominantly lifestyle factors, details of marijuana preparations and the use of marijuana in combination with other drugs) detract from our ability to establish causality. Second, marijuana and other substance use were self-reported and not validated with biological markers. Third, we should collect the information about THC concentration in biological samples (e.g. serum, urine, hair, amniotic fluid, brain fluids) to confirm the exposure amount of marijuana. These above issues probably limited this study to assess the effects of intrauterine exposure to marijuana on placental development.

Acknowledgements

This work was supported by Program for Outstanding Medical Academic Leader of Shanghai (to DT), the National Natural Science Foundation of China (No. 81471461), Program of Shanghai Subject Chief Scientist (No. 14XD1403200), Key Project of Medicine from Science and Technology Commission of Shanghai Municipal (No. 13411950700) and the Key Disciplines Group Construction Project of Pudong Health Bureau of Shanghai (No. PWZxq2014-02).

Disclosure Statement

The authors declare that they have no competing interests.

Reference

- 1 Van Bakel H, Stout JM, Cote AG, Tallon CM, Sharpe AG, Hughes TR, Page JE: The draft genome and transcriptome of *Cannabis sativa*. *Genome Biol* 2011;12:R102.
- 2 Metz TD, Stickrath EH: Marijuana use in pregnancy and lactation: a review of the evidence. *Am J Obstet Gynecol* 2015;213:761-778.
- 3 Vandrey R, Raber JC, Raber ME, Douglass B, Miller C, Bonn-Miller MO: Cannabinoid Dose and Label Accuracy in Edible Medical Cannabis Products. *Jama* 2015;313:2491-2493.
- 4 Hochhauser E, Lahat E, Sultan M, Pappo O, Waldman M, Sarne Y, Shainberg A, Gutman M, Safran M, Ben Ari Z: Ultra Low Dose Delta 9-Tetrahydrocannabinol Protects Mouse Liver from Ischemia Reperfusion Injury. *Cell Physiol Biochem* 2015;36:1971-1981.

- 5 Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI: Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 1990;346:561-564.
- 6 Munro S, Thomas KL, Abu-Shaar M: Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 1993;365:61-65.
- 7 Pertwee RG, Howlett AC, Abood ME, Alexander SP, Di Marzo V, Elphick MR, Greasley PJ, Hansen HS, Kunos G, Mackie K, Mechoulam R, Ross RA: International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB(1) and CB(2). *Pharmacol Rev* 2010;62:588-631.
- 8 Saurel Cubizolles MJ, Prunet C, Blondel B: Cannabis use during pregnancy in France in 2010. *BJOG* 2014;121:971-977.
- 9 Ko JY, Farr SL, Tong VT, Creanga AA, Callaghan WM: Prevalence and patterns of marijuana use among pregnant and nonpregnant women of reproductive age. *Am J Obstet Gynecol* 2015;213:201 e201-201 e210.
- 10 Shen Z, Wu Y, Chen X, Chang X, Zhou Q, Zhou J, Ying H, Zheng J, Duan T, Wang K: Decreased maternal serum 2-methoxyestradiol levels are associated with the development of preeclampsia. *Cell Physiol Biochem* 2014;34:2189-2199.
- 11 Kuczkowski KM: The effects of drug abuse on pregnancy. *Curr Opin Obstet Gynecol* 2007;19:578-585.
- 12 Hill M, Reed K: Pregnancy, breast-feeding, and marijuana: a review article. *Obstet Gynecol Surv* 2013;68:710-718.
- 13 Varner MW, Silver RM, Rowland Hogue CJ, Willinger M, Parker CB, Thorsten VR, Goldenberg RL, Saade GR, Dudley DJ, Coustan D, Stoll B, Bukowski R, Koch MA, Conway D, Pinar H, Reddy UM, Eunice Kennedy Shriver National Institute of Child H, Human Development Stillbirth Collaborative Research N: Association between stillbirth and illicit drug use and smoking during pregnancy. *Obstet Gynecol* 2014;123:113-125.
- 14 Dekker GA, Lee SY, North RA, McCowan LM, Simpson NA, Roberts CT: Risk factors for preterm birth in an international prospective cohort of nulliparous women. *PLoS One* 2012;7:e39154.
- 15 El Marroun H, Tiemeier H, Steegers EA, Jaddoe VW, Hofman A, Verhulst FC, van den Brink W, Huizink AC: Intrauterine cannabis exposure affects fetal growth trajectories: the Generation R Study. *J Am Acad Child Adolesc Psychiatry* 2009;48:1173-1181.
- 16 Janisse JJ, Bailey BA, Ager J, Sokol RJ: Alcohol, tobacco, cocaine, and marijuana use: relative contributions to preterm delivery and fetal growth restriction. *Subst Abus* 2014;35:60-67.
- 17 Luo JY, Fu D, Wu YQ, Gao Y: Inhibition of the JAK2/STAT3/SOSC1 Signaling Pathway Improves Secretion Function of Vascular Endothelial Cells in a Rat Model of Pregnancy-Induced Hypertension. *Cell Physiol Biochem* 2016;40:527-537.
- 18 Liu X, Gu W, Li X: HLA-G regulates the invasive properties of JEG-3 choriocarcinoma cells by controlling STAT3 activation. *Placenta* 2013;34:1044-1052.
- 19 Ngaotepprutaram T, Kaplan BLF, Carney S, Crawford R, Kaminski NE: Suppression by Δ9-tetrahydrocannabinol of the primary immunoglobulin M response by human peripheral blood B cells is associated with impaired STAT3 activation. *Toxicology* 2013;310:84-91.
- 20 Khare M, Taylor AH, Konje JC, Bell SC: Delta9-tetrahydrocannabinol inhibits cytotrophoblast cell proliferation and modulates gene transcription. *Mol Hum Reprod* 2006;12:321-333.
- 21 Huestis MA, Gorelick DA, Heishman SJ, Preston KL, Nelson RA, Moolchan ET, Frank RA: Blockade of effects of smoked marijuana by the CB1-selective cannabinoid receptor antagonist SR141716. *Arch Gen Psychiatry* 2001;58:322-328.
- 22 Rinaldi Carmona M, Barth F, Millan J, Derocq JM, Casellas P, Congy C, Oustric D, Sarran M, Bouaboula M, Calandra B, Portier M, Shire D, Brelviere JC, Le Fur GL: SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J Pharmacol Exp Ther* 1998;284:644-650.
- 23 Shin DS, Kim HN, Shin KD, Yoon YJ, Kim SJ, Han DC, Kwon BM: Cryptotanshinone inhibits constitutive signal transducer and activator of transcription 3 function through blocking the dimerization in DU145 prostate cancer cells. *Cancer Res* 2009;69:193-202.
- 24 Zong S, Li C, Luo C, Zhao X, Liu C, Wang K, Jia W, Bai M, Yin M, Bao S, Guo J, Kang J, Duan T, Zhou Q: Dysregulated expression of IDO may cause unexplained recurrent spontaneous abortion through suppression of trophoblast cell proliferation and migration. *Sci Rep* 2016;6:19916.
- 25 Wasserman E, Tam J, Mechoulam R, Zimmer A, Maor G, Bab I: CB1 cannabinoid receptors mediate endochondral skeletal growth attenuation by Delta9-tetrahydrocannabinol. *Ann N Y Acad Sci* 2015;1335:110-119.

- 26 Jauniaux E, Burton GJ: Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. *Early Hum Dev* 2007;83:699-706.
- 27 Xu T, Yang Q, Liu R, Wang W, Wang S, Liu C, Li J: Ethanol impedes embryo transport and impairs oviduct epithelium. *Toxicology* 2016;357-358:44-51.
- 28 Messiah SE, Ludwig DA, Vidot DC, Accornero VH, Lipshultz SE, Miller TL, Xue L, Bandstra ES: Prenatal Cocaine Exposure and Cardiometabolic Disease Risk Factors in 18- to 20-Year-Old African Americans. *Ethn Dis* 2015;25:419-426.
- 29 Sido JM, Jackson AR, Nagarkatti PS, Nagarkatti M: Marijuana-derived Δ -9-tetrahydrocannabinol suppresses Th1/Th17 cell-mediated delayed-type hypersensitivity through microRNA regulation. *J Mol Med* 2016;94:1039-1051.
- 30 Carter RC, Wainwright H, Molteno CD, Georgieff MK, Dodge NC, Warton F, Meintjes EM, Jacobson JL, Jacobson SW: Alcohol, Methamphetamine, and Marijuana Exposure Have Distinct Effects on the Human Placenta. *Alcoholism: Clin Exp Res* 2016;40:753-764.
- 31 Ortigosa S, Friguls B, Joya X, Martinez S, Marinosa ML, Alameda F, Vall O, Garcia-Algar O: Feto-placental morphological effects of prenatal exposure to drugs of abuse. *Reprod Toxicol* 2012;34:73-79.
- 32 Costa MA, Fonseca BM, Marques F, Teixeira NA, Correia-da-Silva G: The psychoactive compound of Cannabis sativa, Delta(9)-tetrahydrocannabinol (THC) inhibits the human trophoblast cell turnover. *Toxicology* 2015;334:94-103.
- 33 Louei Monfared A, Salati AP: The effects of Carthamus tinctorius L. on placental histomorphology and survival of the neonates in mice. *Avicenna J Phytomed* 2012;2:146-152.
- 34 Battista N, Bari M, Maccarrone M: Endocannabinoids and Reproductive Events in Health and Disease. *Handb Exp Pharmacol* 2015;231:341-365.
- 35 Meccariello R, Battista N, Bradshaw HB, Wang H: Endocannabinoids and reproduction. *Int J Endocrinol* 2014;2014:378069.
- 36 Burston JJ, Wiley JL, Craig AA, Selley DE, Sim-Selley LJ: Regional enhancement of cannabinoid CB1 receptor desensitization in female adolescent rats following repeated Delta-tetrahydrocannabinol exposure. *Br J Pharmacol* 2010;161:103-112.
- 37 Huppertz B: Placental origins of preeclampsia: challenging the current hypothesis. *Hypertension* 2008;51:970-975.
- 38 Kremshofer J, Siwetz M, Berghold VM, Lang I, Huppertz B, Gauster M: A role for GPR55 in human placental venous endothelial cells. *Histochem Cell Biol* 2015;144:49-58.
- 39 Qamri Z, Preet A, Nasser MW, Bass CE, Leone G, Barsky SH, Ganju RK: Synthetic cannabinoid receptor agonists inhibit tumor growth and metastasis of breast cancer. *Mol Cancer Ther* 2009;8:3117-3129.
- 40 Preet A, Qamri Z, Nasser MW, Prasad A, Shilo K, Zou X, Groopman JE, Ganju RK: Cannabinoid receptors, CB1 and CB2, as novel targets for inhibition of non-small cell lung cancer growth and metastasis. *Cancer Prev Res (Phila)* 2011;4:65-75.
- 41 Borg AJ, Yong HE, Lappas M, Degrelle SA, Keogh RJ, Da Silva-Costa F, Fournier T, Abumaree M, Keelan JA, Kalionis B, Murthi P: Decreased STAT3 in human idiopathic fetal growth restriction contributes to trophoblast dysfunction. *Reproduction* 2015;149:523-532.
- 42 Fitzgerald JS, Poehlmann TG, Schleussner E, Markert UR: Trophoblast invasion: the role of intracellular cytokine signalling via signal transducer and activator of transcription 3 (STAT3). *Hum Reprod Update* 2008;14:335-344.