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Oxidative stress caused by a dysregulated Wnt/β-catenin signalling pathway is involved in abnormal placenta formation in pregnant mice with chronic fatigue syndrome

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Research Article

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Summary

Chronic fatigue syndrome (CFS) is characterized by extreme fatigue and disabling symptoms. Women with CFS often have a high risk of gynaecological problems such as irregular menstruation, endometriosis and pelvic pain and sexual dysfunction. Our previous results have shown that, in pregnant mice, CFS significantly decreased the progestational hormone level in serum, as well as learning and memory, and the function of the hypothalamus–pituitary–gonadal axis. In addition, the F1 generation also suffered from congenital hypothyroidism. At present, there has been no report about placenta formation and embryonic development in pregnant mice with CFS. The aim of the present study was to investigate the influence of CFS on the morphology, oxidative stress and Wnt/β-catenin signalling pathway during placenta formation. In this study, we found that CFS decreased the number of implantation sites for blastocysts, and increased the number of absorbed, stillborn and malformed fetuses. The morphology and structure of the placenta were abnormal in pregnant mice with CFS. Further study found that the oxidative stress in serum, uterus and placenta was increased in pregnant mice with CFS, while the levels of antioxidase were decreased. CFS also inhibited the Wnt/β-catenin signalling pathway in the placenta. These results suggested that inhibition of the Wnt/β-catenin signalling pathway and enhanced oxidative stress play an important role in abnormal placentation in pregnant mice with CFS.

Introduction

Chronic fatigue syndrome (CFS), also called myalgic encephalomyelitis, is a complex, chronic and serious illness, characterized by extreme fatigue affecting various body systems (Katafuchi *et al*., 2003). CFS patients usually have sleep disturbance (Josev *et al*., 2017; Pajediene *et al*., 2018), accompanied by multiple clinical symptoms such as decreased motor function, sexual dysfunction, insufficient physical activity, loss of appetite, loss of weight, impaired immune function and imbalance of intestinal flora (Vergauwen *et al*., 2015; van der Schaaf *et al*., 2018). In the 21st century, CFS has become a new killer affecting human physical and mental health. Under various pressures, more and more people are suffering from CFS, and the incidence of CFS is increasing year by year with the rapid pace of work and life in modern society (Jiang *et al*., 2004). CFS is more common in women compared with men (Boneva *et al*., 2015). Epidemiological investigation found that the incidence of CFS was much higher in 31–50-year-old women of reproductive age with a high level of education and work pressure compared with in men (McInnis *et al*., 2014). Female patients with CFS often have multiple gynaecological problems, including menstrual disorders, endometriosis, pelvic pain and sexual dysfunction (Boneva *et al*., 2015; Yeh *et al*., 2018).

Blastocyst implantation and placenta development are dependent on trophoblastic cells. Abnormal differentiation and invasion of placenta trophoblast cells led to spontaneous abortion, preeclampsia, fetal growth restriction, hydatidiform mole and other gestational diseases (Barrientos *et al*., 2017). Recent studies have found that the Wnt/β-catenin signalling pathway is involved in the regulation of placenta formation processes such as decidual transformation of uterine stromal cells, trophoblastic cell invasion, chorioallantoic fusion and reconstruction of maternal and fetal vasculature (Zhang *et al*., 2017; Wang *et al*., 2018). The mental and physical health of women before pregnancy is directly related to the growth and intellectual development of the offspring. Our previous study found that CFS significantly reduced the levels of oestrogen and progesterone in serum, as well as learning and memory. The hypothalamus–pituitary–gonadal axis function was disturbed in female mice with chronic fatigue syndrome (CFS) and before pregnancy. The F1 generation also suffered from congenital hypothyroidism (CH) (Liu and Qian, 2011, Zhao *et al*., 2016a; 2016b). So far, there have been no reports about

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placenta formation and embryo development in CFS pregnant mice. In this study, we aimed to establish a female mouse model of CFS by multiple stimuli, and investigated the influence of CFS on placenta morphology and embryo development and the underlying molecular mechanisms.

Materials and methods

Animal study design

ICR mice were purchased from the laboratory animal centre of Chongqing Tengxin Biotechnology Co., Ltd (Chongqing, China), and raised in the Institute of Laboratory Animal Science in Guizhou University of Traditional Chinese Medicine. After acclimation for 3 days, the female mice were divided randomly into the control group and the CFS group. The mice were fed with a standard pellet diet and distilled water *ad libitum*. The temperature of the animal facility was 22 ± 2°C, and lighting was under a 12 h : 12 h, light : dark cycle (lights on at 8:00 h). The CFS mouse model was induced by multiple different stimuli including confinement, repeated forced swimming, and tail clipping (Zhao *et al.*, 2016a; 2016b). The mice were treated with three different stimuli every day, confinement, forced swimming and tail clipping. For confinement, the mice were placed individually in a centrifuge tube (50 ml) for 10 min for the first 3 days, and 30 min on days 4–35. For repeated forced swimming, each mouse was placed in a cylinder filled with water (about 28°C) for 10 min for the first 3 days, and 30 min on days 4 to 35. For tail clipping, an oval forceps was used to clip the root of the tail for 15 s for 35 days. Moreover, the mice were treated with the following four stresses every week; 4 days were randomly selected from each week. The mice were forced to swim in cold water (about 4°C), stopped feeding for 1 day, stopped water supply for 1 day, and subjected to a reversed cycle of day and night for 1 day. Control mice did not receive any treatment. After successful induction of CFS, the female mice were mated with the control male mice. The mating was confirmed by the presence of a copulatory plug the next morning following mating (1 day post coitum). On days 7–19 post coitum, pregnant mice were sacrificed. Serum and implanted site tissues were collected. Placentas and embryos on days 12 and 14 were isolated and weighed. The implantation sites and uterine tissues were fixed by 4% paraformaldehyde in PBS (pH 7.2) at room temperature for 24 h and embedded in paraffin for histologic examination. The serum was isolated by centrifugation using serum separator tubes.

Haematoxylin and eosin staining

The uterine implantation sites and placenta were fixed overnight in 4% paraformaldehyde (v/v). After washing with 70% ethanol, the tissues were processed, embedded in paraffin, and sectioned. Sections of paraffin-embedded tissues (section thickness, 5 μm) were stained with haematoxylin and eosin. Photographs were obtained from a light microscope (BX51; Olympus, Tokyo, Japan).

Assays of oxidative stress and antioxidase

The oxidative stress and antioxidase levels in serum, uterus and placenta were detected using the corresponding kits. The kits for oxidative stress assay included a reactive oxygen species assay kit (ROS; QY-X16254; Qiaoyu, Shanghai, China), reactive nitrogen assay kit (RNS; QY-Y26312; Qiaoyu, Shanghai, China), malondialdehyde assay kit (MDA; QY-Y23136; Qiaoyu, Shanghai, China). The kits for antioxidase assay included superoxide dismutase assay kit (SOD; QY-S13521; Qiaoyu, Shanghai, China), total antioxidant capacity assay kit (T-AOC; QY-Y61124; Qiaoyu, Shanghai, China), glutathione peroxidase assay kit (GSH-Px; QY-T11542; Qiaoyu, Shanghai, China), peroxidase assay kit (POD; QY-X35410; Qiaoyu, Shanghai, China) and catalase assay kit (CAT; QY-Y32512; Qiaoyu, Shanghai, China).

Quantitative real-time PCR

Total RNA was extracted from placentas on days 12 and 14 using TRIzol regent (DP405-02, Tiangen, Beijing, China) and purified in accordance with the manufacturer's instructions. In total, 2 mg RNA were reverse transcribed with a PrimeScript™ RT reagent kit (RR047B, TaKaRa, Liaoning, China). Real-time PCR was performed using a TaKaRa SYBR® Premix Ex *Taq*™ II kit (RR82LR, TaKaRa, Liaoning, China) and on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems, Foster, CA, USA). Each sample was tested in triplicate in a 20-μl volume for reaction with 2 μl cDNA, 10 μl 2× Master Mix, 1 μl PrimeScript RT Enzyme Mix, 1 μl RT Primer Mix, 4 μl 5× Prime Script Buffer 2 and 4 μl RNase free H_2O. The PCR amplification program was initiated at 95°C for 30 s, followed by 40 thermal cycles of 5 s at 95°C and 40 s at 60°C, annealing temperature for 20 s and 72°C for 20 s. The comparative C_T method was used to analyze the obtained data. The sequences of primers were as follows: Wnt4 Forward: 5′-CATCGAGGAGTGCCAATACCA-3′, reverse: 5′-GGAGGGAGTCCAGTGTGGAA-3′, Wnt5α forward: 5′-GGCGAGCTGTCTACCTGTGG-3′, reverse: 5′-GGCGAACGG GTGACCATAGT-3′; SFRP1 forward: 5′-TGAGGCCATCATT GAACATC-3′, reverse: 5′-TCATCCTCAGTGCAAACTCG-3′; SFRP3 forward: 5′-CAAGGGACACCGTCAATCTT-30, reverse: 5′-CATATCCCAGCGCTTGACTT-30; DKK1 forward: 5′-TCCG AT CATCAGACTGTGCCG-3′, reverse: 5′-TGGGAGCCTTTCC GTTTGTGC-3′; β-catenin forward: 5′-TGGAGGAGATAGTA GAGGGTG-3′, reverse: 5′-AGACATTCGGAATAGGACAGC-3′; β-actin forward: 5′-CGTTGACATCCGTAAAGACCTC-3′, reverse: 5′-ACAGAGTACTTGCGCTCAGGAG-3′. All data were normalized to β-actin and expressed as fold change relative to the control group.

Western blotting

Proteins were extracted from placentas on day 12 and day 14 with a whole cell lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10 mM sodium pyrophosphate, 1.5 mM $MgCl_2$, 100 mM NaF, 10% glycerol, and 1% Triton X-100) containing a cocktail of protease inhibitor (1 mM phenylmethylsulfonylfluoride, 10 mg/ml aprotinin, and 1 mM sodium orthovanadate). Equal amounts of denatured protein were subjected to SDS-PAGE in accordance with standard protocols. Separated proteins were transferred electrophoretically onto PVDF membrane (Millipore, Burlington, MA, USA). After being blocked with 5% skimmed milk for 1 h at room temperature, the membrane was sequentially incubated with primary antibodies against Wnt4 (1:1000, bs-6134R; Bioss Antibodies, Beijing, China), Wnt5α (1:1000, bs-1948R; Bioss Antibodies), SFRP1 (1:500, bs-1303R; Bioss Antibodies), SFRP3 (1:500, bs-1618R; Bioss Antibodies), DKK1 (1:1000, RS-12162R; Bioss Antibodies), β-catenin (1:500, bs-1165R; Bioss Antibodies) and β-actin (1:1000, bs-1165R; Bioss Antibodies) overnight at 4°C, and washed three times (10 min per wash) with TBST. Then, the membrane was

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incubated with horseradish peroxidase-linked secondary antibody (1:5000; Beyotime, Shanghai, China) for 1 h at room temperature in 5% skimmed milk, and washed three times (10 min per wash) with TBST. Immunoreactive bands were detected using an enhanced chemiluminescence kit (Pierce Chemical Co, Rockford, IL, USA). β-Actin was used as an internal control.

Immunohistochemistry

Sections were preincubated with 10% normal goat serum in PBS (pH 7.5) and then incubated with anti-Wnt4 (1:500, bs-6134R; Bioss Antibodies), anti-Wnt5α (1:500, bs-1948R; Bioss Antibodies), anti-SFRP1 (1:400, bs-1303R; Bioss Antibodies), anti-SFRP3 (1:400, bs-1618R; Bioss Antibodies), anti-DKK1 (1:300, RS-12162R; Bioss Antibodies), anti-β-catenin (1:300, bs-1165R; Bioss Antibodies) in 10% normal serum in PBS (pH 7.2) overnight. On the following day, sections were washed in PBS and incubated with a biotinylated secondary antibody (8029 and 8003; ZSGB-BIO, Beijing, China) for 1 h at room temperature. Immunoreactivity was detected using a DAB Substrate kit (ZLI-9017, ZSGB-BIO). Immunoreactivity was visualized as brown staining. Sections incubated with no primary antibody were used as a negative control. Photographs were obtained from a light microscope (BX51; Olympus, Tokyo, Japan).

Statistical analyses

Data were expressed as mean ± standard deviation (SD) and were analyzed by paired Student's *t*-test for comparison between normal and CFS groups. Differences at $P < 0.05$ were considered to be statistically significant.

Results

Comparison of pregnancy status between the control and CFS groups

In a previous study, we found that the hypothalamus–pituitary–gonadal axis (HPG) was disordered in the CFS mouse (Liu and Qian, 2011). Learning and memory in the F1 generation was impaired, and this could be used as an animal model of congenital hypoidism (congenital hypothyroidism, CH) (Liu and Qian, 2011). In this study, we also analyzed the number of implantation sites, stillbirths, live births and others on day 7, day 11, day 15 and day 19 in CFS pregnant mice. As shown in Tables 1 and 2, we found that the number of implantation sites, stillbirths and the lengths of embryos on day 15 and day 19 in CFS pregnant mice were significantly decreased ($P < 0.01$) (Tables 1 and 2).

Comparison of the morphology of placenta between the control and CFS groups in pregnancy mouse

As shown in Fig. 1, we found that the placenta structure of the CFS group began to change on day 10 in CFS pregnant mice. The labyrinth layer of the placenta began to appear on day 10 in the control pregnant mice, while the placenta had not yet differentiated in the CFS group (Fig. 1). As shown in Fig. 2, we found that the area of the three-layer structure of the placenta in the CFS group was smaller compared with that in the normal control group on day 13. The numbers of blood cells in the labyrinth area of the placenta were lower in the CFS group (Fig. 2).

Table 1. Number of implantation sites, stillbirths, live births on day 7, day 11, day 15 and day 19 in normal and CFS pregnant mice

	Normal group ($n = 10$)			Chronic fatigue syndrome group ($n = 10$)		
Days of pregnancy	Number of implantation sites	Number of stillbirths	Number of live births	Number of implantation sites	Number of stillbirths	Number of live births
Day 7	11.33 ± 1.58	0.30 ± 0.48	11.00 ± 1.83	9.11 ± 1.52**	0.40 ± 0.52	8.70 ± 1.57**
Day 11	11.10 ± 1.91	0.40 ± 0.52	10.70 ± 2.11	8.70 ± 1.42**	2.10 ± 0.73**	6.60 ± 1.43**
Day 15	11.00 ± 1.83	0.40 ± 0.51	10.40 ± 1.71	7.10 ± 1.97**	3.40 ± 1.35**	4.10 ± 1.45**
Day 19	10.30 ± 1.95	0.30 ± 0.48	10.30 ± 1.49	6.89 ± 1.26**	3.20 ± 1.34**	4.00 ± 2.46**

*$P < 0.05$, **$P < 0.01$ vs. normal group.

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Table 2. Weight and length during growth of the placenta and embryos on day 15 and day 19 in normal and CFS pregnant mice

The days of pregnancy	Normal group ($n = 10$)				Chronic fatigue syndrome group ($n = 10$)			
	Implantation sites (mg)	Placenta (mg)	embryo (mg)	Embryo length (cm)	Implantation sites (mg)	Placenta (mg)	Embryo (mg)	Embryo length (cm)
Day 15	728.49 ± 34.79	87.34 ± 6.79	299.46 ± 13.93	1.66 ± 0.12	627.55 ± 14.87**	74.35 ± 6.12**	247.21 ± 6.78**	1.31 ± 0.06**
Day 19	1706.69 ± 7.89	143.21 ± 6.60	1384.08 ± 20.05	2.55 ± 0.14	1420.80 ± 64.36**	124.24 ± 10.63**	1243.49 ± 26.76**	1.96 ± 0.12**

*$P < 0.05$, **$P < 0.01$ vs. normal group.

Comparison of oxidative/antioxidant stress index between control group and model group

Differences in the oxidative/antioxidant stress index in pregnant mice in the normal and CFS groups were assessed. Values for the oxidative stress index (ROS, RNS, MDA; Table 3) were significantly lower in the serum and uterus and on day 13 placenta in the normal group compared with CFS group by colorimetric analyses, Further experimental research showed that values for the antioxidant stress index (T-AOC, CAT, SOD, GSH-Px, POD) were higher in the normal control group compared with that in the CFS group ($P < 0.01$) (Table 3).

Alterations of Wnt/β-catenin signalling in placenta during implantation

As mentioned above, these results demonstrated that the numbers of implantation sites and embryo length were decreased and numbers of absorbed fetuses and stillbirths were significantly increased. The morphological structure of the placenta was abnormal. The Wnt pathway is closely associated with implantation and differentiation of trophoblasts (Zhang *et al.*, 2017). We next investigated whether the Wnt signalling pathway in CFS pregnant mice was involved in abnormal placenta formation. As shown in Fig. 3, *SFRP1*, *SFRP3* and *DKK1* mRNA were highly expressed, while *Wnt4*, *Wnt5α* and *β-catenin* were significantly downregulated in the CFS group ($P < 0.01$; Fig. 3*A*,*B*). Western blot analyses revealed that the protein levels of Wnt5α and β-catenin in the placenta were decreased in the CFS group on day 12, while SFRP1 was significantly highly expressed ($P < 0.01$) (Fig. 4*A*). The expression levels of Wnt4, Wnt5α and β-catenin in the placenta were significantly reduced in the CFS pregnant mice on day 14. However, SFRP3 and DKK1 were highly expressed in the CFS group on day 14 ($P < 0.05$) (Fig. 4*B*). As shown in Fig. 5, on day 12 and day 14, most placenta cells from the CFS group were positive for Wnt4, Wnt5α, SFRP1, SFRP3, DKK1 and β-catenin. However, nuclear β-catenin staining was present less often in the CFS group (Fig. 5*A*,*B*). The results demonstrated that the Wnt signalling pathway in the placenta may play a role in abnormal placentation in CFS mice.

Discussion

Recent studies have found that brain function, energy metabolism, acetylcholine and adrenalin signalling were disordered in CFS patients (Mensah *et al.*, 2018; Scheibenbogen *et al.*, 2018; Shan *et al.*, 2018). Epidemiological survey results have shown that 20–25% of the world's population has CFS, with more than 4 million patients in the USA. In China, 10–25% of the urban population and in Britain 1.9% of 16 year olds suffer from this disease (Collin *et al.*, 2016). At present, immune function disorder, central nervous system (CNS) abnormality and oxidative stress are considered to be the basic pathological mechanisms of CFS (Zinn *et al.*, 2018; Cliff *et al.*, 2019; Polli *et al.*, 2019).

The Wnt protein family contains important signalling regulatory proteins and secreted glycoproteins. Wnt signalling is closely related to cell proliferation and differentiation as one of the main pathways that regulates various physiological and biochemical processes such as cell morphology, movement, adhesion, proliferation, differentiation, carcinogenesis

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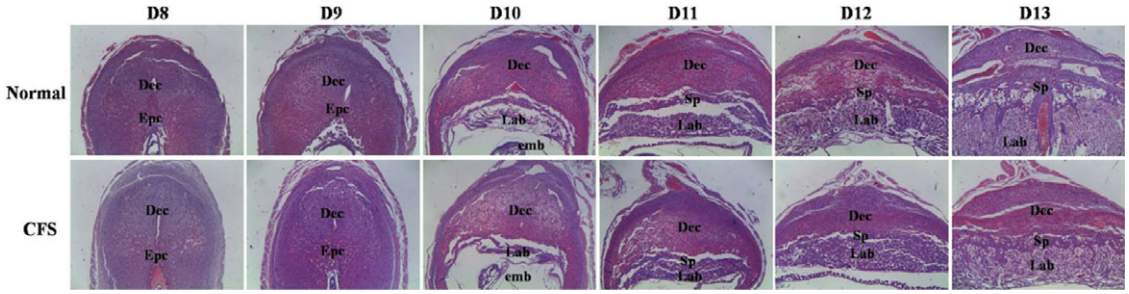

Figure 1. Morphology of placenta from day 8 (D8) to day 13 (D13) in normal and CFS pregnant mice (×40 magnification). Normal, normal group; CFS, chronic fatigue syndrome group; Dec, Decidua basalis; emb, embryo; Epc, ectoplacental cone; Lab, Labyrinth; Sp, spongiotrophoblast.

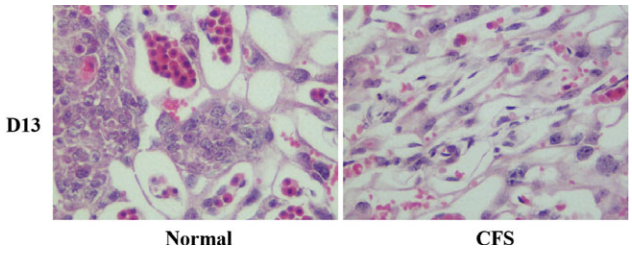

Figure 2. Morphology of labyrinth in placenta on day 13 in normal and CFS pregnant mice (×400 magnification). CFS, chronic fatigue syndrome group; normal, normal group.

and development (Xu *et al.*, 2017). Multiple genes, including *Fox*, *Sox*, *Gata*, *Tead* and *Wnt*, play important roles during placental development. Among them, the Wnt signalling pathway is involved in the regulation of the whole process of placenta formation (Nayeem *et al.*, 2016). Migration and invasion of trophoblast cells are regulated by Wnt signalling through transcription factor T-cell factor-4 (TCF-4) (Meinhardt *et al.*, 2014). Wnt7 is highly expressed in human placental tissues. Wnt5α can inhibit the migration and invasion of HTR-8 cells by reducing integrin β1 levels through activating NF-AT to regulate intercellular adhesion factor-1 (ICAM-1) and vascular cell adhesion factor-1 (VCAM-1) (Herr *et al.*, 2014). The proliferation, differentiation and decidualization of endometrial stromal cells is regulated by Wnt4 (Logan *et al.*, 2018). As inhibitory proteins of the Wnt signalling pathway, secreted frizzled-related proteins 1, 3, 4 (SFRP1, SFRP3, SFRP4) and dickkopf1 (DKKl) play important roles in the formation of the placenta (Zmijanac Partl *et al.*, 2018). It has been found that SFRP1 and SFRP3 are highly expressed in placenta with intrauterine growth restriction (IUGR) (Partl *et al.*, 2014). Low expression of β-catenin protein and high expression of DKK1 and SFRP4 may be involved in the occurrence of severe eclampsia and unexplained recurrent spontaneous abortion (Zhang *et al.*, 2013, 2019). In addition, oxidative stress is involved in the regulation of the Wnt/β-catenin signal pathway by upregulating C/EBP-β, and influencing the activity of matrix metalloproteinases (MMPs), therefore participating in the pathogenesis of preeclampsia (Zhuang *et al.*, 2015). As can be seen from the above findings, oxidative stress and the Wnt/β-catenin signalling pathway play a very important role in the regulation of placenta formation. So far, there have been no studies regarding placental morphology, oxidative stress and Wnt/β-catenin signalling pathway in the CFS placenta. In this study, we established a CFS mouse model in female mice using multiple stimuli (Zhao *et al.*, 2016a; 2016b). This female mouse model described a significant reduction in locomotor activity, spatial memory and learning (Zhao *et al.*, 2016a; 2016b). These results were consistent with previous findings that described the CNS as involved in the pathogenesis of CFS (Glassford, 2017; Ohba *et al.*, 2019). Therefore, we believe that our CFS mouse model was able to simulate the pathology of CFS. Alterations in placenta morphology, oxidative stress and the Wnt/β-catenin pathway were investigated.

In this study, we found that the numbers of implantation sites and stillbirths and the length of embryos on day 15 and day 19 were significantly decreased in CFS pregnant mice (Tables 1 and 2). The labyrinth layer of the placenta began to appear on day 10 day in the normal control pregnant mice, while the placenta was not differentiated in the CFS group (Fig. 1). We also found that the area of the three-layer structure of the placenta in the CFS group was smaller compared with that in the normal control group on day 13. The numbers of blood cells in the labyrinth area of the placenta were decreased in the CFS group (Fig. 2). To further investigate the underlying mechanisms of the abnormal morphology of placenta in CFS pregnant mice, oxidative stress and the Wnt/β-catenin signalling pathway were quantified. Our results showed that oxidative stress was significantly increased in serum, the uterus and the placenta in the CFS group, and might be associated with downregulation of antioxidases in the CFS group ($P < 0.01$). *SFRP1*, *SFRP3* and *DKK1* mRNA were strongly expressed in CFS placenta, while mRNA expression of *Wnt4*, *Wnt5α*, and *β-catenin* was decreased ($P < 0.01$) (Fig. 3*B*). Protein levels of Wnt4, Wnt5α and β-catenin were decreased on day 14 in the CFS placenta. Furthermore, the protein levels of SFRP1 and SFRP3 were decreased ($P < 0.01$) (Fig. 4*B*).

By using a female CFS mouse model, we found that the numbers of implantation sites and length of embryos were decreased. The numbers of absorbed fetuses, stillborn fetuses and malformed fetuses significantly increased over time in CFS pregnant mice. In addition, the placenta had an abnormal morphology. Oxidative stress in serum, uterus and placenta were increased. Levels of antioxidase were decreased in CFS pregnant mice. We also found that the expression levels of Wnt signalling pathway key factor β-catenin protein was significantly decreased, and the expression levels of inhibitory factors SFRP1, SFRP3 and DKK1 were increased in the placenta of CFS pregnant mice.

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Table 3. Indicators of oxidative stress in serum and uterus and on day 13 placenta in normal and pregnant mice

Samples		Normal group ($n = 10$)			Chronic fatigue syndrome group ($n = 10$)		
Oxidative stress index	Indicators	Serum	Uterus	Placenta day 13	Serum	Uterus	Placenta day 13
	Reactive oxygen species (ROS)	0.50 ± 0.01	5.54 ± 0.38	5.74 ± 1.07	4.1 ± 0.29**	44.16 ± 1.78**	43.24 ± 0.42**
	Reactive nitrogen species (RNS)	3.27 ± 0.06	4.26 ± 0.09	5.25 ± 0.15	5.78 ± 0.05**	49.77 ± 0.54**	50.06 ± 0.45**
	Malondialdehyde (MDA)	3.58 ± 0.04	55.42 ± 0.21	51.96 ± 0.83	6.78 ± 0.24**	109.42 ± 2.35**	85.62 ± 0.33**
Antioxidant stress index	Total antioxidant capacity (T-AOC)	18.93 ± 0.35	272.77 ± 16.01	275.03 ± 8.94	7.83 ± 1.25**	83.40 ± 0.92**	126.07 ± 1.79**
	Catalase (CAT)	5.10 ± 0.10	60.29 ± 0.09	155.74 ± 1.07	2.13 ± 0.25**	13.43 ± 0.12**	73.24 ± 0.42**
	Superoxide dismutase (SOD)	4.35 ± 0.03	96.13 ± 5.23	125.85 ± 2.01	0.94 ± 0.10**	34.31 ± 0.94**	55.08 ± 1.06**
	Glutathione peroxidase (GSH-Px)	8.48 ± 0.22	16.57 ± 0.06	15.27 ± 1.34	1.46 ± 0.16**	2.43 ± 0.27**	3.14 ± 0.43**
	Peroxidase (POD)	528.80 ± 12.36	891.40 ± 1.08	954.64 ± 6.93	138.93 ± 14.39**	207.08 ± 3.38**	275.17 ± 8.05**

*$P < 0.05$, **$P < 0.01$ vs. normal group.

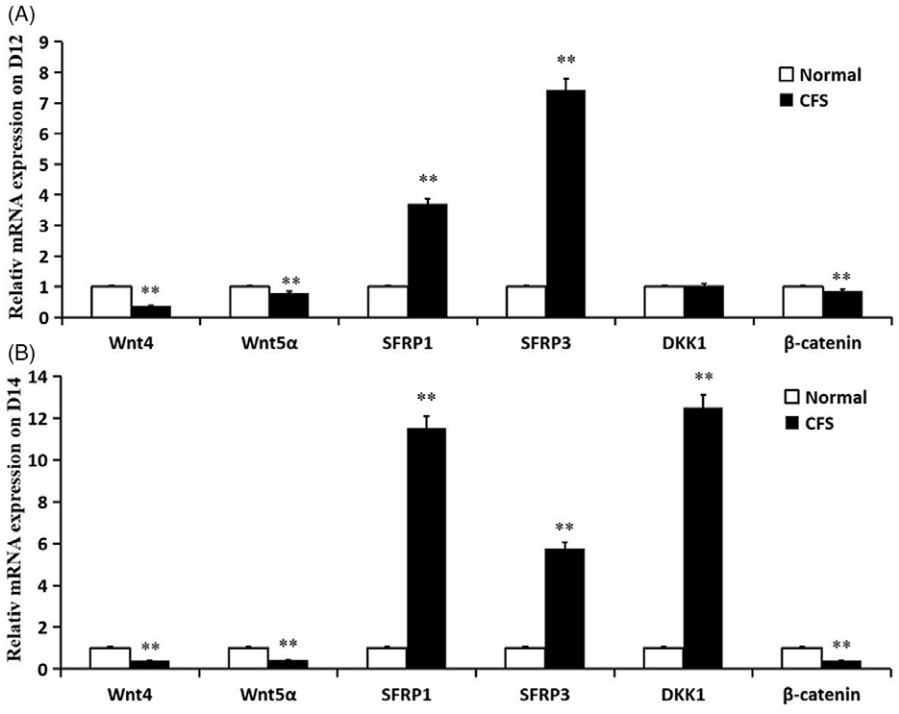

Figure 3. mRNA expression of Wnt/β-catenin associated genes of placenta on day 12 (*A*) and day 14 (*B*) in normal and CFS pregnant mice. $n = 10$ mice per group. CFS, chronic fatigue syndrome group; normal, normal group. *$P < 0.05$, **$P < 0.01$ vs. normal group.

Therefore, we speculated that the abnormal placental structure in CFS pregnant mice was caused by oxidative stress though a dysregulated Wnt/β-catenin signalling pathway, finally resulting in impaired growth and development in the F1 generation in CFS patients. These results shed new light on the influence of CFS on placenta formation. Drugs targeting oxidative stress and the Wnt/β-catenin signalling pathway might be effective for the treatment of CFS-induced abnormalities of the placenta.

In conclusion, multiple gynaecological problems such as irregular menstruation, endometriosis and pelvic pain and the incidence of sexual dysfunction are significantly increased in female patients with CFS. The present study investigated the influence of CFS on placenta formation. Our results showed that CFS caused abnormal formation of the placenta, as revealed by the decreased number of implantation sites and live births, increased numbers of stillbirths, and decreased weights of implantation sites, placenta, and embryos. Further studies found that oxidative stress in serum, the uterus and the placenta were increased in CFS pregnant mice. The levels of antioxidase were decreased. In addition, CFS inhibited the Wnt/β-catenin signalling pathway. Therefore, it can be speculated that abnormal expression of the Wnt/β-catenin signalling pathway and oxidative stress are involved in abnormal formation of the placenta in CFS pregnant mice.

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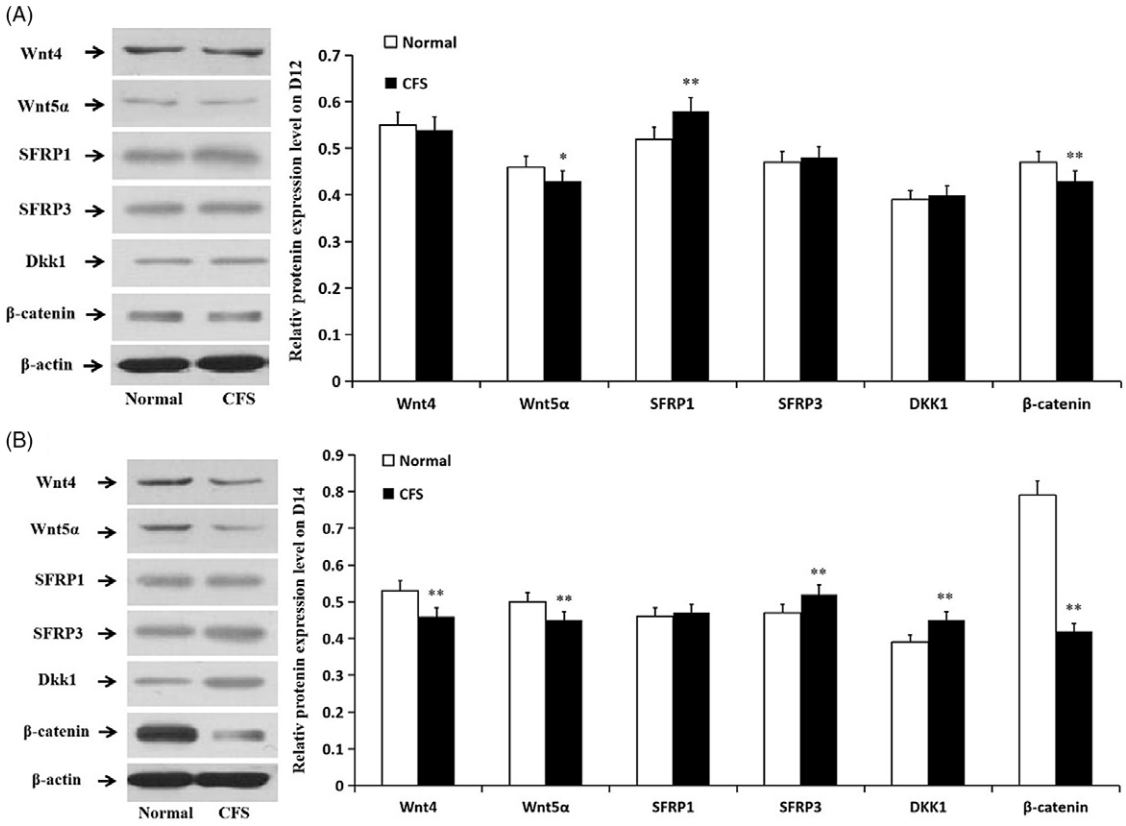

Figure 4. Protein expression of Wnt/β-catenin associated genes of placenta on day 12 (*A*) and day 14 (*B*) in normal and CFS pregnant mice. $n = 10$ mice per group. CFS, chronic fatigue syndrome group; normal, normal group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. normal group.

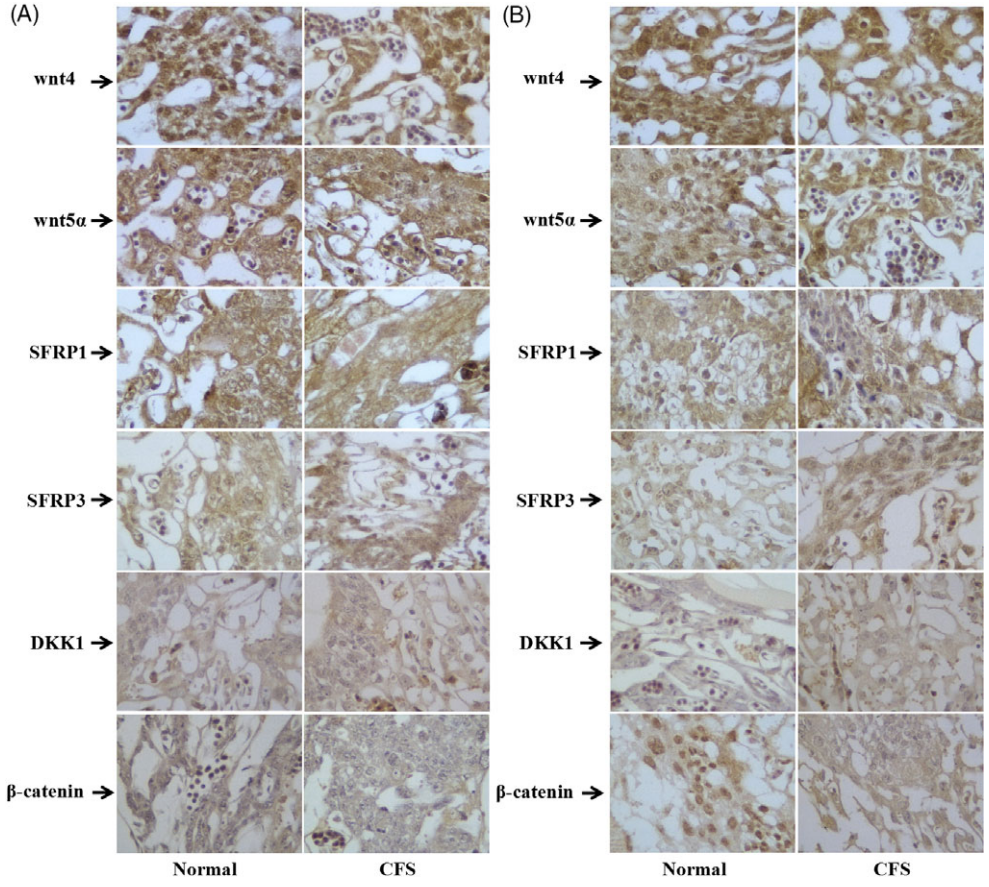

Figure 5. Representative images of immunostaining of Wnt/β-catenin associated proteins in placenta on day 12 (*A*) and day 14 (*B*) in normal and CFS pregnant mice. $n = 10$ mice per group.

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Conflicts of interest. None.

Ethical standards. All procedures related to animal care and treatment were approved by Animal Ethics and Welfare Committee at Guizhou University of Traditional Chinese Medicine.

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