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Effects of modified Bushen Huoxue Prescription combined with active immunotherapy on immunomodulatory functions and pregnancy outcomes of patients with unexplained recurrent spontaneous abortion

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Recurrent spontaneous abortion (RSA) is a common pregnancy disease mostly occurring in the first trimester of pregnancy, and unexplained RSA (URSA) occurs in approximately 40% of RSA patients even after careful examination. Here, we assessed the effects of modified Bushen Huoxue Prescription combined with active immunotherapy on immunomodulatory functions and pregnancy outcomes of URSA patients. Ninety-six eligible URSA patients were randomly divided into control and experimental groups (n=48). Active immunotherapy was performed for control group, while experimental group were treated with modified Bushen Huoxue Prescription combined with active immunotherapy. Counts of peripheral blood helper T lymphocytes 17 (Th17) and regulatory T lymphocytes (Treg), levels of inflammatory factors interleukin-4 (IL-4), IL-10 and IL-17, and mRNA expression levels of retinoic acid-related orphan receptor γt (RORγt) and forkhead box P3 (FoxP3) were compared. Compared with before treatment, ratios of Th17/CD4$^+$ cells and Th17/Treg, IL-17 level and RORγt mRNA level decreased, whereas ratio of Treg/CD4$^+$ cells, IL-4 and IL-10 levels and FoxP3 mRNA level increased in both groups after treatment ($P<0.05$). After treatment, ratios of Th17/CD4$^+$ cells and Th17/Treg, IL-17 level and RORγt mRNA level were lower in experimental group than those in control group, while ratio of Treg/CD4$^+$ cells, IL-4, IL-10 levels and FoxP3 mRNA level were higher in experimental group ($P<0.05$). Modified Bushen Huoxue Prescription combined with active immunotherapy worked well for URSA to improve pregnancy outcomes and lower abortion rate.

Keywords: Active immunotherapy, *Astragalus membranaceus*, *Atractylodes macrocephala*, *Dipsacus asper*, Interleukin, T lymphocytes, *Taxillus chinensis*, Traditional Chinese medicine

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Recurrent spontaneous abortion (RSA) is a common pregnancy disease mostly occurring in the first trimester of pregnancy, with an incidence rate of about 1-2%, and the incidence rate rises continuously in recent years[1]. Its etiological factors are complex, including genetic factors, anatomical factors such as uterine malformations and uterine fibroids, endocrine factors, reproductive tract infection factors, immune factors, thrombosis, smoking, alcoholism, drug abuse and environmental factors. Besides, it fails to know the clear etiological factors in approximately 40% of RSA patients even after careful examination, which is called unexplained RSA (URSA) in such a case[2]. URSA patients have an extremely low successful pregnancy rate since there are no effective treatment plans for them in clinical practice. Therefore, such patients and their families face with tremendous economical and psychological pressure. The incidence of URSA may be closely related to abnormal maternal and fetal immune tolerance in patients, abnormal immune tolerance has an association with the immune imbalance between helper T lymphocyte 17 (Th17) and regulatory T lymphocytes (Treg), and Treg-mediated immune suppression may be a vital player in maternal immune tolerance[3]. For this reason, physicians used active immunotherapy in treating URSA patients in clinical practice. Though, it provides certain curative effects, the treatment failure is still detected in some patients[4]. As the efficacy of immunotherapy remains controversial, finding an effective treatment plan still continues.

Traditional Chinese medicine (TCM) therapy has its unique advantages in regulating the immune function of human body, classic prescriptions, such as Bushen Huoxue Prescription, Yimu Gushen Prescription, and Taiyuan and Shoutai Prescription function well in protecting fetuses and benefiting mothers[5]. In our hospital, the modified Bushen Huoxue Prescription is applied to treat URSA, and a good curative effect has been obtained in clinical practice, without side effects during treatment and adverse effects on fetal health. In the present study, in order to improve the pregnancy outcomes of patients with such a disease and to guide the clinical application of drugs, we explored the effects of the

modified Bushen Huoxue Prescription combined with active immunotherapy on URSA by observing the changes in immune function and pregnancy outcomes of patients.

Materials and Methods

Baseline clinical data

A total of 96 URSA patients aged 22-40 years old and (29.53 ± 4.24) years old on average admitted to and treated in our hospital from January 2017 to March 2019 were enrolled in randomized controlled trial. The sample size was determined based on pre-experiment results. This study was approved by the medical ethics committee of the hospital, and all patients were informed of this study and signed the informed consent. All patients successfully completed the entire study process (no drop-outs).

Their body mass was 42-62 kg, with a mean of (53.82 ± 5.13) kg. The number of abortions was 2-6 times, with an average of (3.63 ± 1.19) times, and the gestational age at abortion was 6-14 weeks and (8.69 ± 2.07) weeks on average. These patients were evenly divided into control group and experimental group using a random number table. The patients in control group were aged 23-41 years old and (29.83 ± 4.66) years old on average, and their body mass was 42-61 kg, with a mean of (54.03 ± 5.22) kg. The number of abortions was 2-6 times, with an average of (3.78 ± 1.29) times, and the gestational age at abortion was 6-14 weeks and (8.73 ± 2.28) weeks on average. The patients in experimental group were aged 22-40 years old and (29.67 ± 4.29) years old on average, and their body mass was 43-62 kg, with a mean of (52.62 ± 5.11) kg. The number of abortions was 2-6 times, with an average of (3.55 ± 1.22) times, and the gestational age at abortion was 6-13 weeks and (8.64 ± 2.27) weeks on average. There were no statistically significant differences in the age, body mass, number of abortions and gestational age at abortion between the two groups of patients (P >0.05), and the basic data were normally distributed and balanced.

Diagnostic criteria

The Western medicine diagnostic criteria were set with reference to the content of RSA as given in Feng *et al.*[6]. URSA refers to ≥2 consecutive times of spontaneous abortion with the same sex partner, excluding the abortion caused by genetic factors, anatomical factors, endocrine, infection and autoimmune abnormality.

The TCM diagnostic criteria were developed based on the syndrome of kidney deficiency and blood stasis[7]. The syndrome is manifested with such symptoms as repeated abortion, soreness and weakness of waist and knees, dizziness and tinnitus, dim complexion, dark purple tongue or tongue with petechia and ecchymosis, and deep thready and hesitant pulse[7].

Inclusion criteria

(i) Patients aged 20-40 years old; (ii) those who had 2 consecutive times or more early spontaneous abortion, with the last abortion occurred within one year; (iii) those who had no history of allergies to drugs in this study; (iv) those who had normal chromosomes and whose sex partner had normal semen and chromosomes; (v) those with negative autoantibodies and antiphospholipid antibodies; (vi) those with normal reproductive endocrine hormones including sex hormones, fasting blood glucose and thyroid function; and (vii) those who did not take hormone drugs in the past 3 months[8].

Exclusion criteria

(i) Patients aged below 20 or over 40 years old; (ii) those with poor compliance in treatment, unable to take drugs according to medical advices or quitting during treatment; (iii) those who and whose sex partner had abnormal results in chromosome karyotype analysis; (iv) those with reproductive tract malformation or organic diseases of reproductive organs based on B-ultrasound examination; (v) those with reproductive tract infections, such as mycoplasma, chlamydia or HIV infections; (vi) those with hepatic, renal, cerebrovascular, cardiovascular or hematopoietic system dysfunction; (vii) those with abnormal results in endocrine examinations (like sex hormone or thyroid function examinations) or TORCH test; (viii) those with mental or psychological illness or unable to communicate normally; (ix) those with contraindications to immunotherapy; (x) those with threatened abortion due to external factors such as falling or swooping down or injuries or environmental factors; or (xi) those with systemic diseases including severe anemia, hypertension or diabetes, or with non-deterministic curative effects of drugs due to smoking and drinking or usage of similar drugs (Chinese medicines for tonifying kidney and promoting blood circulation or hormone medicines) recently[9].

Treatment methods

All patients enrolled were treated with lymphocyte active immunotherapy. According to

methods in literature, the blood was routinely drawn from their husband to test blood type, biochemical and liver function indicators and blood lipid parameters. Blood sources were provided for patients for treatment after confirming that the husband was free of blood-transmitted infectious diseases. If the results suggested blood-transmitted infectious diseases in the husband, a healthy third party without relationship with patients was selected to provide the blood source. Fasting venous blood (20 mL) was collected from the donor, anticoagulated with heparin, and processed according to the instructions of lymphocyte separation medium (Shanghai Hengxin Chemical Reagent Co., Ltd.) to obtain lymphocytes. Then the density was adjusted to 2×10^9-3×10^9 cells/L. Next, fresh lymphocytes were injected intracutaneously on the medial forearm once every 3 weeks, with twice as one course of treatment. The patients were encouraged to become pregnant within 3 months after one course of treatment. After confirming pregnancy, immunotherapy was continued as described above until the gestational age was 12 weeks. While continuing the active immunotherapy after the pregnancy was confirmed, the experimental group was further treated with modified TCM Bushen Huoxue Prescription. The TCM Bushen Huoxue Prescription covered the following medicines: *Salvia miltiorrhiza* Bunge (15 g), Semen Cuscutae (15 g), *Dipsacus asper* (15 g), *Taxillus chinensis* (15 g), Asini Colla Corii (melting by heat) (10 g), *Astragalus membranaceus* (15 g).

Based on the results of the synthesis of the four diagnostic methods for syndrome differentiation and treatment, the modified prescription was adopted for treatment. Specifically, 15 g of charred Cortex Eucommiae and 15 g of charred Radix Sanguisorbae were added if there was excessive vaginal bleeding, 15 g of Cortex Eucommiae was added if there was obvious soreness of waist, 10 g of *Atractylodes macrocephala* and 10 g of Amomi Fructus were added in the case of loose stool, and 10 g of Fructus Ligustri Lucidi and 10 g of *Eclipta prostrata* were added in the case of fire excess from Yin deficiency. The Chinese medicinal materials were provided by the TCM Pharmacy of our hospital and processed into decoction in the same batch by the TCM Preparation Room. Each bag was 100 mL, with 3 bags as one dose. A bag of decoction was separately taken in the morning, at noon and in the evening, with one dose a day, and 15 consecutive days as a course of treatment. The treatment was ended at 12 weeks of pregnancy.

Scoring based on TCM symptoms and signs

The patients in the two groups were scored based on TCM symptoms and signs before and after treatment: (i) soreness and weakness of waist and knees: 0 points for asymptomatic patients, 1 point for patients with mild symptoms and 2 points for patients with obvious symptoms; (ii) dizziness and tinnitus: 0 points for asymptomatic patients, 1 point for patients with mild symptoms and 2 points for patients with obvious symptoms; (iii) dim complexion: 0 points for patients without obvious symptoms, 1 point for patients with mild symptoms and 2 points for patients with obvious symptoms; and (iv) dark purple tongue: 0 points for patients without obvious symptoms, 1 point for patients with mild symptoms and 2 points for patients with obvious symptoms.

Detection of serum Th17 and Treg

Fasting venous blood was collected from the elbow of patients before treatment after pregnancy and at 12 weeks of pregnancy, added with lymphocyte separation medium to separate peripheral blood mononuclear cells (PBMCs). Next, the ratio of Th17/cluster of differentiation 4 $(CD4)^+$ cells, Th17/Treg and ratio of Treg/$CD4^+$ cells in PBMCs were determined through flow cytometry. The differences in the above indicators were compared between patients with successful pregnancy and those with unsuccessful pregnancy in control group and experimental group[10].

Measurement of serum inflammatory factors

Venous blood was drawn from the elbow of patients before and after treatment. Then, the serum was separated to measure serum interleukin-4 (IL-4), IL-10 and IL-17 levels using the corresponding enzyme-linked immunosorbent assay kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., batch number: ML058051)[11].

Detection of Treg transcription factors retinoic acid-related orphan receptor γt (RORγt) and forkhead box P3 (FoxP3) mRNA expressions in PBMCs

Real-time quantitative polymerase chain reaction (PCR) was conducted for detection. Specifically, peripheral blood (400 μL) was collected, anti-coagulated, mixed well with 1200 μL of red blood cell lysis buffer, and centrifuged at 450 rpm for 10 min to obtain PBMCs. Next, total ribonucleic

acids (RNAs) in PBMCs were extracted through TRIzol assay, and mRNA purity was detected. Afterwards, reverse transcription kits (TaKaRa, batch number: 638314) were utilized for reverse transcription of complementary deoxyribonucleic acids (cDNAs). CDNAs (4 μL) were extracted and mixed with SYBR Green PCR master mix. The samples (loading volume: 50 μL) were subjected to PCR amplification under the following conditions: predenaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 30 s and extension at 72°C for 31 s, and final extension at 72°C for 7 min. The relative expressions of the target genes were calculated using $2^{-\Delta\Delta Ct}$. The primers were designed and synthesized by Shanghai Generay Biotech Co., Ltd. RORγt: upstream primer, 5'-AGCAGTAGGGAA TCTTCCA-3'; downstream primer, 5'-CACCGCTAC ACATGGAG-3'. FoxP3: upstream primer, 5'-TCGCG TCCGGTGTGAAAG-3', downstream primer, 5'-CCA CATCCAGCATCCAC-3'. GAPDH: upstream primer, 5'-GTTATACCTTTGGCTCATTGA-3', downstream primer, 5'-ACCAGGGTATCTTAATCCTGTT-3'[12].

Observation of successful pregnancy rate and pregnancy outcomes

The re-pregnancy rate and successful pregnancy rate were compared between the two groups. The criteria for successful pregnancywere set as follows[13]: (i) women with ≥ 28 weeks of pregnancy, intrauterine live fetus based on B-ultrasound, and no normalities in the fetus, placenta and amniotic fluid; and (ii) those who had given birth to normal living infants. The final pregnancy outcomes were compared between the two groups, including the incidence rates of term delivery, premature delivery, fetal growth restriction, gestational hypertension and gestational diabetes mellitus.

Evaluation of safety

Both groups were observed for treatment-related adverse reactions during treatment.

Statistical analysis

SPSS 16.0 software was employed for statistical analysis. Numerical data were expressed as percentage (%), and chi-square (χ^2) test was used for comparisons between groups. Quantitative data were expressed as mean ± standard deviation (x ± s), with two independent samples *t*-test for comparisons of means between two groups and paired *t*-test for comparisons of means before and after intervention within the group. P <0.05 indicated that the difference was statistically significant.

Results and Discussion

Scores of symptoms and signs of kidney deficiency syndrome and blood stasis

The scores of soreness and weakness of waist and knees, dizziness and tinnitus, dim complexion and dark purple tongue significantly declined in both groups after treatment compared with those before treatment (P <0.05). After treatment, such scores were significantly lower in experimental group than those in control group (P <0.05) (Table 1).

Changes in Th17, Treg counts and inflammatory factor levels

Compared with those before treatment, the ratios of Th17/CD4$^+$ cells and Th17/Treg were significantly decreased, whereas the ratio of Treg/CD4$^+$ cells was significantly increased in both groups after treatment (P <0.05). After treatment, the ratios of Th17/CD4$^+$ cells and Th17/Treg were significantly lower in experimental group than those in control group, while the ratio of Treg/CD4$^+$ cells was significantly higher in experimental group than that in control group (P <0.05) (Table 2).

In contrast with those before treatment, IL-4 and IL-10 levels significantly rose, while IL-17 level significantly declined in both groups after treatment (P <0.05). In comparison with control group, experimental group exhibited significantly higher IL-4 and IL-10 levels and a significantly lower IL-17 level after treatment (P <0.05) (Table 2).

Table 1 — Scores of symptoms and signs of the syndrome of kidney deficiency and blood stasis (x ± s)

	Control (n=48)		Experimental (n=48)	
	BT	AT	BT	AT
Soreness and weakness of waist and knees	1.73±0.45	1.45±0.36*	1.70±0.51	1.23±0.48*#
Dizziness and tinnitus	1.66±0.49	1.52±0.56*	1.68±0.53	1.28±0.50*#
Dim complexion	1.59±0.50	1.44±0.33*	1.63±0.42	1.30±0.51*#
Dark purple tongue	1.56±0.50	1.41±0.33*	1.62±0.57	1.29±0.40*#

[BT, Before treatment; AT, After treatment. *P <0.05 *vs*. before treatment within the group; #P <0.05 *vs*. control group]

Table 2 — Changes in Th17, Treg counts and inflammatory factor levels (x ± s)

	Control (n=48)		Experimental (n=48)	
	BT	AT	BT	AT
Th17/CD4$^+$ (%)	4.92±1.38	3.53±1.92*	4.85±1.45	2.11±1.29*#
Treg/CD4$^+$ (%)	4.23±0.58	5.18±0.23*	4.21±0.65	6.31±0.85*#
Th17/Treg	1.16±0.32	0.67±0.14*	1.15±0.28	0.33±0.27*#
IL-4 (ng/L)	28.47±8.97	32.16±7.13*	29.11±8.95	41.54±9.18*#
IL-10 (ng/L)	49.26±9.77	61.78±12.31*	50.84±8.41	91.62±11.63*#
IL-17 (ng/L)	60.39±8.18	52.71±8.63*	61.06±9.73	43.54±7.74*#

[BT, Before treatment; AT, After treatment. *P <0.05 *vs*. before treatment within the group, #P <0.05 *vs*. control group]

Th17/Treg transcription factors RORγt and FoxP3 mRNA expression levels in PBMCs

The mRNA level of FoxP3 was significantly increased, whereas the mRNA level of RORγt was significantly reduced in both groups after treatment compared with those before treatment (P <0.05). After treatment, the mRNA level of FoxP3 was significantly higher, while the mRNA level of RORγt was significantly lower in experimental group than those in control group (P <0.05) (Table 3).

Successful pregnancy rates and pregnancy outcomes

No statistically significant difference was found in the re-pregnancy rate between control group and experimental group after treatment. In comparison with control group, experimental group had a significantly higher successful pregnancy rate and a significantly lower repeated abortion rate (P <0.05). Besides, experimental group displayed a significantly higher term delivery rate and a significantly lower premature delivery rate in contrast with control group (P <0.05). There were no statistically significant differences in the incidence rates of fetal growth restriction, gestational diabetes mellitus and gestational hypertension (Tables 4 and 5).

Adverse reactions

During the entire treatment process, pregnancy reactions such as nausea and vomiting as well as frequent micturition were occasionally observed in some patients, and there were no allergic reactions and other obvious adverse reactions caused by drugs. In addition, no abnormalities were detected in safety-related measurements in the two groups of objects before and after treatment.

Table 3 — Th17/Treg transcription factors RORγt and FoxP3 mRNA expression levels in PBMCs (x ± s)

	Control (n=48)		Experimental (n=48)	
	BT	AT	BT	AT
RORγt mRNA	0.75±0.18	0.51±0.14*	0.80±0.21	0.38±0.13*#
FoxP3 mRNA	0.14±0.09	0.21±0.07*	0.12±0.07	0.33±0.08*#

[BT, Before treatment; AT, After treatment. Relative expressions of target genes were calculated using $2^{-\Delta\Delta Ct}$. *P <0.05 *vs*. before treatment within the group, #P <0.05 *vs*. control group]

Table 4 — Successful pregnancy rates (n=48)

	Repregnancy	Successful pregnancy	Repeated abortion	Unpregnancy
Control	46 (95.83)	37 (77.08)	9 (18.75)	2 (4.20)
Experimental	47 (97.91)	45 (93.75)*	2 (4.16)	1 (2.10)

[*P <0.05 *vs*. control group]

RSA is difined as the loss of a fetus weighing less than 500 g within 20 weeks of pregnancy with the same sexual partner for 2 or more consecutive times, with an incidence rate of 1-5%. In RSA patients, the risk of repeated abortion is close to 40%. As to its treatment, such symptomatic treatments as endocrine regulation, immunotherapy, anticoagulation therapy and anti-infection therapy are adopted based on relevant examination results in clinical practice, and some relative adverse reactions occur during treatment[14]. Moreover, the cause is unknown in half of the cases, namely URSA, and effective Western medicine treatment methods are lacked[15]. TCM therapy has obvious advantages in treating such a disease. In TCM, it is believed that habitual abortion is fundamentally caused by kidney deficiency, and many well-known prescriptions formed during the long-term development of TCM have displayed good curative effects[16]. However, the mechanism has not yet been elucidated, limiting its promotion and application in clinical practice. For this reason, deeply revealing the mechanism of URSA and discovering effective treatment methods are always problematic issues in this field.

The occurrence of URSA, a pathological pregnancy caused by abnormal secretion of cytokines in the body or dysfunction of local cells at the maternal-fetal interface, has a relation to the maternal-fetal immune tolerance mechanism and is the result of immune rejection[17]. Treg, a class of $CD4^+$ cells with the function of negative immunoregulation, can specifically express FoxP3, promote the release of anti-inflammatory factors IL-4 and IL-10 by secreting inhibitory cytokines and maintain autoimmune tolerance. Th17 specifically express RORγt, mainly act as pro-inflammatory factors in the body and participate in the development and progression of immune diseases to some extent[18]. It has been proven that the Th17/Treg immune imbalance affects the occurrence and development of URSA[10]. In this study, it was found that after treatment, the level of Th17 in peripheral blood was decreased, the level of Treg was increased, and the Th17/Treg balance tended to recover in control group and experimental group, obviously improving the immune function of the body, thus affecting pregnancy outcomes. Active

Table 5 — Pregnancy outcomes of patients with successful pregnancy

	Term delivery	Premature delivery	Fetal growth restriction	Gestational hypertension	Gestational DM
Control group (n=37)	30 (81.08)	7 (18.92)	2 (5.41)	3 (8.11)	2 (5.41)
Experimental group (n=45)	43 (95.56)	2 (4.44)*	1 (2.22)	1 (2.22)	3 (6.67)

[*P <0.05 *vs*. control group]

immunotherapy is a common treatment method for URSA. It protects embryos and promotes their healthy development mainly by injecting the lymph injection from the husband of patients or a healthy third party to stimulate the immune system of mothers, block the attack of the immune system on the embryo, prevent antigen rejection, and suppress humoral immunity[19]. The results of this study revealed that the successful pregnancy rate was 77.1% in control group where only active immunotherapy was adopted. However, the repeated abortion rate remains high in the case of active immunotherapy alone for URSA.

There are many reports on TCM therapy in regulating menstruation and preventing miscarriage and modulating the immune balance of the body, indicating that the combination of TCM therapy in treating URSA is expected to raise the successful pregnancy rate and lower the risk of repeated abortion[20]. In TCM, URSA belongs to "habitual abortion", and the etiology mechanism is as follows[21]: it is fundamentally caused by kidney deficiency that results in the inability to nourish the foetus, thus leading to abortion or even repeated abortion. Besides, blood stasis is the syndrome. Kidney-Yang deficiency or internal heat due to kidney-Yin deficiency leads to stagnation of blood stasis, and unsmooth flow of blood gives rise to inability to nourish the foetus and abortion. Repeated abortion aggravates the stagnation of blood stasis, thereby forming a vicious circle. Therefore, the combination of tonifying kidney and promoting blood circulation is the major method for the treatment of this disease. Famous TCM physicians prevent and treat habitual abortion by tonifying kidney and promoting blood circulation, and the representative prescriptions include Guizhi Fuling Pills, Shaofu Zhuyu Decoction and Shoutai Pills[22]. The present study has shown that after treatment, the scores of symptoms and signs of the syndrome of kidney deficiency and blood stasis and the successful pregnancy rate are clearly better in experimental group than those in control group, and the Th17/Treg balance is alleviated. In comparison with control group, experimental group had notably elevated levels of IL-4 and IL -10 and mRNA level of FoxP3, and remarkably lowered IL-17 level and RORγt mRNA level. It suggests that the modified Bushen Huoxue Prescription combined with active immunotherapy is capable of dramatically improving the immune function of patients, regulating the release of inflammatory factors, increasing the successful pregnancy rate of URSA patients, and reducing the repeated abortion rate. Besides, no obvious adverse reactions were found in the two groups during treatment.

Conclusion

Modified Bushen Huoxue Prescription, a concoction of medicinal herbs and traditional chinese medicines, combined with active immunotherapy achieves a definite therapeutic effect on unexplained recurrent spontaneous abortion (URSA) probably by modulating the levels of inflammatory factors and alleviating the Th17/Treg immune imbalance to improve pregnancy outcomes and lower abortion rate, which is also highly safe. However, clinical data with large samples from multiple regions and multiple centers are still required to promote its clinical application.

Conflict of Interest

Authors declare no competing interests.

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