# Effects of Toosendanin on Pregnancy and Uterine Immunity Alterations in Mice

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## Abstract:

This study was conducted to explore the abortifacient effect and the mechanisms of the Chinese herbal medicine component toosendanin, and to elucidate the significance of the Th1 cytokines IFN-gamma and TNF-alpha, CD4+ and CD8+ T lymphocytes in the occurrence of abortion. Graded doses of toosendanin were given by intraperitoneal injection (i.p.) to mice at day 5, 6, 7 of gestation. The levels of Th1 cytokines (IFN-gamma, TNF-alpha) in serum and uterine tissues from mice sacrificed at day 8 were analyzed by enzyme linked immunosorbent assay (ELISA). Presence of T lymphocytes in endometrium was detected by immunohistochemistry. The results revealed that injection of toosendanin could produce a dose-dependent toxicity. The IFN-gamma, TNF-alpha content in serum and uterine tissues were increased significantly. The CD4+ and CD8+ T lymphocytes were also increased in the endometrium of toosendanin treated groups. In conclusion, toosendanin is pregnancy-toxic to animals and it is relevant to the increased contents of IFN-gamma, TNF-alpha and CD4+, CD8+ T lymphocytes.

**Keywords:** Toosendanin | IFN-γ | TNF-α | Pregnancy Toxicity | Phytochemicals | Pharmacodynamics | Medicinal Plants | Toxic Plants

## Article:

### Introduction

The use of the fruit and bark of family *Melia* as digestive tract parasiticide was recorded about two thousand years ago in ancient China. Toosendanin (TSN), a triterpenoid derivative, was extracted from the bark of *Melia toosendan Sieb. et Zucc.* in the 1950's and had been used as an ascarifuge and agricultural insecticide for more than fifty years in China (Shi and Li, 2007). It has been demonstrated that TSN possesses special biological functions as well as considerable various values in scientific research, clinical medicine and agriculture (Shi andWang, 2006). The antifeedant activity of the fruit extract of the *Argentinian Melia azedarach L. (Meliaceae)* was

confirmed by Carpinella and his colleagues (2003). Growth of F1 larvae of the cabbage looper was significantly impaired by toosendanin (Akhtar and Isman, 2003).

In the past few years, there have been more and more reports on adverse effects of plant and vegetable pesticide residues on public health, especially on pregnant females. Some of the pesticides may cause infertility, spontaneous abortion, growth retardation and congenital anomalies. The use of neem (*Azadirachta indica*) seed extracts which contain toosendanin abrogated pregnancy in subhuman primates (Mukherjee *et al.*, 1996a) and rodents (Mukherjee and Talwar, 1996b). A certain dose of toosendanin induced abortion in mice completely (Zhang *et al.*, 2005). In previous studies the authors have demonstrated that the aqueous extract of the bark of*Melia toosendan Sieb. et Zucc* abrogated pregnancy inmice (Zhai *et al.*, 2005). In the present study, toosendanin was given to the pregnant mice at graded dosages. Abortion effect was observed and the uterine immunological parameters were detected in order to evaluate the abortifacient effects of toosendanin and the immunological alterations at the maternal-fetal interface.

## Materials and Methods

### *Animals*

Ten-week old BALB/c mice were purchased from the Experimental Animal Center of the Hebei Medical University, China. The animals were given free access to mouse chow and water, with a 12-hour light cycle from 7:00 to 19:00. Pregnancies were obtained by housing one estrous female with one male overnight, and the female was examined each day in the early morning for the presence of a vaginal plug. The day when the vaginal plug was detected was designated as day 0 of pregnancy.

### *Reagents*

Toosendanin extract (containing 90% of toosendanin) was purchased from Shenzhen Zhuoyuan Developing Company Ltd., China and dissolved in 10% propylene glycol physiological saline at graded concentrations of 10, 20, 30, 40$\mu$g/ml, respectively. Rat anti-mouse CD4+ monoclonal antibody (IgG2b) and rat anti-mouse CD8+ alpha monoclonal antibody (IgG2a) were obtained from Serotec, UK.

### *Animal Treatments*

The pregnant mice (body weight 20.02 ± 1.20 g) were divided into five groups randomly. Group A was kept as the control. Groups B, C, D and E were experimental groups. Mice in group A were given 0.4ml 10% propylene glycol normal saline by intraperitoneal injection (i.p.) at day 5, 6, 7 of gestation.Mice in groupsB,C,DandEwere given 0.4ml of toosendanin solution (the concentrations being 10$\mu$g/ml in group B, 20$\mu$g/ml in group C, 30$\mu$g/ml in group D, 40$\mu$g/ml in group E, respectively by i.p.) at the same days of gestation as in group A.

*Calculation of Embryo Loss Rate and Abortion Rate*

All the pregnant mice were sacrificed by cervical dislocation at day 8 of gestation and the uterus was examined for viable and resorbing embryos. The viable embryos (*V )* were well oxygenated (pink) and showed a well-defined embryonic capsule and placenta. The resorbing embryos (*R)* were usually smaller, showed signs of ischemia, hemorrhage, and often were macerated and black in color without identifiable embryo or placenta. The incidence of embryo loss was presented as a percentage of the contents of the uterus (100*R/(V + R))* (Zhong *et al.*, 2002).The incidence of abortionwas calculated as a percentage of the contents of the miscarriage (100 abortive mice/total mice).

*ELISA Assay for IFN-γ and TNF-α*

At day 8 of gestation, pregnant mice were sacrificed, the uterus was carefully cleansed of fat and the fetus was removed. Uterine lysates were prepared in PBS (pH 7.4) containing phenylmethanesulfanyl fluoride (PMSF, 0.75$\mu$g/ml) in a volume of 6 times of the uterine weight at 4◦C. One quarter of the uterine horn was fixed in Bouin's solution and 6$\mu$m sections were prepared for immunohistochemistry. The serum was isolated routinely and uterine lysates were centrifuged for 15 min at 12,000 r/min at 4◦C and the supernatants were collected for ELISA assay. Using commercial kits of IFN-$\gamma$ and TNF-$\alpha$ (R & D Systems, Minneapolis USA), ELISA assays were performed according to the manufacturer's instructions.

*Immunohistochemistry*

The uterine sections were deparaphinized and hydrated, and stained by using streptavidin immunoperoxidase technique. Briefly, the tissue sections were overlayed with CD4+ or CD8+ antibody after citrate pretreatment, and incubated overnight in a humid chamber at 4◦C. Then the sections were allowed to react with a biotinylated rabbit anti-rat IgG *(*H+L*)* antibody (Vector, UK) in 10% normal calf serum, followed by incubation with horseradish peroxidase conjugated streptavidin (Vector, UK). The tissue sections were washed thoroughly with PBS during each procedure. Finally, the sections were incubated with 0.03% DAB containing 0.05% hydrogen peroxide and mounted.

T cells were observed under a light microscope (magnification 40×*)*. All positive cells in campus visualis were counted. The mean value was pooled from 20 high power fields in each sample, and averaged from 10 mice.

*Statistical Analysis*

Statistical analysis of datawas conducted using SPSS 11.0, analyzed with One-way Analysis of Variance and $\chi$2-test ($p < 0.05$ was taken as significant).

## Results

*The Abortion Effect of Toosendanin*

Mice in group A, pretreated with 10% propylene glycol physiological saline, showed a natural abortion rate of 20%.Mice in group B were pretreated with the dose of 1/60LD50 toosendanin on day 5, 6 and 7 and showed an abortion rate of 50%, and 42.59% embryo resorption rate. Group C has 10 mice and 6 were aborted, the resorption rate being 46.55%. Eight in 10 mice in group D were aborted and most embryos were lost, while a few showed a well-defined embryonic capsule. Group E pretreated with the dose of 1/15 LD50 toosendanin showed an abortion rate of 90%, the embryo resorption rate was 73.33%. The resorbed conceptus was severely macerated and black (Table 1).

*Effects of Toosendanin on Uterine and Serum TNF-α Contents*

Toosendanin elevated the TNF-$\alpha$ levels both in serum and uterine tissues compared to the control ($p < 0.05$ or $p < 0.01$). The TNF-$\alpha$ concentrations in uterous tissues and serum were relatively stable, at 1439.628 ± 163.074 pg/g protein, or 3729 ± 0.144 pg/m. After an increase the concentration of toosendanin, the abortion rate and TNF-$\alpha$ level were elevated from 3.729±0.144 to 55.979±29.753 in serum and from 1439.628±163.074 to 2, 263.128±281.934 in uterus lysate (Table 2).

**Table 1. Different Abortion in Different Groups**

| Groups | Gestation Days 5, 6, 7 | Abortion Rate % | Resorptions % |
|---|---|---|---|
| A | 0.4ml saline, i.p. daily | 20.00(2/10)cDE | 13.79(8/58)BCDE |
| B | 0.4ml TSN (10 μg/ml), i.p. daily | 50.00(5/10) | 42.59(23/54)ADE |
| C | 0.4ml TSN (20 μg/ml), i.p. daily | 60.00(6/10)a | 46.55(17/58)AdE |
| D | 0.4ml TSN (30 μg/ml), i.p. daily | 80.00(8/10)A | 70.37(38/54)ABc |
| E | 0.4ml TSN (40 μg/ml), i.p. daily | 90.00(9/10)A | 73.33(44/60)ABC |

Data with different letters indicate significant differences at $p < 0.01$ (capital letters) or at $p < 0.05$ (small letters).

**Table 2. TNF-$\alpha$ Contents in Serum and Uterine Tissues of Different Groups**

| Groups | Gestation Days 5, 6, 7 | Serum (pg/ml) | Uterine Lysate (pg/g) |
|---|---|---|---|
| A | 0.4ml saline, i.p. | 3.729±0.144bCDE | 1, |

| | | | |
|---|---|---|---|
| | | | 439.628±163.074bCDE |
| B | 0.4ml TSN (10$\mu$g/ml), i.p. daily | 31.938±9.827ad | 1, 893.378±61.236ae |
| C | 0.4ml TSN (20 $\mu$g/ml), i.p. daily | 32.688±11.264Ab | 1, 997.436±271.146A |
| D | 0.4ml TSN (30 $\mu$g/ml), i.p. daily | 43.438±14.965A | 2, 008.374±175.374A |
| E | 0.4ml TSN (40 $\mu$g/ml), i.p. daily | 55.979±29.753Ade | 2, 263.128±281.934Ab |

Values of TNF-$\alpha$ were expressed in pg/mg protein. Data with different superscripts have significant differences at $p < 0.01$ (capital letters) or at $p < 0.05$ (small letters).

*Effects of Toosendanin on Uterine and Serum IFN-γ Contents*

Toosendanin significantly elevated the IFN-$\gamma$ levels both in serum and uterus tissues in comparison to the controls ($p < 0.05$ or $p < 0.01$). The increase of the abortion rate and INF-$\gamma$ concentrations were observed as the toosendanin dosage increased, with the highest level being observed in serum at 56.250±22.991*(*6.000±0.354 in the control) and 4182.402± 608.154 in uterus (2743.998± 169.704 in the control). See Table 3.

*Changes of CD4+ and CD8+ T Lymphocytes in Endometrium Post-TSN Treatment*

In group A, there were fewer CD4+ T cells were less (6.175 ± 0.106) than CD8+ T cells (13.780±0.636), the CD4+/CD8+ ratio was 0.448±0.013. Mice in groups B, C, D and E received different dosages of TSN injection, showed an increasing rate of abortion, with the elevation of CD4+ and CD8+ cell counts. The CD4+ T cell number in group E was 9 times the number in group A ($p < 0.01$) at day 8 of gestation, indicating a close relationship between the CD4+ cells and abortion rate. There was a significant higher CD8+ cell count in TSN treated groups (Table 4).

**Discussion**

There have been few reports on the embryotoxicity of toosendanin in animals. Zhang (2005) and others reported that intraperitoneal injection of toosendanin at 1/10 of LD50 (13.8mg/kg) produced embryotoxicity in Kunmingmice. Aqueous extract of the bark of *Melia toosendan Sieb. et Zucc* abrogated pregnancy in mice (Zhai *et al.*, 2005). In our present study, pregnant BALB/c mice were given toosendanin solution by i.p. injection for 3 consecutive days. The results revealed that the abortion rate was significantly increased in a dose dependent manner with elevation of CD4+, CD8+ T cells and INF-$\gamma$, TNF-$\alpha$ content in uterus.

**Table 3. INF-$\gamma$ Contents in Serum and Uterine Tissues of Different Groups**

| **Groups** | **Gestation Days 5, 6, 7** | **Serum (pg/ml)** | **Uterine (pg/g)** |
|---|---|---|---|

| | | | |
|---|---|---|---|
| A | 0.4ml saline, i.p. | 6.000±0.354bcDE | 2,743.998± 169.704bcdE |
| B | 0.4ml TSN (10$\mu$g/ml), i.p. daily | 34.219±8.646aE | 3,633.600±374.568a |
| C | 0.4ml TSN (20$\mu$g/ml), i.p. daily | 37.813±10.789ae | 3,724.998±421.212a |
| D | 0.4ml TSN (30$\mu$g/ml), i.p. daily | 42.656±15.118A | 3,805.002±243.864a |
| E | 0.4ml TSN (40$\mu$g/ml), i.p. daily | 56.250±22.991ABc | 4,182.402±608.154A |

Values of TNF-$\alpha$ were expressed in pg/mg protein. Data with different letters have significant differences at $p < 0.01$ (capital letters) or at $p < 0.05$ (small letters).

**Table 4. T Lymphocyte Contents in Uterine Tissues in Different Doses of Toosendanin**

| **Groups** | **Gestation day 5, 6, 7** | **CD4+** | **CD8+** | **CD4+/CD8+** |
|---|---|---|---|---|
| A | 0.4ml saline, i.p. daily | 6.175± 0.106bCDE | 13.780± 0.636bCDE | 0.448± 0.013bCDE |
| B | 0.4mlTSN(10$\mu$g/ml), i.p. daily | 11.000± 1.414acDE | 17.000± 1.414aCDE | 0.652± 0.137aDE |
| C | 0.4mlTSN(20$\mu$g/ml), i.p. daily | 15.333± 0.803AbDE | 21.000± 1.000ABDE | 0.730± 0.024ADE |
| D | 0.4mlTSN(30$\mu$g/ml), i.p. daily | 51.295± 1.477ABCe | 38.625± 2.121ABC | 1.327± 0.008ABC |
| E | 0.4mlTSN(40$\mu$g/ml), i.p. daily | 55.850± 2.262ABCd | 41.000± 2.414ABC | 1.362± 0.007ABC |

Data with different letters indicate significant differences at $p < 0.01$ (capital letters) or at $p < 0.05$ (small letters).

One of the most remarkable immunological regulations in reproductive immunology is the maternal immune tolerance toward the fetal semi-allograft during pregnancy. It has been suggested that successful pregnancy is associated with a T-helper 2 (Th2) type phenomenon. During pregnancy, Th1 cytokine production is down-regulated (Clifford *et al.*, 1994). Recently, significantly higher serum levels of Th2 cytokines, IL-6 and IL-10, were detected in normal pregnancy compared to unexplained recurrent pregnancy losses and significantly higher serum level of the Th1 cytokine, IFN-$\gamma$ , was present in women with recurrent pregnancy losses compared to normal pregnancy (Raghupathy *et al.*, 1999). In pregnant mice, the injection of one Th1 cytokine, such as IFN-$\gamma$ , TNF-$\alpha$ and IL-2, or combination of those significantly increased fetal resorption (Chaouat *et al.*, 1990; Clark *et al.*, 2004). By contrast, Th2 cytokines inhibit Th1-induced tissue factor production by monocytes. Studies on IVF (*in vivo* fertilization) patients showed that women with recurrent spontaneous abortion or implantation failure had significantly higher Th1 cytokine expression, i.e., low level of IL-10 and high level of IFN-$\gamma$ and TNF-$\alpha$ (Ng

*et al.*, 2002). In LPS induced abortion modelmice, the IL-10 level was lower (Zhong *et al.*, 2008). Our present study demonstrated that toosendanin induced abortion in mice is closely related to a Th1 cytokine response (IFN-*γ* and TNF-*α)* at the maternal-fetal interface.

TNF-*α* is excreted by macrophages, activated natural killer (NK) cells and mastocytes (Robaye *et al.*, 1991). Injection of TNF-*α* into pregnant mice significantly increased fetal resorption. It has been shown that TNF-*α* production by macrophages is suppressed at the mRNA level during early pregnancy and a significant increase does not occur until at the eighthmonth of gestation (Tranchot-Diallo *et al.*, 1997). TNF-*α*induced a loss of trophoblast viability and combination of TNF-*α* and IFN-*γ* enhanced the cytotoxicity (Yui *et al.*, 1994). TNF-*α* is supposed to suppress the growth of trophoblasts (Todt *et al.*, 1996), possibly by inducing apoptotic changes in these cells (Yui *et al*., 1994). TNF-*α* is present on the proliferating tips of anchoring villi, invasive interstitial cytotrophoblasts, and endovascular trophoblasts which invade spiral arteries (Lea *et al.*, 1997). These findings suggest a role for TNF-*α* in early invasion of trophoblasts. However, a decrease in the release of TNF-*α* from PBMC (peripheral blood mononuclear cell) upon the recognition of HLA-G (human leucocyte antigen) was a consistent finding among normal women, recurrent aborters, and men (Maejima *et al.*, 1997). Perhaps the regulation of TNF-*α* synthesis may determine the reproductive outcome. TNF-*α* expression in CD3+/CD8- cells in infertile women with implantation failures is significantly up-regulated as compared to that of normal controls (Kwak-Kim *et al.*, 2003).

Both T cells (including CD+4Th1 and CD+8Th) and NK cells can produce IFN-*γ* . As a Th1 cytokine, IFN-*γ* contributes to initiation of pregnancy-induced uterine vascular modification, maintenance of decidual integrity, and regulation ofmaturation and senescence of the uterine natural killer cell population (Peschon *et al.*, 1998). IFN-*γ* can be a regulator to proliferation and differentiation of T cell and antigen presenting cells (Zhu *et al.*, 1999;Wang and Zhu, 2002). IFN-*γ* primes and triggers macrophages, which secrete cytokines such as TNF-*α* and IL-2. The cytokines induce infiltratingNKcells to become lymphokine-activated killer cells, which damage the placenta and fetus (Gifford and Lohmann-mathes, 1987). In return, the NK cells producemore IFN-*γ* (Zhu *et al.*, 2005). IFN-*γ* is supposed to be toxic to embryo and trophoblasts (Polgar *et al.*, 1996), a ranged doses of IFN-*γ* (10 ~ 1000 ng/ml) can significantly inhibit viability of trophoblastic cells and secretion of hCG (Wang *et al.*, 2004).

In the present study by using ELISA, we observed that the TNF-*α*, IFN-*γ* concentrations in serum and the supernatant of uterine tissue were significantly higher in mice treated with toosendanin compared to the control group (Tables 2 and 3).High levels ofTNF-*α*and IFN-*γ* are closely associated with the elevated levels of CD4+ cell and this may subsequently exert a negative impact on reproduction (Thum *et al.*, 2007). These results confirms the notion that Th1 cytokines are detrimental to pregnancy maintenance (Raghupathy, 1997).

Pregnancy was terminated successfully in both rodents and primates by use of purified Neem extracts. An increase in CD4 and CD8 cells is noted in spleen at 96 hours and mostly CD8 cells

in mesenteric lymph nodes (Talwar *et al.*, 1997). Treatment causes an elevation of both immunoreactive and bioactive TNF-alpha and gamma-interferon in serum, mesenteric lymph nodes, and foetoplacental tissue (Talwar *et al.*, 1997). Toosendanin was a component of the bark ofMelia toosendan Sieb. et Zucc which is botanically in a close relative of Neem (*Azadirachta indica*). Toosendanin is cytotoxic for some human cancer cell lines derived from different organs. The effect is taken through suppressing the cell cycle progression and inducing cell apoptosis (Zhang *et al.*, 2005). Recent studies showed that toosendanin at low concentrations of $1–10\times10{-}7$Minduced PC12 cells differentiation first and then apoptosis (Tang *et al.*, 2003). Studies suggest that the antibotulismic effect of TSN is achieved by preventing BoNT from approaching its enzymatic substrate, the SNARE protein. It is also found that TSN can induce differentiation and apoptosis in several cell lines, and suppress proliferation of various human cancer cells (Shi and Li, 2007). Other researches suggested that toosendanin causes death of primary rat hepatocytes by mitochondrial dysfunction and caspase activation. Generation of ROS and MAP kinases activation might be involved in this process (Zhang *et al.*, 2008). How toosendanin exerts the pregnancy-toxicity remains further studying.

Cytokines do not act separately but form a complex regulatory network in which modulatory interactions maintain homeostasis between the fetal unit and the maternal immune system. This complex interplay between maternal and fetal immune mechanisms also changes temporally as pregnancy progresses (Marzi *et al.*, 1996; Tranchot-Diallo *et al.*, 1997).When this delicate balance is adversely affected, immunoregulatory mechanisms may be insufficient to restore homeostasis and this may lead to pregnancy failure. Other possible involvement might be the direct embryotoxicity of toosendanin, since there has been reported that toosendanin at 30$\mu$g per mother resulted in embryo death totally (Zhang *et al.*, 2005). There has been no relevant study of the effect of toosendanin on uterine contraction. In this study, the authors postulate that toosendanin disturbed the homeostasis of Th1/Th2 type cytokines by elevating the levels of TNF-$\alpha$ and IFN-$\gamma$ and CD4+/CD8+ ratio resulting in abortion. Since the toosendanin related drugs are widely used as agricultural insecticide, pregnant animals should be kept away from the contaminated grasses or vegetables.

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