



# Effect of transvaginal ultrasound on human chorionic villus cell apoptosis during pregnancy

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Genet. Mol. Res. 14 (4): 18771-18777 (2015)

Received August 12, 2015

Accepted October 16, 2015

Published December 28, 2015

DOI <http://dx.doi.org/10.4238/2015.December.28.26>

**ABSTRACT.** With the advancement of ultrasonic technology in recent years, sonography has become a common medical diagnostic tool, as it has elevated output sonic intensity and elongated exposure time. This study investigates the effect of ultrasound on human chorionic villus cell apoptosis during early pregnancy. Transvaginal ultrasound was performed for a total of 60 women who had undergone induced abortion at our hospital. They were randomly divided into the control, short ultrasound (10 min), and long ultrasound (20 min) groups (N = 20 each). Twenty-four hours after ultrasonic exposure, chorionic villus tissues were extracted during induced abortion, and were tested for cell apoptosis using flow cytometry. Bax and B cell lymphoma-2 (Bcl-2) protein levels were also quantified by immunohistochemistry. We found that the long ultrasound group had significantly higher cell apoptosis rates compared to the short ultrasound group, which in turn had higher rates compared to the control group ( $P < 0.05$  in both cases). Bax protein levels were elevated in both the long and

short ultrasound groups ( $P < 0.05$ ). Bcl-2 proteins in two ultrasound groups, however, were downregulated as compared to those in the control group ( $P < 0.05$ ). It is therefore possible that transvaginal sonography can potentiate the apoptosis of human chorionic villus cells by increasing the Bax/Bcl-2 protein ratio.

**Key words:** Transvaginal ultrasound; Human chorionic villus cells; Cell apoptosis; Bax/Bcl-2

## INTRODUCTION

Cell apoptosis, as one of the programmed cell death mechanisms, is of critical importance for body homeostasis under normal physiological conditions (Longtine et al., 2012). The disruption of normal apoptosis, however, may lead to severe consequences such as premature delivery, which is believed to be related to abnormal apoptosis of placental cells (Bosco et al., 2015). Transvaginal ultrasound has been widely used in gynecologic practice (Ikpeme et al., 2014) due to its inherent advantages, including proximity to the target tissue, higher frequency of the probe, and higher-resolution images (Kubešová et al., 2013). Morphological alterations in chorionic villus cells under ultrasound have been studied; significant changes were observed in microscopic structure after 5-min high frequency exposure under transvaginal sonography but not abdominal sonography until 20 min later. These phenomena raised the issue of the effect of ultrasound on embryonic tissue morphology. This study therefore aimed to investigate if transvaginal ultrasound can regulate apoptosis of human chorionic villus cells, via clinical study of early-pregnant patients undergoing induced abortion.

## MATERIAL AND METHODS

### General information of patients

A total of sixty pregnant women (age: 21-35 years; average age: 28.4 years old) who underwent induced abortion surgery at Shandong Weihai Municipal Hospital between January 2014 and January 2015 were recruited in this study. The average pregnancy time of all patients was 53.8 days. All patients had normal embryo buds and primitive heart beats. The average diameter of gestational sacs was 2.2 cm. None of the participants had any history of miscarriage, exposure to toxicity, radiation or drug abuse. This study has been pre-approved by the ethical committee of our hospital, and written consent was obtained from all subjects involved in the study.

### Grouping and sonography

Patients were randomly divided into three groups: control, short ultrasound, and long ultrasound groups ( $N = 20$  per group). Control patients were not exposed to ultrasound. The patients in the short and long ultrasound groups underwent transvaginal sonography with a 5.0 MHz high frequency probe (VolusonS6, GE, USA). Short ultrasound group patients were exposed for 10 min, while the long ultrasound group received 30 min ultrasound treatment. After 24 h, induced abortion surgery was performed for all patients, during which period chorionic villus tissues were extracted and rinsed in saline. Half of the tissue samples were digested and analyzed by flow

cytometry (Jinghong, China). The other half of tissue samples was fixed in 10% neutral buffered formalin (NBF) for further staining.

### Flow cytometry

Human chorionic villus tissues were first rinsed in PBS and cut into small pieces, which were digested by 0.25% pepsin. Cells were then re-suspended in PBS, centrifuged at 1000 *g* for 15 min, and washed twice. Cells were fixed in 70% ethanol at 4°C overnight. Before loading, cells were prepared suspended in PBS with 50 µg/mL RNaseA. After 30-min incubation at 37°C, cells were stained with propidium iodide (PI) for 30 min. Samples were then loaded onto the flow analyzer (Jinghong, China) to analyze the cell cycle.

### Immunohistochemical (IHC) staining and analysis

Human chorionic villus tissues were embedded in paraffin after NBF fixation. Tissues were then sectioned into 5-µm-thick slices using a microtome (Leica, Germany), and were mounted onto glass slides. After de-waxing and heated antigen retrieval, endogenous peroxidase activity was quenched by 3% hydrogen peroxide. Non-specific binding sites were blocked via incubation with normal goat serum (NGS) at room temperature. Tissue sections were incubated with rabbit anti-human Bax or B cell lymphoma-2 (Bcl-2) polyclonal antibody (1:100, Cell Signaling, USA) for 1 h at room temperature. Goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:100, Cell Signaling) was then added for 10 min. Streptavidin-peroxidase was finally added for 10 min. Hematoxylin was used to counterstain the tissue. Images were captured on an inverted light-field microscope (Olympus, Japan).

Parallel negative controls were stained using PBS instead of primary antibody. Positive controls were obtained by staining known Bax or Bcl-2 positive tissue samples. A positive signal was identified when brown or yellow-brown granules were found in the membrane or cytoplasm of the cell, but not in the nucleus. The results were graded based on the percentage of positive cells in one of the five randomly selected field of views. In detail, negative (-) was defined as when the positive cell percentage was <10%; weak positive (+) staining was defined as when positive cells accounted for 11-25% of total cells; positive (++) was defined as when 26-50% of total cells showed positive signals; strong positive (+++) staining was defined as when more than half of the total cells displayed a positive signal. The latter three of those categories (+, ++ and +++) were included as positive staining.

### Data analysis

The SPSS 17.0 software package was used to analyze all collected data. Data are presented as mean ± standard deviation (SD), and were analyzed by the chi-square test. Differences between groups were determined by analysis of variance (ANOVA). Statistical significance was reached when  $P < 0.05$ .

## RESULTS

### Apoptosis of human chorionic villus cells

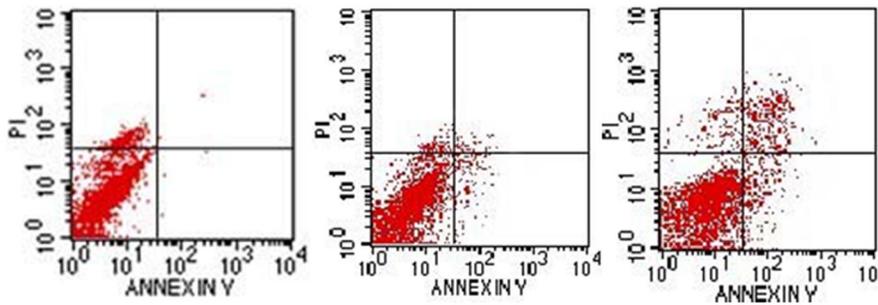
We employed flow cytometry to evaluate apoptosis of human chorionic villus cells in both

control and experimental groups. The results (Table 1, Figure 1) showed significantly greater apoptosis in the long ultrasound group as compared to the short ultrasound group ( $P < 0.05$ ). In addition, cell apoptosis in the short ultrasound group was also significantly higher as compared to that in the control group ( $P < 0.05$ ). Further comparison of early/late stage apoptosis also showed significantly potentiated cell apoptosis in both early and late stage when 30-min ultrasound was applied (Table 2).

**Table 1.** Apoptotic rates of human chorionic villus cells.

Group	Relative cell number			Apoptotic rate (%)
	Normal	Apoptotic	Sum	
Control	9,421	1,901	11,322	16.79
Short ultrasound	11,246	6,492	17,738	36.60*
Long ultrasound	5,104	16,984	22,088	76.89**

\* $P < 0.05$  compared to the control group; \*\* $P < 0.05$  compared to the short ultrasound group.



**Figure 1.** Flow cytometry showing cell apoptosis.

**Table 2.** Early- and late-stage apoptosis.

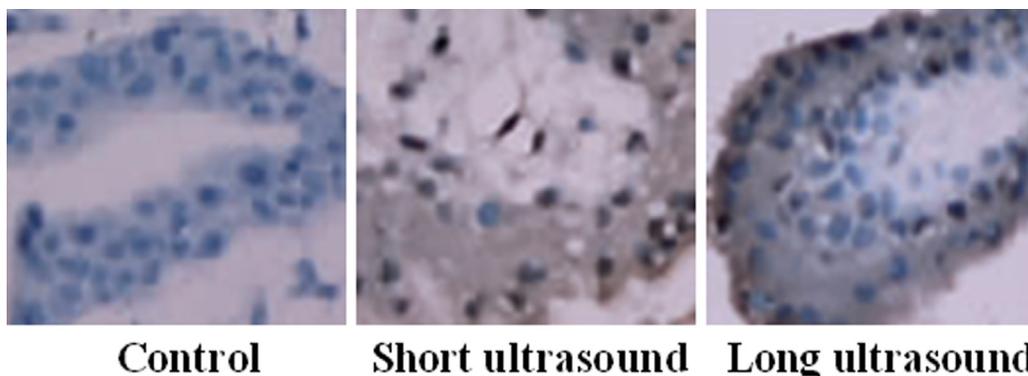
Group	Human chorionic villus cells	
	Early stage	Late stage
Control	0.19 ± 0.04	0.13 ± 0.02
Short ultrasound	1.67 ± 0.59*	3.22 ± 0.24*
Long ultrasound	8.92 ± 0.94**	6.97 ± 0.71**

\* $P < 0.05$  compared to the control group; \*\* $P < 0.05$  compared to the short ultrasound group.

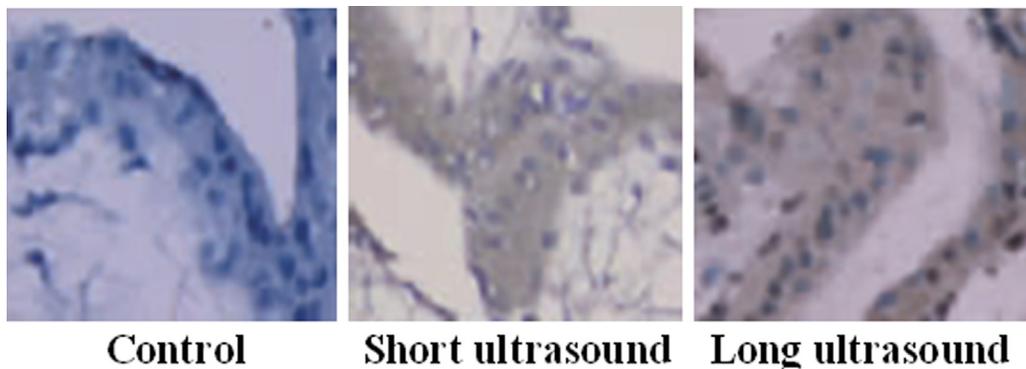
## Bax and Bcl-2 protein expression

We further used IHC staining to detect protein expression of Bax and Bcl-2 in chorionic villus tissues. Microscopic images (Figures 2 and 3) showed membrane and cytosolic expression of those two proteins as brown or yellow granules. In the control group, Bax was mainly expressed in the membrane and cytosol of syncytiotrophoblast cells. In the cells exposed to ultrasound, however, it was distributed in both syncytiotrophoblast cells and cytotrophoblastic cells (Figure 2). Its expression level, as suggested by the staining intensity, increased with elongated exposure time to ultrasound. Bcl-2 protein was expressed in the membrane and cytosol of cytotrophoblastic cells in both the control and the ultrasound groups, the latter of which, however, showed reduction in expression, which was time-dependent (Figure 3).

Further comparisons between the two ultrasound groups (Table 3) showed elevated Bax expression in the long ultrasound group as compared to that in the short ultrasound group (70 vs 50%,  $P < 0.05$ ). Bcl-2 expression was lower in both ultrasound groups as compared to the control, but did not show significant difference between the two experimental groups ( $P > 0.05$ ).



**Figure 2.** Bax protein expression in chorionic villus tissues. Left: control group with weak positive expression (+); middle: short ultrasound group with positive expression (++); right: long ultrasound group with strong positive expression (+++). Magnification: 400X.



**Figure 3.** Bcl-2 protein expression in chorionic villus tissues. Left: control group with weak positive expression (+); middle: short ultrasound group with positive expression (++); right: long ultrasound group with strong positive expression (+++). Magnification: 400X.

**Table 3.** Bax and Bcl-2 protein expression in chorionic villus tissues.

Group	N	Bax				Bcl-2			
		-	++	+++	Positive %	-	++	+++	Positive %
Control	20	17	3	0	15	14	5	1	30
Short Ultrasound	20	10	7	3	50*	16	3	1	20
Long Ultrasound	20	6	9	5	70**	17	3	0	15

\* $P < 0.05$  compared to the control group; \*\* $P < 0.05$  compared to the short ultrasound group. - indicates negative. ++ indicates positive. +++ indicates strong positive.

## DISCUSSION

Apoptosis, a form of programmed cell death, can maintain body homeostasis via elimination of mutated or aging cells (Duaso et al., 2011). Past studies have revealed simultaneous cell proliferation and apoptosis during embryonic and placenta development (Stanek, 2010). Apoptosis of trophoblasts was also observed in mice with premature delivery but not in those with term delivery (Bertucci et al., 2011), suggesting a relationship between cell apoptosis and premature delivery.

Ultrasound has now been widely used in clinics due to its multiple biological functions, including serving mechanic, thermodynamic and cavitation effects (Calzarossa et al., 2013). However, exposure of embryonic mice to color Doppler ultrasound for 30 min has been reported to induce death in myocardial cells (Jia et al., 2005). Transvaginal ultrasound, when compared with the fetal fibronectin screening test, has inherent advantages, including higher frequency, proximity to the target organ, and higher resolution (Tanvir et al., 2014). Recently, molecular effects of transvaginal ultrasound including biochemical functions, gene mutation, cell apoptosis, and morphological alternations have been studied. For example, structural alterations, functional alternations, or even gene mutations of embryos were observed after 10-min high frequency transvaginal ultrasound exposure (Ochiai et al., 2012; Son et al., 2014; Sayrac et al., 2015).

In this study, we found a significantly elevated apoptotic rate in human chorionic villus tissues following long ultrasound exposure, when compared to short ultrasound exposure and the control (76.89 vs 36.60 vs 16.79%). The results suggest that ultrasound exposure may have an effect on cell apoptosis. This is consistent with previous studies where morphological alternations in chorionic cells were observed after 5-min high frequency transvaginal ultrasound or 20-min abdominal ultrasound exposure (Liu et al., 2013). When cells were exposed to 10-min transvaginal high frequency ultrasound, apoptosis increased as well (Ratto et al., 2011), which is consistent with our study results.

Both Bax and Bcl-2 proteins participate in the process of cell death, and are known as key apoptotic proteins (Murphy et al., 2000; Hajiahmadi et al., 2015). Bcl-2 overexpression can inhibit release of cytochrome c, while Bax protein is a positive regulator of cytochrome c and thus of apoptosis (Reed, 2006; Shirali et al., 2013). This study examined Bax and Bcl-2 protein expression by IHC and found membrane and cytosolic expression of both proteins. In brief, Bax protein was expressed in the syncytiotrophoblasts but Bcl-2 proteins were expressed in both syncytiotrophoblasts and cytotrophoblastic cells. We also found increased Bax, but decreased Bcl-2 protein levels with elongated ultrasound exposure, suggesting that disruption of Bax/Bcl-2 ratio may be crucial for initiation of apoptosis (Abarikwu and Farombi, 2015).

In summary, transvaginal ultrasound increases apoptosis in human chorionic villus tissues, possibly via elevating Bax and suppressing Bcl-2 protein expression. However, the threshold protein expression required to induce excessive apoptosis in chorionic villus tissues requires further research.

## Conflicts of interest

The authors declare no conflicts of interest.

## ACKNOWLEDGMENTS

We thank the anonymous reviewers for reviewing this manuscript.

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