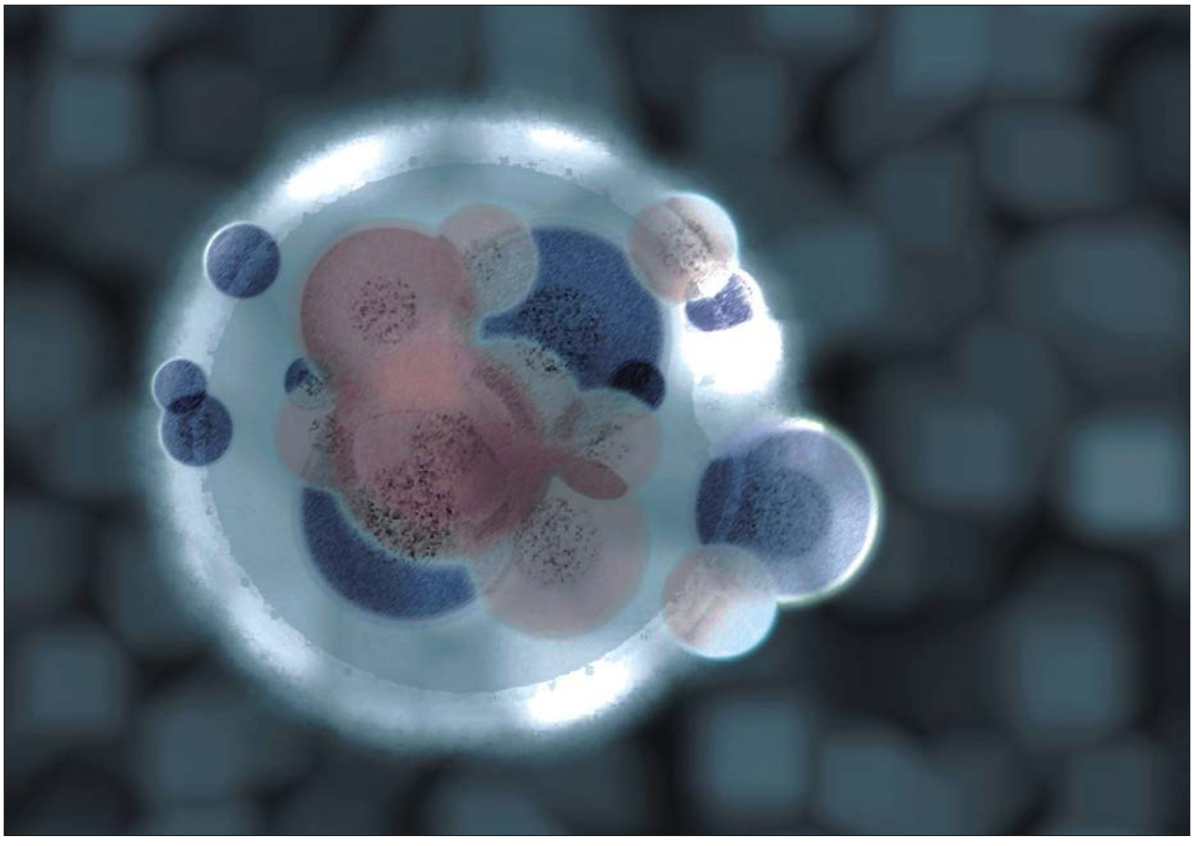

Journal of Biological Sciences

ISSN 1727-3048

science alert

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Journal of Biological Sciences 7 (4): 648-652, 2007
ISSN 1727-3048

The Ultrastructural and Stereological Study of Aqueous Extracts of *Zataria multiflora Boiss* and *Elaeagnus angustifolia* on the Mouse Fetus Stomach

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Abstract: *Zataria multiflora Boiss* (ZmB) and *Elaeagnus angustifolia* (Ea) are used in traditional folk for stomach tonic. Many pregnant women take these plants to treat the digestive problem that is natural during pregnancy. There were no any reported about the effects of these plants in adult and fetus stomach. In order to investigated the probably effects of theses extracts on the fetus digestive system, 28 pregnant BALB/c mice were divided into control, Sham and experimental groups that treated with 200 mg $kg^{\bullet 1}$ aqueous extract of ZmB and 640 mg $kg^{\bullet 1}$ aqueous extract of Ea from 6th to 15th days of pregnancy. The sham group was gavaged with equal amount of distilled water while the control group did not have any treatment. Pregnant mice were sacrificed in 16th day of pregnancy and the fetuses were extracted and their Crown-Rump lengths and weights were measured. Seven fetuses from different mothers were selected and transverse serial sections of their stomach were prepared. The volumes of different layers of stomach were estimated by Cavalieri method. The epithelial and muscular cells of stomach were studied under transmission electron microscopy. There were no significant differences on the volume of stomach after treatment by ZmB but Ea increased the volume of different layers of stomach. No significant differences revealed for weight and CRL of the fetuses. The number of mitochondria of epithelial cells and RER in smooth muscle cells were increased in experimental groups. We concluded that these plants are nonpathogenic and safe and reinforcer for fetuses stomach.

Key words: *Zataria multiflora Boiss*, *Elaeagnus angustifolia*, stomach, stereology, electron microscopy, rat

INTRODUCTION

Zataria multiflora Boiss is a plant belonging to the Labiatae family that mostly grows only in Iran (central and southern of Iran), Pakistan and Afghanistan. This plant with the vernacular name of Avishan Shirazi (in Iran) has several traditional uses such as antiseptic, anesthetic and antispasmodic, carminative and effective to remedy of dyspepsia in since to best of our knowledge there has been no report on teratogenity and effects pregnant women (Zargari, 1990). *Elaeagnus angustifolia* (Ea) is a plant belonging to the Elaeagnaceae family that grows in south eastern and west of Iran. In Traditional medicine, this plant is used for stomach and heart tonic, effective to remedy of skeletal and muscular ailments and act as carminative. Gurbuz *et al*. (2003) reported the antiulcerogenic effect of *Eleagnus multiflora* and Padmavathi *et al*. (2005) reported the anticarcinogenesis effect of *Hippophae rhamnoides* (the plant belonging to Elaeagnacea family) in forestomach but there were no any reported about the effects of these plants in fetus stomach. While there are many pregnant woman take these plants to treat the digestive problem that is natural during pregnancy, this study has initiated of these plant extract on the fetus digestive system. We used of mouse as an animal model to investigate weather these herb extracts affected on the volume of different layers and cell structure of developing fetus stomach.

MATERIALS AND METHODS

Extract preparation: Dried leaf of *Zataria multiflora Boiss* (ZmB) and fruit of *Elaeagnus angustifolia* (Ea) were purchased from a commercial source in Shiraz, Iran. The identities of the plants were confirmed by the Department of Pharmacology, Shahid Beheshti University, Tehran, Iran. The Voucher specimen was preserved for reference in the Herbarium of Pharmacological Department with 620 serial number for ZmB and 117 serial number for Ea. Dried leaf of ZmB and fruit of Ea were powdered and one hundred grams of ZmB was put into a percolator and 700 mL of distilled water was added to the powder for 4 h and one 100 g Ea powder were boiled in 700 mL of distilled water for 15 min. Subsequently, the mixture was filtered

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and concentrated under pressure by a rotary and a dedicator. The yields (w/w) of the aqueous extract of two plants were 20% (g/g).

Animals and extract administration: The adults' male and female BALB/c mice weighting between 25-30 g were obtained from the animal house of Razi Institute of Shiraz. The animals were adapted to the laboratory for a week prior to beginning of the experiments. Animals were maintained at controlled temperature (22-24°C) and a period of 12 h light and 12 h darkness in Biology Department, College of Sciences, Shiraz University. Mice had free access to food and tap water. Animals were weighed before and after experiment. Principles of laboratory care established by the National Institute of Health (NIH publication, 1985) were followed. At 3 am, 3 female mice were matched with 2 male mice. With Descry of vaginal plaque were defined the 0 day of pregnancy.

Twenty eight pregnant mice were divided into 4 groups (7 animals in each group) of control, sham, ZmB treated daily 200 mg kg^{-1} or 6 mg in 0.5 mL distilled water (Hosseizadeh *et al*., 2000) and Ea treated daily 640 mg kg^{-1} or 19.2 mg in 0.5 mL distilled water (Ahmadiani *et al*., 2000). Sham group received equal volumes of distilled water in similar condition and the control group was used to consider of feeding stress. The mentioned dose of extract was administrated orally by needle gavages from 6th to 15th gestation days. At the determined time of 11 pm, food and water were removed for 3 h in all of groups. At the end of this time animals were gavaged with extracts or distilled water in sham group.

Tissue processing and sampling: Pregnant mice at the 16th gestational day were sacrificed under deep anesthesia, with an overdose of anesthetic ether. The 5-10 fetuses of each pregnant mouse were removed and Crown-Rump length and weightings of them were measured. Then seven fetuses of different pregnant mice in each group were fixed in buffer formalin solution (10%) for one week. Fetuses were dissected and their stomach was extracted and paraffin blocks were prepared. From each block 7-10 systematic random sections (5 μm thick) were stained with hematoxylin and eosin (Russ and Dehoff *et al*., 1999). The image of each section was taken, using a digital camera (Moticam model 350, Japan) placed on a light microscope with constant magnification and then transferred to a PC computer. Morphometric study was done using stereological software which was a transparent test system composed of 900 points. Cavalieri method was used for the determination of the volume of the epithelial layer, muscular layer, total thickening of wall and lumen of stomach. The area at the level of the object was confined by the cordless natural pen device and the points hitting the object transect were counted. For determination of inter-point spacing of the point grid, the linear magnification was measured using the image of standard 0.01 μm graticule (Zeiss; Germany) with similar magnifications for all images.

The Coefficient Error (CE) of the Cavalieri method for each block was calculated (Russ and Dehoff *et al*., 1999). If CE was 0-10% or < 25%, the obtained results were valid and >25% were invalid.

Electron microscopy: The stomach of other fetuses were extracted and fixed in Carnovesky solution composed of 4% paraformaldehyde, 25% glutaraldehyde and 0.3 M cacodylate buffer with pH 7.3 (All materials were obtained from Merck Company, Germany), postfixed in 1% osmium tetroxide (Merck, Germany), embedded in TAAB resin (TAAB company, England), sectioned at a thickness of 1 μm (using Reichert-Jung-Ultracut, England) and stained with Toluidine blue. Ultra-thin sections of 60-70 nm thickness of stomach were obtained and stained with uranyl acetate (Merck, Germany) and counter stained with lead citrate (Hunter, 1993) and examined with a transmission electron microscope (LEO 906, Germany).

Statistical analysis: The different volumes of stomach were determined by Mann-Whitney-U test ($\alpha = 0.01$) and the weight and length of fetuses were analyzed by pair t-test ($\alpha = 0.05$).

RESULTS

Animal weight and crown-rump length: There were no any craniofacial malformations in fetuses. We didn't also find any limb abnormality. The CRL and weight of fetuses did not changed by administration of ZmB and Ea extracts compared with control and sham groups (Table 1). Thus apparently, these doses of the extract had no toxic effect on the fetus growth.

The histomorphometrical changes of stomach: Light microscopic studies did not revealed any histological changes in the stomach after administration of the

Table 1: The values (mean±SD) of body weights (grams) and Crown-Rump Length or CRL (mm) of mice fetuses in the control, sham, *Zataria multiflora Boiss* (ZmB) and *Elaeagnus angustifolia* (Ea) treated groups

Group	Control	Sham	ZmB	Ea
CRL	1.58±0.07	1.59±0.07	1.56±0.11	1.55±0.11
Weight	0.42±0.08	0.41±0.04	0.43±0.12	0.42±0.56

Table 2: The volumes (mean±SD) of different layer and total of stomach (Cavalieri method) of mice fetuses in the control, sham, *Zataria multiflora Boiss* (ZmB) and *Elaeagnus angustifolia* (Ea) treated groups

Group	Control	Sham	ZmB	Ea
Total Volume (mm^3)	0.939±0.149	0.942±0.146	0.997±0.243	1.130±0.123 ¤ •
Volume of lumen (mm^3)	0.283±0.069	0.253±0.029	0.303±0.076	0.263±0.057
Volume of Wall thickness (mm^3)	0.656±0.117	0.686±0.125	0.693±0.193	0.843±0.127 ¤ •
Volume of mucosal layer (mm^3)	0.214±0.039	0.224±0.051	0.227±0.076	0.260±0.042 ¤ •
Volume of muscular and serosal layer (mm^3)	0.446±0.083	0.463±0.089	0.466±0.118	0.572±0.086 ¤ •

¤ Significant different from sham group ($p<0.01$), • Significant different from control group ($p<0.01$)

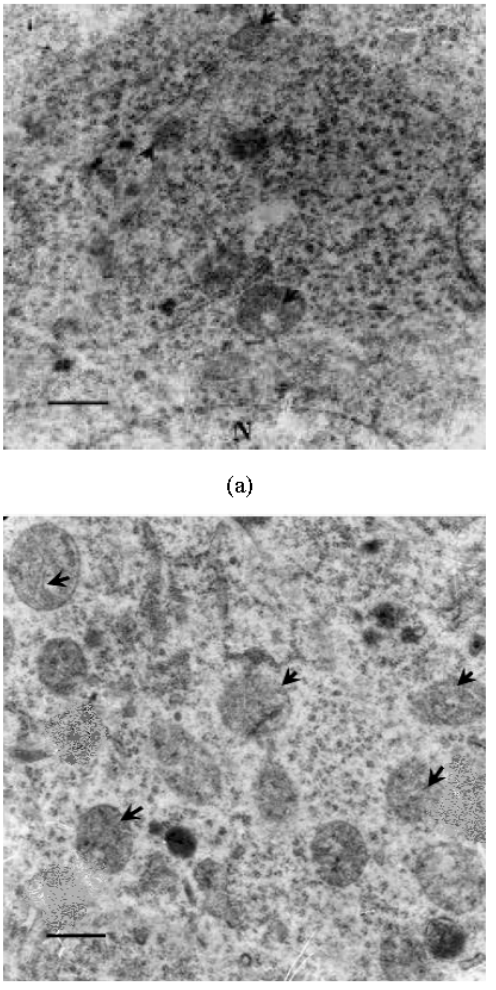
extracts. The stresses that induced by animal handling and gavages was not modified the stomach volume because there was no significant differences between the volume of the stomach in control and sham groups.

There were no significant changes in the volume of mucosal layer, muscularis externa of the mice fetuses' stomach that fed with ZmB compared with control and sham groups. The volume of wall of stomach consists of mucosa, submucosa, muscularis externa and serosa did not showed significant changes compared with control and sham groups statistically too. The total volume of stomach that consists of the volume of the wall and lumen did not changed significantly too (Table 2).

Elaeagnus angustifolia extract increased the total volume of stomach significantly compared with control and sham groups. It was related to the volume of wall of the stomach but not to the lumen. By this mean, we measured the volume of mucosa, muscularis externa plus serosa separately and we noticed the volume of these layers increased significantly compared with control and sham groups (Table 2).

The coefficient error of Cavaleire method was calculated between 1-12%, therefore our results were valid.

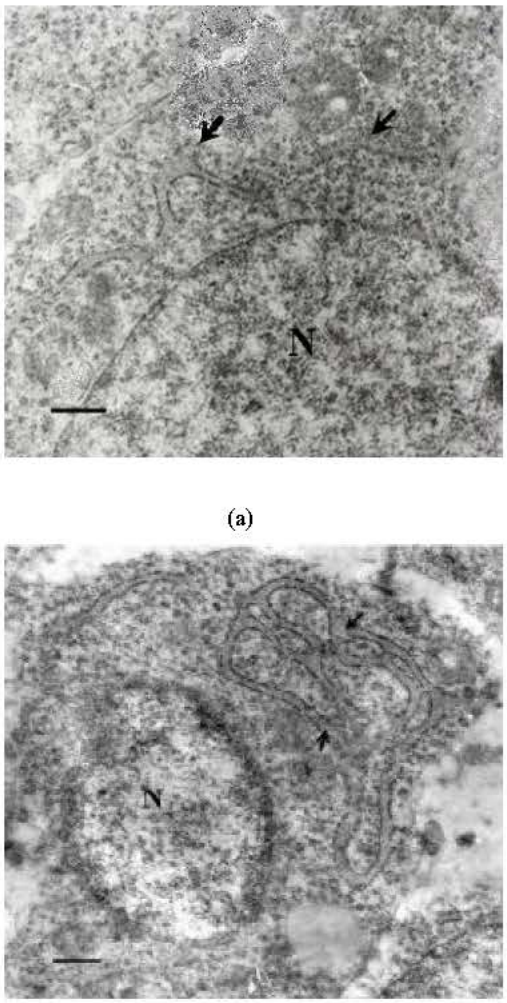
Ultrastructural changes of stomach: After stereological evaluation of different layers of stomach we followed that whether these extracts affected in cellular level or not, so the ultrastructural study of stomach special in epithelium and smooth muscle were done. Stomach has not differentiated completely in 16th day of gestation. The epithelium of mucosal layer was stratified and various cell types of the glands have not differentiated yet. In control and sham groups, the epithelial cells had euchromatic nuclei with prominent nucleoli. A few mitochondria were diffused in cytoplasm. The mitochondria were located far from nucleus. The numerous polysomes were seen and a few rough endoplasmic reticulums were existed near the nucleus (Fig. 1a). In the ZmB and Ea treated groups, the epithelial cells enlarged and contained numerous hypertrophic mitochondria especially with the group that



(a)

(b)

Fig. 1: Electron micrographs of epithelial cells of stomach mouse fetus, a, epithelial cell of sham group. The cytoplasm contains few RER, mitochondria are showed with arrow and b, Epithelial cell of Ea group, please note to the large number of hypertrophic mitochondria (arrow). Scale bars are 0.72 μm



(a)

(b)

Fig. 2: Electron micrographs of smooth muscle cells of mouse fetus stomach (a) Smooth muscle cell in sham group. Please note to RER cisterna (arrow) and (b) smooth muscle cell in Ea group. Please note to spiral arrangement of RER (arrow). Scale bars are 0.72 μm

treated with Ea extract. Because of the cell hypertrophy, the polysomes seemed to be more scattered (Fig. 1b). The smooth muscle cells of muscular layer in ZmB and Ea extract treated groups enlarged compared with control and sham groups. The Rough Endoplasmic Reticulum increased also extremely and unusual arrangements of RER cisterna such as spiral and fingerprint like distinguished specially in Ea treated group. These hypertrophic RER were located far from nuclear membrane. The polysomes in muscle cells of the two extract administrated groups seem larger than the control and sham groups (Fig. 2a and b). The newly formed myofilament bundles were observed in muscle cells in all of groups.

DISCUSSION

The usage of these extracts by pregnant mice can relax the muscle contraction of digestive system that is natural in this period probably by its antioceceptive effect that may be mediated by opioid receptors (Ahmadiani *et al*., 2000) and muscle relaxants activity via flavonoid component (Hosseinzadeh *et al*., 2003) and anti-inflammatory effect against acute and chronic inflammation (Hosseinzadeh *et al*., 2000) and the reduction in response to field stimulation due to a relaxation of the tissue through on intracellular mechanism (Maria and Stephen, 1997). Also *Zataria* had a significant inhibitory effect on Helicobacter psilori reducing both its growth and potent urease activity. Helicobacter pilori is important etiological agent of chronic gastritis, peptic ulceration and gastric cancer in human (Tabak *et al*., 1996). According to our results the *Zataria multiflora Boiss* and *Elaeagnus angustifolia* extracts induced cytoplasmic hypertrosphy in the cells of the most layers of the fetus stomach but *Elaeagnus angustifolia* extract act more strongly. The stereological study fosr determination of volume of different layers of stomach fetuses emphasized this finding too. Meanwhile, no side effects such as growth retardation or induction of abnormalities were observed even in the selected dose that was near the lethal dose of the extracts based on Hosseinzadeh *et al*. (2000 and 2001). We concluded that these herbs can act as reinforcer for stomach fetus and probably the all of its digestive tube. This function were produced by two ways, first by hyperactivity of epithelial cells accompanied with increasing and enlarging of mitochondria it may influence the some activity of epithelial cells such as acid and enzyme secretion after birth that will help to digestion or by further mucus secretion by mucous cell and mucus neck cell of glands that protect the stomach mucosa against ulcer that confirm by antiulcerogenic effect of *Eleagnus multiflora* in adult rats that reported by Gurbuz *et al*. (2003). It may be act on inducing factor or transcription factor that involved in development epithelial cells. Padmavathi *et al*. (2005) showed that *Hippophae rhamnoides* (sea buckthorn) inhibits benzo(a)pyrene-induced forestomach in mouse. They suggest that Hippophae fruit is able to decrease carcinogen-induced forestomach, which might involve up-regulation of phase II and antioxidant enzymes as well

as DNA-binding activity of IRF-1, a known antioncogenic transcription factor causing growth suppression and apoptosis induction for its anticancer effect.

Second by hyperactivity potential of smooth muscle cells in muscular layer of stomach because we find hypertrophic RER in these cells after treatment with aqueous extract of these herbs. Hypertrophic RER will synthesize more myofilament. It acts probably via regulation of genes expression that involved in embryonic cell development but more studies is needed to clarify the properties of these herbs

ACKNOWLEDGMENTS

The authors would like to thank the Office of Vice Chancellor for Research deputy of Shiraz University and Shahid Beheshti University for financial supports and Dr. T. Talaei for editorial assistance.

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