Phytoestrogens levels determination in the cord blood from Malaysia rural and urban populations

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Abstract

This study is a result of an analysis of free and conjugated phytoestrogens daidzein, genistein, daidzin, genistin and coumesterol in human cord blood plasma using LCMS. Cord blood was collected from urban and rural populations of Malaysia (n = 300) to establish a simple preliminary database on the levels of the analyzed compounds in the collected samples. The study also aimed to look at the levels of phytoestrogens in babies during birth as this may have a profound effect on the developmental process. The sample clean up was carried out by solid-phase extraction using C18 column and passed through DEAE sephadex gel before analysis by LCMS. The mean concentrations of total phytoestrogens were daidzein (1.4±2.9 ng/ml), genistein (3.7±2.8 ng/ml), daidzin (3.5±3.1 ng/ml), genistin (19.5±4.2 ng/ml) and coumesterol (3.3±3.3 ng/ml). Distribution of phytoestrogen was found to be higher in samples collected from rural areas compared to that of urban areas.

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Introduction

Phytoestrogens are a diverse group of nonsteroidal plant compounds that can mimic estrogen and are ubiquitous in most plants, fruits and vegetables (Thompson et al., 1991; Griffith and Collison, 2001; Becker et al., 2005; Todaka et al., 2005). They can be found in most foodstuffs related to humans and animals such as seasonings (garlic, aniseed, parsley), legumes (soy, peas, clover), grains (wheat, barley, rice, oat), vegetables (carrots, potatoes, alfalfa) and drinks (tea, coffee). Estrogenic activities of phytoestrogens were first noted as early as 1926 (Murkies et al., 1998). Phytoestrogens can bind to the estrogen receptors, both estrogen alpha (Er-α) and estrogen beta (Er-β) receptors in humans to elicit their effects (Kuiper et al., 1997).

Interestingly, many phytoestrogens seem to have higher affinity to Er-β receptors compared to steroidal estrogens, suggesting that their actions are exerted through different pathways (Setchell, 1998).

Phytoestrogens can be divided into three main groups; isoflavones, coumestans and lignans with isoflavones being the most potent (Figs. 1 and 2).

Epidemiological and experimental data revealed that phytoestrogens may have protective roles against cancer, cardiovascular diseases, osteoporosis and menopause in humans and animals (Thompson, 1995; Adlecureutz and Mazur, 1997; Apers et al., 2004). Although phytoestrogens can mimic natural estrogen, their activity is 10² to 10⁵ times lower than the natural estrogen (Price and Fenwick, 1985; Cassidy et al., 1993). Despite the low estrogenic activity, phytoestrogens are always present in the body in greater amounts than endogenous estrogen (Adlecureutz et al., 1993). However, the bioactive roles...
Fig. 1. Classification of dietary phytoestrogens.

Fig. 2. Chemical structures of the phytoestrogens studied.
of phytoestrogens depend on the amount of circulating endogenous estrogens and the number and type of estrogen receptors present (Cassidy et al., 1994). Phytoestrogens can modulate the estrogen action by acting as agonist and as antagonist at high estrogen concentrations (Whitten et al., 1994). They can reduce estrogen actions by inhibiting several enzymes in estrogen biosynthesis and metabolism (Adlecreutz and Mazur, 1997). Thus, phytoestrogens can act both as an estrogenic or an anti-estrogenic agent.

Recently, there are growing concerns on whether estrogenic compounds in foodstuff may have effects on human health. Phytoestrogens in soy-infant formulas have been reported to potentially interfere with the balance in sex hormones in infants during the developmental stage due to their distinctive sensitivity to estrogenic compounds (Setchell et al., 1997, 1998; Sheehan, 1998; Kuo and Ding, 2004). Dietary phytoestrogens were proved to be beneficial to human health (Cassidy and Faughan, 2000). Adlecreutz and Mazur (1997) reported that incidence of breast and prostate cancers was lower in the Japanese compared to Western populations. Since then, phytoestrogens, naturally occurring estrogen-like compound, are gaining popularity as health supplement. The relation of dietary phytoestrogens and their physiological effects was reviewed by Cornwell et al. (2004).

Various analytical techniques have been used to analyze phytoestrogens using high performance liquid chromatography
(HPLC) or gas chromatography mass spectrometry (GCMS) (Eldrige, 1983; Coward et al., 1993; Wang and Murphy, 1994; Franke et al., 1995; Hutabarat et al., 1998; Griffith and Collison, 2001; Antignac et al., 2004; Apers et al., 2004; Kuo and Ding, 2004; Chan et al., 2006). In the present study, we utilized the liquid chromatography and mass spectrometry (LCMS) detection to investigate the occurrence of isoflavone aglucones (genistein, daidzein), isoflavone glycosides (genistin and daidzin) and coumestans (coumesterol) in the cord blood obtained from mothers delivering babies at selected urban and rural hospitals in Malaysia.

Total isoflavones (conjugated and free forms) and free coumesterol were measured. Our group agreed that it was worthy to conduct our study in specific populations as we expected the environment of the subjects may well affect the levels of phytoestrogens. Thus, the samples were collected from urban and rural areas. This study may allow us to assess the levels of phytoestrogens exposed to infants at birth. Assumption was that phytoestrogens could cross the placental barrier to be present in the fetus. Therefore, we analyzed matching pairs of maternal and cord blood to further investigate fetal exposure to phytoestrogens.

**Experimental**

**Subjects**

For phytoestrogens investigation in cord blood, 300 pregnant Malaysian women who delivered babies from seven hospitals in Malaysia participated voluntarily in this study. The participating hospitals were randomly chosen. The hospitals categorized as urban hospitals were the University Malaya Medical Centre, Kuala Lumpur; General Hospital, Kuala Lumpur; Georgetown General Hospital, Penang and the rural hospitals were Seberang Prai District Hospital, Penang; Cameron Highlands District Hospital, Pahang; Kota Bharu General Hospital, Kelantan; Jerantut District Hospital, Pahang. The criterion used to classify as urban or rural was the city status of the hospital location. Subjects were registered consecutively and no selection was made. The subjects were interviewed and any consumption of food suspected to contain phytoestrogens was recorded in the prepared questionnaires. All subjects consumed ordinary diet until hospitalization and only normal deliveries were selected. All subjects signed informed consent to participate in this study and the protocol was approved by the Ethics Committee of the University of Malaya Medical Centre.

**Samples collection**

12 ml of cord blood samples was collected during deliveries in heparinized tubes. The samples were centrifuged and the plasma separated within 24 h. All samples were stored at −80 °C and transferred in dry ice to the main laboratory in the Department of Pharmacology, University of Malaya, Kuala Lumpur.

<table>
<thead>
<tr>
<th>Compound (ng/ml)</th>
<th>Inter-day (n=6) CV (%)</th>
<th>Intra-day (n=6) CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5  1.5  200.0  400.0</td>
<td>0.5  1.5  200.0  400.0</td>
</tr>
<tr>
<td>Daidzin</td>
<td>14.0 12.8  1.6  4.1</td>
<td>9.2 13.9  1.6  1.1</td>
</tr>
<tr>
<td>Genistin</td>
<td>11.2 10.5  2.8  4.7</td>
<td>11.3 9.8  5.3  1.2</td>
</tr>
<tr>
<td>Daidzein</td>
<td>9.5  13.1  7.4  5.0</td>
<td>12.8 11.1  4.7  5.6</td>
</tr>
<tr>
<td>Genistein</td>
<td>4.6  14.0  7.4  5.5</td>
<td>6.8  8.5  11.3 12.0</td>
</tr>
<tr>
<td>Coumesterol</td>
<td>12.2 10.7  6.7  0.7</td>
<td>11.3 12.4  10.6 6.5</td>
</tr>
</tbody>
</table>
Chromatography

Reference standards for genistein, daidzein and genistin were purchased from Sigma (St. Louis, USA). Daidzin and coumestrol reference standards were purchased from Fluka Chemicals (USA). Chloramphenicol was used as internal standard (IS) and purchased from Sigma (St. Louis, USA). Cord plasma samples were prepared in three phases: (i) solid-phase extraction using Isolute C18, 500 mg, 6 ml reservoir (IST, UK), (ii) solvolysis using dimethylformamide, 6 M HCl and dichloromethane (DCM) and (iii) purification using DEAE-sephadex A-25 gel chromatography. The samples were analyzed on a Shimadzu LCMS8000 system (Shimadzu Corporation, Japan) equipped with ultraviolet (UV) and mass spectrometer (MS) detectors with electrospray ionization (ESI) interface. The aglucones were detected using MS with ion characteristics; daidzein (m/z 253), genistein (m/z 269) and coumestrol (m/z 267). The glycosides genistin and daidzin were detected using UV detector. All values were expressed as ng/ml. Inter- and intra-day precisions of the method were determined using six replicates of quality control (QC) human plasma samples. The extraction and chromatographic procedures enabled the measurement of phytoestrogens in plasma (Figs. 3–5). The performance of the method was summarized in Tables 1 and 2 where the coefficient variation (CV) obtained was from 1.1% to 14.0%, less than 15% of the acceptance limit.

Results

Phytoestrogens in cord blood samples

The concentrations of all five phytoestrogens studied in the samples collected from the seven hospitals were displayed in Fig. 6. The mean concentrations for total phytoestrogens in the cord plasma samples were found to be 3.5±3.1 ng/ml, 19.5±4.2 ng/ml, 1.4±2.9 ng/ml, 3.7±2.8 ng/ml and 3.3±3.3 ng/ml for daidzin, genistin, daidzein, genistein and coumestrol respectively. The highest level of phytoestrogens detected was in the cord plasma of patients from the Kota Bahru General Hospital. The phytoestrogens detected were genistin at 46.6±8.8 ng/ml. Whereas, patients from the University Malaya Medical Centre were recorded to have the lowest level (0.5±3.1 ng/ml) of phytoestrogen in the cord plasma which was for daidzein.

Phytoestrogens levels in relation to intake of phytoestrogens containing food

Dietary intake of mothers during the gestational stage was monitored through the questionnaires. Analysis results of the plasma phytoestrogens levels were compared with patients’ intake of phytoestrogens. The results of the cord plasma phytoestrogens in patients taking food containing phytoestrogens at the frequencies of every meal and hardly consuming phytoestrogens were summarized in Fig. 7. The quantification limit (LOQ) of phytoestrogen was 0.5 ng/ml and the data below the LOQ were calculated as 0.0 ng/ml. The highest concentration of phytoestrogens detected was 55.8±27.7 ng/ml in the consumers of taugeh and the lowest was in consumers of soy-based food (0.9±0.0 ng/ml) and soy-based milk (0.9±7.1 ng/ml). The phytoestrogens detected were genistin and daidzein respectively.

Phytoestrogens levels in the maternal and cord blood

The presence of phytoestrogens in the cord plasma supported the hypothesis that phytoestrogens can cross the placental barrier after metabolism in the mother. Analysis of one hundred and three (103) matching pairs of maternal and cord blood indicated that the target phytoestrogens were detected in the concentration sequence genistin, daidzin, coumestrol, daidzein and genistein. These samples were obtained from mothers delivering babies at the University Malaya Medical Centre, Kuala Lumpur. The results were displayed in Fig. 8. The highest total mean concentrations of phytoestrogens detected were for genistin at 6.1±0.2 ng/ml and 6.0±0.8 ng/ml in the maternal and cord plasma.

Discussion

The differences in diet and lifestyle among the subjects could explain the variations in the total phytoestrogen distribution in patients from urban and rural hospitals, but the study did not show any significant variations in the types of phytoestrogens. Patients from all four rural hospitals showed higher phytoestrogen concentration compared to all three urban hospitals. The concentration of phytoestrogens in the cord blood from Kota Bahru General Hospital was 6-fold higher than in the samples from the University Malaya Medical Centre.

From the results shown in Fig. 6, it can be generalized that in this study, cord plasma samples from rural hospitals generally contained higher concentration of phytoestrogens compared to cord plasma samples from urban hospitals. Samples from the Kota Bahru General Hospital had the highest total mean concentrations of phytoestrogens and samples from the University Malaya Medical Centre had the lowest total mean concentrations of phytoestrogens regardless of the type of phytoestrogens detected. In the rural areas, consumption of plant-based herbal medicines during pregnancy is very common and this may not be commonly practiced in urban areas. Furthermore, rural population usually consumed more vegetables either cooked or raw compared to the urban population.
This could be due to the lower price of vegetables compared to meat and poultry products. Economical concern also influenced the rural population’s dietary intake as animals are usually for trade rather than consumption. There is also the traditional belief that vegetables are healthier than meat.

Asian diet consists mainly of vegetables and legumes. Soy is one of the primary sources of phytoestrogens in the population (Todaka et al., 2005). Studies have been conducted, although few, to assess the exposure of phytoestrogens from cord blood to human fetus (Todaka et al., 2005; Adlecreutz et al., 1999). The issues concerning levels of phytoestrogens in infants are contentious. Once phytoestrogens get into the fetal compartment, phytoestrogens metabolism and repercussion are to be investigated further. The study by Todaka et al., 2005

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**Fig. 6.** Distribution of phytoestrogens in the cord plasma of subjects from the participating hospitals.

**Fig. 7.** Comparison of phytoestrogens concentrations between the cord plasma of subjects consuming food containing phytoestrogens in every meal with subjects hardly consuming food containing phytoestrogens.
correlated the levels of phytoestrogens in the mother and fetus. While the beneficial hormonal or non-hormonal effects of phytoestrogens on human health have been reported (Setchell et al., 1997; Chan et al., 2006; Todaka et al., 2005), there had been reports on the adverse health effects of phytoestrogens, especially during pregnancies (North and Golding, 2000; Becker et al., 2005).

Theoretically, the concentration of phytoestrogens in the cord plasma of patients consuming phytoestrogens at every meal should be higher than in patients who hardly consumed phytoestrogens. However, as in Fig. 7, the results were otherwise. But again, the values could be flawed as the number of subjects that consumed phytoestrogens containing food at every meal was smaller compared to the ‘hardly’ group. It was also noted that genistein and genistin were always detected higher than daidzein and daidzin. The concentrations for coumesterol detected in the cord plasma did not show any trend where similar results were obtained in the distribution of phytoestrogens among subjects. In the current study, we found that the highest level of phytoestrogen was not from soy, which was thought to be the major source of phytoestrogens but through the consumption of bean sprouts or locally known as taugeh. We would not conclude that taugeh is now the main source of phytoestrogens over soy, as in our study, the sample size for these two groups was highly imbalanced where the frequency of taugeh consumption was higher than soy consumption.

All the samples analyzed were found to contain phytoestrogens, especially genistin. Setchell et al. (1997) also obtained the same result whereas Todaka et al. (2005) reported genistin were detected the highest concentration among the phytoestrogens studied. Inter-subject variations including lifestyle, diet, gut microflora and genetic factors continuously influenced the bioavailability of phytoestrogens in the subjects. We believe that metabolism rates played significant role to determining the phytoestrogen levels detected in the cord plasma. Genistein reaches its peak concentration in the plasma about 6 h after intake and has a half-life of about 8 h after the peak (Watanabe et al., 1998). It was unclear when the mothers consumed food containing phytoestrogen before deliveries, but it could have been beyond the half-life. Genistein must have been already metabolized to genistin. On the other hand, genistin is hydrolyzed to genistein. This created a continuous supply for isoflavones genistein and genistin until they are eliminated. Similar reason may apply to cord plasma levels of daidzein and daidzin. Isoflavone daidzein is metabolized to equol by bacteria in the intestine and equol formation is linear with the concentration of gut microflora (Setchell et al., 1997, 2002). Metabolism of isoflavone daidzein to its metabolite equol would be different among the subjects depending on their gut bacterial content. It is suggestive that the levels of daidzein could be related to equol formation. We did not analyze equol or other phytoestrogen metabolites in this study and it is very likely that we had overlooked their presence. The abundance of genistein in this study compared to the other phytoestrogens investigated showed that genistein is more bioavailable than daidzein. For this, pharmacokinetics study needs to be undertaken to confirm the suggestion.

Analysis of cord plasma and corresponding maternal plasma did not produce linear relationship. The total mean level of phytoestrogens in maternal plasma was 18.3 ng/ml and 18.6 ng/ml in cord plasma. The difference was not significant thus no correlation on the relevance of mothers’ diet to the phytoestrogens level in cord could be derived. Nevertheless, detection of phytoestrogens in the cord plasma confirmed that the phytoestrogens have crossed the placental barrier to be in the fetus. Todaka et al. (2005) also reported that phytoestrogens were transferred from mother to fetus but the difference in phytoestrogens concentrations in mothers and infants was not significant. It was then hypothesized, based on this, that the levels of phytoestrogens in both maternal and cord plasma showed no correlation with the frequency of the known phytoestrogens consumption.
References


Conclusion

This study revealed that lifestyle and diet could influence the phytoestrogens levels in the population where traditional rural lifestyle contributed to the high concentration of plasma phytoestrogen. Data from this study and other studies showed the ability of phytoestrogens to be transferred from mother to fetus although the transfer was not significant. Matching maternal and cord blood analysis was only conducted in the samples from the University Malaya Medical Centre which were the subgroup of urban population. Similar analysis should be conducted on the rural population as well for comparison. As this study suggested that eating habit could affect the results, it is best to introduce control in the diet of the subjects participating in this kind of study. It is possible that intake of phytoestrogens during gestation has physiological effects on the fetus. Given the susceptibility of fetus to these phytoestrogens, it is of best interest that the risks associated with fetus exposure to them be studied further.

Acknowledgments

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