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ABSTRACT: The incidence of obesity is increasing rapidly worldwide. Identification of compounds that may decrease fat cell burden will be beneficial in the control of obesity. In the present study we investigated the effect of a combination of grape seed extract, caffeine, genistein, and capsaicin (GCGC) in reducing body weight in obese male zucker rats. Rats were pair-fed on a high fat diet or high fat diet + GCGC for 5-weeks. GCGC caused a significant weight loss due to the depletion of fat depots. GCGC also improved insulin response, reduced plasma glucose and free fatty acids, and increased plasma adiponectin. Adipocytes from rats fed GCGC were more responsive to the various stimulators of lipolysis than the control adipocytes. These results show that a combination of plant-derived products is very effective in reducing body weight and improving insulin sensitivity and glucose response in obese zucker rats. This may have implications in the treatment of overweight and obesity in humans.

KEY WORDS: Body Weight; Caffeine; Capsaicin; Genistein; Grape Seed Extract; Obesity

INTRODUCTION
During the past several decades, obesity has become a public health crisis (Ford et al., 2002, Mokdad et al., 2000) and the incidence of obesity is increasing rapidly in both the industrialized and developing countries. Currently, 67% of Americans are either overweight or obese. Obesity is a major risk factor for cardiovascular disease (CVD), type 2 diabetes, hypertension, and osteoarthritis (World Health Organization, 2000). Major contributing factors for the development of obesity include increased energy intake, reduced physical activity, and decreased consumption of nutrient-rich diet. A potentially effective way of controlling obesity and lessening the risk of CVD and type 2 diabetes is to develop products that modulate fat metabolism or storage by regulating lipogenesis, lipolysis, adipogenesis, and/or fat cell apoptosis.

A number of studies have described the use of plant-derived products for the treatment of obesity, including plant-derived caffeine, phytoestrogens, capsaicin and grape seed extract (Boozer et al., 2002; Goodman-Gruen et al., 2003; Henry et al., 1986; Morino et al., 2003). These products are known to influence one or more aspects of fat cell metabolism (Chen et al., 1994; Jung et al., 1981; Watanabe et al., 1994; Henry et al., 1986; Sukudelska et al., 2000; Moreno et al., 2003). However, the fat cell can compensate for any particular effect caused by any one compound. For example, if lipolysis is induced, the fat cell can compensate by increasing lipogenesis. Moreover, the body could generate more mature fat cells through adipogenesis in response to agents that inhibit lipogenesis and induce lipolysis. Therefore, a combination of compounds that attack multiple determinants of the fat depot should lead to more significant decreases in body fat than if the individual compounds given alone. Therefore, in the present study we investigated the effect of a combination of grape seed extract, caffeine, genistein, and capsaicin in reducing body weight in obese zucker rats.

MATERIALS AND METHODS
Supplements and Diet
The food supplements capsaicin (98% pure), genistein (98% pure), and caffeine were purchased from Sigma-Aldrich, St. Louis, MO and grape seed extract (Leucoselect®) was a gift from Indena, USA, Inc., Seattle, WA. Leucoselect contained catechin (15%), epicatechin gallate, dimers, trimers, tetramers, and their gallates (80%), and pentamers, hexamers, heptamers, and their gallates (5%).

The high fat diet (Table 1) was prepared in the food preparation room at the Louisiana State University (LSU) Health Sciences Center, New Orleans animal care facility. The ingredients were thoroughly mixed together in a mixing bowl fitted with a mechanical stirrer and
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the food was stored at 4 °C. The supplements were added to the high fat diet to provide diet for one week at a time.

Animals
Obese male Zucker rats were obtained from Dr. Johnny Porter, LSU Health Science Center. The rats were fed a high fat base diet (50% fat, 30% protein, and 20% carbohydrate calories) for one week. The rats were then weighed, matched for age and starting weight, and divided into 2 groups of 20 animals each. The 20 animals in each group were separated into 10 pairs based on their initial body weight and one member of each pair was fed an experimental Food A/Diet A or Food B/Diet B (defined in Table 2) while the other member of the pair was fed the high fat diet alone. The daily food intakes of control (treatment-free diet) and treated (treatment diet) animals in the pair were noted. On the following day the control rat received the same weight of treatment-free diet as consumed by its partner under treatment. Therefore, both animals in the pair received same food calories. Weights were followed daily for 5-weeks. At the completion of the feeding protocol, the rats were fasted overnight and sacrificed by decapitation. Trunk blood was collected and serum stored at -80 °C. Epididymal, retroperitoneal, and peri-renal fat depots were dissected from each rat and weighed.

Adipocyte isolation
Adipocytes were isolated from rats fed Diet B for 5-weeks and the corresponding pair-fed controls as described before (Figueroa et al., 2002). Briefly, retroperitoneal fat tissue was dissected from the rats, finely minced, and digested with collagenase (Sigma) in Krebs Ringer bicarbonate (KRB) buffer containing 1% bovine serum albumin (BSA) at 37 °C for 30 min under vigorous shaking. After digestion, the cell suspension was filtered through a nylon filter (Spectrum Laboratories) and washed three times with KRB + 1% BSA. Isolated fat cells were resuspended in KRB with 4% BSA at the rate of 2 g fat tissue per ml.

Lipolysis assay
For lipolysis assay, adipocytes were incubated in 1.5 ml polyethylene microcentrifuge tubes for 120 min with continuous gentle shaking in a water bath at 37 °C. The reaction mixture contained 50 ml adipocytes and different stimulators of lipolysis or buffer and the total volume was made up to 150 ml with KRB. At the end of incubation, the reaction mixture was centrifuged (15,000 g for 20 min) and a portion of the bottom aqueous phase was withdrawn with a long 27-gauge needle without contaminating with adipocytes. The amount of glycerol released in the aqueous phase was measured by the radiometric assay of glycerol as described elsewhere (Figueroa et al., 2002).

Biochemical Assays
Insulin was assayed by ELISA using the ultra sensitive rat insulin ELISA kit from Crystal Chem, Downers Grove, IL. TNF-α and adiponectin were also determined by ELISA using ELISA kits from Biosource, Camarillo, CA and B-Bridge International, Sunnyvale, CA, respectively. Glucose was assayed enzymatically using Liquid Glucose reagent from Pointe Scientific Inc, Lincoln Park, MI. Serum non-esterified fatty acids were determined colorimetrically using the non-esterified fatty acid regent from Waco Chemicals USA, Richmond, VA.

Statistics
We used an analysis of variance (ANOVA) to compare the various studied variables in the groups. When significant, a post hoc unpaired t-test was performed. The results are expressed as mean ± standard deviation (SD). For all comparisons, differences were considered significant at p<0.05. Statistical analyses were carried out using SPSS 15 for Windows.

RESULTS

Body weight
Change in body weight of rats over the duration of the experiment is shown in Figure 1. There was no change in the body weight of animals on Food A and Food B for the first two weeks. However, after that period, there was a steady decline in the body weight of animals in both groups. Towards week 5, the animals in Food A group consuming a combination of grape seed extract, genistein, capsaicin, and caffeine had lost about 30% body weight and the Food B group receiving half the amount of the supplements present

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/Kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>285.0</td>
</tr>
<tr>
<td>Corn starch</td>
<td>90.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
</tr>
<tr>
<td>Crisco</td>
<td>171.1</td>
</tr>
<tr>
<td>Corn oil</td>
<td>40.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>267.6</td>
</tr>
<tr>
<td>Salt mixture</td>
<td>34.0</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>9.7</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.7</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**TABLE 1. Composition of high fat diet**

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>Kcal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>20</td>
</tr>
<tr>
<td>Fat</td>
<td>50</td>
</tr>
<tr>
<td>Protein</td>
<td>30</td>
</tr>
</tbody>
</table>

**TABLE 2. Composition of Food A/Diet A and Food B/Diet B**

<table>
<thead>
<tr>
<th>Food (per Kg of high fat base diet)</th>
<th>Capsaicin</th>
<th>Grape seed extract</th>
<th>Caffeine</th>
<th>Genistein</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20g</td>
<td>600g</td>
<td>12g</td>
<td>600g</td>
</tr>
<tr>
<td>B</td>
<td>10g</td>
<td>300g</td>
<td>6g</td>
<td>300g</td>
</tr>
</tbody>
</table>

**TABLE 2. Composition of Food A/Diet A and Food B/Diet B**

**Adipocyte isolation**
Adipocytes were isolated from rats fed Diet B for 5-weeks and the corresponding pair-fed controls as described before (Figueroa et al., 2002). Briefly, retroperitoneal fat tissue was dissected from the rats, finely minced, and digested with collagensae (Sigma) in Krebs Ringer bicarbonate (KRB) buffer containing 1% bovine serum albumin (BSA) at 37 °C for 30 min under vigorous shaking. After digestion, the cell suspension was filtered through a nylon filter (Spectrum Laboratories) and washed three times with KRB + 1% BSA. Isolated fat cells were resuspended in KRB with 4% BSA at the rate of 2 g fat tissue per ml.

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in Diet A about 16%. Thus, the dietary supplements caused a dose-dependent weight loss.

**FIGURE 1.** Effect of Food A and Food B on body weight. Rats were pair-fed high-fat diet (control) diet and Food A or Food B for 5 weeks and their body weight was recorded periodically.

![Graph showing body weight changes over time for Food A and Food B](image)

Fat depot
The effect of treatment with a combination of grape seed extract, genistein, capsaicin, and caffeine on fat depot weights is shown in Figure 2. The results are expressed as percent fat depot weight of initial body weight. Compared with control rats (high-fat diet), rats receiving food A and food B showed significant decrease in epidydimal fat, retroperitoneal fat, and total combined fat (epidydimal, retropritoneal, and perirenal). However, The perirenal fat depot did not decrease with the treatment.

**TABLE 3.** Effect of Food A on serum levels of glucose, insulin, free fatty acids, and adiponectin

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>Food A</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>203.49 ± 19.47</td>
<td>172.14 ± 18.53</td>
<td>0.031</td>
</tr>
<tr>
<td>Insulin</td>
<td>3.512 ± 3.407</td>
<td>2.42 ± 1.68</td>
<td>NS</td>
</tr>
<tr>
<td>FFA (meq/L)</td>
<td>1.73 ± 0.35</td>
<td>0.954 ± 0.29</td>
<td>0.0001</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>5.49 ± 1.09</td>
<td>15.81 ± 0.45</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Adipocytes isolated from rats fed Food B and the corresponding pair-fed control rats were exposed to various stimulators of lipolysis and lipolysis was measured. The results are shown in Figure 3. Adipocytes from rats treated with Food B showed increased baseline lipolysis compared with control adipocytes. Uniformly, the adipocytes from rats fed Food B were more responsive to the various stimulators of lipolysis, including grape seed extract (one of the supplements) than the control adipocytes. These data demonstrate that treatment with a combination comprising grape seed extract, genistein, capsaicin, and caffeine enhances lipolysis both at baseline and from other stimuli.

**FIGURE 3.** Effect of IBMX and ISO on lipolysis by adipocytes isolated from rats fed Diet B and the corresponding pair-fed control rats. PBS=phosphate-buffered saline, IBMX=isobutyrylmethylxanthine, ISO=isoproterenol, ETOH= ethanol

![Graph showing lipolysis with various stimulators](image)
Although reducing dietary calories combined with regular exercise is an effective way to prevent obesity, only a small percent of people trying to lose weight are able to follow it through. The long-term use of weight control drugs is also questionable because of their serious side effects. The use of various plant extracts in the treatment of obesity is becoming increasingly popular due to their efficacy as well as minimal adverse side effects. This study shows that a combination of plant-derived dietary supplements, including grape seed extract, capsacin, genistein, and caffeine was effective in reducing body weight, decreasing fat depot size, improving insulin and glucose response, raising plasma adiponectin, enhancing fat cell lipolysis, and lowering plasma TNF-α in obese Zucker rats. Individual components of the combination are known to have some effect in causing weight reduction by affecting some aspect of fat metabolism. For example, caffeine has been shown to decrease body fat in genetically obese mice (Chen et al., 1994) and increase oxygen consumption, fat oxidation, and serum free fatty acids in normal and obese humans (Acheson et al., 1980). Moreover, caffeine also activates the sympathetic nervous system, causing thermogenesis (Chen et al., 1994). Thus, although caffeine can reduce body weight, some studies have found elevation of blood pressure with caffeine administration (Noble, 1988). Capsaicin has been shown to stimulate fat oxidation and thermogenesis in humans and rats (Henry et al., 1986). Studies from our laboratory indicate that genistein inhibits adipogenesis in 3T3-L1 fibroblasts (unpublished data). Thermogenesis as well as inhibition of adipogenesis can cause weight loss. However, because of its pungency, capsacin alone has limited usefulness to treat obesity in humans. In healthy, postmenopausal women, genistein consumption was associated with weight loss and reduced body mass index (Goodman-Gruen et al., 2003). Genistein has also been shown to inhibit adipogenesis and stimulate lipolysis in 3T3-L1 adipocytes (Harmon, et al., 2001). Recent studies have shown that grape seed extract is effective in reducing body weight in high fat diet-fed mice and hamsters (Park, et al., 2008; Decorde, et al., 2009) and inhibiting fat mobilizing enzymes in 3T3-L1 adipocytes (Moreno, et al., 2003). As discussed above, although each component of the supplement affects some aspect of fat metabolism and reduces body weight, we hypothesized that a combination of all four compounds will have a greater effect in body weight reduction than each compound alone. Our results show that Food A containing 20 g grape seed extract, 600 mg genistein, 600 mg capsacin, and 12 g caffeine per Kg diet produced a 30% weight loss over 5-weeks. Lowering the concentration of each component by 50% (Food B) still resulted in a 16% weight loss over the same period. Concomitant with the decrease in body weight, we observed a decrease in body fat in the epidydimal and retroperitoneal fat depots in rats fed both Food A and Food B. The retroperitoneal fat depot appears to be very sensitive to the treatment. It is known that it is the retroperitoneal fat depot that has been associated with many of the ill effects of increased adiposity. In this sense, a decrease in this fat depot may carry many health benefits.

Because these animals were pair-fed, their daily caloric intake was similar. Therefore, the weight loss may have resulted from a combination of increased energy expenditure and/or loss and decreased accretion of fat in the body. However, increased energy expenditure did not result from increased physical activity because we did not observe any difference in the daily physical activities of the rats in the control group versus the supplement group. As indicated earlier, caffeine and capsacin in the supplement are known to stimulate thermogenesis and fat oxidation and cause weight loss (Chen et al., 1994; Henry et al., 1986). Moreover, recent studies in experimental animals suggest that grape seed extract may cause weight loss by induction of lipolytic enzymes and/or reducing oxidative stress (Park, et al., 2008; Decorde, et al., 2009). On the other hand, in vitro cell culture studies have shown that grape seed extract inhibits fat-mobilizing enzymes pancreatic lipase, lipoprotein lipase, and hormone-sensitive lipase in 3T3-L1 adipocytes (Moreno, et al., 2003). This might be useful to limit dietary fat absorption and prevent fat deposition in the adipose tissue.

Our study shows that in the rats fed the combination diet weight loss was accompanied by improved insulin sensitivity, and reduced fasting glucose and free fatty acids. Because obesity is a major risk factor for insulin resistance, reduction in body weight produced by our combination of supplements is most likely responsible for the improved glucose and insulin sensitivity.

Plasma free fatty acid concentration decreased significantly in the rats receiving the supplements. Elevated levels of free fatty acids in plasma are associated with insulin resistance. Free fatty acids are thought to inhibit insulin-mediated glucose uptake in muscle through initial inhibition of pyruvate dehydrogenase (Randle, et al., 1963). However, more recent studies suggest that free fatty acids induce insulin resistance by inhibiting glucose transport/phosphorylation which causes reduced muscle glycogen synthesis and glucose oxidation (Roden, et al., 1996). Therefore, the improvement in insulin and glucose response in our study is probably due to a reduction in plasma free fatty acids by the dietary supplements.

It is known that weight loss can be achieved by influencing different aspects of adipocyte metabolism. Our results show that one mechanism by which our combination diet may induce weight reduction is by stimulating fat cell lipolysis. Compared with the adipocytes from control pair-fed rats, adipocytes from rats fed Food B showed increased baseline lipolysis as well as enhanced lipolysis from various stimuli. One or more component of the combination may be responsible for the enhanced lipolysis. Increased lipolysis will result in reduced fat cell volume and adiposity.

The combination diet containing grape seed extract, genistein, capsacin, and caffeine produced a decrease in serum TNF-α and a significant elevation in serum adiponectin. Obesity is considered an inflammatory state and TNF-α is known to cause inflammation. TNF-α exerts its inflammatory action, at least in part, through the activation of NF-κB, which in turn up regulates such genes as iNOS, cytokines, and adhesion molecules (Ruan et al., 2002). TNF-α also decreases adiponectin gene expression and secretion (Fasshauer et al., 2002). In contrast, adiponectin protects against TNF-α- induced NF-κB activation in macrophages and...
endothelial cells (Wulster-Radcliffe et al., 2004). Thus, TNF-α and adiponectin act as counterbalancing factors controlling NF-κB activation. Therefore, the combination diet-induced decrease in TNF-α together with a corresponding increase in adiponectin in our study will help reduce the inflammatory state associated with obesity.

Insulin resistance is usually associated with low plasma adiponectin levels and adiponectin administration improves insulin action (Chandran et al., 2003; Diez, et al., 2003). Also, adiponectin deficiency produces insulin resistance and higher plasma levels of free fatty acids (Kubota, et al., 2002; Maeda, et al., 2002) and overexpression of adiponectin improves insulin sensitivity and plasma free fatty acids (Combs, et al., 2004). Moreover, plasma adiponectin levels rise following weight loss or treatment with thiazolidinediones (Chandran et al., 2003; Diez, et al., 2003). Therefore, it is reasonable to suggest that the rise in plasma adiponectin in the rats treated with the supplements is key to the improved insulin and plasma free fatty acid response in these animals. Adiponectin activates AMPK/malonyl-CoA signaling and modulates NF-κB pathway that result in increased fatty acid oxidation, glucose utilization, and improved insulin response (Chandran et al., 2003; Diez, et al., 2003).

In summary, our study shows that plant-derived dietary supplements were effective in reducing body weight and alleviating some of the co-morbidities associated with overweight and obesity. These results may have implications in the treatment of obesity in humans.

CONFLICT OF INTEREST STATEMENT

All the authors of this article attest that there was no potential conflict of interest that could have influenced this work, attest that no author's institution has contracts relating to this research through which it or any other organization may stand to gain financially now or in the future, attest that no other agreements of authors or their institutions could be seen as involved a financial interest in this work. However, the authors own a patent on the use of the supplements used in the study. All authors have reviewed the contents of the manuscript being submitted, approve its contents and validate the accuracy of the data.

REFERENCES


ABSTRACT: Diet containing N-nitroso compound has been introduced as an environmental factor in the etiology of brain tumor with incidence of 6-8 per 100,000 in USA. Previous studies have not compared the incidence rate of brain tumor in two regions having different dietary pattern. To address this gap, a study designed to find out the incidence of brain tumor in Qazvin city (Iran), which has different dietary pattern from western society. Using newly diagnosed brain tumor, the incidence rate of brain tumor determined during years 2007-8 and dietary pattern of region extracted from previous conducted studies. This study revealed that, although there was different dietary pattern in urban and rural area of study, but the brain tumor incidence was 4 per 100,000 in both regions. Also, the dietary pattern in the region was different from western societies, but the brain tumor incidence rate was comparable with those of USA. We suggest that there might be other environmental factor affecting etiology of brain tumor too.

KEY WORDS: Brain Tumor, Diet, Epidemiology, and Tumor Incidence

INTRODUCTION

Diet is thought to be an important environmental factor in the etiology of several major cancers including brain cancer with an incidence of 6-8 per 100,000 in USA (SEER, 2008; Bunin et al., 1993; Chen, 2002; Pereira, 2001). However, after decades of research in the field of nutritional epidemiology there are still inconsistent results on the relationships between diet and brain tumor, which affect adults and children and is a significant cause of mortality and morbidity. Nitrite exposure has been hypothesized as a risk factor whereas nutrient inhibitors such as vitamin C found in fruits and vegetables are considered to reduce the risk (Terry et al., 2009). Several studies have found maternal intake of folates as a factor to reduce the incidence of brain tumor and also serum level of ascorbic acid and α and γ tocopherol in adults inversely has been related to brain tumor (Hunchark et al., 2004). Consumption of cured meats the primary source of dietary N-nitroso compound (NOC) and their precursor are considered a potential factor in pathogenesis of brain tumor (Hunchark et al. 2004; Lencioni, 1999; Preston-Martin, 1982). NOC are largely formed endogenously and have been shown carcinogenic. Furthermore these compounds are considered important in childhood brain tumor pathogenesis through maternal consumption of cured meat during pregnancy (Scanlan, 1983; Huncharek, 2004)). Among the cured meats, especially cooked bacon, concentrations of 10-100 μg kg⁻¹ have been found. This would correspond to consumption of 1 μg of nitrosodimethylamine (NDMA) in a 100-g portion. Much higher concentrations of NDMA (but lower ones of other nitrosamines) have been found in Japanese smoked and cured fish (more than 100 μg kg⁻¹) (Lencioni, 1999). Beer is one source of NDMA, in which as much as 70 μg l⁻¹ has been reported in some types of German beer, although usual levels are much lower (10 or 5 μg l⁻¹); this could mean a considerable intake for a heavy beer drinker of several liters per day (Lencioni, 1999). Food frequency questionnaire (FFQ) study showed that, American people consume around 96±53 g protein a day which is mainly from meats products and also they drink alcohol, which results in more exposure to N-nitrose, compounds (Stuff, 2009). Therefore the aim of this study was to find out the incidence of brain tumor in population whose 60- 65% of their energy intake comes from carbohydrate sources and 10-12% from protein sources (Azizi, 2007) with rare source of cured meat and alcohol.

METHODS

Information on newly diagnosed brain tumor among adult population of rural and urban area occurring during 2 years period from 21 of March 2007 to 20 of December 2008 was obtained from district surgery hospital in Qazvin Province. The province covers 15821 km² in North West of Tehran (120 Km from Tehran). The reporting areas included in this analysis were 4 townships
Diet and Brain Tumor

(Qazwin, Takestan, Abyek, Bou'in-Zahra) and 16 small towns with 44 rural districts, and 1543 villages. Population information extracted from local vital records office (SCI, 2008). Brain tumor incidences were calculated for years 2008 and 2007, as cases per 100,000 persons. The rates were calculated for population \( \geq 15 \) years old. Dietary and food consumption pattern of area obtained from previous study conducted in the province (Ghaffari, 1999). Signed consent form for releasing data was obtained from participant patients. Research committee of Shahid Rajaee hospital approved the procedure.

**RESULTS**

The population of the Qazvin province was 1.16 million and 1.18 million people in year 2007 and 2008 respectively, of which 68.05% lived in the cities and 31.95% lived in the villages. Concerning the sex-ratio, the ratio of men to women was 50.7 to 49.3%. About 60% of population was \( \geq 15 \) years old (SCI, 2008) (table 1). As table 2 shows incidence of brain tumor among adults \( \geq 15 \) year was 3.9 and 4.2 per 100,000 in year 2007 and 2008 respectively. Incidence of brain tumor among men and women was similar (4 per 100,000) and also combined incidence in years 2007 and 2008 in rural and urban area was similar (4 per 100,000). Extracted data from previous dietary survey in the region in the table 3 shows that, consumption of meat among urban population was as low as 30 g a day and frequency of cured meat and N-nitroso compound containing products consumption was once a year. In rural area there was no cured meat consumption (table 3).

**TABLE 1. Demographic information of studied area**

<table>
<thead>
<tr>
<th>YEAR</th>
<th>RURAL POPULATION (%)</th>
<th>URBAN POPULATION (%)</th>
<th>TOTAL POPULATION</th>
<th>SEX RATIO M/F (%)</th>
<th>TOTAL POPULATION ( &lt;15 ) YEAR</th>
<th>TOTAL POPULATION ( \geq 15 ) YEAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>371,200 (32%)</td>
<td>788,800 (68%)</td>
<td>1,160,000</td>
<td>50.7/49.3</td>
<td>464,000</td>
<td>696,000</td>
</tr>
<tr>
<td>2008</td>
<td>377,600 (32%)</td>
<td>802,400 (68%)</td>
<td>1,180,000</td>
<td>50.7/49.3</td>
<td>472,000</td>
<td>708,000</td>
</tr>
</tbody>
</table>

**TABLE 2. Number and incidence of diagnosed brain tumor among adults (\( \geq 15 \) year) per 100,000 populations**

<table>
<thead>
<tr>
<th>YEAR</th>
<th>MALE URBAN (RURAL)</th>
<th>FEMALE URBAN (RURAL)</th>
<th>TOTAL DIAGNOSED TUMORS (=15 YEAR)</th>
<th>INCIDENCE (URBAN)</th>
<th>INCIDENCE (RURAL)</th>
<th>INCIDENCE (TOTAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>10/3</td>
<td>9.5</td>
<td>27</td>
<td>4</td>
<td>3.6</td>
<td>3.9</td>
</tr>
<tr>
<td>2008</td>
<td>11/4</td>
<td>9.6</td>
<td>30</td>
<td>4.15</td>
<td>4.4</td>
<td>4.2</td>
</tr>
<tr>
<td>Combined 2007-8</td>
<td>21/7</td>
<td>18.11</td>
<td>57</td>
<td>4.08</td>
<td>4</td>
<td>4.05</td>
</tr>
</tbody>
</table>

**TABLE 3. Daily energy, macronutrient intake and meat consumption of adults in urban and rural area**

<table>
<thead>
<tr>
<th>URBAN/RURAL</th>
<th>ENERGY INTAKE (KCAL)</th>
<th>CARBOHY DRATE % (KCAL)</th>
<th>FAT % (KCAL)</th>
<th>PROTEIN % (KCAL)</th>
<th>RED MEAT (GR)</th>
<th>POULTRY (G)</th>
<th>FREQUENCY OF CURED MEAT CONSUMPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urban</td>
<td>1700±600</td>
<td>60</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>100</td>
<td>Once a year</td>
</tr>
<tr>
<td>Rural</td>
<td>2500</td>
<td>65</td>
<td>25</td>
<td>10</td>
<td>15</td>
<td>50</td>
<td>Never</td>
</tr>
</tbody>
</table>

**Discussion**

This study showed that the incidence of brain tumor was 4 per 100000 in adults which was similar among men and women and also in rural and urban area. The incidence rate was lower than that was reported in USA (SEER, 2008). The observed differences can be attributed to increased diagnostic method of brain tumor in USA and European countries and also existence of accurate brain tumor registry system in USA. In several studies brain tumor has been related to consumption of cured meat which contains N-nitroso compound. High sources of N-nitroso compound food groups are included beef, sausage, bacon, wine, hot dog, ham, and beer (Stuff et al., 2009). Using food frequency questionnair (FFQ) and also 7-day food records in Americans showed that a greater proportion of individuals reporting intake of foods high in N-nitroso compound (Stuff et al., 2009). In American population study the median (25th and 75th percentile) of the intake for N-nitroso compound ranged from 0.001-15 µg/day (Stuff et al., 2009). Similar results have been found in study conducted by Brunner et al. (2001) in adult men and women in England in which correlation between energy and alcohol intake and intake of N-nitroso compound were found. In population of our study the consumption of foods containing N-nitros compound ie cured meats and alcohol was very low (Ghaffari, 1999). In a dietary survey in the region of our study, it has been shown that the consumption of cured meat products in urban area was once a month and no consumption of cured meats and alcohol were seen in rural area (Ghaffari, 1999). Although there was significant difference in dietary pattern of western society and population of Qazvin province in terms of the N-nitroso compound intake, but the incidence of brain tumor was similar.

In conclusion consumption of foods having low concentration of N-nitroso compound in rural area and having brain tumor incidence as high as urban area and comparison of diet habit and brain tumor incidence in rural area with urban area and also comparing diet habit of American people with population of our study leads to this result that there might be other important environmental factors which may contribut to pathogenesis of brain tumor too.
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CONFLICT OF INTEREST: There is no conflict of interest

REFERENCES


Prenatal Exposure to Equol Decreases Body Weight and Depressive-Like Behaviors in Male and Female Offspring

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ABSTRACT: A critical period in rat reproductive development occurs during gestational days 12-21. During this interval hormonal factors can alter reproductive development and postnatal behavioral expression in rats. Pregnant rats were injected with one of five treatments. At birth pups were examined for external morphological differences between sexes. Behavioral analysis [Porsolt Forced Swim test (PFST)] was performed on postnatal day 29. Prenatal equol exposure at high (10.5 mg/kg) or supra-pharmacological (63.0 mg/kg) doses during late gestation: a) significantly decreased body weight in the mothers and newborns, b) did not alter genital development or DHT levels in either the male or female offspring and c) significantly decreased depressive-like behaviors in the PFST. These results suggest that exposure to high or supra-pharmacological doses during late gestation do not negatively affect pup growth, reproductive development, or depressive-related behavior in the rat model. Notably, depressive-behaviors in the offspring were significantly decreased compared to controls.

KEY WORDS: Behavior, Body Weight, Depression, Phytoestrogen, Prenatal Equol, Rat

INTRODUCTION

A critical period of reproductive development in rats occurs prenatally between gestational day 12 to birth (Foster and Harris, 2005). During this time period the administration of antiandrogens or testosterone can alter reproductive development in male rats (Foster and Harris, 2005; Welsh et al., 2008). Neonatal estrogen exposure can also influence reproductive functions of male rats as adults (Goyal et al., 2003). Substances similar to, or involving estrogen hormone action include bisphenol A (BPA) found in plastic bottles, estrogen receptor subtype-specific agonists such as propyl pyrazole triol (PPT) or diarylpropionitrile (DPN), and certain phytoestrogens such as coumestrol have gained high-profile investigative attention recently (Safe, 2000; Patisaul and Bateman, 2008; Whitten et al., 1995).

Phytoestrogens are plant-derived, non-steroidal molecules that have structural and functional similarity to 17β-estradiol (Knight and Eeden, 1996) with affinities for estrogen receptor beta (ER-β) greater than estrogen receptor alpha (ER-α) (Kuiper et al., 1998). Previous studies have shown that ERα is critical for reproductive function as demonstrated by the lack of fecundity in ERα knockout mice (Hewitt and Korach, 2003). Whereas, ERβ does not appear to be critical for reproductive function, since ERβ knockout females have reduced ovulatory function but remain fertile (Hewitt and Korach, 2003). Estrogen receptor beta (ERβ) may be involved in regulating brain development and behavioral expression such as having positive influences on synaptic plasticity, emotional behavior (decreasing anxiety/depression) and neuroprotection (Krezel et al., 2001; Frye and Wawrzynki, 2003; Walf and Frye, 2006; Cordey and Pike, 2005).

Of particular interest is the isoflavone metabolite equol, which is derived by the intestinal conversion from daidzein, and has an affinity for ERβ (Setchell et al., 2002; Setchell et al., 2005). Equol is also a selective androgen modulator (SAM), having the ability to bind specifically 5α-dihydrotestosterone (5α-DHT) and inhibit its potent androgen hormone actions (Lund et al., 2004). However, 5α-DHT is important in the development of male morphology (Mcintyre et al., 2001). If 5α-DHT’s hormone action is altered by prenatal exposure to equol it may potentially influence male external genital development.

In rats, during pregnancy, alpha-fetoprotein binds all circulating maternal estrogens protecting the offspring from this hormonal influence (Garreau et al., 1991). However, equol has the ability to stimulate and bind alpha-fetoprotein (Garreau et al., 1991). Finally, our previous results along with other investigative studies show that phytoestrogens, including equol, cross from mother to baby through the rat placenta during late gestation and from mother to offspring via
the mother’s milk (Weber et al., 2001a). Maternal to fetal/newborn transfer has been shown in human studies examining serum isoflavone levels when circulating concentrations are high enough for this transfer to occur (Adlercreutz et al., 1999).

The objectives of this study were: 1) based upon the known weight-reducing influence of soy isoflavone molecules via consumption on body weight and adipose tissue deposition previously reported in rats (Lephart et al., 2004a), we hypothesized that mothers treated with equol during late pregnancy may display lower body weights along with their offspring, 2) since equol has the ability to bind 5α-DHT we tested whether equol may alter external genital development and 3) because equol binds ERβ we examined if the expression of depressive-like behaviors of the offspring may be reduced.

MATERIALS AND METHODS

Reagents
Dimethyl sulfoxide (DMSO) (HPLC grade) and flutamide were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and equol (purity > 99 %) was obtained from LC Laboratories (Woburn, MA, USA).

Animals
Thirty-six female and ten male Long-Evans rats were purchased from Charles River Laboratories (Wilmington, MA, USA) at 55 days-old. They were given ad libitum access to a low phytoestrogen diet (see diet section below) and tap water. The animals were maintained on 12-hour light/dark cycle (lights on 6 am to 6 pm) and the light intensity or illumination of the room was approximately 80 footcandles.

The rats were divided into five groups for injection treatments (see below). The pregnant mothers were weighed on gestational days 0, 6, 12, and 20. Starting at gestational day 14 the rats received daily (subcutaneous- s.c.) injections through gestational day 20. After delivery, the mothers were again weighed and then a subset of the litters were sacrificed. For those animals sacrificed, maternal trunk blood was collected and abdominal white adipose tissue weight was recorded. The offspring were counted, weighed, anogenital distance (AGD) measured in millimeters using microcalipers (± 0.1 mm), and trunk blood collected by sex based on AGD parameters. If unable to determine newborn sex from its AGD length then the pelvic cavity was opened and the presence of the testes was examined by a dissecting microscope in order to confirm the sex. Some of the mothers and offspring from each treatment group, except the flutamide group, were kept alive and allowed to continue to grow. [Almost all flutamide-treated newborns were examined by pelvic cavity dissection to determine sex.] The pups were weaned at postnatal day 21 and moved into group cages (4 to 5 animals per cage) according to gender and treatment. Each treatment group remained on the low phytoestrogen diet until behavioral testing was performed at 29 days post-birth.

Diet
Before their arrival in our laboratory, (from the supplier, Charles River, Wilmington, MA, USA), the male and female rats were fed a diet containing approximately 200-ppm of isoflavones, (previously analyzed by our laboratory) (Weber et al., 2001b). Upon arrival, all rats were placed on a low phytoestrogen diet called by this laboratory the Phyto-free diet [purchased from Zeigler Bros. Inc. (Gardners, PA, USA), catalog # 541200-12-00, Rodent PHY RDC]. This diet has about 10 ppm of isoflavones, as determined by HPLC (Weber et al., 2001a; Weber et al., 2001b).

Mating
The rats were allowed ten-to-fourteen days to adapt to their new surroundings before breeding. The males were put into cages that have wiring along the bottom to allow the feces to drop onto cardboard sheeting below the cage. One female was placed into each cage over night and the following morning and/or evening the cardboard below the cage was examined for a vaginal plug consisting of the ejaculate signifying that the male successfully inseminated the female. After insemination the female body weight was recorded and the date marked as day zero of pregnancy (@ 8/9 am or 5/6 pm). Once a female was inseminated she was removed from the breeding cage and another female was introduced to the male. This was done until the vaginal plug(s) were found for all of the rats. All of the male rats were sexually active and there were approximately 2 to 4 pregnancies obtained per male among all of the treatment groups.

Treatments
The female rats were divided into five groups (weight-matched) before the injection treatments were started: 1) Non-injected Controls, 2) dimethyl sulfoxide (DMSO)-injected Controls, 3) Equol at 10.5 mg/Kg (in DMSO), 4) Equol at 63.0 mg/Kg (in DMSO) and 5) Flutamide (90 mg/Kg, androgen-receptor blocker) in ethanol. [Flutamide is only soluble in ethanol, while equol is soluble in DMSO]. The equol 10.5 dose represented a rodent diet rich in phytoestrogens, while the equol 63.0 dose represented a supra-pharmaceutical (63.0 mg/Kg) dose. These differing doses were administered to see if equol could alter male genitalia. Flutamide was used as a positive control when examining male genital development. Flutamide is an androgen receptor blocker and when administered prenatally causes malformation of the male reproductive tract (McIntyre et al., 2001).

On day 14 of gestation the mothers from the treatment groups were injected daily with 0.1 cc of their specific treatment through day 20 of pregnancy. The mothers were injected subcutaneously at the nape of the neck after being carefully wrapped in cloth to help the mother be more comfortable and secure while minimizing prenatal stress. Afterward each animal was gently placed back into her cage. All treatments stopped the day before the expected delivery at gestational day 21.

Porsolt Forced-Swim Test
In the present study, behavior was assessed in the young offspring by the Porsolt forced-swim test at day 29 before the females would have started their estrous cycles and thus making comparisons between the males and females easier to interpret. Females normally have a greater tendency toward depression compared to males (Walf and Frye, 2006). The Porsolt, or forced swimming test, (also known as the behavioral despair test) is the most commonly used test for assessment of depressive-like behaviors in rodent models. The test is frequently used to measure the effect of antidepressant drugs on behavior (Porsolt...
Serum Phytoestrogen Levels
Blood was collected from both mothers and pups sacrificed (at least 6 hrs) after birth and serum was prepared. Serum was analyzed via gas chromatography/mass spectrometry (GC/MS) for the presence of the isoflavones genistein, daidzein, and equol in both the pups and mothers (ng/ml) (Weber et al., 2001b; Setchell et al., 1987). Maternal and newborn samples were not run simultaneously but internal control samples were within normal ranges for each assay. The maternal samples represented 3 to 5 litters by treatment group. The newborn serum was pooled by treatment group and at least two samples were analyzed by HPLC by treatment. Each fetal sample represented approximately 9 individual animals.

DHT Levels
Serum from mother and newborn pups were examined for 5α-DHT levels using a DHT ELISA kit (Cat # 1940) obtained from Alpha Diagnostic International (San Antonio, TX, USA) in a single assay. The newborn serum was pooled by treatment group and at least two samples were analyzed by HPLC by treatment. All values are expressed as the mean ± the standard error of the mean.

Statistics
All data were first analyzed by one-way analysis of variance. Where appropriate, pairwise comparisons were analyzed using the Newman–Keuls-test. All statistics were run using the Minitab statistical software, p < 0.05 was considered significant. All results are presented as MEANS ± SEM in all of the graphs and significant differences are marked.

RESULTS

Maternal
There were no significant differences in litter size, female/male birth ratio, or gestational length among the treatment groups that were tested (p<0.177, p<0.617, p<0.242 respectively, data not shown graphically).

Offspring Body Weight
Female body weight changes and weight gain are shown in Table 2 while male body weight changes and weight gain are shown in Table 3.

Birth Weight

TABLE 1. Maternal Weight Changes. ** Values significantly less than DMSO control values (p<0.05). All values are expressed as the mean ± the standard error of the mean.

<table>
<thead>
<tr>
<th>FEMALES</th>
<th>NON-INJECT</th>
<th>DMSO</th>
<th>EQUOL 10.5</th>
<th>EQUOL 63.0</th>
<th>FLUTAMIDE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=3</td>
<td>N=4</td>
<td>N=6</td>
<td>N=6</td>
<td>N=5</td>
</tr>
<tr>
<td>Plug Weight</td>
<td>244.0 ± 25.4</td>
<td>222.9 ± 7.2</td>
<td>229.6 ± 6.1</td>
<td>224.2 ± 2.8</td>
<td>230 ± 5.3</td>
</tr>
<tr>
<td>Day 20 Weight</td>
<td>387.7 ± 42.9</td>
<td>386.6 ± 11.0</td>
<td>405.6 ± 9.3</td>
<td>357.7 ± 10.5**</td>
<td>392.6 ± 7.8</td>
</tr>
<tr>
<td>After Delivery Weight</td>
<td>336.6 ± 22.1</td>
<td>353.8 ± 30.7</td>
<td>325.9 ± 11.7</td>
<td>279.2 ± 9.6**</td>
<td>356.5 ± 37.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Non-Inject</th>
<th>DMSO</th>
<th>Equol 10.5</th>
<th>Equol 63.0</th>
<th>FLUTAMIDE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=9</td>
<td>n=10</td>
<td>n=16</td>
<td>n=16</td>
<td>n=5</td>
</tr>
<tr>
<td>Birth to Day 29 Weight</td>
<td>76.5 ± 1.3</td>
<td>74.3 ± 1.3</td>
<td>79.6 ± 1.6</td>
<td>87.5 ± 0.6 #</td>
<td>N/A</td>
</tr>
<tr>
<td>Day 29 Body Weight</td>
<td>82.4 ± 1.3</td>
<td>79.5 ± 1.3</td>
<td>85.2 ± 1.6</td>
<td>92.4 ± 0.5 #</td>
<td>N/A</td>
</tr>
</tbody>
</table>

TABLE 2. Female Offspring Weight Changes From Birth to Postnatal Day 29. Because the flutamide treated animals were sacrificed at birth, no postnatal data is presented; this is indicated by (N/A). *Value is significantly greater than other treatment groups (p<0.001). **Value is significantly greater than the Equol 63.0 treatment group and DMSO control animals (p<0.01). #Value is significantly less than DMSO control animals (p<0.05). 

<table>
<thead>
<tr>
<th>MALES</th>
<th>NON-INJECT</th>
<th>DMSO</th>
<th>Equol 10.5</th>
<th>Equol 63.0</th>
<th>FLUTAMIDE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=30</td>
<td>N=34</td>
<td>N=37</td>
<td>N=38</td>
<td>N=36</td>
</tr>
<tr>
<td>Birth Weight</td>
<td>6.22 ± 0.065*</td>
<td>5.59 ± 0.106</td>
<td>5.76 ± 0.144</td>
<td>5.20 ± 0.112 +</td>
<td>5.96 ± 0.066 **</td>
</tr>
<tr>
<td>Birth to Day 29</td>
<td>83.14 ± 0.7</td>
<td>88.2 ± 0.9</td>
<td>85.8 ± 1.6</td>
<td>98.2 ± 0.8 #</td>
<td>N/A</td>
</tr>
<tr>
<td>Day 29 Body Weight</td>
<td>89.4 ± 0.67</td>
<td>93.9 ± 0.96</td>
<td>91.4 ± 1.56</td>
<td>103.4 ± 0.80 #</td>
<td>N/A</td>
</tr>
</tbody>
</table>

TABLE 3. Male Offspring Body Weight Changes From Birth to Postnatal Day 29. Because the flutamide treated animals were sacrificed at birth, no postnatal data is presented; this is indicated by (N/A). *Value is significantly greater than other treatment groups (p<0.001). **Value is significantly greater than DMSO control animals (p<0.004). +Value is significantly greater than all other treatment groups (p<0.001-0.02). All values are expressed as the mean ± the standard error of the mean.
Female
At birth the female non-injected control group and female flutamide group both weighed significantly more than any other group (p<0.001). Also the equol 63.0 treated animals displayed the lowest birth weights that were significantly less than non-injected or DMSO control values (p<0.001 and p<0.027 respectively). Finally, female birth weights were significantly greater in the equol 10.5 group compared to equol 63.0 and DMSO controls (p<0.001 and p<0.013 respectively).

Male
At birth the male non-injected control group weighed significantly more than all other groups (ranging from p<0.001-0.007). The male equol 63.0 group weighed significantly less than all other groups (p<0.001-0.013). The DMSO and equol 10.5 groups were not significantly different from each other. The flutamide group was significantly less than the non-injected controls (p<0.007) but significantly greater than the DMSO controls (p<0.004).

Weight Gain from Birth to Postnatal Day 29

Female
The equol 63.0 group gained significantly more weight than all other treatment groups (p<0.006, p<0.001, p<0.022 respectively see Table 2).

Male
The equol 63.0 group gained significantly more weight than the non-injected controls, DMSO controls, and equol 10.5 group (ranging from p<0.016 to p<0.026 see Table 3). The non-injected controls, DMSO controls, and equol 10.5 groups were not significantly different from one another (p<0.762).

Postnatal Day 29 Body Weight

Female
The equol 63.0 group weighed significantly more than all other groups (p<0.002 see Table 2). The other groups were not significantly different from each other (p<0.489).

Male
The equol 63.0 group weighed significantly more than all other groups (p<0.002-0.039 see Table 3).

Maternal Serum Phytoestrogen Levels
Maternal serum levels of equol, genistein, daidzein were obtained from gas chromatography/mass spectrometry (GC/MS) analysis. These results are shown in Figure 1A. The equol 63.0 groups had the highest level of serum equol (p<0.001) compared to all other treatment groups. The equol 10.5 group was significantly greater compared to non-injected and DMSO control values (p<0.028). Finally, the serum levels of daidzein or genistein were not significantly different among any of the treatment groups.

FIGURE 1. (A) Maternal Serum Isoflavone Levels Measured Postnatal Day 1. ** The Equol 63.0 treatment group had significantly higher serum equol levels than all other treatment groups (p<0.001). # The Equol 10.5 treatment group had significantly greater serum equol levels than both the control groups and the flutamide treatment group (p<0.05). All values are expressed as the mean ± the standard error of the mean. (We did not test the significance of the fluctuations in genistein and daidzein; the changes were relatively minor and not the focus of this study). (B) Newborn Female Serum Isoflavone Levels Measured Postnatal Day 1. ** The Equol 63.0 and Equol 10.5 treatment groups’ serum equol levels were significantly greater than both the control groups and the flutamide treatment group (p<0.001). The Equol 63.0 treatment group and the Equol 10.5 treatment group’s serum equol levels were significantly different from each other (p<0.01). All values are expressed as the mean ± the standard error of the mean. (We did not test the significance of the fluctuations in genistein and daidzein; the changes were relatively minor and not the focus of this study). (C) Newborn Male Serum Isoflavone Levels Measured Postnatal Day 1. ** The Equol 63.0 and Equol 10.5 treatment groups’ serum equol levels were significantly greater than both the control groups and the flutamide treatment group (p<0.004). The Equol 63.0 treatment group and the Equol 10.5 treatment group’s serum equol levels were significantly different from each other (p<0.04). All values are expressed as the mean ± the standard error of the mean. (We did not test the significance of the fluctuations in genistein and daidzein; the changes were relatively minor and not the focus of this study).
Newborn Serum Phytoestrogen Levels

Newborn pup serum levels of equol, genistein, and daidzein were obtained using gas chromatography/mass spectrometry (GC/MS) analysis. The results are shown in Figures 1B and 1C for females and males respectively. At least two samples from each group were analyzed, however, each sample represents 9 animals so there are approximately 18 animals per treatment group. As expected the equol 63.0 group had the highest levels of equol in both the male and female pups. All of the equol treated groups had significantly more equol in the serum than the non-injected controls, DMSO controls, and flutamide groups (p<0.004 to 0.001 males and females respectively). The equol concentrations increased in a step-wise manner as dosage increased and all equol groups were significantly different from each other in both males and females (p<0.01 to 0.04).

Newborn Anogenital Distance (AGD) Standardized by Body Weight (AGD/BW)

These results are shown in Figure 2. The AGD/BW ratios (millimeters divided by body weight in grams) showed that in all the groups the males and females were significantly different from each other (p<0.001) except for the flutamide group where the male and female ratios were not significantly different from each other (p<0.208). Flutamide treated males had the smallest AGD/BW ratio which was expected and served as a positive anti-androgen control.

Newborn Serum DHT Levels

Female and male DHT levels are shown in Figures 3A and 3B respectively.

FIGURE 2. Pup Anogenital Distance to Animal Body Weight Ratio When Measured at Birth. Only the flutamide treatment group demonstrated no significant differences in pup anogenital distance to animal body weight ratio between male and female pups. Anogenital distance measured in mm. Body weight measured in grams. N refers to the number of pups in each group. All values are expressed as the mean ± the standard error of the mean.

Female

The non-injected control female DHT levels were significantly lower than all other groups (p<0.009-0.05) except DMSO controls (p<0.202). The female flutamide fetal DHT levels were the highest but not significantly different than any other group except the non-injected controls (p<0.021).

Male

As shown in figure 3B, the male fetal DHT levels among all the treatment groups tested were not significantly different from each other. In summary, there were no significant differences in female or male DHT levels among the DMSO and equol-treated groups.
Day 29 Forced Swim Test

Females

The DMSO controls were significantly more immobile than the non-injected controls, equol 10.5 and equol 63.0 animals (p<0.042, p<0.029, p<0.017 respectively) as shown in Figure 4A. The equol 63.0 animals' total distance traveled was significantly greater than the DMSO controls (p<0.041) as shown in Figure 4B. In summary, the equol 63.0 group displayed the lowest levels of depressive-like behaviors compared to DMSO values.

FIGURE 4. (A) Postnatal Day 29 Female Offspring Porsolt Forced Swim Test Time Spent Immobile. **The females in the DMSO treatment group spent significantly more time immobile than all other treatment groups (p<0.01-05). All values are expressed as the mean ± the standard error of the mean. (B) Postnatal Day 29 Female Offspring Porsolt Forced Swim Test Total Distance Travelled. **The Equol 63.0 treatment group animals swam significantly further than the DMSO control animals (p<0.05). All values are expressed as the mean ± the standard error of the mean.

Males

The equol 63.0 group spent less time immobile than all of the other groups. This group was significantly less immobile than the DMSO controls (p<0.010) as seen in Figure 5A. The equol 63.0 treated animals swam the greatest distance compared to all other treatment groups though this distance was not significant as shown in Figure 5B. In summary, the male data was similar to the female data where the equol 63.0 group displayed the lowest levels of depressive-like behaviors compared to DMSO values.

FIGURE 5. (A) Postnatal Day 29 Male Offspring Porsolt Forced Swim Test Time Spent Immobile. **The Equol 63.0 treatment group males spent significantly less time immobile than the DMSO control males (p<0.01). All values are expressed as the mean ± the standard error of the mean. (B) Postnatal Day 29 Male Offspring Porsolt Forced Swim Test Total Distance Travelled. There were no significant differences between any of the male treatment groups. All values are expressed as the mean ± the standard error of the mean.

DISCUSSION

Soy-derived isoflavone consumption in humans is estimated at 50 mg/day in Asian countries, while Western consumption is approximately 3 – 5 mg/day (Lephart et al., 2004a; Munro et al., 2003). The long historical dietary exposure to soy and isoflavone-derived molecules in humans worldwide is well established without adverse effects (Munro et al., 2003; Hoikkala et al., 2007). Many
animal studies have examined several isoflavone compounds within a large range of doses on developmental or reproductive parameters where no harmful toxicological observations were made (Medlock et al., 1995; Thompson et al., 1984; Wood et al., 2006). However, a potential nutritional link to brain and behavioral parameters has not been studied in reference to equol's unique biochemical properties that influence hormone actions.

In regards to a nutritional link, we previously demonstrated that rats exposed to a phytoestrogen-rich diet postnatally gain significantly less body weight compared to animals fed a diet low in phytoestrogens where the major isoflavone metabolite is equol (Lephart et al., 2004a; Bu and Lephart, 2006). Because few studies have investigated the impact of isoflavones during late pregnancy (Weber et al., 2001a) we undertook the present study to examine this isoflavone metabolite and study this potential nutritional/behavioral link.

Mothers in the supra-pharmacological equol dose group (@ 63.0 mg/kg) gained significantly less weight then all other treatment groups (except the non-injected control animals). This trend was also seen in the male and female offspring. The higher the equol dose the lower the birth weight. Whether or not this situation is due to a decrease in food/water intake and/or an increase in metabolism is unknown. Previous studies suggest that during postnatal development there is adequate food/water intake with potentially significant increases in metabolic parameters (Lephart et al., 2004a). One recent study examining equol administration in adult ovariectomized rats (Rachon et al., 2007) and in another study that employed a rich soy-based ingredient where a 2000 mg/kg/day dose (where equol represented 0.65 % of this dose or approximately 13 mg) (Matulka et al., 2009) confirm the present results in reference to equol’s influence on body weight.

Equol has been reported as a SAM with the ability to bind and inhibit the action of 5α-DHT (Lund et al., 2004a). If a 5α-DHT inhibitor or other androgen receptor blocker (like flutamide) is administered during late gestation male reproductive development is altered with female-like external genital morphology as measured by the AGD in rodents (Foster and Harris, 2005; McIntyre et al., 2001; Lephart and Husmann 1993). When equol was administered during this critical time period there were no changes in external genital morphology of the male pups, nor were serum 5α-DHT levels altered in the male or female newborns. This indicates that 5α-DHT functioned normally even in the presence of high pharmacological equol levels reaching approximately 7,770 ng/ml or 32 μM in maternal and around 1,000 ng/ml or about 4μM in the newborn serum samples. These doses are much higher compared to humans consuming soy based products in an Asian diet (Adlercreutz and Mazur, 1997). For example, total maternal plasma isoflavonoid concentrations of Japanese women at birth range between 19 to 744 nM (mean 232 nM) while in cord plasma isoflavones range from 58 to 831 nM (mean 299 nM) (Adlercreutz et al., 1999). The 10.5 mg/kg equol dose yielded equol levels (at 894 nM for maternal and 886 nM for newborn values) that are more than 3 to 4-times higher compared to the human birth data which suggests that human dietary consumption of soy food products may never approach this dosage. In this experiment while the flutamide-treated pups had the smallest AGDs, and the equol-treated groups did not display significantly shortened AGDs even at the highest dose. The lack of altering 5α-DHT levels in this study may be due to the presence and action of alpha-fetoprotein in the rodent model that can be stimulated by equol and then in turn bind equol at relatively high concentrations (Garreau et al., 1991).

Our laboratory has reported that feeding young adult or mid-aged male or female rats a diet rich in isoflavones significantly decreases anxiety levels as assessed by the elevated plus maze (Lund and Lephart, 2001; Lephart et al., 2004b). However, recent studies by Patisaul and Bateman 2008 indicate that phytoestrogens may increase anxiety levels in intact male rats during postnatal development at a dose of 10 mg/kg/day in newborn pups for four days that may represent a high level of exposure in reference to human data.

In the present results, it is the female behaviors that appear to be more affected by equol administration than the male behaviors. For example, both the high (10.5 mg/kg) and supra-pharmacological (63.0 mg/kg) equol-dosed female animals displayed less depressive-related behaviors while only the supra-pharmacological (63.0 mg/kg) male animals displayed significantly lower depressive-related behaviors. In other words, these prepubertal animals displayed more mobility when tested in the forced-swim test during early postnatal development. To support this notion, recent unpublished data from our laboratory suggests that mid-aged (> 300 day-old) non-cycling female rats fed a diet low in isoflavones display depressive-like behaviors in the Porsolt swim test when compared to controls fed a phytoestrogen-rich diet. But when the mid-aged female rats on the diet low in isoflavones were subsequently administered equol at 2.5 mg/day (or 5.0 mg/kg) (for approximately 1 week), the depressive-like behaviors were significantly decreased (compared to pre-equol treatment values) and comparable to control phytoestrogen-rich fed animals. Also of interest is the fact that while the supra-pharmacological male and female prenatally equol-treated animals gained the most weight postnatally, these animals were the most mobile in the Porsolt forced swim test.

It does appear that the manner of administration of treatments to the animals had an affect. In the female (and male) rat swim tests it was observed the animals that expressed the least depressive related behaviors were the equol-dosed groups as well as the non-injected control animals. These three groups were significantly greater than the DMSO-control group in the female rats while only the supra-pharmacological dose in the males was significant. Considering the mode of equol administration, the DMSO-vehicle injections to the maternal rats had a tendency for the offspring to express more depressive-related behaviors. This could be a result of the stress of injections to the mother since prenatal stress has a tendency to increase likelihood of depressive-related behaviors (Frye and Wawrzycki, 2003). We do not believe the DMSO vehicle played a role in prenatal stress since it is considered a safe (skin penetrating agent) and has desirable uses in medical settings (Jacob and de al Torre, 2009).

In summary, it appears that prenatal exposure during
late pregnancy to high equol doses in rats does not alter newborn DHT levels in males or females or alter external male genital development. High prenatal equol doses also decrease maternal and newborn body weight. After the treatments stopped the prena tally equol-treated animals gained the most weight during early postnatal development but still displayed the lowest levels of depressive-like behaviors. Injecting the pregnant animals appeared to cause prenatal stress; however, both the high and supra-pharmacological equol doses appeared to restore depressive-related behavioral levels to that of untreated-control rats. In order to avoid prenatal stress complications in future studies equol should be administered in the maternal diet. Therefore, further research is warranted to investigate the properties of equol and its potentially beneficial influence via nutritional sources and its link to behavioral expression.

COMPETING INTERESTS
The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS
Crystal Blake- participated in the experiment design, performed the experiments, analyzed the data and authored the text, etc; Kim Fabick- performed the experiments and also assisted in several aspects of the study; Kenneth Setchell- performed the isoflavone blood analysis; Trent Lund- performed the experiments, data analysis of Porsolt results; Edwin Lephart- participated in the experiment design, performed the experiments, data analysis, authoring of the text, etc.

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REFERENCES


Prenatal Equol and Depressive-related Behavior


ABSTRACT: With many nutraceutical interventions designed to slow cognitive aging, there is a need for computerised tests that can detect small cognitive changes that may occur in response to these interventions. A battery of 13 computerised cognitive tasks was developed to capture the range of cognitive functions that decline with age. One hundred and twenty adults aged 21 to 86 years, with a MMSE score >27 completed the test battery. Accuracy and response time were measured. Regression analysis revealed age-related decrements in cognitive performance for all tasks. Performance accuracy for the Spatial Working Memory task and speed of response for Spatial Working Memory, Contextual Memory and Immediate Recognition tasks showed the greatest age-related decline. The tasks showed good test-retest reliability and correlated with other commonly used neuropsychological tests in aging research. With the sensitivity of this cognitive test battery to aging, it may be useful in future studies investigating cognitive improvements in response to nutraceutical interventions in older adults.

KEY WORDS: Age-Related Cognitive Decline, Cognitive Aging, Computerised Testing, Nutraceutical Intervention

INTRODUCTION

With the population aging at a rapid rate, there is increasing interest in slowing the cognitive decline associated with increasing age. Both pharmacological interventions (e.g. cholinesterase inhibitors) and nutraceutical interventions (e.g. Ginkgo biloba) are purported to improve or stabilise cognitive functions that decline with age. In order to test the efficacy of these interventions, tasks must be used which target the cognitive functions most susceptible to cognitive decline and which are sensitive enough to capture very small changes that may occur in response to these interventions.
Computerised cognitive testing of cognitive aging

intensive tasks, this decline begins as early as in the twenties (Park et al., 2002). Evidence from brain imaging studies indicates that spatial abilities are susceptible to decline (Cabeza et al., 2005). Additionally, Merrick et al. (2004) found significant age effects mainly with visuospatial tasks compared to verbal tasks.

Despite reports of general cognitive decline with age, older individuals can sometimes perform with the same level of accuracy as younger people if given enough time to complete a task (Brebion, 2001). However, when timing is restricted, accuracy might be impaired if there is insufficient time to complete all of the mental processes required for the task (Salthouse, 1996). Thus, there is a trade-off: speed is forfeited for greater accuracy, or vice-versa. Accordingly it is important when investigating age-related cognitive decline to measure both variables in order to obtain a more complete representation of an individual’s cognitive functioning.

Computerised tasks are expedient because they offer both speed and accuracy measures. They can also provide millisecond sensitivity, which enables the detection of very small changes that may occur early in the course of cognitive decline or in response to a therapy or intervention. Moreover, computerised tasks can score performances quickly and accurately, can provide multiple formats for longitudinal studies or multiple testing sessions, and provide a consistent approach for presentation of stimuli and delivery of task instructions. This opportunity is not afforded by the more traditional neuropsychological tests such as the Mini Mental State Examination (MMSE) (Folstein et al., 1975) and the Wechsler Adult Intelligence Scales (WAIS) (Wechsler, 1981).

There are numerous computerised test batteries that have been used to measure cognitive processes. Some of these have focussed on the measurement of the effects of pharmacological and nutraceutical compounds (Ferguson et al., 2003; Wesnes et al., 2000) and others have been used as a diagnostic tool for early detection of dementia and Alzheimer’s disease (Egerhazi et al., 2007). Some batteries that are currently in use in elderly populations were not designed specifically for an elderly population or are biased toward the identification of overt dementia (Anderson et al., 2006) and do not attempt to capture the range of cognitive functions that decline in normal aging. A number of these task batteries are commercial products and so accessibility is limited. Moreover, very few cognitive batteries have been developed to specifically target cognitive decline. Normative data is also limited.

There are numerous new nutraceutical interventions currently being used and tested in elderly patients which claim to target the cognitive processes that decline with age (Fotuhi et al., 2009; Fusco et al., 2007; Jia et al., 2008). When researching such interventions, it is important that the test battery has millisecond sensitivity in order to measure the subtle changes that may occur in response to the intervention. Also, it is important that the test battery include fluid measures of intelligence that have been shown to decline with age and thus have potential to improve or stabilise with such interventions.

The present study used 13 moderately demanding computerised tasks that assessed speed and accuracy of cognitive functions in adults of various ages with the aim of determining which tasks are sensitive to the effects of aging. It was hypothesised that the tasks would be reliable over time with little or no practice effects. High correlations (r>-.6) were considered adequate test-retest reliability. It was hypothesised that all tasks would demonstrate some decline in performance with increasing age, particularly the tasks more sensitive to aging such as Spatial Working Memory and Contextual Memory (episodic memory). Furthermore, it was hypothesised that age effects would remain apparent after controlling for the underlying effects of processing speed.

MATERIALS AND METHODS

Subjects

One hundred and twenty participants (57 female, 63 male) were recruited via advertisements in local newspapers and university bulletin boards. Ages ranged from 21 to 86 years (M=53.1, SD=16.0). The mean level of education for the sample was 15.3 years (SD=3.2). Participants were screened for cognitive impairment using the MMSE and all participants had a score of 27 or higher. Participants were non-smokers, had no history of head injury or neurological disorder and were not suffering any clinically diagnosed depressive disorder. No participant was excluded based on initial screening. Thirty-five participants (22 female, 13 male) additionally completed the validation tasks and 34 subjects (23 female, 11 male) returned to complete a follow-up session (test-retest) approximately one to four weeks after the initial session. When possible, testing took place at the same time of the day as the first session and participants completed an alternate version of the battery. The study had approval from the Swinburne University Human Research Ethics Committee and all participants gave written informed consent.

Equipment and materials

The tasks consisted of thirteen screen-based cognitive tasks that were developed based on the cognitive and neuroimaging literature focussing on processes that are most likely to decline with age. Simple and Choice Reaction Time tasks (explained below) were included in the battery to control for motor effects and decision time being a component of other tasks. These tasks have been used in previous studies investigating nutraceutical effects on cognition (Pipingas et al., 2008).

Testing was conducted on a 17-inch colour monitor using a DOS-based computer software package and took approximately 50 minutes to complete. Stimulus timing and participant response were accurate to one millisecond, with stimulus presentation synchronised to the screen refresh rate (Paredes et al., 1990). Participants responded using a 4-button box, with each button a different colour and arranged in the positions of compass poles (top = yellow etc). The investigator read the task instructions from a manual to ensure consistent explanation of the tasks. Each task was preceded by a short practice trial and participants were given an opportunity to ask questions. Except where otherwise indicated, tasks are described here in the order presented to participants. A selection of participants returned for a second visit and completed an alternate form of the tasks.

Immediate/Delayed Word Recall

Participants were presented with a list of 12 words that were presented one at a time in the centre of the screen. Each word was presented for 2-seconds, with an inter-stimulus interval (ISI) of 1-second. Participants were then given two minutes to record with pen and paper as many words as they could remember (Immediate
condition). At the end of the testing session, typically 45 minutes later, they were again given two minutes to record remembered words from the original studied list (Delayed condition).

Simple Reaction Time
Participants responded with a right button press to the appearance of a single white square at the centre of the screen. Thirty targets were presented with a randomised inter-stimulus interval (ISI) to avoid anticipation effects.

Choice Reaction Time
Participants responded with a left (blue) or right (red) button press to the appearance of a blue triangle or red square respectively. Presentation order and ISI were randomised to avoid anticipation effects. This task was used as a measure of visual perceptual decision time.

Immediate/Delayed Recognition
Participants were asked to study a series of 40 abstract images presented serially in the centre of the screen for 3-seconds each with no ISI. On completion, another series of images was presented, half of which were from the studied series and half were new (Immediate condition). Participants indicated with a right (yes) or left (no) button press whether or not they recognised the image from the studied series. This task was repeated at the end of the testing session with the remaining 20 images from the studied series and another 20 new images (Delayed condition). Because abstract patterns are difficult to verbalise, the task can be described as a measure of non-verbal recognition memory.

Visual Vigilance
Participants were required to initially memorise a single digit target number appearing in the centre of the screen for 2-seconds. A series of numbers was then presented one at a time in the centre of the screen for 0.6-seconds each, with no ISI. Each time the target number appeared in the series, participants were required to respond as quickly as possible with a right button press. The appearance of the target was randomised and the probability was set at 20%, resulting in 30 possible targets from a total of 150 stimuli presented. The time taken to respond was used as a measure of visual vigilance or sustained attention.

N-Back Working Memory
In each of these tasks a series of letters were presented one at a time in the centre of the screen for 1.3-seconds, with an ISI of 0.2-seconds. Participants responded with a right button press each time a letter was the same as the previous letter (1-Back condition), or the same as the letter two letters before (2-Back condition). A target probability of 20% was set, resulting in 15 possible targets from a total of 75 stimuli presented. Due to the requirement to hold changing information in the short term memory store, this task was used as a measure of working memory.

Stroop Colour-Word
The test consisted of two congruent and two incongruent trials, presented alternately. Stimulus words were randomly presented (RED, BLUE, GREEN, YELLOW) in either congruent or incongruent colours for 1.7-seconds, with an ISI of 0.5-seconds. Participants responded by pressing one of four buttons corresponding to the colour of the word, irrespective of what the word read. This task was used as a measure of executive function and more specifically inhibition; participants had to inhibit the automatic reading response.

Spatial Working Memory
In each trial participants were presented with a 4 x 4 white grid on a black background, with six grid positions containing white squares. Participants were given 3 seconds to remember where the white squares were located. The grid became blank and a series of four white squares were sequentially displayed in various grid positions for 2-seconds each. Participants responded with a yes/no response to indicate whether each square matched a position that was originally filled. In total, participants completed 14 trials, each of which was separated by a blank screen displayed for 2-seconds. Each trial was set such that two out of the four locations in the response series corresponded to the original grid locations, and two did not. The task required participants to hold spatial information in a store that has previously been described as working memory (Baddeley, 2003).

Contextual Memory
A series of 20 everyday images were presented at the top/bottom/ left/right of the screen for 3-seconds each with no ISI. On completion of the series the same images were displayed again in randomised order in the centre of the screen for 2-seconds each with no ISI. Participants responded with a top/bottom/left/right button press to indicate the original location of each image. This task required participants to recall the spatial context of the original presentation and was used as a measure of episodic memory. Upon completion of the SUCCAB computerised tasks, a subset of participants completed the validation tasks, described below in the order of presentation.

Logical Memory I (From the Weschler Memory Scale)
In each of the three parts of this test, participants listened to a paragraph and immediately after were instructed to repeat as much of the story as they could remember. The first paragraph was about a woman named Anna and the second and third paragraphs were the same and about a man named Joe. Participants were scored on the number of correct ideas they remembered. This task has been described as the purest measure of episodic memory (Ritchie et al., 1993)

Logical Memory II (From the Weschler Memory Scale)
During the first two parts, participants repeated back as much of the stories that they were read in Logical Memory I. During the third part, participants were presented with 15 statements on each story and they had to indicate whether the statement was true or false.

Trail Making Test (TMT) - Part A
Part A Participants were given a piece of paper with the numbers 1-25 surrounded by circles written randomly on the page. Participants were instructed to follow the numbers as quickly as possible in ascending order starting at one and finishing at 25 by drawing straight lines between each number, without lifting the pen from the paper. If a participant missed a number/letter, they were instructed to go back and correct the order. Participants were scored on the time taken to complete the task.
**Digit Symbol-Coding (from the WAIS III)**

Participants were presented with an A4 piece of paper with a key on the top consisting of numbers 1-9 with a corresponding symbol under each number. Below were rows of random numbers with blank squares below each number, and participants were given two minutes to fill in the blank squares with the corresponding symbol. Participants were not allowed to skip any numbers or rows.

**Statistical Analyses**

For the Immediate and Delayed Word Recall tasks, only accuracy was analysed. For Simple and Choice Reaction Time, Visual Vigilance, 1-Back and Stroop tasks, ceiling effects were anticipated for accuracy data and thus only response time was analysed. Both accuracy and speed of response were analysed for the remaining computerised tasks. Test-retest reliability was assessed using Pearson’s Product-Moment correlations (r) between Time 1 (T1) and Time 2 (T2). Practice effects were analysed by testing the significance of the difference between the mean of T1 and the mean of T2 with paired t-test comparisons. Accuracy and response time for the tasks were correlated with some of the widely used neuropsychological tests in aging research. Sequential multiple regression analyses were performed using SPSS software version 13 (SPSS Inc. Chicago) for all cognitive measures. Number of years of education was included in all regression models because education often correlates with cognitive performance (Anstey and Christensen, 2000) and education levels may differ between older and younger adults. For accuracy measures, education and age were entered sequentially into each model as the predictor variables. For the response time measures, education and age were entered sequentially for the Simple and Choice Reaction Time tasks only. For the remaining response time tasks, education, Choice Reaction Time and age were entered sequentially into each model. The Choice Reaction Time task was chosen as a baseline measure of processing speed to control for the effects of motor response and simple decision time that might be evident in all tasks susceptible to global cognitive slowing. For the Stroop tasks, an additional variable was created by subtracting each score on the Congruent task from the score on the Incongruent task. The extra time taken to respond to the Incongruent task is thought to represent the inhibition of automatic word reading known as the Stroop interference effect (Wecker et al., 2000).

**RESULTS**

Preliminary analysis confirmed that age was correlated with education, with older participants generally having fewer years of education than younger participants (r = -.29, p=.002). Minimum and maximum scores, means, standard deviations, and correlation with age and each accuracy and response time measure are presented in Table 1. Age was significantly correlated with accuracy and response time on all tasks. Figure 1 illustrates the relationship between age and performance for selected cognitive tasks.

The results from the test-retest reliability and practice effects are shown in Table 2. All performance accuracy measures showed moderate to high correlations, and all except one were significant at p<.001. The only significant practice effect was found for the Spatial Working Memory task. The majority of the response time measures showed high and significant correlations at p<.001. Only the Immediate Recognition, Stroop tasks and Contextual Memory task showed

### Table 1. Minimum and Maximum Scores, Means, Standard Deviations and Correlation with Age for Cognitive Measures.

<table>
<thead>
<tr>
<th>Min</th>
<th>Max</th>
<th>Mean (±SD)</th>
<th>Correlation with Age r</th>
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</thead>
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<tr>
<td><strong>Accuracy (%)</strong></td>
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<tr>
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<tr>
<td>Choice Reaction Time</td>
<td>77</td>
<td>100</td>
<td>96.0 (4.9)</td>
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<td>Immediate Recognition</td>
<td>38</td>
<td>95</td>
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<td>169</td>
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<td>256 (45)</td>
</tr>
<tr>
<td>Choice Reaction Time</td>
<td>240</td>
<td>648</td>
<td>448 (68)</td>
</tr>
<tr>
<td>Immediate Recognition</td>
<td>734</td>
<td>1445</td>
<td>1064 (134)</td>
</tr>
<tr>
<td>Visual Vigilance</td>
<td>304</td>
<td>551</td>
<td>397 (45)</td>
</tr>
<tr>
<td>1-Back Working Memory</td>
<td>285</td>
<td>586</td>
<td>399 (63)</td>
</tr>
<tr>
<td>2-Back Working Memory</td>
<td>341</td>
<td>916</td>
<td>581 (122)</td>
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<td>Stroop Congruent</td>
<td>553</td>
<td>1313</td>
<td>888 (186)</td>
</tr>
<tr>
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<td>528</td>
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<td>894 (184)</td>
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<td>Stroop Interference Effect</td>
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<td>128 (115)</td>
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<tr>
<td>Spatial Working Memory</td>
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<td>1635</td>
<td>963 (211)</td>
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<td>654</td>
<td>1424</td>
<td>1017 (160)</td>
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<td>Delayed Recognition</td>
<td>777</td>
<td>1319</td>
<td>1028 (122)</td>
</tr>
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</table>

**FIGURE 1. Relationship between Cognitive Performance and Age for Selected Cognitive Tasks.**
significant practice effects. The validity of the tasks was explored using correlations between the computerised tasks and the validation tests, shown in Table 3. The Logical Memory Tests correlated with the memory tasks (word and picture recall and recognition tasks) and not with the attention/executive function-type tasks (N-Back, Stroop and Spatial Working Memory). Both the Trail making Test and Digit Symbol Coding task showed strong correlations with the computerised tasks.

Results of regression models for accuracy measures are presented in Table 4. After controlling for education, age significantly predicted performance accuracy for all analysed tasks. Change in $R^2$ was largest for Spatial Working Memory, reflecting the greatest decline in this cognitive measure with increasing age.

Results of regression models for response time measures are presented in Table 4. For Simple and Choice Reaction Time, age significantly predicted performance after controlling for education, with change in $R^2$ values reflecting slower response time with increasing age. After controlling for education and Choice Reaction Time, age significantly predicted performance on all tasks except 1-Back and 2-Back Working Memory. Change in $R^2$ values for age were highest in the Spatial Working Memory, Stroop Incongruent and Contextual Memory tasks, indicating that age had a greater impact on response time for these tasks.

Results of regression models also confirmed the importance of taking into account education and processing speed when investigating cognitive

### TABLE 3. Correlations Between SUCCAB Tasks and Validation Tasks. $^* p< .05, ^{**} p< .001, N=34$

<table>
<thead>
<tr>
<th></th>
<th>Logical memory 1 total score</th>
<th>Logical memory 2 total score</th>
<th>Trailmaking A</th>
<th>Digit Symbol Coding</th>
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<tr>
<td>Accuracy</td>
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<td>.421**</td>
<td>.423**</td>
<td>-.497**</td>
<td>.572**</td>
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<td>.431**</td>
<td>.511**</td>
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<td>.422**</td>
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<td>.372**</td>
<td>.464**</td>
<td>-.584**</td>
<td>.669**</td>
</tr>
<tr>
<td>2-back</td>
<td>.215</td>
<td>.0196</td>
<td>-.232</td>
<td>.510**</td>
</tr>
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<td>Incongruent stroop</td>
<td>.201</td>
<td>.23</td>
<td>-.556**</td>
<td>.637**</td>
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<td>.18</td>
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<td>.757**</td>
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<td>Contextual memory</td>
<td>.259*</td>
<td>.338**</td>
<td>-.498**</td>
<td>.603**</td>
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<td>.410**</td>
<td>.389**</td>
<td>-.465**</td>
<td>.453**</td>
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<td>Response Time</td>
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<tr>
<td>Simple RT</td>
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<td>.767**</td>
<td>-.792**</td>
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<td>Visual vigilance</td>
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<td>1-back</td>
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<td>.676**</td>
<td>-.696**</td>
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<td>.627**</td>
<td>-.745**</td>
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TABLE 4. Sequential Regression Models for Cognitive Measures. *p<.05, **p<.01, ***p<.001, N=120

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<thead>
<tr>
<th></th>
<th>Adjusted R² (whole model)</th>
<th>R² Change</th>
<th>F Change</th>
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<td>Immediate Word Recall</td>
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<td>Education</td>
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<tr>
<td>Age</td>
<td>.066</td>
<td>.067</td>
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<td>.079</td>
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<tr>
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<td>.094</td>
<td>.082</td>
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</table>

The current study examined age-related cognitive changes using a battery of computerised tests. The reliability hypotheses were supported in that there were good test-retest coefficients (ranging from .42-.93) and practice effects were only observed for a few of the measures. As anticipated, age significantly predicted performance for all tasks in the battery, with the strongest effects demonstrated in performance accuracy for Spatial Working Memory, and in response time for Spatial Working Memory, Contextual Memory and Stroop Incongruent tasks. Once potentially confounding effects of education and processing speed were taken into account, age significantly predicted performance for all tasks with the exception of the 1-Back and 2-Back Working Memory tasks.

The practice effects were observed for reaction time measures of the more complex tasks (Immediate Recognition, Stroop, and Contextual Memory). Therefore it is recommended that an additional practice version (or longer initial practice) be given to participants for these tasks so that performance can stabilise more rapidly, eliminating (or reducing) the practice effects. The Spatial Working Memory task was the only task that showed practice effects for the performance accuracy measure but not for the reaction time measure. In all tasks there is a trade off between accuracy and reaction time (Salthouse, 1996). It seems that in the case of the Spatial Working Memory task, participants may appear to concentrate more on accuracy, and less on speed, resulting in the observed practice effect for aging. Significant results were demonstrated for the control variables, education and Choice Reaction Time. For the accuracy measures, education was significant in the Spatial Working Memory and Immediate Recognition tasks, with borderline significance in the Delayed Recognition task. For response time measures, education was a significant predictor in Simple Reaction Time, Spatial Working Memory, Contextual Memory, Immediate Recognition and both Stroop tasks, with borderline significance in the Stroop Interference variable. As expected, the Choice Reaction Time task significantly contributed to performance on all response time measures, indicating that performance in these tasks was partly mediated by processing speed and simple decision making.

DISCUSSION
The current study examined age-related cognitive changes using a battery of computerised tests. The reliability hypotheses were supported in that there were good test-retest coefficients (ranging from .42-.93) and practice effects were only observed for a few of the measures. As anticipated, age significantly predicted performance for all tasks in the battery, with the strongest effects demonstrated in performance accuracy for Spatial Working Memory, and in response time for Spatial Working Memory, Contextual Memory and Stroop Incongruent tasks. Once potentially confounding effects of education and processing speed were taken into account, age significantly predicted performance for all tasks with the exception of the 1-Back and 2-Back Working Memory tasks.

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accuracy.

The computerised tasks were validated against established pencil-and-paper neuropsychological tests that have demonstrated sensitivity to individual differences in performance and cognitive decline (Golski et al., 1998). They have also previously been used as validation tests for measures of cognitive functions that decline with age (Salthouse et al., 2003; Wilson et al., 2002). Results revealed strong correlations with the validation tests. The computerized tasks measuring recall and recognition correlated with the Logical Memory tests, measures of episodic memory that have been previously shown to decline with age (Haaland et al., 2003). Both the accuracy and reaction time measures of the computerized tasks correlated with the Trail making Test and the Digit Symbol Coding Test, measures of executive function, attention and processing speed (Wecker et al., 2000). These strong correlations reveal that the computerised tasks capture a combination of cognitive functions also captured in these widely used tests and are a valid measure of the cognitive functions that decline with age.

Spatial Working Memory appeared to show the most robust aging effects, with age-related impairment evident for both response time and accuracy measures. Age-related impairment has previously been demonstrated in tasks that measure other spatial abilities, for example mental rotation and navigation (Driscoll et al., 2005) and memory for object arrangements (Cherry and Park, 1993). The spatial recognition task in the Cambridge Automated Neuropsychological Test Battery (CANTAB) similarly demonstrated impaired performance with age (Rabbitt and Lowe, 2000). However, there is little cognitive research investigating spatial working memory. The present task suggests that with increasing age it becomes increasingly difficult to hold spatial information in working memory. Given the strong sensitivity of this task to cognitive aging it could be a useful tool in future aging research to measure the degree of cognitive decline and to examine the efficacy of interventions.

Robust aging effects were evident in speed of response for the Contextual Memory task, with significant effects also demonstrated for accuracy. This is consistent with previous research that demonstrates a decline in episodic memory with increasing age using a similar task (Anderson et al., 2006; Nilsson, 2003). Poorer performance on episodic memory tasks may also predict future cognitive impairment or dementia (Albert et al., 2007; Collie and Maruff, 2000), therefore this type of task is an important measure in cognitive aging research.

The Immediate and Delayed Recognition tasks similarly demonstrated age effects for accuracy, with stronger effects evident for response time on these tasks. Generally, previous research has demonstrated that older individuals perform better on recognition tasks than in free recall (Davis et al., 2003) and this was also demonstrated here, with greater accuracy on the recognition tasks than word recall in the group overall. Accuracy in the Delayed Word Recall task demonstrated a stronger age effect than Immediate Word Recall. This finding is consistent with research investigating free recall of word lists that suggests that the short-term memory store is less affected by aging than longer-term memory (Ward and Mayer, 2005).

The current study demonstrated that performance on the Stroop tasks were both correlated with age, an effect that has been demonstrated in several studies in the past (Cohn et al., 1984; Van der Elst et al., 2006; Wecker et al., 2000; West, 2004). However, there has been some discussion as to whether such decrements in inhibitory function are merely due to the effects of age on processing speed (Salthouse and Meinz, 1995). The present study indicated that processing speed accounted for a large proportion of variance in the Stroop Incongruent task. The Interference Effect variable was also significantly related to age, however the relationship was much smaller, as indicated by a small significant effect after accounting for education. In a previous study, researchers argued that the Stroop ‘interference effect’ becomes more pronounced with age and reflects a decline in executive function (Van der Elst et al., 2006). Moreover, the authors suggested that the degree of decline is influenced by the individual’s level of education. This notion is not inconsistent with the findings of the present study, with education having a borderline significant relationship with the Interference variable, and a significant relationship with the Stroop Incongruent task.

Examining the results of the computerised battery as a whole, most of the tasks showed age-related decline. The N-Back tasks showed no age-related decline and were not reliable measures and should therefore be excluded from future studies using this test battery. Overall, the tasks appear to be robust measures of the cognitive functions that decline with age, as apparent from the strong correlations with widely used validation tests and the strong age effects. This advocates the use of the test battery in studies investigating the efficacy of intervention designed to improve or preserve cognition in elderly people. The response time measures were more sensitive to age than accuracy measures, with the exception of Spatial Working Memory. This could be due to the instructions for this task that asked participants to prioritise accuracy over speed. However the present study demonstrates the need to use tests that are sensitive to response time as well as accuracy; if only accuracy measures were recorded then important information regarding age-related cognitive decline would have been overlooked. Speed of response is thus a critical measure for aging research; not just as a measure of general cognitive slowing but also as a measure that can be used to investigate decline in specific cognitive functions. The significant results for education as a control variable also highlight the need to consider education when examining the effects of aging on cognitive tasks. Future studies examining the test battery should gather normative data for different age groups and for patient populations such as dementia, schizophrenia and frontal lobe patients.

The present study has demonstrated the sensitivity of a series of computerised cognitive tasks to age-related cognitive decline. The tasks are easy-to-use and provide a comprehensive assessment of cognitive functions using both performance accuracy and response time measures. This study has also emphasized the importance of examining spatial working memory and contextual memory when assessing age-related decline. The sensitivity of the cognitive battery advocates its use in research that investigates cognitive interventions in older people; it may have capacity to detect the subtle changes that are
found in response to pharmaceutical or other interventions.

REFERENCES


Neuropsychologist 19, 45-54.


ABSTRACT: Grape varieties differ markedly in the range of phenolic compounds and antioxidant activity showing health promoting activities like cardio protective, hepatoprotective, neuroprotective, anticarcinogenic, anti-inflammatory actions. Considering the huge biodiversity of grape varieties and their immense functional attributes, Agharkar Research Institute has developed few hybrid varieties suitable for Indian conditions being drought resistant, disease resistant and having pleasant color and taste. This study was undertaken to investigate the antioxidant activity of 14 hybrid grape varieties (designated as V5-V18) with respect to four established American 'juice grape' varieties cultivated in India (V1- vitis labrusca, V2-vitis labrusca, V3- Vitis champini and V9- vitis labrusca). Trolox equivalent antioxidant capacity, ferrous iron chelation activity, vitamin C and polyphenol contents were found to be higher in fresh samples of V7 and V10, than those of V1, V2, V3 and V9. In particular, V10 contained the highest phenolics (1360 mg Gallic acid equivalent per 100g); TEAC (2373 mM Trolox equivalent /100 g of fresh weight); ferrous iron chelation activity (1407 mM EDTA equivalent /100 g) and vitamin C (29.2 mg/100g). This study demonstrates that grape hybrids V7 and V10 are potential sources of nutraceutical phenolics and can thus be utilized as functional foods.

KEYWORDS: FICA, hybrids, Juice grapes, polyphenols, TEAC and vitamin C

INTRODUCTION
Grapes are the source of large number of nutraceuticals including resveratrol and have been suggested to have cardiovascular benefits, cancer chemo preventive activity, skin cancer prevention and protective action against other less prevalent but devastating illnesses such as Alzheimer's disease and urinary bladder dysfunction (Pezzuto JM. 2008). Cultivated grapes have been studied all around the world but Indian juice grape germplasm and hybrids have not been much explored for their nutraceutical potential (Puspa et al. 2008). Secondly, there have been limited reports on the comparative characterization of antioxidants for American juice grapes cultivated in Indian climatic conditions and hybrids (Agte et al. 2003). Hence, the objectives of this study were 1) to evaluate the total phenolic content and antioxidant activities of the fresh fruit and juice and 2) to compare the four American 'juice grape' varieties with fourteen ARI hybrids for changes in their nutraceutical activities.

Materials and Methods
American grape types used for juice making (n=4) and ARI- hybrids (n=14) were harvested during March-2007 and March-2009 i.e. at two consecutive seasons at the ARI farm located at Hol near Pune city (Table 1). While harvesting, morphological characters like development of color, softness of the berry, total soluble solids (T.S.S.) and taste were considered. All these parameters for harvesting of grapes were as developed and standardized over the years. The samples, when ready for picking, were brought to ARI laboratory for the further processing. Initial observations i.e. berry characters such as berry weight, berry color, berry shape, length, diameter, seeds per berry, total soluble solids, juice color, acidity, pH were noted. The fruits were washed and juice was extracted by crushing the whole fruits including seeds in a blender and pressing the homogenate through muslin cloth. Both fresh fruits and Juice of the grapes were preserved at -20°C and used for determining antioxidant activity.

Table 1. Parent and hybrid grape varieties used in this study

<table>
<thead>
<tr>
<th>VARIETY</th>
<th>GRAPE VARIETIES AND HYBRIDS</th>
<th>NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>Vitis labrusca Buckl and sweet water</td>
<td>Bucklandsweet water</td>
</tr>
<tr>
<td>V2</td>
<td>Vitis labrusca Isabella</td>
<td>Isabela</td>
</tr>
<tr>
<td>V3</td>
<td>Vitis champini Champanel</td>
<td>Champanel</td>
</tr>
<tr>
<td>V4</td>
<td>rotundifolia X vinifera (JAM X KS8)</td>
<td>ARI - 177</td>
</tr>
<tr>
<td>V5</td>
<td>labrusca X vinifera (CHS X CAT)</td>
<td>ARI - 144</td>
</tr>
<tr>
<td>V6</td>
<td>rotundifolia X vinifera (KLS X JAM)</td>
<td>ARI - 1301</td>
</tr>
<tr>
<td>V7</td>
<td>labrusca X vinifera (CAT X BS)</td>
<td>ARI - 516</td>
</tr>
<tr>
<td>V8</td>
<td>labrusca X vinifera (ANB X CAT)</td>
<td>ARI - 245</td>
</tr>
<tr>
<td>V9</td>
<td>vinifera X vinifera (DJ X RR)</td>
<td>ARI - 27</td>
</tr>
<tr>
<td>V10</td>
<td>champini X vinifera (CMP X CAT)</td>
<td>ARI - 417</td>
</tr>
<tr>
<td>V11</td>
<td>labrusca X vinifera</td>
<td>ARI - 501</td>
</tr>
<tr>
<td>V12</td>
<td>labrusca X vinifera (BB X GUL)</td>
<td>ARI - 422</td>
</tr>
<tr>
<td>V13</td>
<td>champini X vinifera (CMP X GUL)</td>
<td>ARI - 181</td>
</tr>
<tr>
<td>V14</td>
<td>labrusca X vinifera (CON X CHS)</td>
<td>ARI - 250</td>
</tr>
<tr>
<td>V15</td>
<td>vinifera X vinifera (RR X CAL)</td>
<td>ARI - 311</td>
</tr>
<tr>
<td>V16</td>
<td>labrusca X vinifera (CAI X BHO)</td>
<td>ARI - 317</td>
</tr>
<tr>
<td>V17</td>
<td>rotundifolia X vinifera (JAM X GUL)</td>
<td>ARI - 40</td>
</tr>
</tbody>
</table>

Table 1. Parent and hybrid grape varieties used in this study
Determination of vitamin C was done using reduction of 2, 6 dichloro phenol indophenol (DCPIP) as previously reported (Agte et al. 1995). Vitamin C equivalents as DCPIP dye reducing potential of juice and fresh samples of 18 grape varieties was expressed as mg per 100g of sample weight.

Total phenolic content was determined using the FolinCiocalteu’s colorimetric assay (Agte et al. 1995). In brief, 0.5 mL aliquot of the prepared extract was diluted five times, of which 100 μL aliquot was taken for further analysis. The 100 μL aliquot was mixed with 1 mL phenol reagent, 1 mL 10% sodium bicarbonate, and 4 mL distilled water. The mixture was allowed to stand for 1 h in the dark. The total phenolic concentration was calculated from a calibration curve of gallic acid with absorbance at 760 nm. Results were expressed as mg gallic acid equivalents (GAE)/g fresh weight of the samples.

Ferrous ion chelating activity (FICA) was determined as described by Yamaguchi et al. (2000). Briefly, sample was extracted in 1% sodium dodecyl sulphate (SDS). To 0.5 ml of the extract, equal volumes of FeSO₄ solution (1mM in 1% SDS), Tris- HCl buffer (0.1 M, pH=7.4) and α, α’- bipyridyl (0.1% in 0.2 M HCl) followed by 0.4 ml of Hydroxyl amine hydrochloride (10% in 1% SDS) and mixed thoroughly and then diluted with 2.6 ml of distilled water. The available ferrous ions form the reddish pink colored complex with α, α’- bipyridyl. Thus, the decrease in the absorbance in presence of sample extract was measured at 522 nm and the ferrous ion chelating ability of sample was expressed as mM of EDTA equivalents per 100 g of sample. EDTA was used to generate the standard curve.

The Trolox equivalent antioxidant activity (TEAC) was estimated using the ABTS’ system (2, 2’ azinobis 3-ethylbenzothiazoline 6-sulphonic acid) as per the method of Zielindka and Szawara-Nowak (2007). ABTS’ was prepared by oxidizing 5 mM of ABTS, with manganese dioxide in PBS (pH 7.4) at ambient temperature for 2 hours in dark. The ABTS antioxidant reaction mixture contained 1.0 mL of ABTS’ with an absorbance of 0.85 at 734 nm and 50 μL of test sample extract in methanol or trolox (0.1 mg/mL in methanol) as a positive control. Calibration curve of Trolox (0.1mg/mL) was plotted (r²=0.997) and results were expressed as mg of Trolox equivalent ABTS’ radical scavenging activity per 2.5 gm of sample.

Statistical analysis

All measurements were made in triplicates. Significant differences between means were computed using analysis of variance (ANOVA) and computing the critical difference (C.D.) at probability of 0.05, 0.01 and 0.001. Computations were done using Excel 2007 for Windows XP.

RESULTS

The data presented in Table 2 show mean and standard deviation (S.D.) for TEAC, vitamin C, FICA and total polyphenols of the fresh fruits and juice from all of all the 18 different types of grapes. There was a large variability within the four ‘American juice’ varieties (V1, V2, V3 and V9) for all the antioxidant indices. ANOVA indicated significant differences between 4 ‘American juice’ varieties for all the 3 antioxidant indices (p<0.0001) and marginally significant differences for vitamin C contents (p=0.1). V9 and V3 were found to be superior to V2 and V1.

Figure 1. Effect of hybridization on contents of vitamin C. Differences between V7 and its parent varieties (V1 and V9) were highly significant as indicated by two way ANOVA (p<0.001)

Figure 2. Effect of hybridization on contents of polyphenols. Differences between V7 and its parent varieties (V1 and V9) were highly significant as indicated by two way ANOVA (p<0.001)

Figure 3. Effect of hybridization on TEAC. Differences between V7 and its parent varieties (V1 and V9) were highly significant as indicated by two way ANOVA (p<0.001)

Figure 4. Effect of hybridization on FICA. Differences between V7 and its parent varieties (V1 and V9) were highly significant as indicated by two way ANOVA (p<0.001)
The total polyphenols, TEAC and FICA of fresh fruit samples of the 14 hybrid varieties were significantly higher than that for whole fruits of four juice varieties (p<0.001) but differences were non significant for juice samples (Table 2). On the other hand, average vitamin C for juice of the 14 hybrid varieties was found to be significantly higher than that for four juice varieties (p<0.05) but similar in case of whole fruits. These data indicated superior performance of hybrids as compared to juice varieties. Secondly, there was a lower retention of polyphenols, TEAC and FICA activity but better retention of vitamin C in juice than whole fruits.

Table 3. Ranking of hybrid grape fruits and juices with respect to their antioxidant potential against individual American juice varieties. Only the varieties showing significant differences have been ranked.

Table 4. Correlations of Vitamin C, Total polyphenols, TEAC and FICA of grape varieties. * p<0.05, ** p<0.01. *** p<0.001
Vitamin C, total polyphenols, TEAC and FICA with their respective levels of significance. The correlations (r) for juice were significant for all the 4 parameters. In case of whole fruit samples, only TEAC and polyphenols were significantly correlated (r=0.81, p<0.001). From the significance of values of correlation coefficients, it can be inferred that the grape varieties having higher vitamin C and polyphenols content in juice showed higher radical scavenging activity.

All the parent varieties of the 14 hybrids were not available for analysis. But for V7, a promising hybrid, data of the parent varieties was available. Figures 1-4 show the performance of V7 as against the parents, V1 and V9. The results of two way ANOVA indicated significant increase in all the four study parameters of antioxidant potential between parents and hybrid indicating superior performance of the hybrid than the parents (p<0.001).

DISCUSSION

*Vitis vinifera*, the grapes used for wine, though native to southern Europe and Western Asia is widely cultivated in India. Grape seed and skin are the waste products generated during manufacture of juice for wine making but contain several active polyphenolic components. An overview of the pharmacological, toxicological, clinical studies of grape and its active components has been reported (Nassiri and Hosseinzadeh, 2009). In light of increasing prevalence of the non communicable diseases, development of local grape hybrids with improved nutraceutical potentials carries significance for formulating the health food products. Antioxidant activity of berry phenolics, in addition to other mechanisms, may contribute to human health, but Grape varieties differ markedly in the range of phenolic compounds and antioxidant activity. Flavan-3-ols, anthocyanins, and hydroxycinnamates are the main components of purple grape juice showing health promoting activities like cardio protective, hepatoprotective, neuroprotective, anticarcinogenic, anti-inflammatory (Mullen et al. 2007). The possible relationship remains yet to be scientifically substantiated (Heinonen 2007).

In the present study, we have assessed four aspects of antioxidant activity i.e. total polyphenols, vitamin C, TEAC and FICA. It has been noticed that the hybrids had better nutraceutical potential than the American juice varieties of *V. labrusca* cultivar. By evaluating the nutraceuticals for the possible benefits of hybrid and juice grapes, we were able to assess the value of these natural resources in Indian climatic conditions. The present results can help to promote their cultivation in and outside India.

Antioxidant activity of 4 Indian grape cultivars varying in their skin color, seed and polyphenol content (Bangalore blue, Pandhari sahebi, Sharad seedless and Thompson seedless) and their components have been reported wherein polyphenols from Sharad were more potent as antioxidant than Thompson, showing IC50 values of 1250 +/- 30 and 2650 +/- 125 mg/ml, respectively (Pakhale et al. 2007). In another study, the antioxidant potential of 11 grapes varieties from India and nearby Asian countries indicated variety ‘Mango’ to be the most potent followed by Sharad Seedless (Kedage et al. 2007). Antioxidant activities of grape seed extracts with two different free radical scavenging methods, ABTS [2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] and DPPH (2,2-diphenyl-picrylhydrazyl) assays, using Trolox equivalent as standards, have also been investigated (Oktay et al. 2009). TEAC of fresh grape fruits and juice extracts investigated in the present study expressed as mM of TEAC per 100 g of sample weight were much higher for hybrids (1007 +/- 477) and their juice varieties (586 +/- 116) but these could not be compared with the reported values due to different ways of reporting the values. Still, our study indicated that all the active principles in fresh fruits of hybrids are not completely extractable in the juice except vitamin C. These results give experimental support about the advice of consuming of whole fruit rather than juice for getting more benefit of the antioxidants.

FICA has the significance with respect to antiinflammatory, anticancer and cardio protective activities of functional foods including grapes. FICA of fresh grape fruits and juice extracts expressed as mM EDTA equivalent FICA per 100 g of sample weight was highest in V10 followed by V7. Our results for fresh grape juice samples gave similar high correlations not only with total polyphenols (r=0.60, P < 0.01) but also with levels of vitamin C (r=0.70, P < 0.001) and FICA (r=0.60, P < 0.01). Although similar data of associations for grapes was not available, ABTS radical scavenging activity of apple was strongly related with the total polyphenolic content determined both by the spectrophotometric and Folin-Ciocalteu methods. For fresh fruit (R² = 0.72; P < 0.01) and for juice (R² = 0.55; P < 0.05) (Lamperi et al. 2008).

The gallic acid equivalent total phenolic content of whole fruits of hybrids (506 +/- 292) was 1.5 times higher than juice grape varieties (361 +/- 100). Ethyl acetate extracts of seeds originating from nine Hellenic native and international *Vitis vinifera* varieties cultivated in Greece were screened for their contents of characteristic polyphenols. Total content varied from 55.1 to 964 mg per 100 g of seeds, the average being 380 mg per 100 g (Ramila et al. 2005). Our values are based on whole fruit analysis but show the similar range. However, these values were lower than our earlier reported values on hybrids of Thomson seedless and Sonaka (Agte et al. 2003). Vitamin C content as mg per 100g of sample weight on the other hand, was higher in hybrids reported in the present study than earlier reported hybrids of Thomson seedless and Sonaka (Agte et al. 2003). For the hybrids V7 and V10, the values of vitamin C were found to be very high. Since these hybrids have higher levels of other 3 antioxidant indices, these need further attention.

Drying of all the grape samples resulted in considerable loss of antioxidant activity in terms of 4 indices suggesting consumption of fresh grapes during their season. Secondly juice also had lower antioxidant activity than the whole fruits indicating preference for eating the whole fruit rather than the juice. Though the loss on drying was found highest in V7 and V10, it was interesting to note that even after drying, grapes have retained 31 % of the vitamin C contents and 44% of TEAC.

Multiple comparison test based on ANOVA and CD for all four antioxidant parameters have shown that among all 14 hybrids variety V7 and V10 were superior to the other hybrids as well as...
A great amount of in vitro evidence exists, showing that berry phenolics are powerful antioxidants. However, the antioxidant effect of berry phenolics is strongly dependent on the choice of the type. Present results indicate promise in 2 hybrid varieties as sources of antioxidants. The effect of these hybrids on other functional attributes remains to be seen. Also the in vivo antioxidant effects of their consumption need to be assessed.

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CONFLICT-OF-INTEREST STATEMENT

All authors wish to report that there are no potential conflicts of interest, including specific financial interests relevant to the subject of their manuscript.

REFERENCES


Oktay, Y., Emre, B. and Nevzat A. (2009) Antioxidative activities of grape (Vitis vinifera) seed extracts obtained from different varieties grown in Turkey International Journal of Food Science and Technology 43, 154 – 159


Yamaguchi, F., Ariga, T., Yoshimura, Y. and Nakazawa, H.

FEED EFFICIENCY OF RATS MAINTAINED ON A DIET WITH VARIED CASEIN TO GLUTEN RATIOS

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ABSTRACT: The contribution of cereal protein (gluten, limited in lysine) to total protein intake varies between countries and individuals and the effect of such a variation on weight gain is not clear. Thus, the objective of this study was to determine the effect of altering protein quality, achieved by varying the casein to gluten ratio of the diet, on the feed efficiency of rats. For this purpose, male Sprague-Dawley rats were randomly divided into five groups based on the casein and gluten content of the diet that constituted the sole protein sources and covered 20% of the total energy needs. Food intake and body weight were monitored every two days for a period of six weeks. The results showed that the high gluten groups had the lowest body weight, weight gain, food intake and feed efficiency. During the experimental period, both food intake per 100g body weight and feed efficiency of the pure gluten group were constant, whereas that of the other groups decreased with time. It can be concluded that under status of sufficient percentage of energy being provided from protein using two sources that more than 25% of protein from gluten would be needed for adequate growth. In addition, results indicate that the adoption of a cereal-based diet, particularly during the growth period, may require higher levels of energy intake (due to reduced feed efficiency) or even lysine supplementation.

KEYWORDS: Casein, feed efficiency, lysine, rats, wheat gluten

INTRODUCTION

The quantity and the quality of proteins consumed around the globe are known to vary considerably from one place to another. Western and developed societies rely mainly on animal sources for proteins (Singh et al., 2003), while developing societies generally depend upon plant sources for proteins (Hussain et al., 2004) resulting in a considerable variation of the dietary essential amino acid supply. However, the proposition of either increasing the contribution of plant proteins at the expense of animal proteins in Western diets or being directed towards adopting vegetarianism as an approach to reduce the risks of developing several chronic diseases is a growing issue (Hu, 2003; Lea et al., 2006). The contribution of plant proteins to the total protein intake is estimated to represent 100% in vegans, 66.4% in vegetarians and 41% in omnivores with cereal grains being the major plant protein source (Millward, 1999). Despite their quantitative importance, the quality of cereal proteins in the diet has not been given much consideration. Cereals, consumed principally in the form of wheat, are primarily composed of carbohydrates and only moderate amounts of proteins (8-12% on a weight basis) (Bos et al., 2005). Not only is the protein content of wheat low, but it is also categorized as being of low quality due to the fact that its protein portion, gluten, is limiting in the essential amino acid lysine (Hoffman and McNeil, 1949; Rice et al., 1960). Lysine is the first limiting amino acid in other frequently consumed cereal foods including rice, corn, oats, and potatoes (Bos et al., 2005). Moreover, it has been stated that published protein requirements are based on the amount of dietary protein that would also supply adequate amounts of lysine (Ball, 2007). On the other hand, proteins of animal sources contain relatively high amounts of lysine and, thus, are able to complement the lower lysine content of wheat proteins when adequately integrated into the diet.

It has been well documented that rats and young children maintained on a diet in which gluten is the only source of protein would suffer from growth impairment (Graham et al., 1981; Munaver and Harper, 1959). Moreover, these diets tend to be low in their protein content (less than 10% of the total energy). However, it is still not clear whether alterations in the animal to plant protein ratio would have an impact on growth and affect feed efficiency [weight gain (g) per 100g food intake] while maintaining an adequate percentage of energy from protein. Thus, the present study was designed to investigate the consequence of altering the casein to gluten ratio of an isocaloric diet, supplying an adequate percentage of energy from protein, on the feed efficiency of male Sprague-Dawley rats.
MATERIAL AND METHODS

Animal Housing

Six-week-old male Sprague-Dawley rats (American University of Beirut, Lebanon) were individually housed in wire-bottomed cages enabling the collection of food spillage and waste. Room temperature was maintained at 22°C ± 1°C with a 12-hour light:12-hour dark cycle (lights on 8:00 am). Rats were allowed a seven day adaptation period where they had free access to water and were fed ad libitum a semi-synthetic control diet (Table 1) (Obeid et al., 2005) to familiarize with both the environment and the powdered food before being shifted to their corresponding diets. All procedures were approved by the institutional animal care and use committee (IACUC) of the American University of Beirut.

Experimental Protocol

Subsequent to the adaptation period, rats were randomly divided into five groups and placed on their respective diets for a period of six weeks. The groups were named according to the protein source combination they received. The percentage of energy from protein was fixed at 20% but the source was manipulated by altering the ratio of casein to gluten which constituted the sole protein sources. The percentage contribution of gluten and casein to the energy content of the different diets were as follows: C20:G0, C15:G5, C10:G10, C5:G15, C0:G20. The two protein sources were incorporated in the diets by replacing with an appropriate amount of wheat starch due to the difference in the percentage of protein in each protein source (Table I). The energy content and macronutrient distribution (supplying 20% of energy as protein, 23% as fat and 57% as carbohydrate) was similar among the different experimental diets.

Food intake and body weight were measured every two days at the same time of the day. Efficiency of feed utilization was expressed as weight gain (g) per 100g of food consumed (g). Relative food intake was expressed as food intake (g) per 100g body weight for the same day. At the end of the six-week experimental period, rats were fasted overnight and the following morning (between 8:00 a.m. and 10:00 a.m.) were tube fed a 3 ml liquid meal containing 1.00g of their respective diets and sacrificed one hour later by decapitation. Blood samples were collected from the neck vessels in EDTA tubes; plasma was separated and stored at -80 ºC until analysis. Liver and epididymal fat pad were excised, immediately frozen in liquid nitrogen and stored at -80 ºC until weighing.

Plasma Analysis

Postprandial plasma glucose and triglycerides were determined using an enzymatic colorimetric method on the Vitros DT 60II Chemistry System (Ortho-Clinical Diagnostics, Johnson & Johnson, New York). Postprandial plasma insulin was measured using the Rat/Mouse Insulin Elisa Kit 96-Well Plate for the quantification of non-radioactive insulin provided by LINCO Research, Inc. USA.

TABLE 1. Diet composition of the experimental diets. a Contains 87% protein; b Contains 76% protein; c 57% CHO- 20% Protein- 23% Fat; * AIN-93G mineral mix and AIN-76A vitamin mix; C20:G0: consists of 20% Casein and 0% Gluten as the protein source; C15:G5: consists of 15% Casein and 5% Gluten as the protein source; C10:G10: consists of 10% Casein and 10% Gluten as the protein source; C5:G15: consists of 5% Casein and 15% Gluten as the protein source; C0:G20: consists of 0% Casein and 20% Gluten as the protein source.

<table>
<thead>
<tr>
<th>INGREDIENTS (G/KG)</th>
<th>CONTROL</th>
<th>C20:G0</th>
<th>C15:G5</th>
<th>C10:G10</th>
<th>C5:G15</th>
<th>C0:G20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein a</td>
<td>198</td>
<td>230</td>
<td>172.4</td>
<td>114.9</td>
<td>57.5</td>
<td>—</td>
</tr>
<tr>
<td>Wheat gluten b</td>
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<td>568</td>
<td>559.8</td>
<td>551.5</td>
<td>544</td>
<td>538</td>
</tr>
<tr>
<td>Wheat Starch</td>
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<td>2</td>
<td>2</td>
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<tr>
<td>DL-Methionine</td>
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<td>35</td>
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<td>Mineral mix*</td>
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</tr>
<tr>
<td>Alphacel</td>
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<td>55</td>
<td>55</td>
<td>55</td>
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<td>55</td>
</tr>
<tr>
<td>Maize oil</td>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Lysine content</td>
<td>12.7</td>
<td>14.8</td>
<td>12.6</td>
<td>10.3</td>
<td>8.1</td>
<td>5.8</td>
</tr>
<tr>
<td>Gross energy (Kcal/g) c</td>
<td>3.93</td>
<td>4.00</td>
<td>4.01</td>
<td>4.01</td>
<td>4.02</td>
<td>4.03</td>
</tr>
</tbody>
</table>

TABLE 2. Mean body weights (g) of rats during the six-week experimental period. a,C (%): G (%): numbers in between brackets reflect the percentage (wt/wt) contribution of each protein source in the diet; C20:G0, 20% casein + 0% gluten; C15:G5, 15% casein + 5% gluten; C10:G10, 10% casein + 10% gluten; C5:G15, 5% casein + 15% gluten; C0:G20, 0% casein + 20% gluten; Values are mean ± SEM; Values in the same row with different superscripts are significantly different based on Fisher’s pairwise comparison (p<0.05).

<table>
<thead>
<tr>
<th>GROUP (C(%):G(%)) a</th>
<th>C20:G0</th>
<th>C15:G5</th>
<th>C10:G10</th>
<th>C5:G15</th>
<th>C0:G20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>n=9</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=9</td>
</tr>
<tr>
<td>Initial</td>
<td>202.10 ± 10.10</td>
<td>198.18 ± 9.21</td>
<td>198.76 ± 8.82</td>
<td>197.83 ± 8.43</td>
<td>202.48 ± 8.96</td>
</tr>
<tr>
<td>Day 10</td>
<td>267.50 ± 13.90</td>
<td>268.60 ± 10.80</td>
<td>265.10 ± 1.30</td>
<td>256.20 ± 11.10</td>
<td>226.38 ± 9.65</td>
</tr>
<tr>
<td>Day 20</td>
<td>345.90 ± 14.40</td>
<td>346.00 ± 10.90</td>
<td>336.70 ± 12.50</td>
<td>319.60 ± 11.20</td>
<td>248.50 ± 10.60</td>
</tr>
<tr>
<td>Day 30</td>
<td>399.26 ± 16.20</td>
<td>398.69 ± 13.00</td>
<td>380.48 ± 13.60</td>
<td>370.36 ± 11.40</td>
<td>275.54 ± 11.80</td>
</tr>
<tr>
<td>Final body weight</td>
<td>446.80 ± 19.00</td>
<td>444.30 ± 16.80</td>
<td>426.30 ± 18.10</td>
<td>411.10 ± 13.20</td>
<td>314.20 ± 13.80</td>
</tr>
</tbody>
</table>

ANOVA P-VALUE
Data related to food intake and weight gain were pooled in 10 day categories to minimize the effect of daily variation. Data are expressed as means ± SE of all values. Data analysis was performed using the MINITAB 13.1 software program. Results were analyzed by one way analysis of variance (ANOVA), and specific comparisons were made between each of the five groups using Fisher’s pairwise comparisons. A probability of less than 0.05 was considered to be significant.

RESULTS

Body Weight

Initial body weight was similar among the different groups (Table 2). However, discrepancies in body weight were observed starting from day 20 and onwards, where that of the C0:G20 group (pure gluten group) was significantly lower than that of the other groups with a 30% reduction as compared to the C20:G0 group (pure casein group). No significant difference in body weight was found among the other groups throughout the experiment though. When compared with the C20:G0 group, final body weight of the C5:G15 group was slightly but not significantly reduced (8%), whereas that of the C0:G20 group was significantly lower (30%) (Table 7). Thus, only absolute and not partial replacement of casein with gluten in the diet resulted in a significant decrease in body weight.

Weight Gain

Weight gain of the different groups was found to decrease with time with the exception of the C0:G20 group in which weight gain was constant across the experimental period; this lead to a similarity in weight gain between groups in the last 10 experimental days (Table 3). Weight gain of rats in which gluten contributed to more than 10% of the energy (or 50% of energy from protein; C10:G10 group) was altered, especially in the first 30 days of experimental period. A small, but significant, reduction was observed in the C5:G15 group, while that of the pure gluten group was severely reduced. The reduction was more pronounced in the early stage of the experimental period, where the growth rate is highest. Overall weight gain of the C5:G15 group was reduced by about 13%, while that of the C0:G20 group was reduced by about 55% as compared to the C20:G0 group (Table 7).

Food Intake

A small increase in food intake was observed with time. Rats were eating a comparable amount of food on the first ten days (Table 4). However, discrepancies were observed on the second and third ten days; both the C5:G15 group and the C0:G20 group had significant lower intakes (by 13.8%) as compared to both the C20:G0 and the C15:G5 group. No statistical significance in food intake was observed in the last 10 days. Overall food intake (Table 7) showed that both a 75% (C5:G15) and total replacement of casein

<table>
<thead>
<tr>
<th>GROUP (C(%):G(%))*</th>
<th>C20:G0</th>
<th>C15:G5</th>
<th>C10:G10</th>
<th>C5:G15</th>
<th>C0:G20</th>
<th>ANOVA P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=9</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td>First 10 days</td>
<td>20.73±1.02\text{y}</td>
<td>19.51±0.85\text{y}</td>
<td>19.44±1.04</td>
<td>18.79±0.83</td>
<td>18.04±0.92\text{y}</td>
<td>0.335</td>
</tr>
<tr>
<td>Second 10 days</td>
<td>23.32±0.73\text{a}</td>
<td>22.97±0.56\text{a}</td>
<td>21.95±0.83\text{a}</td>
<td>20.66±0.64\text{a}</td>
<td>19.26±0.73\text{a}</td>
<td>0.001</td>
</tr>
<tr>
<td>Third 10 days</td>
<td>24.31±0.79\text{z}</td>
<td>24.85±0.95\text{z}</td>
<td>22.50±0.66\text{z}</td>
<td>21.27±0.53\text{z}</td>
<td>20.21±0.70\text{z}</td>
<td>0.000</td>
</tr>
<tr>
<td>Last 10 days</td>
<td>24.01±0.93\text{z}</td>
<td>23.73±1.02\text{z}</td>
<td>22.25±1.16</td>
<td>20.81±0.75</td>
<td>21.23±0.76\text{z}</td>
<td>0.066</td>
</tr>
<tr>
<td>ANOVA P-value</td>
<td>0.027</td>
<td>0.001</td>
<td>0.105</td>
<td>0.083</td>
<td>0.042</td>
<td></td>
</tr>
</tbody>
</table>

* C(%):G(%): numbers in between brackets reflect the percentage (wt/wt) contribution of each protein source in the diet; C20:G0, 20% casein + 0% gluten; C15:G5, 15% casein + 5% gluten; C10:G10, 10% casein + 10% gluten; C5:G15, 5% casein + 15% gluten; C0:G20, 0% casein + 20% gluten; Values are mean ± SEM; \text{a, b, c} Values in the same row with different superscripts are significantly different based on Fisher’s pairwise comparison (p<0.05); \text{y, z} Values in the same column with different superscripts are significantly different based on Fisher’s pairwise comparison (p<0.05)
(C0:G20) with gluten resulted in significant reductions in food intake by 11.7% and 14.8% respectively as compared to the C20:G0 group.

**Relative Food Intake**

Relative food intake or food intake (g) per 100g body weight was calculated in order to account for the difference in body weight between the groups (Table 5). Relative food intake of the different groups seemed to decrease with time, except for the pure gluten group, which maintained a constant relative food intake. All groups, except that of the pure gluten group, were found to have a similar rate of relative food intake reduction. The sustenance of relative food intake of the pure gluten group resulted in a significant increase in its value as compared with the other groups starting from day 20 till the end of experimental period. In addition, overall relative food intake of the pure gluten groups was higher than that of the other groups (Table 7).

### TABLE 5. Mean relative food intake (g/100g body weight) of rats during the six-week experimental period.

<table>
<thead>
<tr>
<th>GROUP (C(%):G(%))*</th>
<th>C20:G0</th>
<th>C15:G5</th>
<th>C10:G10</th>
<th>C5:G15</th>
<th>C0:G20</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=9</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td>First 10 days</td>
<td>24.30±0.22 w</td>
<td>22.87±0.75 w</td>
<td>22.05±0.47 w</td>
<td>19.36±0.17 w</td>
<td>7.21±1.10 w</td>
</tr>
<tr>
<td>Second 10 days</td>
<td>18.45±1.18 x</td>
<td>15.25±0.58 x</td>
<td>13.82±0.47 x</td>
<td>13.97±0.47 x</td>
<td>5.21±0.83 x</td>
</tr>
<tr>
<td>Third 10 days</td>
<td>11.01±0.46 y</td>
<td>10.78±0.53 y</td>
<td>10.27±0.48 y</td>
<td>11.22±1.34 y</td>
<td>6.14±0.70 y</td>
</tr>
<tr>
<td>Last 10 days</td>
<td>4.63±1.94 a</td>
<td>6.45±0.33 a</td>
<td>6.83±0.69 a</td>
<td>7.08±0.87 a</td>
<td>6.69±0.47 a</td>
</tr>
<tr>
<td>ANOVA P-value</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.234</td>
</tr>
</tbody>
</table>

### TABLE 6. Mean feed efficiency (weight gain (g)/100g food intake) of rats during the six-week experimental period.

<table>
<thead>
<tr>
<th>GROUP (C(%):G(%))*</th>
<th>C20:G0</th>
<th>C15:G5</th>
<th>C10:G10</th>
<th>C5:G15</th>
<th>C0:G20</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=9</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td>First 10 days</td>
<td>6.12±0.29 a</td>
<td>6.18±0.30 a</td>
<td>5.96±0.25 a</td>
<td>5.31±0.20 a</td>
<td>2.79±0.19 a</td>
</tr>
<tr>
<td>Second 10 days</td>
<td>23.09±0.81 a</td>
<td>22.76±0.82 a</td>
<td>21.53±0.89 a</td>
<td>20.38±0.63 a</td>
<td>19.68±0.71 a</td>
</tr>
<tr>
<td>Third 10 days</td>
<td>7.16±0.09 a</td>
<td>7.13±0.12 a</td>
<td>6.92±0.12 a</td>
<td>6.77±0.14 a</td>
<td>7.68±0.19 b</td>
</tr>
<tr>
<td>Last 10 days</td>
<td>4.63±1.94 a</td>
<td>6.45±0.33 a</td>
<td>6.83±0.69 a</td>
<td>7.08±0.87 a</td>
<td>6.69±0.47 a</td>
</tr>
</tbody>
</table>

### TABLE 7. Final body weight (g) and average weight gain (g/day), food intake (g/day), relative food intake (g/100g body weight) of rats during the six-week experimental period.

<table>
<thead>
<tr>
<th>GROUP (C(%):G(%))*</th>
<th>C20:G0</th>
<th>C15:G5</th>
<th>C10:G10</th>
<th>C5:G15</th>
<th>C0:G20</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=9</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td>Weight gain (g/day)</td>
<td>7.16±0.09 a</td>
<td>7.13±0.12 a</td>
<td>6.92±0.12 a</td>
<td>6.77±0.14 a</td>
<td>7.68±0.19 b</td>
</tr>
</tbody>
</table>

**Feed Efficiency**

Feed efficiency (Table 6) of the different groups decreased sharply with time except for that of the pure gluten group. A low feed efficiency was present in the pure gluten group from the time of gluten introduction and this was sustained over the experimental period. Such sustenance resulted in a difference in feed efficiency between the pure gluten group and other groups at the different time periods, except for the last 10 experimental days. At the same time, feed efficiency of the C5:G15 group was found to be slightly lower than that of the pure casein group during the first 20 experimental days. Total replacement of casein with gluten significantly induced about a 70% reduction in the feed efficiency in the first 10 experimental days and the gap was reduced with time, in which
feed efficiency became similar.

Overall, total replacement of casein with gluten reduced feed efficiency by more than 50%, while 75% replacement induced about 10% reduction, but no reduction was observed with replacement of 50% or less (Table 7).

**TABLE 8. Mean organ weights (g) of rats fed on their respective diets after the six-week experimental period.**

*C(%):G(%): the numbers in between brackets reflect the percentage (wt/wt) contribution of each protein source in the diet; C20:G0, 20% casein + 0% gluten; C15:G5, 15% casein + 5% gluten; C10:G10, 10% casein + 10% gluten; C5:G15, 5% casein + 15% gluten; C0:G20, 0% casein + 20% gluten; Values are mean ± SEM; * Values in the same row with different superscripts are significantly different based on Fisher’s pairwise comparison; (p<0.05); BW: Body Weight; EFP: Epididymal Fat Pad.

<table>
<thead>
<tr>
<th>GROUP (C(%):G(%))</th>
<th>C20:G0</th>
<th>C15:G5</th>
<th>C10:G10</th>
<th>C5:G15</th>
<th>C0:G20</th>
<th>ANOVA P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>n=9</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>13.20±0.70*</td>
<td>12.70±0.67*</td>
<td>11.81±0.59*</td>
<td>11.40±0.39*</td>
<td>7.76±0.44*</td>
<td>0.000</td>
</tr>
<tr>
<td>Weight (g)/100g BW</td>
<td>2.89±0.06*</td>
<td>2.86±0.07*</td>
<td>2.76±0.04*</td>
<td>2.80±0.06*</td>
<td>2.46±0.05*</td>
<td>0.000</td>
</tr>
<tr>
<td>EFP</td>
<td>n=9</td>
<td>n=8</td>
<td>n=8</td>
<td>n=8</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>7.67±1.21*</td>
<td>7.66±1.18*</td>
<td>6.58±0.90*</td>
<td>6.26±0.92*</td>
<td>3.84±0.46*</td>
<td>0.032</td>
</tr>
<tr>
<td>Weight (g)/100g BW</td>
<td>1.64±0.20</td>
<td>1.68±0.19</td>
<td>1.51±0.14</td>
<td>1.50±0.17</td>
<td>1.20±0.09</td>
<td>0.201</td>
</tr>
</tbody>
</table>

Liver and Epididymal Fat Pad (EFP) Weights (Table 8)

Liver weight (grams) seems to decrease with increased gluten contribution to the diet, in which that of the C5:G15 group was slightly but significantly lower (by 13.6%) than that of the C20:G0 group, while that of the pure gluten group was highly reduced (by 32%) as compared to the pure casein group. Liver weight expressed as percentage of total body weight was mainly reduced in the pure gluten group as compared to the other groups. Epididymal fat pad weight of the pure gluten group was significantly lower than that of the other groups. But, when expressed as per 100g body weight no significant difference was observed between the groups, although that of the pure gluten was slightly lower.

**Plasma Insulin, Glucose and Triglycerides (Table 9)**

Postprandial plasma insulin levels (1 hour after tube feeding 1.00 gram of the appropriate diet) were similar between the different groups. However, postprandial plasma glucose of the C10:G10 group was lower than that of the other groups and this was statistically significant with both the C20:G0 and C15:G5 groups only. Postprandial plasma triglyceride levels were not significantly different between the groups, although that of the pure gluten group was observed to be slightly lower.

**TABLE 9. Mean postprandial plasma insulin, glucose and triglycerides values one hour after tube feeding 1.00g of the respective experimental diets at the end of the six-week experimental period.**

*C(%):G(%): the numbers in between brackets reflect the percentage (wt/wt) contribution of each protein source in the diet; C20:G0, 20% casein + 0% gluten; C15:G5, 15% casein + 5% gluten; C10:G10, 10% casein + 10% gluten; C5:G15, 5% casein + 15% gluten; C0:G20, 0% casein + 20% gluten; Values are mean ± SEM; * Values in the same row with different superscripts are significantly different based on Fisher’s pairwise comparison; (p<0.05).

<table>
<thead>
<tr>
<th>GROUP (C(%):G(%))</th>
<th>C20:G0</th>
<th>C15:G5</th>
<th>C10:G10</th>
<th>C5:G15</th>
<th>C0:G20</th>
<th>ANOVA P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (ng/dl)</td>
<td>1.81±0.33</td>
<td>1.89±0.40</td>
<td>1.18±0.16</td>
<td>1.57±0.34</td>
<td>1.50±0.47</td>
<td>0.590</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>176.63±5.06*</td>
<td>180.71±6.14*</td>
<td>151.86±4.55*</td>
<td>168.00±7.42*</td>
<td>164.78±5.47*</td>
<td>0.015</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>49.71±5.89</td>
<td>51.86±4.39</td>
<td>50.71±2.02</td>
<td>59.57±6.30</td>
<td>39.78±3.37</td>
<td>0.347</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Food intake and thus protein intake is known to vary between countries and among individuals. Food quality is largely determined by food availability in developing countries unlike that of developed countries where it is mainly related to the eating habits of individuals. In developed countries, the association between animal based diets and the prevalence of chronic diseases (obesity, diabetes, CVD, hypertension etc) encouraged a lot of individuals to adopt a plant based diet. Plant proteins are known to be of low quality and their effect on growth and maintenance is not fully clear. The present study was designed to examine the consequence of altering the protein quality of both an isocaloric and isonitrogenous diet (20% of energy from protein) on the feed efficiency of male rats. The ratio of casein to gluten was altered and this resulted in a major alteration in the lysine content, in which that of the high gluten groups was below the recommended level.

A small but significant reduction in food intake was observed in the high gluten groups (C5:G15 and C0:G20), especially the pure gluten group. This is likely to have been attributed to the low protein quality and is in line with other studies where rats were reported to consume lower amounts of food when maintained on a diet of low protein quality (Aoyama and Wada, 1999). A decrease in plasma lysine ensuing from a low lysine diet results in lower concentrations of the limiting amino acid in the brain; this fact is believed to be associated with a decrease in total food intake (Harper et al., 1964). The anterior piriform cortex part of the brain contains an amino acid chemosensory system and is thought to mediate the anorectic response associated with low plasma lysine levels (Gietzen et al., 1998).

Moreover, competition between amino acids for uptake at the blood brain barrier occurs between the limiting amino acid and elevated plasma levels of other...
essential amino acids that are found in greater proportions in the diet (Harper et al., 1970; Rogers and Leung, 1973). Hrupka and colleagues (1999) found that when rats are offered a choice of two diets that vary in their lysine content, they tend to choose the diet containing adequate lysine levels in a way as to achieve an intake of lysine that is consistent with normal growth and that will restore food intake. On the other hand, the sustenance of relative food intake (g/100g body weight) in the pure gluten group, unlike other groups, resembles that of a low protein diet (Mercer et al., 1981). Relative food intake was reported to increase with decreased protein level of rats fed isocaloric diets containing graded levels of protein (Mercer et al., 1981). This may have been related to the presence of a compensatory mechanism to combat the decrease in body weight.

The observed reduction in weight gain of the high gluten groups (C5:G15 and C0:G20) was mainly pronounced in the early stages (younger age) and this fact is in line with other studies (Mokady and Einav, 1978). The similarity in the percentage of energy provided from protein between the diets indicates that such a reduction was mainly the result of protein quality. This is supported by the fact that when rats were fed wheat gluten fortified with as little as 0.2% lysine, weight gain was significantly improved as compared to those fed wheat alone (Bahl and Venkitasubramanian, 1977).

Similar to weight gain, feed efficiency was reduced in the high gluten groups, and this was highly pronounced in the pure gluten group. At the same time, feed efficiency of the pure gluten group was constant all throughout the experiment in which the difference appeared in the early stages of growth. This may relate to the fact that the requirement of lysine in relation to most other essential amino acids is much greater for the growing rat than for maintenance of the adult rat (Said and Hegsted, 1970). In a study where growing rats were fed a diet with the protein source being exclusively provided from casein, soybean, lactalbumin or wheat gluten each at a level of 10% of the total energy, wheat gluten expressed the lowest weight gain and feed efficiency while casein was the highest; this was exhibited by a 87% and 84% decrease respectively as compared to casein (Herzberg and Rogerson, 1984). In the pure gluten group, the observed comparable reduction between weight gain and feed efficiency (54% and 56.8% respectively), in the midst of a minor reduction in absolute food intake, indicates that weight gain reduction was mainly attributed to the decrease in feed efficiency.

Feeding rats diets that are deficient in any single essential amino acid usually results in body weight loss and a reduction in total food intake but an increase in relative food intake (Tulp et al., 1979). Diets deficient in amino acids tend to result in lower circulating levels of the anabolic hormone, insulin-like growth factor-I (IGF-I), independent of insulin levels (Maiter, 1989) and a lysine deficient diet was reported to cause a significant decline in plasma levels of IGF-I, as compared to a control diet (Takenaka et al., 2000). In addition, low quality proteins are thought to induce a greater thermogenic effect than high quality proteins, in which surplus amino acids are disposed off by increased oxidation when the dietary protein is not balanced (Millward and Rivers, 1988), while a more balanced diet seems to lower metabolic rate (Swick and Gribskov, 1983). Increased thermogenesis induced by low quality proteins is thought to be a means by which rats would be able to consume more food in an attempt to improve their protein intake while avoiding the risk of becoming obese (Stock MJ, 1999). Interestingly, lysine-deficient diets were reported to produce the least decrease in body weight among the essential amino acids (Sidransky and Baba, 1960). This is due to the presence of a conservation capacity; in fact the rate of lysine catabolism of rats maintained on lysine-deficient diet was reported to be lower than the rate of threonine catabolism of rats fed a threonine deficient diet (Yamashita and Ashida, 1969). However, such a conservation capacity is not likely to prevent the inhibition of protein synthesis (Canfield and Chyti, 1978). Thus, it is possible that the capacity of lysine retention is greater than that of other essential amino acids and this may have ameliorated the rate of weight loss.

Protein or amino acids are known to affect body weight and food intake control but the mechanism(s) of action is/are not clear and may relate to several factors including an increase in energy expenditure and satiety (Halton and Hu, 2004; Poitier et al., 2009). Proteins are the most thermogenic or energy inefficient macronutrients (Halton and Hu, 2004; Westerterp-Plantenga, 2003). Their high diet-induced thermogenesis (DIT) capacity may in turn become translated into satiety feelings (Halton and Hu, 2004). Moreover, several peripheral and central satiety hormones were reported to be altered by protein ingestion and specific amino acids are believed to be involved in this process (Poitier et al., 2009). Under a high or low quality protein diet there will be a surplus of amino acids which are disposed off through oxidation. This would in turn contribute to thermogenesis and satiety. Therefore, under situations of sufficient protein contribution to energy, the improvement in protein quality will decrease the quantity of surplus amino acids and this in turn will impact thermogenesis and satiety. Under a protein contribution to energy of 20%, the availability of more than 5% in the form of animal protein (casein) was found to be sufficient to maintain growth and development. The lowering of such a percentage seems to negatively impact growth especially and this was utmost as the age decreased.

On the other hand, the slight increase in insulin and glucose concentrations of the high casein (C20:G0 and C15:G5 groups) and high gluten groups (C5:G15 and C0:G20 groups) as compared to the C10:G10 group may have been attributed to an improved insulin sensitivity by the latter group. It is well known that proteins and amino acids have insulinemic properties and this seems to vary depending on the type of protein or amino acid (Nilsson et al., 2004). Lysine, when ingested with glucose, was reported to decrease glucose without affecting insulin (Kalogeropoulo et al., 2009). However, this effect does not seem to be linear especially that glucose concentration was observed to be the lowest in the C10:G10 group, such postulation is in line with others where the inclusion of different lysine levels to diet with varied casein content failed to affect fasting plasma glucose (Hevia et al., 1980). Thus, the protein combination could have induced such an effect rather than the lysine content per se. This fact recommends further investigation in order to optimize both insulinemic and glycemic responses. Moreover, lysine deficient
diets were reported to alter lipid status by increasing lipid accumulation in plasma and tissues and these effects were modified by the inclusion of lysine in the diet. These alterations were proposed to be related to the capacity to synthesize carnitine (essential for fatty acid oxidation), which requires lysine as a precursor (Bahl and Venkitasubramanian, 1977; Nilsson et al., 2004; Hevia et al., 1980; Khan and Bamji, 1979). In addition, serum triglyceride was reported to be altered by the percentage of casein in the diet, in which the highest levels (about 100 mg/dl) were obtained at 15% and this was reduced by half (about 50 mg/dl) at 30%. Inclusion of lysine in the diet caused a slight but not significant increase in fasting serum triglyceride levels, especially at the higher lysine groups (Nilsson et al., 2004). Our data showed that the adoption of a 20% protein diet resulted in a triglyceride concentration similar to that of the 30% casein diet and this was not affected by the type of protein (gluten or casein). Therefore, our data and that of others (Nilsson et al., 2004) indicated that the manipulation of the gluten to casein ratio (lysine levels) does not seem to have an impact on plasma triglyceride status.

Thus, it can be inferred that the adoption of a plant-based diet, especially one that is dependent entirely on cereals, is likely to affect growth and development in infants. The introduction of a minimum of 5% of energy from protein is likely to present such an effect, especially under sufficient protein intake. The reduction in growth was mainly related to a decrease in feed efficiency and this may imply that under low quality protein, energy requirement should be increased.

ACKNOWLEDGEMENT

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REFERENCES


ABSTRACT: St. John’s wort (Hypericum perforatum) is one of the popular phytomedicines. Beneficial effects of this herb in the treatment of mild to moderate depression are well known. Also, we recently described protective effects of H. perforatum against memory impairments caused by chronic stress. It is also a mild nootropic per se. In this study we tested a hypothesis that St. John’s wort alleviates age-related long-term memory impairment. Middle-aged rats (84-weeks-old) displayed a significant (p<0.05) decline in the retrieval of passive avoidance behaviour showing thus increased forgetting. Long-term administration of H. perforatum (350 mg/kg for 21 days) effectively prevented this decline making the avoidance latency in the middle-aged rats as good as young animals [250 s and 236 s respectively, both significantly (p<0.01) better than that in the middle-aged rats (121 s)]. These data point to alleviation of the age-related memory decline by St. John’s wort.

KEYWORDS: Aging, Elevated plus maze, Learning, Memory, Open field, Passive avoidance, Rat, St. John’s wort

INTRODUCTION
Age-related injuries begin to accumulate once biological development of the body is finished. Several brain transformations have been described in aging manifested in cognition, learning ability and memory that usually decline with age. The cholinergic neurotransmission and cholinergic neurons in central nervous system become more vulnerable to damage and degeneration because adaptive mechanisms, which maintain dynamic equilibrium of several processes and functions are not able to compensate for adverse effects of aging. In consequence mild cognitive impairment and afterwards dementia, including that of Alzheimer’s type may occur (Counts and Mufson, 2005).

Cholinergic neurons in brain are important for acquisition and consolidation of a diversity of learned behaviors (Miranda and Bermúdez-Rattoni, 2007). The appreciable clinical evidence shows that blocking muscarinic receptors leads to impairment of working memory and reference memory, attention and decision making processes, and also to altered sensory processing (Giacobini, 1996).

The pharmacologic strategies to combat age-related cognitive disorders have been aimed to improve cholinergic transmission. Increasing endogenous levels of ACh by inhibiting acetylcholinesterase (AChE) were found to be the most effective among the various approaches in senile dementia (Giacobini, 1996).

These drugs, however, cause several adverse effects, that limit their therapeutic value (Giacobini, 1996). In this context, use of natural more safe inhibitors of AChE might be an interesting alternative.

In our earlier studies we showed that Hypericum perforatum could prevent cognition deficits caused by chronic stress. Based on these (Trofimiuk et al. 2005; Trofimiuk and Braszko, 2008) as well as the other (Ferreira et al., 2006; Re et al., 2003) data an action of H. perforatum in inhibiting AChE appears possible.

The amygdala is the limbic structure essential for coordination the autonomic and endocrine responses to emotional states (Shekhar et al., 2005). It plays a role in passive avoidance performance and creation of emotional memory. The amygdala complex receives cholinergic innervations from the basal forebrain (Klein and Yakel, 2006). Cholinergic drugs have been shown to regulate passive avoidance performance via the amygdale (Riekkinen et al., 1993). Accordingly, Klein and Yakel (2006) showed that functional nicotinic ACh receptor-mediated processes in amygdala might play a significant role in fear and aversively motivated memory.

This study was aimed at testing a novel strategy of fighting age-related changes in cognitive functions by using Hypericum perforatum. Passive avoidance test was used because of its sensitivity in detection decline of amygdala-dependent memory in rodents.

MATERIALS AND METHODS

Animals
This study used 41 two-month old (140-150g each) and 40
ninteen-month old (600-800g each) male Wistar rats. They were maintained in a temperature (23°C) and humidity (50-60%RH) controlled vivarium in groups of five or three under a constant 12/12 h light/dark schedule (lights on at 7:00 a.m.) with free access to the standard lab chow and tap water.

Drugs

Dried crude herb of *H. perforatum* (Labofarm, Poland) in the form of a brown powder, standardized to 0.3% hypericins was used in all experiments. Its constituents were naphthodiantrones (hypericin and its derivatives), phloroglucinols (up to 6% hyperforin), flavonoids 2-4% (rutine, quercetine, quercitrine, isoquercitin, biapigenin, hyperoside) and procyanidins 8-12% (procyanidin, catechin, epicatechin polymers). It was suspended in a 2% carboxymethyl-cellulose (2% CMC) and administered at the dose of 350 mg kg-1 daily for three weeks by gastrointestinal gavage (p.o.) in the volume of 1.5 ml kg-1. This dose corresponds to the human recommended daily dose of 0.9 g total hypericins (Widy-Tyszkiewicz et al. 2002).

Animals were divided into four groups treated as follows: 1) 21 young rats received 2% CMC p.o. (Young); 2) 20 middle-aged rats received 2% CMC (Aged); 3) 20 young rats received 350 mg kg-1 *H. perforatum* p.o. in 2% CMC (Young+Hp); 4) 20 middle-aged rats received 350 mg kg-1 *H. perforatum* p.o. in 2% CMC (Aged+Hp).

The experimental procedures were carried out according to the European Council Directive of 24 November 1986 (6/609/EEC) and were approved by the Local Ethics Commission for Animal Experimentation.

Behavioral tests

**Open field.**

The next day after ending three weeks of administration drug or vehicle animals were tested in an open field for assessment of psychomotor as well musculo-skeletal aspects of their performance. Locomotor exploratory activity was measured in the open field which was a square 100cm x 100cm white floor divided by eight lines into 25 equal squares and surrounded by a 47cm high wall (Braszko et al. 1987). Four plastic bars, 20cm high, were designed as objects of possible animal’s interest and fixed perpendicularly, parallel to each other, in four line crossings, in the central area of the floor. A rat was placed in the center of the floor and, following 1 min. of adaptation, crossings, rearings and bar approaches were counted manually for 5 min.

**Elevated plus-maze.**

Anxiety was evaluated, next day after assessment of open field performance, in an elevated ‘plus’ maze (made of gray colored wooden planks) consisted of four arms, 50 x 10cm (length x width). Two arms (closed) had 40cm high walls, covered with removable lid, and the two remaining arms (open) had no walls, such that the open or closed arms were opposite to each other. The maze was elevated to a height of 50cm from the floor. Rats were placed for 5 min. in a pretest arena (60 x 60 x 35cm, construed of the same material) prior to exposure to the maze. This step allowed facilitation of exploratory behavior. The experimental procedure was similar to that described by Pellow et al. (1985). Immediately after the pretest exposure rats were placed in the center of the elevated ‘plus’ maze facing one of the open arms. During the 5 min. test period the following measures were taken: the number of entries into the open and closed arms and the time spent in the open and closed arms. An entry was defined as all four feet into one arm. An increase in open arms entries and increase in time spent in open arms were interpreted as indicative of potential anxiolytic activity.

**Passive avoidance.**

Passive avoidance behavior was studied in a one trial learning, step-tough situation (Ader et al., 1972), which utilizes the natural preference of rats for dark environments. The apparatus consists of the platform (250 x 80mm) connected with a dark compartment – a metal box (400 x 400 x 400mm) with an opening (60 x 100mm) in the middle of the frontal wall length. After a 2-min. habituation to the dark compartment, the rat was placed on the illuminated platform and allowed to enter the dark compartment. Two more approach trials were given on the following day with a 2-min. interval. At the end of the second trial unavoidable scrambled electric foot shock (0.25 mA, AC, 2 s) was delivered through the grid floor of the dark compartment (learning trial). Retention of the passive avoidance response was tested 24-hours later by placing the animal on the platform and measuring the latency to re-enter the dark compartment to a maximum of 300 s. The four rats were excluded from test probe after training sessions because they did not effectively learn the rules of that test. As a result, their data for the PAB are not available.

**Statistical analysis.**

Data were presented as means ± standard error of mean (S.E.M.). One-way analysis of variance (ANOVA), followed by Bonferroni test for chosen group comparisons, was applied for mean performance of the rats in the open field, elevated plus-maze and passive avoidance tests. The probability level less than 0.05 was accepted as significant.

**RESULTS**

**Effects of aging and *H. perforatum* administration on passive avoidance behavior**

ANOVA of the results obtained in the passive avoidance test yielded F (3,36)=5.721 (p<0.001) showing thus statistically significant differences between the groups (Fig. 1). Post-hoc comparisons in preselected pairs with Bonferroni test revealed that middle-aged rats re-entered dark part of the apparatus significantly earlier then both young (p<0.05), and treated with *H. perforatum* (p<0.01) middle-aged rats. This pattern showed that substantial adverse effects of aging on learning
and avoidance behavior were abolished by the simultaneous *H. perforatum* administration.

**FIGURE 1.** Effects of aging and long-term *H. perforatum* treatment on the passive avoidance behaviour in passive avoidance test. Columns represent means ± S.E.M. of the re-entry latencies obtained from *n* rats indicated at the bottom of the figure. *p*<0.05 vs. Young; **p**<0.01 vs. Aged.

**DISCUSSION**

In this study we showed that *H. perforatum* effectively restored cognitive functions impaired by aging and significantly reduced age-related forgetting in rats. Aging caused significant motor impairment as tested by crossings, rearings and bar approaches in the open field (*p*<0.001 for all) but did not influence anxiety as tested in the elevated plus-maze.

The memory functions were tested in the passive avoidance paradigm, which is widely utilized for testing learning and memory in rats. In the passive avoidance test, animals on exposure to first trial acquire the information that entry into dark chamber results in noxious experience of electric shock. The
cognitive ability of the animal is reflected in avoiding the entry into dark chamber (a judgment based on successful retention and recalling of the acquired information). It was interesting to find in the present study that the re-entry latencies in the aged rats were significantly lower as compared to the young rats. It was still more interesting to note that treatment with Hypericum perforatum significantly improved learning and memory in older rats as shown by increase in retention latency to enter in the passive avoidance test.

Acetylcholine has an essential role in the central nervous system and its implication in the learning and memory as well as neurodegenerative disorders is undeniable. Although huge amounts of existing data support the participation of cholinergic neurotransmission in modulating diverse forms of learning and memory (Parent and Baxter, 2004), the possibility that alterations in the cholinergic system are also involved in the aversive learning has been verified (Riekkinen et al., 1993). Cholinergic augmentation, especially inhibitors of AChE, is basis of dementia therapy (Enz et al. 1993; Giacobini, 1996; Siddiqui and Wagstaff, 2006). There is the growing evidence about potential anti-AChE activity of H. perforatum and reduction of the degradation rate of ACh (Ferreira et al., 2006; Re et al., 2003). Therefore we could speculate that this mechanism of action is responsible for the improving the passive avoidance learning performance in older rats in this study.

Nevertheless, the systemic administration of 5-HT1A receptor antagonists can facilitate aversive learning by enhancing hippocampal/cortical cholinergic and glutamatergic neurotransmissions (Madjid et al., 2006). Hippocampus, prefrontal cortex and amygdala are the brain regions most related to aversive learning (Mamiya et al., 2009). 5-HT1A receptors are located presynaptically as somatodendritic receptors in the raphe nucleus and postsynaptically in limbic and cortical regions (Hamon et al., 1990). The 5-HT1A receptor in the agranular insular cortex (a limbic-related cortex) has been reported to be involved in the consolidation of memory for inhibitory avoidance in rats (Mello e Souza et al., 2001). It has been demonstrated that H. perforatum normalizes number and sensitivity of the 5-HT1A receptors in brain (Butterweck et al., 2001; Yu, 2000). Therefore, it is highly probable that this mechanism of action of H. perforatum is included in facilitating passive avoidance performance in aged rats.

In order to study the relationship between anxiety and cognition, the elevated plus-maze test was carried out. There is profound evidence that cognitive processes and anxiety are interrelated (Goswami et al., 1996). The elevated plus-maze is among the most popular behavioral methods of measuring anxiety. In the present study we did not observe any influence of aging as well as Hypericum perforatum treatment on anxiety. Although other authors (Torras-Garcia et al., 2005) reported that aging is associated with a decrease in anxiety because increase in the number of entries in open arm by old rats as compared to young rats was observed. Our results are in line with observations of Andrade et al. (2003) showing lower level of elevated plus-maze exploration by older rats than young ones but in our study we did not see statistically significant changes in anxiety behaviour apart from its role in cognition.

Spontaneous open field activity includes a variety of responses that could be interpreted as indices of exploration, arousal, locomotion, anxiety and emotionality. In particular, increased locomotion (horizontal, forward or ambulatory activity) and vertical activity (rearing) characterize rat behavioral response to novelty. In this study we showed that aging is associated with decrease of locomotor activity in rats. Treatment with H. perforatum did not influence this negative impact of aging. It was interesting to note that H. perforatum medication could decrease some aspects of locomotion in young rats (decreased number of rearings). Number of rearings may indicate not only locomotor activity but also mirror e.g., the level of arousal or anxiety, but the results obtained in the elevated plus-maze did not show that Hypericum perforatum treatment or aging could evoke anxiogenic behavior.

CONCLUSION

In conclusion it appears that H. perforatum offers a safe and not expensive alternative in treating learning and memory deficits associated with aging.

ACKNOWLEDGEMENTS

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REFERENCES

Ader, R., Weijnen, J.A.W.M., Moleman, P. (1972) Retention of passive avoidance response as function of the intensity and duration of electric shock. Psychonomic Science 26, 125-129


H. Perforatum alleviates forgetting
ABSTRACT: The relationship between diabetes and selenium (Se) compounds has been certified. The aim of our experiment was to use carrageenan and sodium selenite to produce κ-selenocarrageenan oligosaccharides (SCOs), which should have antidiabetic and antioxidative effects. The reaction was carried out at 60°C under acidic conditions. The product was characterized with UV spectroscopy, NMR spectroscopy, infrared spectroscopy, flame atomic absorption spectrometry (FASS) and thin layer chromatography (TLC). Our results showed that some sulfated groups on the site of the 4-sulfated-β-D-galactose units (G4) were replaced by sodium selenite and produced SCOs. The SCOs were used as a Se supplement in alloxan-induced diabetic rats, and its antidiabetic and antioxidative effects were examined. We found that SCOs could reduce the glucose concentration and restore the alloxan-induced damage to the pancreas islet, potentially via its antioxidative activity. In conclusion, we prepared κ-selenocarrageenan oligosaccharides with antidiabetic and antioxidative activities.

KEY WORDS: Antidiabetic; Antioxidative; Carrageenan; K-Selenocarrageenan Oligosaccharides (SCOs).

INTRODUCTION

Selenium is a trace element whose essential requirement in mammals was discovered in 1957 by Schwarz and Foltz. Scientific reports have shown that selenium has a large number of biological functions in humans, the most important action being its antioxidative effect because it forms selenocysteine, which is part of the active center of the glutathione peroxidase enzyme (GSH-Px). This enzyme eliminates the free radicals of the phospholipids of the membrane during oxidative stress (Chappuis and Poupon, 1991; Levander and Burk, 1994). Consequently, a deficiency in Se would provoke impairment of GSH-Px activity and result in peroxidation stress in the body, thereby increasing the risk of degenerative diseases (Chan et al., 1998; Hartman et al., 2002; Levander and Burk, 1994; Papp et al., 2007; Tato Rocha et al., 1994; Tapiero et al., 2003; Zeng and Combs, 2008). Diabetes is known as one of many degenerative diseases specifically related to the impaired homeostasis of certain elements such as selenium (Marcason, 2008; Navarro-Alarcon et al., 1999b; Simonoff and Simonoff, 1991). More recently, the origin of cell damage undergone by diabetics was attributed to free radicals (Figuerola, 1992). Hence, it is important for diabetic patients to supplement selenium and increase the antioxidative capability of the body. Selenium exists both in inorganic and organic forms. Due to its low physiological activity, high toxicity and mutagenic action, the utilization of inorganic selenium is restricted in the biomedicine field. The production of inorganic selenium with nontoxic and highly antioxidative properties is therefore crucial for treating degenerative disease such as diabetes.

Carrageenan is the main cell wall component of various marine red algae. This anionic polymer belongs to a family composed of alternating 3-linked β- and 4-linked α-galactopyranose. The anionic polymers differ in the occurrence of an α-3, 6-anhydro bridge in the 4-linked residues and in the number and position of sulfate esterification per repeating disaccharide. Carrageenan has been regarded as a safe food additive by the Food and Drug Administration in the United States. Due to its special structure and presence of a sulfated group, carrageenan has shown various biological activities, such as antiviral, antitumor and anticoagulant properties (Cáceres et al., 2000; Carlucci et al., 1997; Duarte et al., 2001; Noda et al., 1990). Carrageean is a large molecular saccharide, and its poor dissolubility and absorbability restrict its application in the biomedical field. However, an oligosaccharide of carrageenan has more potential in biomedical area with respect to good dissolubility, absorbability and higher biological activities. Some researchers have shown that oligocarrageenans have antioxidative activity
in vivo and in vitro (Mou et al., 2003; Yuan et al., 2005), demonstrating that the antioxidative mechanism of the sulfated compound is different to that of the selenium compound. Free radicals are harmful to diabetic patients and cause the reduction of Se concentrations in the serum (Navarro-Alarcon et al., 1999b; Schlienger et al., 1988; Simonoff and Simonoff, 1991; Twardowska Saуча et al., 1994). We therefore designed our experiment to prepare κ-selenocarrageenan oligosaccharides (SCOs) using κ-carrageenan as a safe starting material while expecting the product to share both the properties of the oligocarrageenan and the selenium compound. We use the SCOs as a Se supplement for diabetic rats and examined its hypoglycemic and antioxidative activities.

MATERIALS AND METHODS

Materials

κ-carrageenan was obtained from Jiangsu ChangHang Hydrocolloid Scientific Co. Ltd (Jiangsu China). Sixty-five-week-old female mice, weighing 20±3 g, were purchased from the Experimental Animal Center of Dalian Medical University and maintained in a standard animal room for a week before the experiment started. All other reagents were of analytical grade.

Preparation of κ-selenocarrageenan oligosaccharides

Carrageenan (1 g) and sodium selenite (1 g) were dissolved in 100 ml of 0.5% sulfuric acid solution. The reaction system was heated to 60°C and stirred for 4 h. The reaction was quenched by adding 10% of sodium hydroxide solution; the pH of this system was adjusted to 7 at room temperature. The product was condensed by vacuum pump and then dialyzed in a 500-Da cut-off bag filter. κ-selenocarrageenan oligosaccharides (SCOs) were obtained by freeze-drying the product of dialysis.

Measurement of the Se content in SCOs

The Se content in SCOs was measured by the method of flame atomic absorption spectrometry (FASS). A Carl Zeiss (Jena, Germany) Model AAS3 flame atomic absorption spectrometer equipped with a 10-cm air-acetylene burner head assembly and an IBM-PC compatible computer was used throughout the study. A Se hollow cathode lamp was used as the radiation source. No background correction was required in this mode of operation. Operating parameters were set according to the reference method protocol of Matusiewicz and Krawczyk (2007).

UV spectroscopy, NMR spectroscopy and infrared spectroscopy

Sodium selenite (1 mg), κ-carrageenan oligosaccharides (1 mg) and κ-selenocarrageenan oligosaccharides (1 mg) were dissolved in 1 ml of distilled water each. The absorption of the solutions was measured by a UV spectrometer set at a wavelength range of 190 nm to 300 nm.

For NMR analysis, κ-carrageenan oligosaccharides (50 mg) and κ-selenocarrageenan oligosaccharides (50 mg) were dissolved in 500 μl of high-quality D2O (99.96%), containing 0.1 μl acetone. 1H-NMR experiments were carried out at 500 MHz and 13C-NMR at 150 MHz on a Bruker Avance DRX-500 (Bruker Co., Ltd., Switzerland) spectrometer with a 5-mm 1H probe.

An infrared spectrum of κ-carrageenan oligosaccharides (0.5 mg) and κ-selenocarrageenan oligosaccharides (0.5 mg) was taken on a Perkin-Elmer instrument (PerkinElmer Instrument Co., Ltd. USA) as KBr pellets at room temperature.

Measurement of the average molecular weight of SCOs and TLC assay

The average molecular weight of SCOs was assayed with the method of 3,5-dinitrosalicylic (DNS). Briefly, 1 mg of SCOs was dissolved in 1 ml of distilled water and boiled with 4 ml of DNS solution for 10 min. After cooling to room temperature (25°C), the mixed solution was transferred to a 96-well microplate and analyzed spectrometrically at 530 nm with the microplate reader. The molar concentration of SCOs was obtained according to the standard curve prepared with a series of dilutions (0-20 mol/ml) of lactose by the DNS method, and the average molecular weight was calculated by the mass of SCOs divided by its molar concentration.

The SCOs, carrageenan and lactose were developed on TLC using a solvent system of n-butanol/acidic acid/H2O in a ratio of 2:2:1 (v/v). The SCOs was visualized with an amidobenzene-diphenylamine solution at 80°C for 20 min.

Diabetic rats and experimental groups

Diabetic rats were induced with a single intravenous injection of alloxan (32 mg/kg, bw; Sigma), dissolved in physiological saline. Five days later, the plasma glucose concentration was determined in blood samples obtained from rats after being fed. Non-diabetic (<14.7 mmol glucose/l) or extremely diabetic (>35.5 mmol glucose/l) rats were excluded from this study. Diabetic rats were randomly allocated to four groups (n=12 per group). Three groups were used as experimental groups, with intragastric administration of SCOs supplied at a dose of 50 mg/kg/day, 200 mg/kg/day or 500 mg/kg/day for 14 days. One group of diabetic and normal rats was used as two control groups and was treated with physiological saline.

Test of glucose concentration and antioxidative activity in serum

After the treatment of SCOs for 14 days, glucose, superoxide dismutase (SOD) activity, glutathione peroxidase enzyme activity (GSH-Px) and the content of malondialdehyde (MDA) in rat sera were measured using commercial kits purchased from Nanjing Jiancheng Co., Ltd. of Nanjing University.

Histopathological observations

After treatment with SCOs for 14 days, the animals were killed and their pancreases removed. The organs were fixed in a formalin solution for 24 h and then embedded in paraffin. Sections were cut at a 5-μm thickness and stained.
with hematoxylin and eosin. The sections were then viewed under a light microscope to detect eventual histopathological changes.

Statistical analysis

The results are presented as the mean ± standard deviation (SD). The data followed a normal distribution. A two-way analysis of variance was performed using a Student's t-test. P < 0.05 was selected as a statistically significant difference, and P < 0.01 was selected as an extremely statistically significant difference.

RESULTS

Characteristics of κ-selenocarrageenan oligosaccharides (SCOs)

Under the acidic conditions, κ-carrageenan not only reacted with sodium selenite to produce κ-selenocarrageenan but also was hydrolyzed to oligosaccharides. Thus, the final products of the reaction were κ-selenocarrageenan oligosaccharides (SCOs). SCOs are a pink powder that could not dissolve in organic solvent. The DNS result shows that the average SCOs molecular weight is 1.43 kDa. According to the results of flame atomic absorption spectrometry, the Se content of the SCOs is 30 µg/mg. Figure 1 displays the result of thin-layer chromatography (TLC): SCOs is seen to distribute between lactose and carrageenan but is closer to lactose.

FIGURE 1. TLC of a κ-selenocarrageenan oligosaccharides (SCOs) sample. A: Lactose; B: κ-selenocarrageenan oligosaccharides (SCOs); C: κ-carrageenan.

The results of UV spectrum analysis are shown in Figure 2. There is no absorption peak between 190 nm and 300 nm for the oligocarrageenan, but sodium selenite has one absorption peak at 210 nm. SCOs also have an absorption peak at 210 nm, similar to that of sodium selenite, indicating that the product of SCOs contains the Se element.

FIGURE 2. UV spectrum of a κ-selenocarrageenan oligosaccharides (SCOs) sample.

Infrared spectra of oligocarrageenan and SCOs are shown in Figure 3. Clearly, the infrared spectra of SCOs are similar to that of oligocarrageenan. The absorption band at 850 cm⁻¹ indicates that the sulfate group is attached to the C-4 position of galactose. However, this absorption band shifted to 857.72 cm⁻¹ in the SCOs infrared spectral data. This shift may be caused by the selenious group reacted on the oligocarrageenan.

The ¹H NMR and ¹³C NMR spectra of oligocarrageenan and SCOs are given in Figure 4 and Table 1. The ¹H NMR and ¹³C NMR spectra of oligocarrageenan are consistent with previously published data (Tojo et al., 2003; Van de Velde et al., 2004). The ¹H NMR and ¹³C NMR spectra of SCOs are similar to that of oligocarrageenan, indicating that the base structure of SCOs is similar to that of oligocarrageenan. In the ¹H NMR spectrum of SCOs, the signal at 4.87 ppm was attributed to the anomeric proton of the 4-sulfated-β-D-galactose units (G4). However, another new signal at 4.85 ppm was observed in the ¹H NMR spectrum of SCO compared to that of oligocarrageenan (Fig 4).

FIGURE 3. Infrared spectra of oligocarrageenan and κ-selenocarrageenan oligosaccharides. A: Oligocarrageenan; B: κ-selenocarrageenan oligosaccharides.
of SCOs (showed in Fig 4 by red arrow and in Table 1). Taken together, the NMR spectra of SCO indicate that some sulfated groups were replaced by the selenious group on the site of the 4-sulfated-β-D-galactose unit.

FIGURE 4. NMR spectrum of κ-oligocarrageenan and κ-selenocarrageenan oligosaccharides (SCOs). A: 1H NMR spectrum of κ-oligocarrageenan; B: 1H NMR spectrum of SCOs; C: 13C NMR spectrum of κ-oligocarrageenan; D: 13C NMR spectrum of SCOs.

Table 1. The chemical shifts of oligocarrageenan and κ-selenocarrageenan oligosaccharides (SCOs) in 13C-NMR spectra

<table>
<thead>
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<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
<th>G6</th>
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<td>70.02</td>
<td>78.75</td>
<td>73.61</td>
<td>75.10</td>
<td>61.61</td>
</tr>
<tr>
<td>SCOs</td>
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<td>70.01</td>
<td>78.73</td>
<td>71.10</td>
<td>75.10</td>
<td>61.60</td>
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<tr>
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<td>95.14</td>
<td>71.46</td>
<td>79.54</td>
<td>78.64</td>
<td>77.02</td>
<td>69.78</td>
</tr>
</tbody>
</table>

Note: G represent 1-3-β-D-4-sulfate-D-galactose, A represent (1-4)-α-3, 6-anhydro bridge-D-galactose

The antidiabetic activity of SCO
The blood glucose concentration of experimental animals after treatment with alloxan was higher than that of control animals. Diabetic rats showed representative symptoms, such as consuming more food, more drink, excreting more urine and losing weight. The antidiabetic activity of SCOs is shown in Figure 5. After treatment with different doses of SCOs for 14 days, the blood glucose concentration of the experimental groups decreased and reached almost normal levels, compared to that of the diabetic group. It is clear that the highest degrading effect occurred using the dosage of 50 mg/kg SCOs. The representative symptoms of diabetes (namely consuming more food, more drink and excreting more urine) disappeared, and the experimental rats stopped losing weight after treatment with SCOs (Fig 5B). Together, these results indicate that SCOs could decrease the blood glucose concentration and demonstrated antidiabetic activity in diabetic rats.

FIGURE 5. Antidiabetic activity of SCOs for diabetic rat. A: Effect of SCOs on the glucose content in rat serum; B: Effect of SCOs on rat weight. ∗represents P<0.01 in a t-test compared to the diabetic rat group (n=12), ∗∗represents P<0.05 in a t-test compared to the diabetic rat group (n=12).

The antioxidant activity of SCOs for diabetes rat
The superoxide dismutase (SOD) activity, glutathione peroxidase enzyme activity (GSH-Px) and content of malondialdehyde (MDA) in the serum of experimental rats were measured after treatment with different doses of SCOs for 14 days; these results are shown in Figure 6. SCOs enhanced the SOD and GSH-Px activity in rat sera after treatment for 14 days. For SOD, the highest enhancement activity was observed
at the dosage of 50 mg/kg SCO; the enhancement activity decreased with increasing SCOs dosage. Since Se is a part of the active center of GSH-Px, SCOs could enhance the activity of GSH-Px; the highest enhancement activity was observed at the SCOs dosage of 200 mg/kg. MDA is the final product of the cell membrane peroxidation and has been regarded as one marker of oxidation stress in the cell membrane. Alloxan, a strong oxidant, can injure pancreatic islet cells and cause the animals to show diabetic symptoms. The MDA content in rat sera was increased significantly when induced by alloxan injury, indicating that the diabetic rats were under peroxidation stress. The MDA content of the experimental animals was reduced to or close to normal levels after treatment with SCOs for 14 days.

**Histopathological observations of the pancreas islet**

**FIGURE 6. Effect of SCOs on antioxidase activity and MDA content in rat serum.** A: Effect of SCOs on the activity of SOD; B: Effect of SCOs on the activity of GSH-Px; C: Effect of SCOs on the MDA content in rat serum. * represents \( P<0.01 \) in a \( t \)-test compared to the diabetic rat group \( (n=12) \), ** represents \( P<0.05 \) in a \( t \)-test compared to the diabetic rat group \( (n=12) \).

The histology of pancreas islet cells of the experimental rats is shown in Figure 7. The pancreas islet of normal rats is intact, the boundary is clear, and the pancreas islet cells contain normal elliptical nuclei (Figure 7A). For the diabetic rat, however, the islet shows signs of deterioration and appears shrunken with an unclear boundary. The cells are in a state of disarray, show anomalous structure with irregular nuclei, and some cells with inflammation are observed in the pancreas islet (Figure 7B). For the 50-mg/kg and 200-mg/kg treatment groups, the pancreas islet is still intact with a clear boundary, and the cells are full with normal elliptical nuclei, but some inflammatory cells are also observed. For the 500-mg/kg groups, no inflammatory cells could be observed in the pancreas islet.

**DISCUSSION**

\( \kappa \)-carrageenan is a type of sulfated polysaccharide extracted from red marine algae, which consists of alternating 3-linked \( \beta \)-D-galactose (G units) and 4-linked 3,6-anhydro-D-galactose (A units). Previous research revealed that \( \kappa \)-oligocarrageenan has antioxidative activity \textit{in vivo} and \textit{in vitro} (Mou, 2003; Yuan 2005). Selenium is an important part of the active center of the glutathione peroxidase enzyme (GSH-Px) (Chappuis and Poupon, 1991; Levander and Burk, 1994). It is therefore essential to supplement Se element when a state of peroxidation occurs in the body. Diabetes is one of the degenerative diseases with a disturbance in Se homeostasis and damage caused by free radicals (Marcason, 2008; Navarro-Alarcon et al., 1999b; Simonoff and Simonoff, 1991). Hence, it is important for diabetic patients to supplement selenium and increase the antioxidative activity of the body. Considering the different antioxidative mechanism of sulfate and selenium compounds (Battin and Brumaghim, 2009), we designed this experiment to produce sulfated seleno-oligosaccharides with \( \kappa \)-carrageenan and measured their antidiabetic and antioxidative activity.

Under acidic conditions, carrageenan can be hydrolyzed into...
oligocarrageenan. The results of DNS and TLC indicate that the product is a low molecular weight carrageenan. The results of FAAS, UV and IR spectra show that oligocarrageenan has both a sulfate group and a selenium element. Hence, the product of our experiment is an oligocarrageenan with selenium, which contains properties of both compounds and is named κ-selenocarrageenan oligosaccharides (SCOs). According to the 1H and 13C NMR spectra, we deduce that some sulfated groups on the site of the 4-sulfated-β-D-galactose units (G4) were replaced by sodium selenite. The supposed structure of the product and the reaction principle are provided in Figure 8.

Alloxan, a strong oxidant, can injure the pancreatic islet cells (Fig 7B) and induce hyperglycemic symptoms (Fig 5A). After treatment with SCOs for 14 days, the glucose concentration in the serum of experimental rat groups decreased to or were close to the normal level (Fig 5A), and the injury to pancreas islet cells was overcome (Fig 7). Together, these results indicate that SCOs has an antidiabetic effect for alloxan-induced diabetic animals and that the best effect was observed at a dosage of 50 mg/kg. SOD and GSH-Px are two important oxidases in animals and can protect tissue from damage by free radicals. MDA is a marker of the oxidation state of the body. It is clear that SCOs can enhance the activity of SOD and GSH-Px and reduce the MDA content in diabetic sera (Fig 6). The different enhancement of SOD and GSH-Px may be due to the different effect of the sulfated group and Se of SCOs. These results certify that SCOs has antioxidative activity in vivo and that the antioxidative activity maybe due to both the sulfate group and selenium element of SCOs. The injury of pancreas islet cells is due to the peroxidation induced by alloxan in the body. The antioxidative effect of SCOs is in accord with the antioxidative activity of SCO. Thus, we can conclude that the antidiabetic effect of SCO is due to its antioxidative activity.

In conclusion, we prepared κ-selenocarrageenan oligosaccharides (SCOs) using carrageenan. The product not only has a sulfate group but also contains a Se element and shows antioxidative activity. The SCOs can decrease the blood glucose level to almost normal and overcome the damage to the pancreas islet cells that is induced in alloxan-induced diabetic rats. The antidiabetic effect of SCOs may be due to its antioxidative activity.

ACKNOWLEDGEMENT

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REFERENCES


ABSTRACT: Research has shown that regular breakfast cereal consumption is associated with better well-being (subjective reports of health and functioning). There is also evidence that a high fibre diet is associated with increased well-being. The present study involved a secondary analysis of baseline data from Smith et al. (2001). Initial analyses examined associations between high fibre intake and well-being (emotional distress, fatigue, cognitive difficulties and somatic symptoms). The results showed that high fibre intake was associated with increased well-being. Subsequent analyses examined whether the effects of total fibre intake could be accounted for by ingestion of specific sources of fibre, namely breakfast cereal and fruit/vegetables. The results showed that it was the breakfast cereal that was largely responsible for the increased well-being. Digestive problems are also associated with reduced well-being and a second set of analyses examined whether the benefits of fibre were due to a reduction in digestive problems. The results showed that digestive problems reduced well-being but these effects were independent of the effects of fibre.

KEY WORDS: Breakfast cereal, fibre, digestive problems, well-being.

INTRODUCTION

A number of scientific studies have shown a link between eating a high fibre diet and a range of health benefits (e.g. cardiovascular health, Pereira et al., 2004; diabetes, Liu et al., 2003; digestive health, Jefferson, 2005; weight loss, Ludwig et al., 1999; and cancer, Bingham, 2006). Research has also shown that increasing dietary fibre from wheat bran cereals decreases fatigue and increases energy (Smith et al., 2001). The first aim of the present study was to conduct secondary analyses of an existing database (Smith et al., 2001) to examine associations between total fibre intake and well-being. Smith 2005 has discussed the concept of well-being and argues that it should cover many dimensions. In the present analyses emotional distress, fatigue, cognitive difficulties and somatic symptoms (Ray et al., 1992) were examined as these covered more dimensions than many measures of physical and mental health. A second aim of the analyses was to determine whether specific sources of dietary fibre, namely breakfast cereals and fruit/vegetables also had positive effects on well-being.

Consumption of breakfast is often considered one of the important health-related behaviours (Berkman and Breslow, 1983 and Rice and Duncan, 1985) and there has been considerable research into its effects. Smith (1998) has found an association between regular consumption of breakfast cereal and well-being. The results showed that those who consumed a cereal breakfast each day were less depressed, less emotionally distressed and had lower levels of perceived stress than less regular consumers. These beneficial effects of breakfast have been found in samples aged from eight to eighty years (Smith, 1998; Smith, 1999; Smith 2003; Smith, in press).

A high intake of fruit and vegetables is recognised as a major contributor to good health (Eikenberry and Smith, 2004). It has been recognised as protective for many conditions, including cardiovascular disease (Djousse et al., 2004) and cancer (Cox et al., 2000). Indeed, a high intake of fruit and vegetables has been shown to be inversely related to mortality in men and women (Khaw et al., 2001). Furthermore, a recent study has shown that higher fruit and vegetable consumption is associated with better self-reported physical functional health (Myint et al., 2007). However, the effects of fruit and vegetable intake on other aspects of well-being were less clear. This issue was examined in the present study.

Fruit and vegetable consumption could influence well-being through numerous biological mechanisms. One mechanism is through the fibre content. Soluble fibre is mainly found in fruits and vegetables. Insoluble fibre or roughage also includes pectins from fruit and vegetables. Similarly, lignin consists of the tough fibrous parts of plant cell walls such as stalks and skins. The present study examined whether fibre from fruits and vegetables had the same effect on well-being as total fibre intake. Fibre influences...
digestive functioning and the next section shows that digestive problems are associated with reduced well-being.

There is considerable evidence that constipation and other disorders of bowel functioning are significant causes of morbidity (Garrow and James, 1993). Changes in dietary pattern are one of the main causes of the high prevalence of these disorders although the aetiology can be complex (Taylor, 1990). Dietary fibre can improve bowel functioning and wheat bran is the fibre of choice for the treatment of constipation because of its ability to increase faecal bulk (Gray, 1995). Indeed, the addition of wheat bran to the diet prevents constipation in up to 60% of elderly patients (Hull et al., 1980). A study of over 1,000 patients suffering from constipation has shown wheat bran to be effective in over 80% of the sample (Frexinos, 2004). Overall, there is ample evidence to show that high fibre cereals have beneficial effects on bowel functioning.

Bowel functioning is associated with other aspects of health. For example, when a person is stressed their bowel habit often changes. Patients with constipation have increased psychopathology (Merkel et al., 1993). We have recently confirmed these findings in an epidemiological study of a community sample of over 14,000 volunteers (Smith, submitted). The present study examined associations between fibre intake, digestive function and well-being.

There are several possible explanations for the above findings. The first explanation is that it is well-being that determines food choice rather than the other way around. This view is supported by the literature on abnormal dietary intake in patients with depression (Christensen and Somers, 1996). However, intervention studies show that high fibre breakfast cereal consumption is associated with improved well-being (Smith et al., 2001) and it is now important to identify the mechanisms that underlie this effect. One plausible mechanism is that consumption of high fibre breakfast cereal improves digestive functioning which in turn improves mental health. The present study examined this issue and whether different sources of fibre produce similar or different effects. This was achieved by a secondary analysis of a database containing information on intake of different sources of fibre, digestive problems and aspects of well-being.

METHODS

The study was approved by the Cardiff University School of Psychology Ethics Committee and carried out with the informed consent of the volunteers.

Questionnaires

Volunteers completed the following questionnaires:

(1) Fibre intake questionnaire (Kellogg’s 1994). This is shown in Table 1. It assesses fibre intake and weights the different sources for their fibre content. Total fibre intake, fibre from breakfast cereals and fibre from fruit and vegetables were used in the present analyses.

(2) Well-being measures. Measures of emotional distress, fatigue, cognitive difficulties and somatic symptoms were obtained from the Profile of Fatigue Related States Questionnaire.

(3) Digestive Problems. Digestive problems were measured using the questionnaire shown in Table 2.

### Table 1. Fibre intake questionnaire and responses (Scores for each response are shown below) over the last 7 days

<table>
<thead>
<tr>
<th>What kind of breakfast cereal did you regularly eat?</th>
<th>All-Bran, other high bran cereal</th>
<th>Puffed wheat, bran flakes, wheat biscuits, shredded wheat, wheat other whole wheat flakes, cereal, oat bran flakes</th>
<th>Muesli</th>
<th>Corn flakes, Rice Krispies, other cereal</th>
<th>Don’t eat breakfast cereal</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>50</td>
<td>50</td>
<td>20</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>On a typical weekday, how many slices of bread did you eat? (a roll counts as two slices of bread)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>What sort of bread did you usually eat?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wholemeal (not wholemeal)</td>
</tr>
<tr>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>On a typical weekday, how many biscuits would you eat?</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 or more</td>
</tr>
<tr>
<td>6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>How many times a week did you eat these foods?</th>
</tr>
</thead>
<tbody>
<tr>
<td>A = Twice or more day</td>
</tr>
<tr>
<td>B = Once a day</td>
</tr>
<tr>
<td>C = 5-6 times a week</td>
</tr>
<tr>
<td>D = 3-4 times a week</td>
</tr>
<tr>
<td>E = Twice a week</td>
</tr>
<tr>
<td>F = Once a week</td>
</tr>
<tr>
<td>F = Once a fortnight</td>
</tr>
<tr>
<td>H = Less than once a fortnight</td>
</tr>
</tbody>
</table>
TABLE 1. Continued

<table>
<thead>
<tr>
<th>Product</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baked beans</td>
<td>140</td>
<td>70</td>
<td>50</td>
<td>30</td>
<td>20</td>
<td>10</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Breakfast cereal</td>
<td>112</td>
<td>56</td>
<td>40</td>
<td>24</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Fresh fruit</td>
<td>70</td>
<td>35</td>
<td>25</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Tinned fruit</td>
<td>70</td>
<td>35</td>
<td>25</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Dried fruit</td>
<td>70</td>
<td>35</td>
<td>25</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Leafy vegetables, e.g. cabbage</td>
<td>42</td>
<td>21</td>
<td>15</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Root vegetables, e.g. carrots</td>
<td>42</td>
<td>21</td>
<td>15</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Jacket potatoes</td>
<td>28</td>
<td>14</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Boiled potatoes</td>
<td>28</td>
<td>14</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Mashed potatoes</td>
<td>28</td>
<td>14</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Roast potatoes</td>
<td>28</td>
<td>14</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chips</td>
<td>28</td>
<td>14</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Biscuits</td>
<td>28</td>
<td>14</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Rice</td>
<td>14</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Pasta</td>
<td>14</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Crispbreads</td>
<td>14</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Crisps</td>
<td>14</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Salads</td>
<td>14</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

TABLE 2. Bowel function evaluation

Using this scale, we'd like to ask you about some physical symptoms that people often experience. For each of the following eleven symptom we would like you to indicate how much that problem has bothered or distressed you during the past seven days, including today. For each, we'd like you to answer by writing 0 = not at all, 1 = a little bit, 2 = moderately, 3 = quite a bit, or 4 = extremely in the blank space.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>_________</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Constipation</td>
<td>_________</td>
</tr>
<tr>
<td>2. Diarrhoea</td>
<td>_________</td>
</tr>
<tr>
<td>3. Indigestion</td>
<td>_________</td>
</tr>
<tr>
<td>4. Nausea and/or vomiting</td>
<td>_________</td>
</tr>
<tr>
<td>5. Stomach pains (e.g. cramps)</td>
<td>_________</td>
</tr>
<tr>
<td>6. Flatulence</td>
<td>_________</td>
</tr>
<tr>
<td>7. Poor appetite</td>
<td>_________</td>
</tr>
<tr>
<td>8. Weight loss/feeling slimmer</td>
<td>_________</td>
</tr>
<tr>
<td>9. Pain in Bowels</td>
<td>_________</td>
</tr>
<tr>
<td>10. Incomplete evacuation of bowels</td>
<td>_________</td>
</tr>
<tr>
<td>11. Bloatedness</td>
<td>_________</td>
</tr>
</tbody>
</table>
Sample and Study Subjects

Details of the sample (demographics and fibre intake) are shown in Table 3.

TABLE 3. Demographic characteristics and fibre intake of the participants (N = 139).

<table>
<thead>
<tr>
<th>Age:</th>
<th>Mean = 52.3 years</th>
<th>s.d. 14.6 range 30 - 80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender:</td>
<td>Male: 32.4%</td>
<td>Female: 67.6%</td>
</tr>
<tr>
<td>Marital status:</td>
<td>Single: 15.8%</td>
<td>Married: 55.4%</td>
</tr>
<tr>
<td>Ethnicity:</td>
<td>White: 96.4%</td>
<td></td>
</tr>
<tr>
<td>Education:</td>
<td>Left school before 16: 27.7%</td>
<td>CSE’s/O’ levels: 39.4%</td>
</tr>
<tr>
<td>Employment:</td>
<td>Full-time: 31.2%</td>
<td>Part-time: 20.3%</td>
</tr>
<tr>
<td>Total fibre intake</td>
<td>Range: 32 to 590.</td>
<td>Quartile 1: 142</td>
</tr>
<tr>
<td>Fibre from breakfast cerea</td>
<td>Range = 0 to 100.</td>
<td>Quartile 1: 0</td>
</tr>
<tr>
<td>Soluble fibre from fruit and vegetables</td>
<td>Range = 0 to 307.</td>
<td>Quartile 1: 46</td>
</tr>
</tbody>
</table>

RESULTS

Total fibre consumption and well-being

The initial analyses of variance examined associations between total fibre intake and the well-being measures. There were significant effects of fibre intake on emotional distress, fatigue and cognitive difficulties. Higher fibre intake was associated with significantly greater well-being (see Table 4).

TABLE 4. Total fibre intake and well-being (scores are means, s.e.s in parentheses)

<table>
<thead>
<tr>
<th>Total fibre intake</th>
<th>Measures of well-being</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st quartile</td>
<td>2nd quartile</td>
</tr>
<tr>
<td>Emotional distress</td>
<td>42.1 (3.6)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>35.7 (2.9)</td>
</tr>
<tr>
<td>Cognitive difficulty</td>
<td>28.9 (2.4)</td>
</tr>
<tr>
<td>Somatic symptoms</td>
<td>33.7 (2.4)</td>
</tr>
</tbody>
</table>

Results from analyses of variance:

Emotional distress: Fibre intake: F 3,135 = 3.17 p < 0.05
Fatigue: Fibre intake: F 3,135 = 2.60 p = 0.05
Cognitive difficulty: Fibre intake: F 3,135 = 3.62 p < 0.05
Somatic symptoms: Fibre intake: F 3,135 = 1.65 p > 0.05

The beneficial effects of fibre can be seen most clearly by comparing those with the lowest and highest intakes (see Figures 1-3).

FIGURE 1. Fibre intake and emotional distress (bottom quartile versus top quartile; scores are the means, standard errors shown as bars. High scores = more emotional distress).

FIGURE 2. Fibre intake and fatigue (bottom quartile versus top quartile; scores are the means, standard errors shown as bars. High scores = more fatigue).
The second set of analyses examined the association between fibre from breakfast cereal and well-being. An identical pattern of significant effects as in the total fibre analyses was obtained (see Table 5).

The next set of analyses examined the association between fibre from fruit and vegetables and well-being. None of the measures of well-being showed a significant effect of fibre intake (see Table 6).

Table 5. Fibre from breakfast cereal and well-being (scores are means, s.e.s in parentheses)

<table>
<thead>
<tr>
<th>Fibre</th>
<th>Measures of well-being</th>
<th>Below median</th>
<th>Above median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emotional distress</td>
<td>41.7 (2.6)</td>
<td>33.0 (1.8)</td>
<td></td>
</tr>
<tr>
<td>Fatigue</td>
<td>34.5 (2.2)</td>
<td>27.2 (1.6)</td>
<td></td>
</tr>
<tr>
<td>Cognitive difficulty</td>
<td>28.9 (1.6)</td>
<td>23.9 (1.2)</td>
<td></td>
</tr>
<tr>
<td>Somatic symptoms</td>
<td>32.8 (1.6)</td>
<td>29.3 (1.5)</td>
<td></td>
</tr>
</tbody>
</table>

Results from analyses of variance:
- Emotional distress: Fibre intake: $F_{1,137} = 7.85$, $p < 0.01$
- Fatigue: Fibre intake: $F_{1,137} = 7.57$, $p < 0.01$
- Cognitive difficulty: Fibre intake: $F_{1,137} = 6.20$, $p < 0.05$
- Somatic symptoms: Fibre intake: $F_{1,137} = 2.60$, $p > 0.05$

Table 6. Fibre from fruit and vegetables and well-being (scores are means, s.e.s in parentheses)

<table>
<thead>
<tr>
<th>Fibre intake</th>
<th>Measures of well-being</th>
<th>1st quartile</th>
<th>2nd quartile</th>
<th>3rd quartile</th>
<th>4th quartile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emotional distress</td>
<td>40.3 (3.5)</td>
<td>37.8 (3.2)</td>
<td>34.1 (2.7)</td>
<td>34.8 (3.2)</td>
<td></td>
</tr>
<tr>
<td>Fatigue</td>
<td>35.2 (2.8)</td>
<td>29.9 (2.6)</td>
<td>25.9 (2.3)</td>
<td>30.4 (2.8)</td>
<td></td>
</tr>
<tr>
<td>Cognitive difficulty</td>
<td>28.0 (2.2)</td>
<td>26.8 (2.0)</td>
<td>25.5 (1.9)</td>
<td>24.0 (1.9)</td>
<td></td>
</tr>
<tr>
<td>Somatic symptoms</td>
<td>33.0 (2.5)</td>
<td>29.9 (2.4)</td>
<td>29.5 (1.8)</td>
<td>30.9 (2.0)</td>
<td></td>
</tr>
</tbody>
</table>

Results from analyses of variance:
- Emotional distress: Fibre intake: $F_{3,135} < 1$
- Fatigue: Fibre intake: $F_{3,135} = 2.11$, $p > 0.05$
- Cognitive difficulty: Fibre intake: $F_{3,135} < 1$
- Somatic symptoms: Fibre intake: $F_{3,135} < 1$

Bowel function and health/mood outcomes

Constipation

In the following analyses volunteers were sub-divided into those reporting no constipation and those reporting some signs. Those who were constipated reported that they were significantly more fatigued ($F_{1,137} = 8.21$, $p < 0.005$ – see Figure 4), had more cognitive difficulties (problems of memory, attention, action) ($F_{1,137} = 6.03$, $p < 0.05$ – see Figure 5), and more somatic (physical) symptoms ($F_{1,137} = 13.4$, $p < 0.0005$ – see Figure 6). These results confirm earlier findings although there was a lack of significant associations with mental health measures.
Loss of appetite

Volunteers were sub-divided into those who reported they were not bothered by loss of appetite and those who were bothered to some extent. Those who perceived they had lost their appetite reported significantly more emotional distress ($F_{1, 137}=22.05, p < 0.0005$ – see Figure 7), more fatigue ($F_{1, 137}=12.21, p < 0.0005$ – see Figure 8), somatic symptoms ($F_{1, 137}=15.21, p < 0.0005$), Loss of appetite: mean=36.3, s.e. =2.4; no loss: mean=28.6, s.e. =1.1) and cognitive difficulties ($F_{1, 137}=15.2, p < 0.0005$, Loss of appetite: mean=31.9, s.e=2.2; no loss: mean=23.7, s.e. =1.02).

Flatulence

Volunteers were sub-divided into those who reported little/no flatulence and those who reported moderate/or more flatulence. Analyses revealed that those reporting greater flatulence felt significantly more fatigued ($F_{1, 137}=4.05, p < 0.05$ – see Figure 9) and had more somatic symptoms ($F_{1, 137}=6.62, p < 0.05$ – see Figure 10).

These results show that the different digestive symptoms were associated with specific profiles of reduced well-being. The next set of analyses examined whether the effects of fibre reflected removal of the negative effects of the digestive problems.

Analyses examining fibre intake and digestive problems

These analyses confirmed the effects described above and showed no interactions between fibre intake and digestive problems. This suggests that effects of fibre intake on well-being cannot be totally explained by effects of the fibre on preventing digestive symptoms.

FIGURE 7. Loss of appetite and emotional distress (scores are the means, s.e.s shown as bars. High scores=greater emotional distress)
DISCUSSION

The results from this study have confirmed that a high fibre diet is associated with increased well-being, as indicated by better mental health, more energy and better cognitive functioning. This effect did not reflect a reduction in digestive problems (constipation, flatulence, and loss of appetite), although bowel functioning was independently related to well-being outcomes. Different types of bowel problem were associated with different outcomes. For example, constipation was associated with fatigue, cognitive difficulties and somatic symptoms (but not mental health problems) whereas flatulence was only associated with fatigue and somatic symptoms. This confirms other research on minor symptoms which shows that different forms of pathology have different CNS effects (Smith et al., 1987).

The second series of analyses examined associations between specific sources of fibre and the well-being outcomes. Dietary surveys (Henderson et al., 2002) show that cereal foods provide 42% of fibre intake. Fruit and vegetables (including potatoes) provide a similar percentage of fibre intake. The analyses examined whether fibre from breakfast cereals and fruit and vegetables both improved well-being. The results showed that higher fibre intake from breakfast cereals was associated with better well-being whereas fibre from fruit and vegetables failed to have a significant effect on well-being. The beneficial effects of breakfast cereals on well-being confirm previous cross-sectional results and findings from intervention studies. The absence of an association between fruit and vegetable intake and well-being requires further investigation. The last results suggest that consumption of fruit and vegetables may influence health through different mechanisms to fibre. Neither source of fibre appears to influence well-being by counteracting the negative effects of digestive symptoms. This does not mean that the benefits do not reflect changes in the brain-gut axis. Rather it suggests that the changes are at a sub-clinical level not a change in clinical symptoms. There are many possible mechanisms that could be involved (Gomez-Pinilla, 2008). The next section describes a possible mechanism that has been put forward to account for improved well-being following ingestion of high fibre breakfast cereal.

The following account describes two mechanisms that could account for the beneficial effects of high fibre breakfast cereal found in cross-sectional and intervention studies. Fibre is fermented to short chain fatty acids by gut flora. Acetate goes to muscle and ATP generated. Gut fermentation and subsequent use of SCFA contributes to about 10% of a person’s energy requirements. This explanation plausibly accounts for the rapid effect of fibre and the magnitude of the effect (about a 10% increase in reported energy). The second possible mechanism is detoxification. Clostridia are known to form neurotoxins and these come from protein metabolism not from carbohydrate or fibre. Fibre stimulates benign flora (bifidobacteria, lactobacilli) which cannot make toxins. This process is slow compared to the fermentation effects and could possibly explain the greater (both in terms of magnitude and type) effects observed in cross-sectional analyses. This view can now be tested by examining whether high fibre breakfast cereal has a pre-biotic effect.
In conclusion, the present study has demonstrated associations between total fibre intake and different aspects of well-being. These effects were also observed when fibre intake from breakfast cereal was considered. In contrast, fibre from fruit and vegetables did not produce the same effects. The mechanisms underlying these effects remain unknown but they do not reflect removal of the negative influence of digestive symptoms.

ACKNOWLEDGEMENTS
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REFERENCES


Smith AP In Press. An investigation of the effects of breakfast cereals on alertness, cognitive function and other aspects of the well-being of children. *Nutritional Neuroscience*.


