Growth of Human Colon Cancer Cells in Nude Mice is Delayed by Ketogenic Diet With or Without Omega-3 Fatty Acids and Medium-chain Triglycerides

Guang-Wei Hao1&, Yu-Sheng Chen1&, De-Ming He2, Hai-Yu Wang1, Guo-Hao Wu1, Bo Zhang1*

Abstract

**Background:** Tumors are largely unable to metabolize ketone bodies for energy due to various deficiencies in one or both of the key mitochondrial enzymes, which may provide a rationale for therapeutic strategies that inhibit tumor growth by administration of a ketogenic diet with average protein but low in carbohydrates and high in fat. **Materials and Methods:** Thirty-six male BALB/C nude mice were injected subcutaneously with tumor cells of the colon cancer cell line HCT116. The animals were then randomly split into three feeding groups and fed either a ketogenic diet rich in omega-3 fatty acids and MCT (MKD group; n=12) or lard only (LKD group; n=12) or a standard diet (SD group; n=12) ad libitum. Experiments were ended upon attainment of the target tumor volume of 600 mm³ to 700 mm³. The three diets were compared for tumor growth and survival time (interval between tumor cell injection and attainment of target tumor volume). **Results:** The tumor growth in the MKD and LKD groups was significantly delayed compared to that in the SD group. **Conclusions:** Application of an unrestricted ketogenic diet delayed tumor growth in a mouse xenograft model. Further studies are needed to address the mechanism of this diet intervention and the impact on other tumor-relevant parameters such as invasion and metastasis.

Keywords: Ketogenic diet - colon cancer - ω-3 fatty acids - MCT - lard

Asian Pac J Cancer Prev. 16 (5), 2061-2068

Introduction

Colorectal cancer which has become a major disease that threatens human health is the third most commonly diagnosed cancer in males and the second in females (Jemal et al., 2011; Amin et al., 2012). Although the incidence of colorectal cancer is generally reported together, 72% can be separated into colon cancer. Over the past decades, significant progress has been achieved in the treatment of colorectal cancer by advances in surgery, radiotherapy, and systemic treatment. Surgery with curative intent is the main treatment of stages I-III colon cancer, and chemicals which have side effects can also be used in many cases. A dietary intervention relied heavily on ketone bodies for energy supply has proved to be effective in many cancers such as malignant brain tumor (Seyfried et al., 2012), prostate cancer (Masko et al., 2010; Kim et al., 2012) and gastric cancer (Otto et al., 2008).

Most malignant tumors are largely dependent on glucose for their growth and survival, but they are unable to metabolise ketone bodies for energy production (Walenta et al., 2000). A ketogenic diet restricts the glucose supply while providing the body with adequate energy substrates in the form of fat for generating ketone bodies. In 1995 Nebeling and coworkers described the long-term management of paediatric astrocytoma patients by a ketogenic diet. In addition to its beneficial effect on tumor growth, the diet improved the patients’ nutritional status (Nebeling et al., 1995).

Ketogenic diets aim to induce ketosis, a physiological response of the body to limited dietary carbohydrate intake with consequent exhaustion of the glycogen content in liver and skeletal muscle resulting in the body’s use of fat for energy. During ketosis, the liver starts to degrade fatty acids and to form acetyl-CoA in fatty acid oxidation. Acetyl-CoA can then be diverted into the ketone bodies acetoacetate and β-hydroxybutyrate (β-OHB), the major ketone body in plasma (Veech et al., 2004). Ketone bodies are transported from the liver to other tissues where they can be reconverted to acetyl-CoA. Although glucose is the preferred fuel, ketone bodies can supply 2% to 6% of the body’s energy needs after an overnight fast and 30% to 40% after a 3-day fast.

When applied under caloric restriction, different kinds of ketogenic diet supplemented with either lard or soybean oil has been shown to have an inhibitory effect
on tumor growth (Mukherjee et al., 1999; Oleksyszyn et al., 2011; Akram et al., 2013). However, under caloric restriction the diets led to a dramatic weight loss in contrast to ad libitum feeding. Several groups have focused on the impact of lipid oils on both tumor growth and body weight. Without caloric restriction, an anti-tumor effect was demonstrated for diets rich in omega-3 fatty acids and medium chain (6-12 carbons) triglycerides (MCT), which are not present in lard and safflower oil (Nebeling et al., 1995; Kimoto et al., 1998; Kwan et al., 2014). Different researchers have repeatedly shown in animal experiments that the growth of human cancer xenografts can be slowed by omega-3 fatty acid-enriched diets (Hardman et al., 2002; Hardman et al., 2007; Wang et al., 2014). A recent study revealed that the risk of prostate cancer can be modulated by the dietary omega-6/omega-3 polyunsaturated fatty acids ratio in prostate-specific Pten knockout mice (Berquin et al., 2007). Since Pten acts as a suppressor of Akt signalling, which itself is intimately linked to the glycolytic phenotype, these experiments provide a link between lipid and glucose metabolism in pathological conditions.

To determine the impact of a ketogenic diet rich in different kinds of fatty acids on tumors exhibiting aerobic glycolysis, we compared the effects on growth and survival of a nutritionally balanced carbohydrate-restricted diet supplemented with a mixture of vegetable oils and oil extracts possessing elevated levels of polyunsaturated omega-3 fatty acid and MCT (MKD) or rich in lard (LKD) with those of a standard diet (SD) in a mouse xenograft model. We report a tumor-suppressive effect of these diets with respect to growth, necrosis, and neovascularization of subcutaneously implanted tumors in nude mice, feeding ad libitum.

Materials and Methods

Animals

Thirty-six male nude mice (4 to 6-week-old males) of the BALB/C strain obtained from SLAC laboratory animal corporation (Shanghai, China) were maintained in groups of twelve animals per cage in laminar flow hoods and ad libitum animal care. (China, Law for the Protection of Animals).

Tumor cells of the human cell line HCT116

We used cells of the human colon cancer cell line HCT116, which were kindly provided by institute of biochemistry and biology, Chinese academy of science. The cell line is available from the cell bank of Chinese academy of science (shanghai, China). The tumor cells were cultured as a monolayer in McCoy’s 5A medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum.

In vivo growth of cell line HCT116 colon cancer cells

A freshly thawed tumor cell aliquot was cultured for up to twelve passages in vitro not more than 3 weeks prior to injection into nude mice. All mice received tumor cells from the same cell passage. The cultured cells (nearly 70-80% confluence) were harvested with trypsin EDTA (PAA). About 9×10⁶ cells were inoculated subcutaneously in the right side of the back near the neck. Tumor nodules appeared approximately 3-5 days following cell injection. All data presented is based on these tumor nodules. No animal died from tumor growth. The tumor size was measured with calipers and the tumor volume (mm³) was calculated using the ellipsoid formula A² × B × π/6, where A represents the smaller diameter. Endpoint for the experiments was attainment of a tumor volume between 600 and 700 mm³ (target tumor volume), with the interval between subcutaneous tumor cell inoculation and the endpoint defined as the survival time. Tumors reaching the target tumor volume were dissected and the final tumor volumes were determined. Subsequently, they were cut through the median, one part was fixed in formalin and embedded in paraffin, while the other part was embedded in Tissue-Tek and snap frozen in liquid nitrogen.

Diets and feeding

All mice received a nutritionally balanced diet provided by the special animal feed manufacturer SLAC laboratory animal corporation (Shanghai, China) prior to the inoculation of tumor cells. This standard diet was supplied in pellets delivering 14.7 kJ/g gross energy and consisting of 5.28% fat, 22.1% protein, and 52% carbohydrates (Table 1). The ketogenic diets consist of a mixture of fresh, high quality food homogenized into a paste using a standard food processor and delivers 31.1kJ/g gross energy. The overall macronutrient composition of each ketogenic diet, expressed as a percentage of total grams, was as follows: MKD and LKD (gram: 69% fat, 3% carbohydrate and 20% protein). Both ketogenic diets were aliquoted in petri dishes under sterile conditions and stored at -20°C. Two dishes per twelve animals were thawed overnight at +4°C prior to feeding and the calory-intake was calculated by the calory-intake of the SD group in the past 24 hours. Following tumor cell injection on day 0 the animals (n=36) were randomly split into three equal feeding groups: standard diet (SD) and ketogenic diets (MKD and LKD as described before). Tumor size and body weight of all animals were measured every second/third/fourth day.

Materials and Methods

Table 1. Composition of the Standard (SD) and Ketogenic Diets (KD) Used in this Study

<table>
<thead>
<tr>
<th></th>
<th>SD</th>
<th>MKD</th>
<th>LKD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>22.1</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Fat</td>
<td>0</td>
<td>36.2</td>
<td>0</td>
</tr>
<tr>
<td>MCT</td>
<td>21.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Omega-3</td>
<td>5.28</td>
<td>11</td>
<td>69</td>
</tr>
<tr>
<td>lard</td>
<td>52</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>2.16</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>AN-76 mineral mixture</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AN-76 fiber mixture</td>
<td>4.12</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Energy density(kj/g)</td>
<td>14.7</td>
<td>31.1</td>
<td>31.1</td>
</tr>
<tr>
<td>Total(gram)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Ketogenic ratio 0.07:1 3:1 3:1
Measurement of plasma glucose, β-hydroxybutyrate (β-OHB), serum insulin and blood lipids

Blood glucose and β-OHB levels were measured on the day of tumor cell injection (day 0) and every week thereafter until the last day of the experiments before tumor resection. Measurements were done with a blood glucose and ketone monitoring system (Optium Xceed, Abbott Laboratories, Abbott Diabetes care, U.S.A.) and corresponding test strips (Abbott Diabetes care Ltd, Witney, Oxon, UK) using 2 μl of peripheral blood collected from a snipped tail vein of each animal. Serum insulin and blood lipids were tested by the clinical laboratory of our hospital on the day of sacrifice.

Determination of the size of necrosis

Sections corresponding to the median line were stained with haematoxyline-eosine and photographed with a Nikon D90 Single-lens reflex digital camera. Images of each whole section were imported into photoshop software and the complete area of necrosis per section was quantified through the pixel method. Briefly, the statistical data of both the necrosis area and total area in pixel can be showed in the histogram, therefore the percentage of necrosis area to total area can be determined.

Microvessel analysis

Cryosections of dissected tumors were stained with a PECAM-1 antibody (bioworld, America), final dilution 1:100. Microvessel density was quantified by examining areas of vascular hot spots in high power fields (hpf, 200 x) as previously described(Weidner et al., 1991). Briefly, sections were scanned at 40 x and at 100 x for the localization of vascular hot spots. Blood vessels were counted at 200 x in the three most non-necrotic vascular areas of the tumor. The values of the three sections were averaged for each HCT116 tumor.

Statistical analysis

SPSS16.0, GraphPad Prism 5.0 software was used for statistical analyses. Body weight, tumor growth, plasma glucose, blood ketone levels, necrotic areas and microvessels were analysed by Kruskal-Wallis and Mann-Whitney U test to show significant differences between different groups after the nonparametric rank order test. Probability values below 0.05 were considered significant.

Results

Course of body weights

All animals of the KD groups readily accepted the unrestricted ketogenic diet and showed a steady increase in body weight over a period of up to 45 days (Figure 1). The mean body weights of the SD, MKD and LKD animals prior to tumor cell injection were 19.5±0.9 g vs 19.6±0.9 g vs 19.9±0.9 g (difference not significant, \( p > 0.05 \)), respectively, and at day 22: 24.6±1.2 g vs 24.7±1.2 g vs 24.9±1.2 g (difference not significant, \( p > 0.05 \)).

Analyses of tumor growth

All 36 nude mice receiving a subcutaneous injection of tumor cells of the colon cancer cell line HCT116 showed growth of solid tumors in 12 days. The initial day of tumor palpability was earliest in the SD group (3 days) and latest in the MKD group (5 days), and the median time of tumor palpability in SD, MKD and LKD groups are 6, 8 and 8 days, respectively (Figure 2A). On the day of tumor cell injection, animals of the MKD and LKD group were switched from the standard diet to the ketogenic diet as described before. Tumor progression was followed by measuring tumor volume over time. The difference between the median tumor volume curves of MKD/LKD and SD animals is significant (\( p < 0.05 \), Figure 2B), while the difference between MKD and LKD group is not that significant (\( p > 0.05 \), Figure 2B). Kruskal-Wallis and Mann-Whitney U test analyses for the mean tumor volumes in different groups in day 13, 16 and 19 showed delayed tumor growth in both MKD and LKD group than that of the SD group (\( p < 0.05 \)), although there is a tendency that the tumor growth in MKD group is much slower than LKD group, the difference is not significant (\( p > 0.05 \)).

Animal survival

The last animal in the SD group reached the endpoint on day 29 after tumor cell injection (Figutr 3). At this time point, only 3 of 12 animals in the MKD group reached the target tumor volume and 3 of 12 in the LKD group, too. The last animal of the MKD and LKD group reached the endpoint on day 45 and 43, respectively. The mean survival time of animals in the SD group was 24.8±3.1 days, in the MKD group 35.1±7.6 days while in the LKD group 33.8±6.7 days. Overall, application of the unrestricted ketogenic diet with different kinds of fatty acid was highly significantly associated with survival (\( p < 0.05 \), Figure 3), but the difference between MKD and LKD group was not significant (\( p > 0.05 \), Figure 3).

Influence of diet on serum glucose, β-OHB, insulin levels and lipid profiles

Mice of both the MKD and LKD group achieved ketosis within seven days after feeding was started and their β-OHB levels remained continuously higher than those of the SD animals after day 7 (\( p < 0.05 \), Figure
consistent with studies showing that a ketogenic diet does not lower plasma glucose levels when given \textit{ad libitum} (Kimoto et al., 1998). The serum insulin levels at the end of experiment were slightly reduced in MKD/LKD animals in comparison to SD animals, but the difference was not significant (Figure 4C, $p>0.05$). In addition, there were no significant changes in triglycerides in all three diet groups, but with a rise in total cholesterol and high-density lipoprotein cholesterol together with low-density

Figure 2A: Influence of the Ketogenic Diets on Tumor Take. The tumor take of the MKD and LKD group is delayed than that of the SD group, but the difference between MKD and LKD is not significant. The horizontal line represent median day of tumor palpability; 2B: Influence of the Ketogenic Diets on Tumor Growth. Values are expressed as the median of each group. Curves only extend to 25 days in SD group and 34 days in MKD/LKD group because after this time, >50% of the mice fed with different diets had been sacrificed, respectively, and therefore the median tumor volume is not meaningful. A: Day 13; B: Day 16; C: Day 19. Upper and lower box borders represent 25th and 75th percentile values, respectively. Horizontal line within the box represents the median value. Upper and lower whiskers correspond to 95th and 5th percentile values, respectively.

Figure 3. Influence of the Ketogenic Diets on Animal Survival Times. Data are expressed as Kaplan-Meier survival curves (n=12 mice per group). Survival in the MKD and LKD group was significantly prolonged compared to that in the SD group ($p<0.05$), but the difference between MKD and LKD group was not significant ($p>0.05$).

4A). In contrast, no significant difference was noted between the blood glucose levels of the MKD, LKD and SD groups ($p>0.05$, Figure 4B). These findings are
lipoprotein cholesterol in MKD and LKD group compared to SD group, but the difference between MKD and LKD group was not significant (p>0.05, Figure 4D).

**Influence of diet on tumor necrotic area and tumor vascularity**

Histology of tumor nodules from animals of the MKD and LKD group exhibited highly significantly larger necrotic areas (53.0% and 51.2% of total area in median, respectively) than tumors of the SD group (26.4% in median, p=0.000), but the difference between MKD and LKD group is not significant (p>0.05, Figure 5A).

To determine if ketogenic diet with different kinds of fatty acids influenced angiogenesis, we used CD31 immunostaining to examine blood vessel densities in the HCT116 colon cancers. The number of blood vessels in the SD group was noticeably more than the MKD and LKD groups (p<0.05, Figure 5B). Also, vessel density under 200× high power field was significantly less in the MKD and LKD groups than in the SD group (p<0.05, Figure 10). Although there was a tendency that the vessel density in the MKD group was less than that of the LKD group, the difference was not significant (p>0.05).

**Discussion**

This study was designed to test whether a ketogenic diet with different kinds of fatty acids can inhibit the growth of tumors of the human colon cancer cell line HCT116 in a xenograft model. The ketogenic diet used here provides average protein and is low in carbohydrates and high in fat enriched with omega-3 fatty acids and MCT (MKD) or with lard only (LKD). Compared to the applied standard diet, the unrestricted ketogenic diet (both LKD and MKD) had a retarding effect on tumor growth and resulted in larger necrotic areas within the tumors. Although the difference is not significant, there is a tendency that MKD is more efficient than LKD. Blood glucose levels in MKD and LKD group were unaltered, while their ketone body levels were significantly elevated compared to those of the SD group. In contrast to most conventional cancer chemotherapies, which indiscriminately target both normal cells and tumor cells, ketogenic diets are the only known therapies that can target tumor cells while enhancing the health and vitality of normal cells (Nebeling et al., 1995; Seyfried et al., 2005).

In this regard, the ketogenic diet for cancer management stands apart from all conventional therapeutic approaches. The observation that unrestricted access to a ketogenic diet retarded tumor growth contrasts with data on another ketogenic diet, KetoCal, a commercially available diet for children with epilepsy (Oleksyszyn et al., 2011). The therapeutic effect of KetoCal on tumor growth was apparent in adult mice only when their caloric intake was restricted, which resulted in a 20%-23% loss in body weight within eight days after start of feeding (Oleksyszyn et al., 2011). In contrast to the calorically restricted KetoCal diet, we observed neither significant weight loss nor reduced blood glucose levels in our animals, although the tumor suppressive effects of the diets were comparable. Our data therefore suggest that an effective metabolic tumor therapy is not necessarily accompanied by reduced blood glucose levels. A possible cause of the observed delaying effect of the ketogenic diet on tumor growth is that tumors are largely unable to metabolize ketone bodies for energy due to various deficiencies in one or both of the key mitochondrial enzymes, β-hydroxybutyrate dehydrogenase (β-OHBDDH) and succinyl-CoA: 3-ketoacid CoA transferase (SCOT) (Tisdale et al., 1984; Sawai et al., 2004; Jiang et al., 2013; Poff et al., 2014). The deficiencies in these enzymes are important for tumor management when glucose is not sufficient and when cells would require ketone bodies for energy. This is most evident from the analysis of tumor growth in the MKD and LKD groups where tumor growth was delayed under ketosis because the SCOT and
β-OHBDH deficiencies would prevent tumor cells from using ketones as an alternative fuel thus metabolically isolating the tumor cells from the normal cells. Another possible cause of the observed delaying effect of the ketogenic diet on tumor growth is the high levels of omega-3 fatty acids and MCT in the diet. An antitumor effect has been demonstrated for both omega-3 fatty acids and MCT in patients and experimental models (Nebeling et al., 1995; Kimoto et al., 1998; Hardman et al., 2002; Wang et al., 2014; Kwan et al., 2014).

Cancer patients with advanced incurable cancer are typically threatened by cancer cachexia, characterized by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism, which leads to progressive weight loss, mainly due to loss of skeletal muscle mass (with or without loss of fat mass), and anorexia (Giordano et al., 2005; Fearon et al., 2011). Although cancer cachexia accounts for about 20% of cancer deaths, its underlying mechanisms are not well known in detail (Ifitikhar et al., 2011; Mondello et al., 2014). To improve the quality of life and survival time of incurable patients, it is important to avert the onset of cachexia. Colorically restricted diets are therefore not suitable as treatment for these patients. Ketogenic diets, however, with high fat, adequate protein and low carbohydrates, have been shown to prevent or limit the protein catabolism in skeletal muscle (Freeman et al., 2007). In 1999, Barber et al. reported that an energy dense nutritional supplement rich in fish oil could increase the body weight of cachectic cancer patients (Barber et al., 1999). A non-restricted ketogenic diet may thus indeed be capable of benefiting cachectic cancer patients when supplemented with adequate lipids. The ketogenic diet (both MKD and LKD) described in our study induced both a slight increase in body weight and a slower growth rate of human tumor cells in nude mice.

In our study, the changes in triglycerides in all three diet groups were not significant, but total TCHO, HDL-c and LDL-c in MKD and LKD group elevated greatly compared to SD group, and this is not satisfactory. But a pilot study effects of a ketogenic diet on the quality of life in 16 patients with advanced cancer showed no significant change in triglycerides and a decrease in TCHO, LDL and LDL/HDL (Schmidt et al., 2011), and the lipid profiles changed significantly with reductions in triglycerides, total cholesterol and LDL-c along with a rise in HDL-c levels which all reached significance in 106 Italian council employees (Paoli et al., 2011). A possible explanation maybe that the tumor-bearing nude mice cannot make full use of ketone bodies so that most of the hydroxymethyl glutaryl coenzyme A (HMG-Co A), a substrate can be transferred into both ketone bodies and cholesterol, are transferred into cholesterol, and further studies are needed to prove it. But at least the ketogenic diet has a good effect on the lipid profiles of human beings.

Tumors of both the MKD and LKD group were characterized by significantly larger necrotic areas than those of the SD group. This finding may be explained by the restricted glucose supply in the MKD and LKD group. However, we did not find significant differences in blood glucose levels of MKD, LKD and SD animals. This observation indicates that glucose is synthesised from noncarbohydrate precursors by a process called gluconeogenesis. One possible explanation for the significantly delayed tumor growth despite constant blood glucose levels in mice of the KD group is the ability of ketogenic diets to significantly reduce blood insulin levels (Sharman et al., 2002). It is widely accepted that frequently elevated levels of insulin can stimulate tumor growth (Beck et al., 1989; Lu et al., 2014). We found slightly reduced insulin levels in MKD and LKD animals, but the difference to insulin levels of SD animals was not significant.

Another possible explanation for the antitumor effect of the ketogenic diets is their ability to delay tumor take. Following tumor cell injection the animals of the MKD and LKD group were fed with ketogenic diets. Kruskal-Wallis and Mann-Whitney U test analyses showed that the time of tumor palpability are longer in both MKD and LKD groups compared to that of the SD group, but the difference between MKD and LKD group is not that significant. The observation that the ketogenic diets delay the tumor cell take could be clinically significant for prevention of metastatic tumor cell take. However, further studies are required to accurately discriminate the effects of the ketogenic diets.

The significantly larger necrotic areas in the centre of tumors grown in the MKD and LKD mice correlate well with the reduced microvessel density in these tumors. The suppression of neovascularization may be provoked by reduced levels of lactate/pyruvate in glucose starved tumor cells, which are able to stimulate angiogenesis via HIF-1-mediated trans-activation of VEGF (Lu et al., 2002) as well as by the anti-angiogenic effect of omega-3 fatty acids (Yang et al., 1998; Connor et al., 2007). Suppressed neovascularisation may further inhibit an adequate supply of glucose to the centre of the tumors. In aggressive tumor cells such a severe limitation of substrate produces a state termed ‘metabolic catastrophe’, which enhances necrosis. The therapeutic induction of metabolic catastrophe was recently proposed as an approach to killing “unkillable” tumor cells (Jin et al., 2007). Since glucose-fermenting tumor cells have been shown to have substantially enhanced resistance to several anticancer drugs (Xu et al., 2005), ketogenic diet therapy may represent an effective approach for targeting fermentative cell populations.

In conclusions, in this pilot study we demonstrate that a carbohydrate-restricted diet supplemented with lipids rich in omega-3 fatty acids and MCT or lard only delays the growth of glucose fermenting tumors. The effect did not depend on caloric restriction and there was no loss of body weight. From our study we find out that the MKD is more efficient than LKD, but more samples are needed. Further studies are needed to address the mechanism of this diet intervention and the impact on other tumor-relevant functions such as invasive growth and metastasis. The ketogenic diets described here may provide a promising strategy for anti-tumor therapy in the future.

Acknowledgements

We thank Chinese Academy of Science for the cells.
and Qing-Yang Meng and Qiu-Lei Xi for their help with my experiments and all the people who have helped us in the duration of the experiment. Thank you very much. There is no potential competing interest with other people or organizations of our study.

References


DOI:http://dx.doi.org/10.7314/APJCP.2015.16.5.2061


