Reviewer 01

Comments and Suggestions for Authors

The results of the in vivo study showed that a mixture of omega-3 and omega-6 reduces melanoma growth are novel and interesting. However, the experimental procedure did not use purified fatty acids but used fish oil for omega-3 and soybean oil for omega-6. These oils contain other fatty acids aside from omega-3 and omega-6 fatty acids. Therefore, the conclusion that a mixture of omega-3 and omega-6 reduces melanoma growth is questionable. The conclusion should be revised in that oral administration of 1:1 mixture of fish oil and soybean oil reduces melanoma growth in mice but fish oil alone or soybean oil alone is ineffective.

Author response: Thank you so much for these comments. We would like to mention that although the information about the full composition of fatty acids contained in the fish and soybean oils used in this study can not be provided by us, the information provided by the manufacturer (Naturalis®, Nutrição & Farma LTDA, Brazil) is that the fish oil is rich in omega-3 since there are a total of 1.6g of polyunsaturated fatty acids in 2g of the oil, 1.1g corresponding to eicosapentaenoic acid (EPA) and 0.2g to docosahexaenoic acid (DHA). In this way, more than 80% of this oil is composed by omega-3 polyunsaturated fatty acids. Regarding the soybean oil, according to the information provided by the manufacturer (Soya Distribuidora de Produtos Alimentícios LTDA) it is rich in omega-6 since there are a total of 6.7g of polyunsaturated fatty acids in 12.3g of the oil, 6g corresponding to linoleic acid (omega-6) and 0.7g to linolenic acid (omega-3). So, more than 89% of this oil is composed of omega-6 polyunsaturated fatty acids. These pieces of information were added in the sentence related to oils description (page 9, lines 305 - 312). However, the reviewer is right, since this information was now added into the manuscript, emphasizing that these oils are enriched, but now exclusively composed by omega-3 or -6. In addition, we believe that it is also important to clarify that, the reason to use these oils from these companies is that they are commercially available in Brazil.

Although the data presented above can support our conclusion that the oral administration of mixture of omega-3-rich fish oil and omega-6-rich soybean oil in a ratio of 1:1 was able to alter the release of inflammatory mediators that are essential for a microenvironment favorable to the melanoma growth, we agree with the suggestion and changed the conclusion for the sentence “The oral administration of 1:1 mixture of fish oil and soybean oil was able to alter the release of inflammatory mediators that are essential for a microenvironment favorable to the melanoma growth in mice, whereas fish oil or soybean oil alone was ineffective.”

The dose of the oils used in the study was 10 μl per mouse by gavage (line 327). How is it possible to administer this volume accurately to a mouse using a syringe and a gavage needle with a dead volume which could be 1 or 10 μl depending upon the gauge of the gavage needle. Part of the 10 μl dose of the oil will just remain in the dead volume of the needle.
Author response: Thank you so much for this appointment. It is important to clarify that the gavage treatment was performed using a number 23 steel gavage tube and a 1.0-ml microsyringe. In addition, to minimize the loss of dead volume, initially, we added a volume of 500 μl of each oil used in this study into the syringe and after the steel gavage tube was coupled to the syringe. After that, we discarded a volume until to guarantee that the oil dripped through steel gavage tube. Only after this, we performed the treatment of each animal carefully. These procedures were carried out during the treatment by gavage.

The sentence: “The gavage treatment was performed using a number 23 steel gavage tube and a 1.0-ml microsyringe.” was added into the manuscript (page 9, lines 337 - 338).

The in vitro study using melanoma cells in 96-well cell culture plates is questionable. The oils are virtually insoluble in aqueous media. The culture media contain 10% FBS which may increase the solubility of the oils in the media. The findings of the dose-response study using 5, 10, 20 and 40 μl of oil per well may be meaningless if 5 μl is already saturating when added to 0.2 ml of culture medium/well. The volume of culture medium per well was not stated in the Methods section but 0.2 μl/well is a standard volume used in cell culture using 96-well plates. What is the solubility of the oils in RPMI 1640 medium with 10% FBS?

Author response: Even though long-chain fatty acid solubilization may be difficult due to low solubility in aqueous solutions, some strategies employed to solve this issue usually include the use of organic solvents, heating, dimethyl sulfoxide (DMSO), and cell culture media containing albumin. It is widely accepted that animal serum, the original vehicle for providing lipids to cells in culture, uses proteins as carriers of every lipid required by mammalian systems. Fetal bovine serum (FBS), the most common serum in cell culture, contains very high levels of lipids (approximately 300 mg/mL of cholesterol and 30 mg/mL of oleic acid and the major lipid carrier proteins in sera include the albumin, a globular protein with many distinct hydrophobic moieties and which represents over 50% of the protein in serum, because albumin is high solubility in aqueous solutions. Albumin has six (or possibly seven) high-affinity binding sites for FAs and is thus an efficient carrier serving to substantially increase the solubility of fatty acids in aqueous solutions. It was demonstrated that in a mixture containing arachidonate, linoleate, oleate, palmitate, and stearate, with the total molar fractions of, 10, 15, 24, 21, and 29%, it was found that the free FA fractions observed were 23, 21, 20, 2 and 15%, respectively. This outcome is expected since the solubility and albumin affinity for each type of fatty acid is different. It is worth to mention that the fraction of unbound FFAs accessible for cellular uptake depends on the ratio of total FFAs to albumin. Thus, the biological effect of FFAs can be augmented by increasing the FFA concentration or by decreasing the bovine serum albumin (BSA) concentration. The unbound FFA concentration is also determined by the relative affinities of the FFAs for albumin. For example, when using a total FFA concentration of 0.5 mM in the presence of 1% w/v (151 μM) of albumin, corresponding to an FFA/albumin molar ratio of 3.3, the
theoretical unbound palmitate and oleate concentrations are 27 nM and 47 nM, respectively.

In addition, the sentence “Cell culture was performed by addition of 100μL of RPMI 1640 medium supplemented with 10% fetal bovine serum containing five thousand B16F10 melanoma cells (5x10^3 cells) per well in 96-well cell culture plaque. After 24h, different amounts (40, 20, 10 and 5μL) of fish oil rich in omega-3 or soybean oil rich in omega-6 or a mixture of these oils in a ratio of 1:1 were added to the B16F10 cell culture, final volume of each well was standardized to 200μL and the cell culture was incubated for 48h.” was revised and added in main text (page 9, lines 313-318).