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LETTER TO THE EDITOR

An essay on superoxide dismutase, 2-methoxyestradiol, and the proper uses of scientific methods

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A paper recently published by Li et al. (2015) significantly confuses an already confused issue. This essay attempts to clear the accumulated confusion and especially the one that results from that paper.

Fifteen years ago Huang et al. (2000) reported that 2-methoxyestradiol (ME) inhibits superoxide dismutase(s) [SOD(s)] Cu, Zn SOD, and Mn SOD. Soon thereafter, we (Kachadourian et al. 2001) concluded that, in fact, ME does not inhibit SODs. It should be stated that the conflict concerns the ability of ME to directly inhibit the activity of SOD under the conditions of the in vitro assays and not on what might happen in vivo or in cell cultures.

We suspected that the purported inhibition of the SODs by ME is actually caused by an artifact in the assay used by Huang et al. (2000) which leads to ME mimicking the effects of inhibitors of SOD, without actually being an inhibitor. We were able to reproduce their results using the same assay method that they used. However, ME did not "inhibit" SOD in several other assays, each of which was based on different principles and the usage of different superoxide generating systems and of different superoxide detectors. The logical conclusion is that ME is not an inhibitor of SOD.

Later, two groups (Golab et al. 2003; Florczak et al. 2009) concluded that their findings support the view that ME inhibits SOD. Let us examine whether this is the case. They used essentially the same method as the one used by Huang et al. except that XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide); was replaced by similar tetrazolium salts such as INT (2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride). However, they did not use other methods. In addition, in some of the cases SOD activity from cancer cells incubated with ME was studied and in such cases the effect of ME might be indirect as actually suggested (Golab et al. 2003). Therefore, the data in these two papers do not refute any of the conflicting views. On the other hand, our results do not contradict the thesis that ME decreases the activity of SODs in vivo or in cultured (cancer) cells by thus far an unknown mechanism.

The most recent claim that ME inhibits SOD (Mn SOD from *Clostridium difficile*) was made by Li et al. (2015). While in both, Huang's et al. and Li's et al. assays superoxide anion radical ($O_2^{\cdot-}$) was generated by xanthine oxidase (XO) plus xanthine (X), the first group used XTT, and the second, cytochrome c (cyt c), as detectors for $O_2^{\cdot-}$. A more detailed inspection of the Li et al. paper follows.

In Fig. 4 panel B, Li et al. followed the reduction of cytochrome c at 550 nm (A550) as a function of the concentration of SOD (Mn SOD) after 30 min of reaction.

In the absence of ME, A550 nm was ~0.17 in the absence of SOD, while in the presence of ~0.02 μg/ml SOD A550 nm was slightly over 0.11 and in the presence of ~0.16 μg/ml SOD A550 was also slightly over 0.11.

Thus, these authors report that an eightfold range SOD concentration inhibited the reduction of cytochrome

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c equally. This must mean that at both concentrations SOD managed to scavenge virtually all of the $O_2^{\cdot-}$, outcompeting cyt c almost completely. This being the case, their results establish that the reduction of cyt c seen in the absence of SOD on this figure is ~60 % $O_2^{\cdot-}$ independent (0.11/0.17 × 100 = ~60 %) and only ~40 % is $O_2^{\cdot-}$dependent. However, native XO does not reduce cyt c directly, and aerobically almost 100 % of the reduction of cyt c is mediated by $O_2^{\cdot-}$. Yet there are forms that are able of reducing cyt c directly, deflavo XO being one of them (Anda et al. 1972). Li et all, obviously had a kind of XO (obtained from Sigma) capable of reducing cyt c directly. In this regard, we recommend XO that can be isolated by the method of Waud et al. (1975). Commercial preparation of xanthine oxidase should be tested for the presence of any significant superoxide-independent pathway for reduction of cytochrome c.

The most important conclusion of our analysis results from the solution of the following paradox. According to Fig. 4 panel C the "inhibition" of SOD caused by 0.1 mM ME is ~60 %. This means that the activity of 0.16 μg/ml SOD which is 60 % inhibited, should be equal to the activity of 0.064 μg/ml non-inhibited SOD (0.16 × 0.4 = 0.064). However, as already mentioned, even 0.02 μg/ml SOD, scavenges practically all $O_2^{\cdot-}$, and A550 was 0.11 at both SOD concentrations, 0.02 and 0.16 μg/ml. Hence, 0.16 μg/ml SOD which is inhibited 60 % must give an A550 of 0.11. What however can be seen on panel B is that A550, in the presence of 0.16 μg/ml SOD and 0.1 mM ME, is 0.14!

It has to be concluded that Li et al. actually provide evidence supporting the view that the purported inhibition of SOD by ME is caused by an artifact in the assay used.

There are numerous other serious problems in their paper. Thus, it should be recommended, in the absence of any reason to do otherwise, that the classical method of McCord and Fridovich used by Li et al. should be performed as described there for reasons explained there and elsewhere (McCord and Fridovich 1969; Crapo et al. 1978). This includes measuring the initial rates of cyt c reduction, rather than the accumulation of reduced cyt c for 30 min and even in some experiments for one hour. For example, the activity of their SOD is likely seriously overestimated due to the presence of $O_2^{\cdot-}$ independent reduction of cyt c and to the measuring of reduced cyt c accumulation rather than the initial rate of cyt c reduction. This criticism may be extended to the work of Huang et al., who also measured the accumulation of XTT for 30 and even 60 min rather than the initial rate of reduction and in this way led Li et al. to perform their assay in the same faulty manner.

The results presented on Fig. 4 panel A, purportedly presenting the extent of the inhibition of SOD by 1 μM ME, as a function of the concentration of SOD, raise a number of questions for which it is difficult to find a rational answer. Thus: how is 1 μM ME inhibiting SOD by as much as 70 % on panel A, when according to panel C it did not practically inhibit at that concentration? Why is ME inhibiting SOD more when the latter is at higher concentrations? The approximate molecular weight of dimeric MnSODs is ~40,000. At 0.25 mg/ml MnSOD, its concentration is ~6 μM. How was 1 μM ME able to inhibit a sixfold higher concentration of MnSOD to the extent of 70 % even if one molecule ME completely inactivates one molecule SOD?

Finally, Li et al. found that ME does not interfere with the reduction of cyt c by XO + X in the absence of SOD, while Huang et al. stated that the opposite is true. Thus, those interested in these matters should try to verify which view is closer to the truth before using that method for establishing whether ME is a real inhibitor of SODs.

We are forced to question both the validity of the data presented and the conclusions of Li et al. That leaves our previous demonstration (Kachadourian et al. 2001), that ME does not directly inhibit SOD still standing.

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Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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