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RESEARCH ARTICLE

Influence of Anticoagulants on Determination of H_2O_2 Levels in Blood: Comparison of Citrate and EDTA

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Abstract

Background: H_2O_2 is supposed to be part of the laboratory test panel for assessment of oxidative stress. This molecule contributes to oxidative stress by reacting with Fe^{2+} ions to produce hydroxyl radicals. Anticoagulants, including citrate and EDTA chelate Fe^{2+}, possibly have implications in oxidative stress studies especially when H_2O_2 is measured.

Objective: This brief commentary is on the measurability of H_2O_2 in citrated and EDTA blood samples.

Method: Laboratory evaluation was performed using blood samples of 30 sheep collected in EDTA and citrate tubes. Samples were centrifuged, and plasma separated. H_2O_2 was determined using the Biotech H_2O_2 560 assay protocol on plasma. The ratio of EDTA to iron was also estimated to help interpret levels of H_2O_2 in EDTA blood.

Results: There was measurable H_2O_2 reaction in citrate samples, but not in the EDTA blood. Based on brand sterile hematology tubes containing 1.8 mg EDTA per mL blood; approximately 4:1 ratio of EDTA to iron is estimated. Presuming this calculated ratio is a reflection of the true iron levels in the sheep's blood, the test results support the suggestion that ratio of EDTA to iron is greater than 1:1.

Conclusion: Evidence is hereby presented to advance that EDTA anticoagulant is unsuitable for laboratory testing of H_2O_2 in plasma. Further studies may be needed to ascertain the effects of different anticoagulants on H_2O_2 in whole blood and haemolysate to see how these can be compared to plasma. This is relevant to establish a standard protocol for integration of H_2O_2 test for assessment of full oxidative stress panel in clinical practice.

Keywords

Anticoagulants, Blood samples, Diagnostic lab, Hydrogen peroxide, Oxidative stress

Introduction

H_2O_2 contributes to oxidative stress by reacting with Fe^{2+} ions via the Fenton reaction to produce hydroxyl radicals [1,2]. Blood contains iron either as free component of haemoglobin (Fe^{2+}) or the Fe^{3+} bound to transferrin. This underlies speculation that the levels of Fe^{2+}, by virtue of their reacting with H_2O_2, are targets of oxidants and are determinants of oxidative stress. Classical example is the methaemoglobinaemia [3], which is a significant clinical condition caused by oxidative stress reaction [4,5].

Citrate and EDTA are coagulants that chelate cations such as Fe^{2+}; and could possibly affect total oxidant including H_2O_2 measurements. In an investigation of the rates of reactions of H_2O_2 with different iron complexes including EDTA-Fe^{2+} and citrate-Fe^{2+}, it was found that in the absence of NO, oxidation of EDTA-Fe^{2+} complex by H_2O_2 to form Fe^{3+} was nearly twice as fast as the reaction of H_2O_2 with the citrate-Fe^{2+} complex. Studies have shown that in the presence of 0.48 µM NO, the reaction rate of H_2O_2 can be reduced to a near complete stop with the EDTA-Fe^{2+} complex while oxidation of the citrate-Fe^{2+} complex may also drastically slow, but not to the same extent as the EDTA-Fe^{2+} complex [6]. Therefore, it is possible that the minimum level of H_2O_2 detectable in citrated blood may be undetectable in the EDTA blood; and this has implications in oxidative stress studies, especially when H_2O_2 is to be the measured clinical parameter. Since both EDTA and citrate chelate iron that otherwise potentially react with H_2O_2 to cause

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oxidative stress [1,2], it is pertinent to establish the suitability of anticoagulants for the laboratory blood tubes where H_2O_2 is measured. Though, it is pertinent to note that EDTA may have paradoxical stimulatory and inhibitory effects on the Fenton reaction.

It is pertinent to since it has been observed that in the presence of Fe^{2+} and H_2O_2, thiochrome was oxidized, while the addition of excess EDTA resulted in inhibition of oxidation, possibly due to the chelation of iron with EDTA [7-11]. Akagawa and Suyama reported similar results, where oxidation via the Fenton reaction, in this case, of H_2O_2 and Cu^{2+} had inhibition through action of EDTA. EDTA is involved in oxidative reactions e.g. deamination and this is perhaps due to the depression of hydroxyl radical formation and competition between e.g. the protein and EDTA for reaction of hydroxyl radical [1].

Conversely, EDTA along with other Fenton oxidants such as Fe^{2+} and H_2O_2 are believed to stimulate oxidation *in vivo* [2]. Inhibition or stimulation of oxidation via the Fenton reaction perhaps depends on the ratio of EDTA to iron. A ratio of EDTA to iron that is higher than 1:1 inhibits oxidation, while lower ratios stimulate it [11]. Studies by Baron, et al. [2] support this, as the 1:1 of EDTA/iron ratio in their study resulted in stimulation of protein oxidation. Furthermore, they found that EDTA in the presence of small oxidizable molecules decreased the level of protein oxidation, while in the absence of these molecules, EDTA increased oxidation. This reflects the suggestion that EDTA acts as a competitor with proteins for reaction of hydroxyl radical [1]. The numerous nature and quantities of oxidizable molecules in biological samples would therefore have implications on oxidative stress measurements.

Aim

The objective of this basic science commentary is to advance the influence of anticoagulants EDTA and citrate on H_2O_2 levels in blood. This is with a view to contribute to the discourse on establishing protocol for full oxidative stress evaluation in clinical practice.

Methods

The study was approved by postgraduate research office of the Faculty of Engineering, Health, Science and the Environment of Charles Darwin University as Undergraduate Training and Research Opportunity Program (UTROP). The ethical concerns were verified by Animal Welfare Officer from the Office of Research and Innovation of the University.

As reported at Australian Institute of Medical Scientists conference [12]. Sheep blood was collected at the Berrima Veterinary Laboratory (Northern Territory) by their standard protocol. Blood samples were collected from 30 sheep; each sheep blood was collected in EDTA and in a citrate anticoagulant. These were transported back to the laboratory in an esky cooler and immediately centrifuged at 2500 g for 5 minutes and plasma separated and kept.

The measurement of H_2O_2 was determined using the Bioxytech H_2O_2 560 from Oxis ResearchTM, which were purchased through Sapphire Biosciences Australia. Assay protocol on plasma was based on a simple principle of colorimetric reaction readable on spectrophotometer. Blood samples were collected over a two week period and tested within 24 hours of collection. The cells and plasma were kept at 4 °C until required for testing on the same day of sample collection.

The effects of citrate and EDTA anticoagulants on H_2O_2 levels in blood were investigated. It was hypothesized that EDTA is not suitable anticoagulant for blood sample meant to measure H_2O_2 since EDTA inhibits H_2O_2-Fe^{2+} Fenton reaction.

First, the thirty sheep blood samples were collected into EDTA and citrate tubes. H_2O_2 was determined by Biotech assay method. Second, EDTA/iron ratio was estimated to aid explains measurement of H_2O_2 in EDTA-anticoagulated blood by using sheep reference ranges for haemoglobin and serum iron. Taking into consideration the actions of EDTA on oxidation specifically the 1/1 ratio of EDTA to iron [2,10], we determined our own ratio to aid explain measurement of H_2O_2 in EDTA anticoagulated blood. Since iron level was not measured, the ratio used was approximate and based on the following. Sheep reference ranges for haemoglobin and serum iron are 8-16 g/dL and 166-222 ug/dL, respectively [13].

- As an estimate, the study used the means of these ranges, 12 g/dL (0.12 g/mL) for haemoglobin and 194 µg/dL (1.94 µg/mL) for serum iron, to consider the total iron levels in sheep blood.
- Though the measurement of H_2O_2 was in plasma, the effects of EDTA would have been occurring as soon as the whole blood was collected and mixed with the EDTA lining the tube, thus haemoglobin iron levels was included in the ratio.
- Assuming that one gram of haemoglobin contains 3.47 mg of iron [14], in 1 mL of blood there is 0.4164 mg iron per mL blood. Serum iron accounts for 1.94 µg (0.00194 mg) of iron in whole blood, however the oxidative action of the Fenton reaction is due to Fe^{2+} rather than its oxidized form, Fe^{3+}, so this this value was not included.

Results

It was observed that H_2O_2 reaction color change occurred in the citrate specimen, but not in the EDTA specimen. Figure 1 shows that while blue coloration indicated H_2O_2 reaction in the citrate samples; all drains from cuvettes of the EDTA specimens appeared yellow signifying lack of reaction in EDTA specimens. Based on

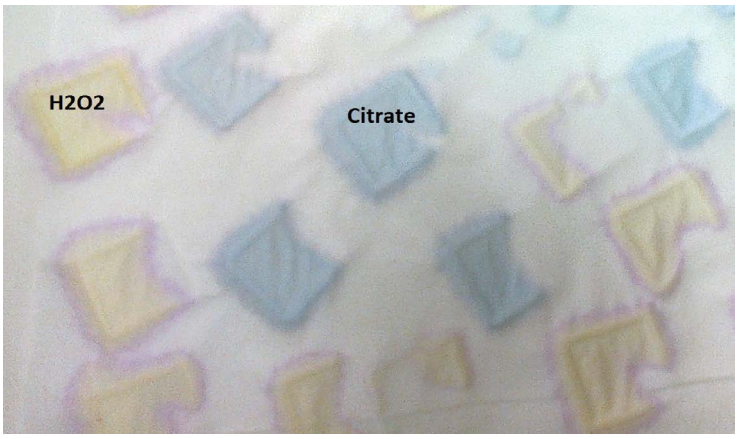

Figure 1: H_2O_2 reaction colors from drains of cuvettes of citrate and EDTA samples.

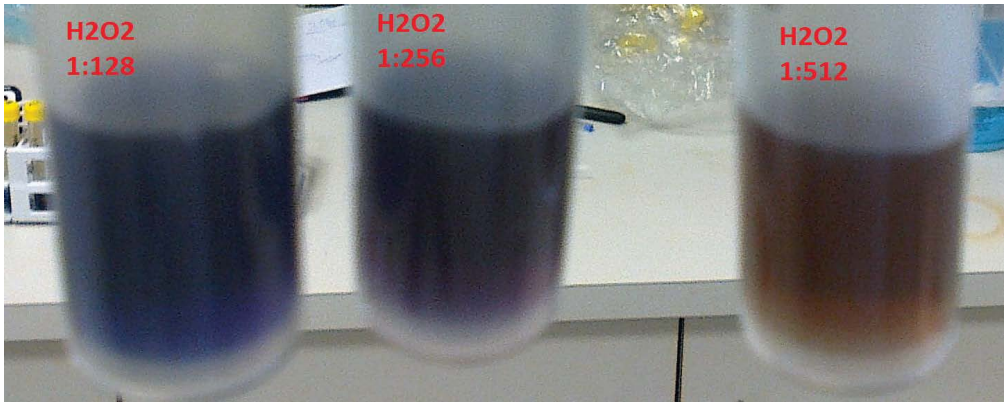

Figure 2: Gradient H_2O_2 reaction colors standard dilutions.

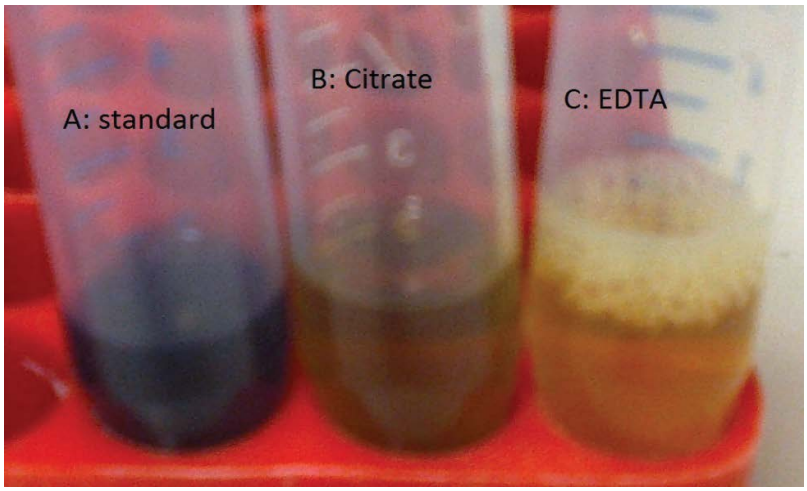

Figure 3: Differential H_2O_2 reaction colors of citrate vs. EDTA.

the observation of the first week (Figure 1), the second week's batch was performed with intent to confirm reactions while visual comparisons were also done. Figure 2 shows dilution of standard solution - that there is gradient reduction in intensity of blue coloration i.e. bluish indication of H_2O_2 reaction reduced as the dilution of standard increased towards 1/512 (Figure 2). When equal 1:4 dilutions of the anticoagulated plasma samples were compared to a 1:4 standard dilution, Figure 3 shows blue colour reaction in

- Standard solution: Very strong - requiring further dilution for colorimetric reading
- Citrate sample: Visible - could be read by the colorimeter
- EDTA: Non-existent and undetectable by colorimeter at recommended OD.

Brand sterile hematology tubes contain 1.8 mg EDTA per mL blood, which contains approximately 0.5 mg of iron in men [14] and a little less in sheep [13]. Thus, we have a ratio of EDTA to iron 1.8/0.42 simplified to 4.3/1. Even if we include our serum iron to our total iron, this

ratio still comes to 4.3/1. Presuming this calculated ratio is a reflection of the true iron levels in our sheep blood, our results therefore support the suggestion that ratio of EDTA to iron higher than 1/1.

Discussion

The results revealed that no apparent measurable H_2O_2 in the EDTA blood sample. This possibly implicates a role of EDTA in H_2O_2 inhibition. Based on the consideration of the paradoxical actions of EDTA, we decided to determine our own ratio hopefully to aid in explaining our inability to measure H_2O_2 in EDTA-anticoagulated blood. The EDTA BD Vactutainer® brand sterile hematology tubes contain 1.8 mg EDTA per mL blood. Thus, we have a ratio of EDTA to iron 1.8/0.42 simplified to 4.3/1. Even if we include our serum iron to our total iron, this ratio still comes to approximately 4/1. Presuming this calculated ratio is a reflection of the true iron levels in our sheep blood; our results support the suggestion that when ratio of EDTA to iron is higher than 1/1, causes inhibition of oxidation. Thus, with the assumption that inhibition of oxidation by EDTA is via inhibition of the H_2O_2-Fe^{2+} Fenton reaction, this could explain why H_2O_2 was not detectable in EDTA-anticoagulated sheep blood.

It has been observed that EDTA and heparin give false low result of oxidative stress parameters, probably due to their antioxidant properties. Therefore, it is already known or cautioned that in clinical setting these anticoagulants should be used in processing laboratory samples with care [15]. While heparin has been used for assessment of malondialdehyde as well as reduced glutathione [16,17], it is commonly encouraged to avoid using EDTA as precautionary measure [18-20]. It is hereby re-articulated more specifically that EDTA did not allow for identification of H_2O_2 where citrate anticoagulant did.

Thus, this basic clinical science commentary propagates two points what is already known, or indicated in literature, that EDTA has paradoxical behaviour in physiological reactions. What is being further added is that EDTA as a laboratory reagent-anticoagulant may never be useful for evaluation of H_2O_2. The significance is in bringing oxidative stress research to clinical diagnostic practice. Obviously, concentration may differ between plasma, serum and whole blood specimens i.e. similar to some other clinical biochemistry analytes [20]. Therefore, studies are needed to elucidate the influence of different anticoagulants on blood levels of oxidative stress indices, including the effects on time after blood collection on H_2O_2.

Conclusion

This paper advances that EDTA anticoagulant is unsuitable for testing H_2O_2 in plasma since level of H_2O_2 is dependent on EDTA/iron ratio. Further studies are needed on the effects of different anticoagulants on oxidative stress indices measurements and such studies should include the effects on time after blood collection on indices such as H_2O_2. These studies need to investigate whole blood as well as haemolysate to see how observations compare plasma.

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