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Brief Report

Heparin or EDTA; anticoagulant of choice in free radical estimation?

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Abstract
Physiological levels of reactive oxygen species (ROS) are needed for various normal processes like cellular immunity, sperm function, capacitation, acrosome reaction, proper embryonic development and implantation, cell signalling, DNA repair activation and gene transcription. Hence anything that creates imbalance in the ROS homeostasis would affect these essential physiological processes. Heparin is a sulfated anionic glycosaminoglycan widely used as an anticoagulant in conditions such as acute coronary syndrome, atrial fibrillation, deep-vein thrombosis and pulmonary embolism, cardiopulmonary bypass for heart surgery and hemofiltration. Ethylenediaminetetraacetic acid (EDTA) is a widely used anticoagulant which is also used in medical conditions like heavy metal poisoning for chelation therapy. To measure the ROS levels in the whole blood we employed a simple chemiluminescence assay using luminol as a chemiluminescent probe. To avoid clotting of blood while estimating free radical levels we used heparin and EDTA as anticoagulants. We observed a significant difference in ROS levels between the two readings using heparin and EDTA. We wish to state that though heparin and EDTA are used as anticoagulants they cause a marked reduction (false low) in the readings due to their antioxidant properties. EDTA caused a much more drastic and significant decline in ROS levels as compared to heparin. Thus in clinical setting they should be used with caution; after assessment of ROS levels so that the redox sensitive physiological reactions may not be impaired and it may not decrease rate of transcription of several genes.

INTRODUCTION

Reactive oxygen species (ROS) are defined as chemically reactive compounds containing oxygen such as peroxides and superoxides. ROS are formed as a natural by-product in the body and have important roles in cell signaling, DNA repair activation and homeostasis [1-3]. Physiological levels of ROS are necessary to maintain normal cell function but when the level of ROS increases beyond the antioxidant capacity of the body it results in a condition known as oxidative stress. Oxidative stress has been implicated in the pathogenesis of many human diseases such as atherosclerosis, cancer, diabetes, liver damage, rheumatoid arthritis, cataract, AIDS, inflammatory bowel disease, central nervous system disorders, Parkinson's disease, motor neuron disease, infertility and recurrent spontaneous abortions [4-6]. Antioxidants are compounds capable of inhibiting oxidation of other compounds. They do this by getting oxidized themselves. There are complex systems of enzymatic (catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase) and nonenzymatic (selenium, glutathione, vitamins A, C and E, ceruloplasmin, and GSH) antioxidants in the body which maintain physiological levels of free radicals [7, 8].

Heparin and ethylenediaminetetraacetic acid (EDTA) are anticoagulants widely used in clinical practice but due to their structural aspects they also act as antioxidants. Heparin is a highly-sulfated glycosaminoglycan. It is widely used as an injectable anticoagulant, and has the highest negative charge density of any known biological molecule [9]. It is used to form an inner anticoagulant surface on various experimental and medical devices such as test tubes and renal dialysis machines.

Heparin and heparin sulfates have anionic groups which can interact with cations in the physiological environment. So, these molecules can be thought of affecting free radical activity by modulating the cationic environment around the free radicals. Heparin inhibits Fe-catalysed free radical peroxidation of linolenic acid. It inhibits the generation of conjugated dienes and thiobarbituric acid responsive products in a reaction containing linolenic acid and iron [10]. Heparin mediates the release of superoxide dismutase in the proximity of the endothelial cells of the blood vessels. It also acts as a free radical sink and accumulates oxygen free radicals and the metal ions iron and copper that otherwise act as oxidizing agents [11, 12]. Since heparin is generally used as an anticoagulant in conditions such as acute coronary syndrome, atrial fibrillation, deep-vein thrombosis and pulmonary embolism, cardiopulmonary bypass for heart surgery and hemofiltration, investigations should be performed to estimate ROS levels as its administration in these conditions can decline the ROS levels below the normal physiological range and disrupt various physiological functions. As described earlier physiological levels of ROS are required for various processes in the body.

EDTA is a polyamino carboxylic acid. Its conjugate base is named ethylenediaminetetraacetate. It is widely used as a hexadentate ("six-toothed") ligand and chelating agent due to its ability to "sequester" metal ions such as Ca^{2+} and $Fe3^{+}$. EDTA finds its importance as a routinely used laboratory anticoagulant. After binding to EDTA metal ions lose their reactivity. EDTA is available as several salts, notably disodium EDTA and calcium disodium EDTA. Metal ions like Mn, Fe and Ni are involved in the dismutation of superoxide catalysed by super oxide dismutase. EDTA owing to its metal chelating property can interfere in the activity of this enzyme and others involved in ROS homeostasis in the body and hence can have profound effects on the internal ROS production and thereby adversely affect several physiological functions.

In this pilot study we measured ROS levels in the whole blood using a chemiluminescence method employing luminol as a probe. Whole blood was collected in EDTA vials and vials flushed with heparin and ROS levels were measured. A sharp decline in the ROS level was observed in the whole blood with EDTA as compared to whole blood using heparin as an anticoagulant. In this study we emphasize that while estimating ROS levels it is important to use heparin as anticoagulant rather than EDTA as it gives false low readings. In addition we issue a word of caution for clinicians who use EDTA for various therapeutic interventions that it may adversely affect several redox sensitive reactions and thus ROS levels should be monitored prior to and during the course of its use

MATERIALS AND METHODS

Subjects

The study included 15 healthy individuals falling in the age group of 20-35 years. Subjects with recent (3 months) history of fever, infection or systemic illness were excluded from the study. Proforma were filled and all subjects were enrolled after taking written informed consent and ethical approval.

Analysis of reactive oxygen species

Blood was collected by venoipuncture and 0.5 ml of the blood was transferred into microcentrifuge tubes containing heparin and 3.5 ml into EDTA vials. 0.5 ml of the blood from the EDTA vial was kept for ROS estimation and the rest was sent for the measurement of other blood parameters - total leucocyte count (TLC), differential count and erythrocyte sedimentation rate (ESR) - in order to adjust the chemiluminescence produced during one minute in relative light units (RLU) per 10^4 neutrophils. To measure the ROS levels in whole blood samples we adopted a luminol enhanced chemiluminescence method, with slight modification [6, 13]. Chemiluminescence was recorded using single detector luminometer (Sirius, Berthold Detection Systems GmbH, Pforzheim, Germany). Luminol acts as a signal amplifier molecule that reacts with oxygen species produced by neutrophils in the whole blood to produce an excited reaction intermediate. This excited reaction product emits light when it returns to its ground state. 400 µl of whole blood (with heparin and with EDTA) was taken in the tube and the readings were measured for 10 min. After 10 min, 10 µl of luminol (5-amino-2,3,-dihydro-1,4-phthalazinedione; Sigma-Aldrich, Czech Republic) was added to the tube and readings were recorded again for 10 min. The reading without luminol was subtracted from the reading with luminol to obtain the original value. H_2O_2 was used as a positive control. All measurements were done in triplicates and the average values were expressed as RLU/minute/10^4 neutrophil counts as neutrophils are the major source of ROS in the blood. The mean ROS levels of whole blood with heparin and with EDTA were compared using unpaired *t*-test and p value less than 0.001 was considered significant.

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Table 1. Blood parameters and whole blood ROS levels with heparin and EDTA

Subject number	Total leukocyte count (TLC, x 10^3/ml)	Neutrophil (N, %)	Lymphocyte (L, %)	Monocyte (M, %)	Eosinophil (E, %)	Basophil (B, %)	Erythrocyte sedimentation rate (ESR)	ROS levels with heparin	ROS levels with EDTA
1	5600	66	25	8	1	0.4	7	2020	3.5
2	6800	57.2	31.3	7.9	3.5	0.1	10	1086.4	0.87
3	4800	72	17.8	8.4	1.5	0.3	13	3140	2.8
4	8400	49	36.8	7.1	6.5	0.6	14	1409.2	3.4
5	8100	62.1	31.2	4.1	2.3	0.3	11	3440	46
6	8200	63.9	17.7	7.3	10.1	1	8	616.5	45.9
7	8400	74	21.1	2.8	1.8	0.3	11	276	1.6
8	7800	49	35	8	3	0.5	12	2618	2.1
9	8500	53	38	2	4	0.6	10	1438	1.7
10	5400	55	40	4	1	0.4	10	2866	4
11	7400	67	27	3	3	0.5	5	1682	4.5
12	9400	52	35	6	4	0.3	10	1235	1.67
13	6600	69	28	2	1	1	2	1433	1.8
14	6500	68	24	4	4	0.8	5	2161	3.1
15	8400	54	30	6	5	0.5	2	3939	6.2

ROS levels measured by luminol enhanced chemiluminescence assay and expressed as RLU/minute/10^4 neutrophils. A drastic decline in the ROS level was observed in the whole blood with EDTA. Normal Ranges of blood parameters: TLC, 4-11 x 10^9/l; N, 40-80%; L, 20-40%; M, 2-10%; E, 1-6%; B, <1-2%; ESR, 0-20 mm/h (modified Westergreen method)[14].

RESULTS

ROS levels were measured for both heparinized and EDTA whole blood samples. Chemiluminescence was observed every 1 second for 10 min and expressed as RLU/minute/10^4 neutrophils. The mean ROS levels in EDTA whole blood and heparinised whole blood were 8.6 and 1974.94 RLU per minute per 10^4 neutrophils, respectively. A sharp and significant ($p < 0.001$) decline was seen in the ROS levels with EDTA whole blood sample as compared to whole blood samples with heparin (Table 1).

DISCUSSION

Heparin and EDTA are anticoagulants which are widely used in clinical practice. Since years heparin was exploited as an anticoagulant only but now it is well established that it also plays significant roles in physiological processes like trophoblast function, megakaryocyte differentiation of bone marrow cells, and increasing the efficiency of embryo implantation in assisted reproductive technology. It is very well documented by various groups that heparin acts as a free radical sink and serves as an antioxidant. We have also found similar results on the inhibition of luminol dependent chemiluminescence in whole blood using heparin. Heparin is generally administered in conditions such as acute coronary syndrome, atrial fibrillation, deep-vein thrombosis and pulmonary embolism, cardiopulmonary bypass for heart surgery and hemofiltration to prevent the formation of emboli or clot due to its anticoagulant property. Since ROS are essential in various metabolic processes in the body ROS levels should be analysed before and after use of heparin keeping its antioxidant property in view, so that redox sensitive reactions are not adversely affected. It should be used in such quantities that its antioxidant effects are minimal.

Like heparin, EDTA is a widely used anticoagulant. EDTA is used as an antidote in cases of mercury and lead poisoning [15]. It is used to remove excess iron from the body. This therapy otherwise known as “chelation therapy” is used to treat the complication of repeated blood transfusions as in thalassemia. EDTA reduces oxidative DNA damage and lipid peroxidation [16]. EDTA finds its importance in evaluating kidney function. For this purpose chromium salt of EDTA [Cr(EDTA)] is given intravenously. Glomerular filtration rate is evaluated by monitoring the filtration of the complex [Cr(EDTA)] into the urine [17]. EDTA is used extensively in the analysis of blood. It is an anticoagulant of choice for complete blood counts (full blood examination). However in this study we found a sharp and significant decline in ROS levels in blood stored in EDTA and thus also highlight the need to increase awareness among clinicians that administration of EDTA for various clinical interventions should be done with caution as it can reduce ROS levels below normal physiological values adversely affecting various vital processes requiring normal ROS levels. So, there should be constant monitoring of ROS levels before and during EDTA therapy. Though both heparin and EDTA are used as anticoagulants however for estimation of ROS levels we believe that heparin is the anticoagulant of choice as EDTA caused a very significant decline in ROS levels.

The mean ROS levels detected using EDTA were significantly lower than that analysed using heparin as an anticoagulant and thus heparin should be used while estimating free radical levels in blood. We recommend that EDTA should be used with caution for various therapeutic interventions with constant monitoring before and during therapy as rapid and significant decline in ROS levels may disrupt and adversely affect gene transcription and several physiological functions.

CONFLICTS OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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