In Vitro and in Vivo Measurements of ROS Scavenging Activity and White Blood Cells Activity by Chemiluminescence of a New Selena-Diazole Derivative Compare to Dipyrone Activity

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Abstract. Reactive oxygen species ROS scavenging activity of whole Blood and White Blood Cells were measured. The estimation depends on the principle of luminol oxidation by ROS produced during phagocytosis. The study aimed to evaluate ROS production of Whole Blood and WBCs activity. CL measured using Luminol-dependent CL reader, and the recording system is Scaler Time ST7. The female rats in four groups received T and/or Di, or received only 2mL of DW for 30 days. CL Invitro antioxidant activity the result exhibited T&Di group was significant reduction in CL. Invivo CL of T group CL activity of whole blood was significant elevated CL than all groups. While T&Di group CL activity was reduced essentially than T, Di group; Functional WBC/100cells invitro CL activity of T&Di decreased significantly than DW CL while the Di, and T groups had no important changed than DW group WBCs CL activity. The results of WBCs invivo CL were T&Di activity was significantly higher than DW activity. Also, in Di the CL activity of WBCs significantly elevated than DW. While T the WBC activity showed non-significant alteration with DW. It was concluded that T compound had a potent ROS scavenging activity with reduced WBCs activity.

Keywards: selenadiazole, Dipyrone, Chemiluminescence, ROS, WBCs

1. Introduction

Vital physiological role of selenium is the potent antioxidant activity of many selenoproteins in mammals. Some of selenoproteins are well known functions, for example thioredoxin reductase (TrxR), glutathione peroxidase , and iodothyronine deiodinases, the functions of other proteins are not identified include selenoproteins T ,X, Y[1]. Synthetic compounds of

organoselenium have been planned to achieve larger chemo protection ability with less side effects[2]. Compounds of organic selenium have substantially greater bioavailability, significantly less toxic than inorganic compounds for example selenomethonine bioavailability more than selenite up to twice, also they are more effective that stimulated to synthesis, identifications and created of novel selenium pharmaceuticals used as anticancer, antimicrobials, and antioxidant[3]. Inadequate Se, principally Se-proteins, is related with frequent human diseases including malignancy, diabetes mellitus, cardio-vascular, and immune system conditions[4].

Antioxidants act at low concentrations and declining the capability of free radicles to react with surrounding cells and tissues. Antioxidants decrease energy of free atoms or give up their electrons render them more stable. Also, antioxidant may interrupt the oxidizing chain to reduce the destructive effects of active radicles[5]. The main source of all radicals in functioning cells in human and animal is the reaction of one-electron reduction of molecular oxygen, which is initiate production of superoxide anion-radical from either the cytoplasmic membrane NADPH-oxidase complex or membrane of endoplasmic reticulum[6].

Assay of whole-blood Chemiluminescence is a simple and rapid technique of reactive oxygen species by circulating leukocytes production measurement, used to assess the total antioxidant activity (TAA) in biological tissues like serum, saliva, and invitro such as in green tea. Whole-blood phagocytes may better reflect the host physiological state because isolation of cells can alter the viability, activity, and receptor expression of cells. Though, when the CL assesses is applied to whole blood, the measurement of CL intensity is related to numerous causes; First, Whole-blood assay as with isolated cells, is likely to be related to CL activity/phagocyte. The influence of monocytes CL activity to the Whole-blood a response is likely negligible because of their low numbers in the blood. So, this examine mainly measures the activity of PMNs. Second, the Whole-blood assay is related to blood PMN numbers with isolated phagocytes [7].

Luminol frequently defined as permeating cells, thus it identifies a number of intracellular ROS such as $H_2O_2^-$, HO^-, O_2^-, HOCl, furthermore RNS like NO^-, $ONOO^-$, intra and extra cellular. While lucigenin unable to permeate cells, thus it detects only extracellular O_2^-, in order to have a broad representation of ROS production, it must be used with other probes [8]. The potent (ROS) created by phagocytes is able to oxidizing luminol CL indicator, and production of CL's light bursts. Luminol can react with the (ROS) generated during Phagocytosis to produce an activated intermediate state that emits light upon returning to the ground state. Luminol-amplified CL activity can be simplified by a formula:
[Luminol + ROS peroxide catalyst N2+amino-phthalate ion +Light.]

This technique of luminol-amplified CL is a sensitive system, authorizing the use of less than 104 phagocytes per assay [9]. In addition CL measurement being a relatively rapid assay to perform, a main advantages of CL is that it can be achieved on low-volume sample of blood 1–50 µL. Additional advantage is that evaluation of the circulating phagocyte with no isolation required[10].

Dipyrone is a sodium salt of antipyrinyl methylaminomethane-sulfonic acid, in fact it’s a pro-drug, of pyrazolone derivative NSAIDs. It’s a white crystalline powder, identical soluble in both water and alcohol. Described as very effective antipyretic, painkiller, but neglected anti-inflammatory effects[11]. Dipyrone or metamizole is a contentious drug, due to the hazard of its agranulocytosis side effect; therefor it’s excluded for human use.

2. Materials and Methods

2.1 Antioxidant measurements by Chemiluminescence (CL)

CL measured using Luminol-dependent CL reader; comprise of two simple systems. Light detection system and recording system. Light detection system; this part used to identify the output of light, and to transmit it to electrical sign that can be measured. It consists of two parts; Glass cap (80mL), in which the CL solution and samples and
Photomultiplier tube (PMT) RCA313034-20, include cathode made up of Sb-CS3. The tube retained inside a tight cover to prevent the outside light.
The recording system: The recording system is Scaler Time ST7 made in British Nuclear Enterprise. The system includes high voltage supplier about 0-2 KV; high pulsation analyzer, and a time controlled by operations process.

2.2 Preparation of blood sample

Forty female rats divided in four groups, three groups received 50mg/kg BW of T and/or Di, while control rats received only 2mL of DW for 30 days. Blood collected with sodium citrate collected tubes. The tubes kept at 37ºC until the assay was start, which commonly measured within half an hour after collection. Also blood collected in EDTA anticoagulant tubes for WBC count.

2.3 Chemiluminescence (CL) measurement of ROS scavenging

The measurement depends on the principle of luminol oxidation by ROS produced during phagocytosis. Luminol-dependent CL in stimulated granulocytes in whole blood was measured using an ultra –high-sensitive photon counting system. The reaction mixture composed as (0.2) ml of (T) and/ or Di, was added to small beaker only invirto study. NaOH (100)µL with luminol (0.2)mL. Than whole blood (20) µL from untreated rats for in vitro study or from each group for invivo measurement.

2.4 WBC /100 cells activity measurement by CL

The results of CL in the peak height curve were estimated, the results analysis were performed with SPSS statistical software version 10. ANOVA analysis of variance probability value of < 0.05 was considered to be statistically significant.

$$\text{functional activity/ 100 cells} = \frac{\text{Peak hight}}{\text{WBC Count}} * 100$$

WBC count was using Hematology analyzer, Count60. All the measurements were estimated in mm peak height and related to the same number of cells i.e.(100cells) for the purpose of the comparison between the two groups[9].

3. Results

3.1 Invitro and invivo luminol CL measurements of whole blood scavenging of ROS activity:

It is apparent from this table (1) and figure (1) that showed invitro and invivo CL results as in figure (2). Invitro there was non- important decreased of (T) (170.250±70.007) and Di (170.77± 78.638) groups CL value than in DW group (193.28± 148.607). However T&Di group (101.183±23.071) showed significant reduction in CL.
In contrast Invivo CL activity showed an increment in CL activity of both T (349.62±67.522) and Di (239.067± 14.061) group than activity of DW (193.28± 148.607); but only CL activity T (349.62±67.522) was statistically significant. While, T&Di group CL activity was reduced non-significantly; it was illustrated in figure (2); nevertheless significantly reduced than T group value. Table (1) also, shows there is a significant difference between invitro and invivo for each treatment.

3.2 WBC /100 cells activity measurement by CL

In each experiment total blood volume (0.02 ml) was kept constant, but the WBC number for stimulation was varied. The amplitude of each peak was affected by WBC count, the highest peak (mm) divided on the WBCs count of the same sample. WBC/100cells invitro CL activity of T&Di decreased significant (2.153± 0.491) than DW CL (4.1123 ± 0.63237), while the Di and T had no important changed (3.633± 1.673) and (3.622± 1.489) respectively than DW group WBCs CL activity. The results were as illustrated in table (2).

WBCs invivo CL activity; T&Di activity was (18.838±15.048) which is significantly higher than DW group activity (4.1123 ± 3.162). Also in Di group the CL activity of WBCs significantly elevated to (8.954±5.283) than DW group. While T group the WBC activity (5.180± 1.000) shows non-significant alteration with DW g; however there was significant reduction of CL of WBC of (T) group than in T&Di groups. The results were revealed in figure (3), and (4)

Table (1) Shows invitro and invivo comparison of chemiluminscence of whole blood activity of different treatment groups of rats treated with Di and/or T compound.

Treated groups	Mean invitro CL of whole blood (µV)±SD	Mean invivo of whole blood CL(µV)±SD	T test significances
DW	193.28± 148.607 A	193.280± 148.607 A	NS
Di	170.77± 78.638 A	239.067± 14.061 A	S
T	170.250±70.007 A	349.62±67.522 B	S
T&Di	101.183±23.071 B	155.413±124.145 A C	S
LSD	69.067	83.653	

Means bearing different capital letters vertically differs significantly at 0.05 level (P<0.05).NS= Not significant, S= Significant horizontally (P<0.05)

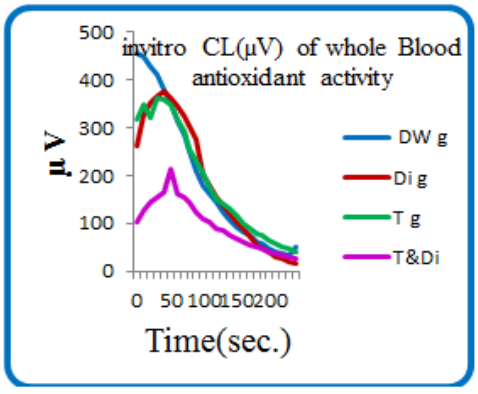

Figure1: invitro CL activity of Whole Blood

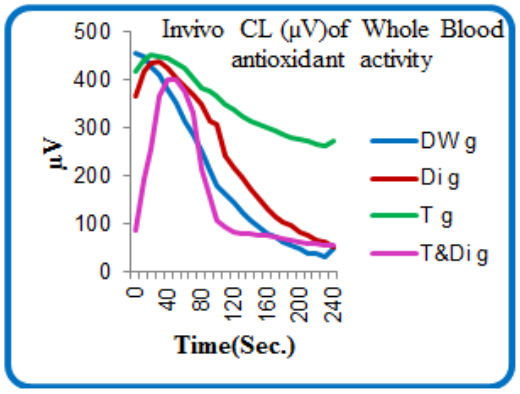

Figure 2: invivo CL activity of Whole Blood

Table (2). showed invitro and invivo functional Chemiluminescence WBC activity of different treatment groups of rats treated with Di and / or T compound.

Treatment group	Invitro CL of WBCs activity (mm/10^3*100/1000) Mean ± SD		Invivo CL of WBC activity (mm/10^3*100/1000) Mean ± SD		t- test siginificacy
DW	4.1123 ± 3.162	A	4.1123 ± 3.16	A	N.S
Di	3.633± 1.673	A	8.954±5.283	B	S
T	3.622± 1.489	A	5.180± 1.000	AB	S
T&Di	2.153± 0.491	B	18.838±15.048	C	S
LSD	1.47		4.841		

NS = Not significant, S= Siginificant horizontally at 0.05 level. Means bearing different capital letters vertically differs significantly at 0.05 level (P<0.05).

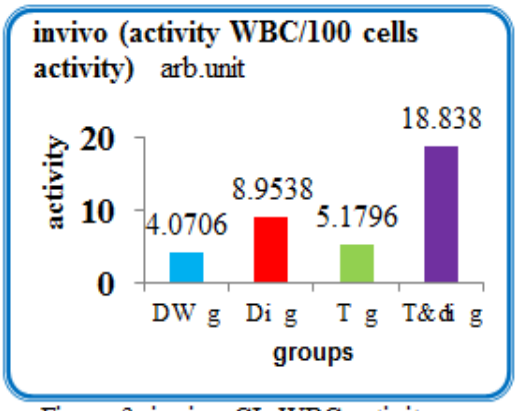

Figure 3: invivo CL-WBC activity

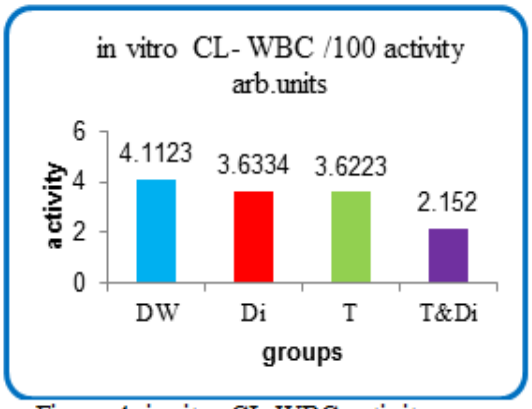

Figure 4: invitro CL-WBC activity

4. Discussion

4.1 Invitro and invivo luminol CL measurements of whole blood antioxidant activity:
Chemiluminescence CL has been used to estimate antioxidant and anti-phagocytes activity in biological liquids. The assay includes the CL substrate luminal, which used as a phagocyte CL activator, adding of luminol increases cells CL intensity to 1000 times [6]. Emission of light arises when hydrogen peroxidase oxidized luminol. This method can quantify capacity of the antioxidant substance that reduces the light output. This process includes the steps of: initiating and allowing a CL reaction development [12]. Pervious study conducted by [13] reported that measurement of CL rates must be within 15 min, because the production of ROS decrease intensely with 60 min, in the present study the time for sample CL measurement was directly measured and only first 4 min because Di group CL assay decline dramatically after 4 min. Also the temperature maintained about 37ºC, because phagocytosis directly related to environmental temperature [14].

CL can be actually sensitive in distinguish low-level reactions because it provides a detectable response under the detection limit of greatest chemical analyzes, the results affected by many factors such as blood dilution, environmental temperature, and time required for CL measurement. The reaction combination comprised of 2 ml CL inducer, NaOH (0.2 mL) and luminol (0.2 mL), in a (5 mL) beaker. whole blood (0.02 mL) was added to this mixture and agitated to mix well prior to transferring to photon counting system cuvette , where the temperature must be kept at 37ºC[15], also another study [14] conclude that, though the antioxidant might inhibit the CL by reducing Xanthine oxidase (XO), it inhibits oxidation of luminol, and reduce excited aminophthalate, in addition to antiradical effects, therefore some reactions need for CL inducer like Barium sulfate($BaSO_4$). Another factor affecting CL measurement was the low dilution of blood sample (1:5); various cellular components of blood influence the results. In the current study the blood dilution was 1:20 to minimized non-granulocytes WBC cells effects. Blood samples kept in 37ºC and examined within 10min after blood collection, the period for CL activity calculations restricted by first 4 min due to the limited activity of Di which decline rapidly. The differences between invitro and invivo CL activity might be due to long term treatment which affected the antioxidant activity.

However, there have been no controlled studies which compare differences in invivo and invitro CL activity, also the experimental data are rather controversial, few studied were carried to study both Dipyrone CL activity and there is no general agreement about whole blood CL activity of organo- selenium compound. However the results in the present study agree with study by [16], Se-deficient mice reduced H_2O_2 production and the least extent of CL activity, while Se-high mice high CL production. The invivo Whole Blood CL results showed significant increase in CL activity of T which might be due to long term administration results in increase selenium containing enzymes including GPx. Di also showed significant increased. A study by[17] agree with the results of present study, the authors reported that Di prevented ROO and HO-induced luminol oxidation in a concentration in a dose dependent manner.

4.2 CL WBC activity

CL depends on respiratory burst and leukocyte phagocytosis; all types of leukocytes have ability of respiratory burst and produced ROS. Oxygen-derived free radicals like $1O_2$ singlet molecular oxygen (SMO), O_2^- superoxide anion radical and OH^-hydroxyl radical are a very high reactive species. The determination of radical species is not simple due to different chemical properties recognition, their short lifetimes and evaluation is difficult. Non- isolated-granulocytes were verified for their phagocytic action in whole blood to reflect the invivo stimulation of granulocytes. This method of luminol improved CL is a sensitive system, allowing the use of > 10^4 phagocytes per assay[18](Bedouhène, Moulti-Mati et al. 2017).

Decline of Chemiluminescence strength derived from luminol and superoxide anion radical, created from xanthine oxidase (XO) has been studied as a screening technique for antioxidants determination in real samples[19]. CL procedure can quantify antioxidant capacity of a substance, the light output was reduced. In the present study CL activity of WBC reported significant differences between invivo and invitro results. Invitro WBC/100cells invitro CL activity of T&Di decreased significantly than DW group WBC/CL, while the Di and T groups had no important changed of WBCs CL activity than in DW group /WBC-CL.

WBCs invivo CL activity was T&Di group activity a significantly higher than DW group activity. Also in Di group the CL activity of WBCs significantly elevated than DW group. While T group the WBC activity showed non-significant alteration with DW group; however there was a significant reduction of CL of WBC of T group than in T&Di groups. All treated groups showed significant alteration between invitro and invivo WBC/100 cells activity. From the results it was clear that there was a paradoxical activity of WBC/100 cells of T&Di group invitro decreased significantly, while invitro highly increased than other study groups.

A study [20] concluded that addition of Se can avoid destruction through removal of toxic constituents and free radicals and also can reduce neutrophils production of ROS and lipid peroxidation, as the results of current study. These results of the present study also agree with the findings of study by [7], which included that Phenylselenocarbonyl)-piperidine and N,N-diethyl-4-chloroselenobenzamide also effectively scavenged (O_2^-). Selenaurea compound showed inhibitory CL activity, due to incubation with PMNs for 5 min prior to addition of stimulator.

A study of intra- and extracellular ROS was measured by using a luminol- CL assay ROS scavengers [8] recorded that Di reduced formation of extracellular ROS production by inhibition neutrophil extracellular traps(NETs) released, which depend on NADPH oxidase and myeloperoxidase (MPO)enzymes but no effects on intracellular antioxidant enzyme. From the previous data we can explain the antioxidant effects of dipyrone invivo. In contrast a study reported that Di active neutrophil burst inhibitors[17].

Conflict of interest: no thing declared

5. References

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