

## Hydroxyl Radical ( $^{\circ}\text{OH}$ ) Scavenger Power of Tris (hydroxymethyl) Compared to Phosphate Buffer

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### Abstract

Tris and phosphate buffer are regularly used in experimental investigations. These buffers might have radical scavenger properties toward different kinds of Reactive Oxygen Species (ROS) produced in solutions during chemical reactions like Fenton reaction and gamma radiolysis. Hydroxyl radicals ( $^{\circ}\text{OH}$ ) are the most reactive and oxidizing agents having a great potential in oxidization of macromolecules like DNA and proteins. This *in vitro* study was aimed to evaluate radio-protective effects of Tris and phosphate buffer toward hydroxyl radicals generated by Fenton reaction. Hence,  $^{\circ}\text{OH}$  radicals were produced using a mixture of Hydrogen Peroxide and Ferrous Sulfate, called Fenton system. Human serum albumin ( $500\mu\text{M}$ ) was prepared in Tris (10mM) and phosphate buffer (10mM), separately. These two samples were incubated with Fenton reaction (Ferrous Sulfate + Hydrogen peroxide) (10 mM) for 30 minutes and carbonyl groups were quantified by spectrophotometric carbonylation assay. The results of this study revealed the values of  $1.04 \pm 0.02$  and  $1.73 \pm 0.03$  for Tris and phosphate buffer treated samples, respectively. In conclusion, these findings confirmed that Tris buffer is a stronger radical scavenger toward  $^{\circ}\text{OH}$  radicals than phosphate buffer.

**Keywords:** ROS, Fenton reaction, hydroxyl radicals, carbonylation assay

### 1. Introduction

Since several years ago, Tris and phosphate buffer have been used prevalently in most of experimental investigations due to their powerful buffering function in the range of physiological pH. These buffers have been frequently selected in protection of many chemical and biological systems, and also used in several examinations linked to free radicals and oxidative stress *in vivo* and *in vitro* studies (Cullis, Elsy, Fan, & Symons, 1993; Good et al., 1966; Greenwald & Moy, 1980). It is well documented that buffers have a profound impact on the tertiary and quaternary structure of proteins (Ugwu & Apte, 2004). Moreover, antioxidant properties of some buffers are explained due to their metal binding affinity (Porasuphatana, Weaver, Budzichowski, Tsai, & Rosen, 2001). During the study of effects of oxidative stress on different biological systems and reactions of free radicals, which are generated after gamma radiolysis, with various macromolecules like DNA and proteins, it is completely visible that different types of buffers give unequal results which complicate interpretation of our findings. In radiation biology and radiation protection field, also, these buffers are crucial in preparation of protein, DNA and other macromolecules during irradiation. The study of the reaction of these macromolecules with hydroxyl radicals ( $^{\circ}\text{OH}$ ) was therefore initiated in several experiments (Hicks & Gebicki, 1986). It was cleared that sodium phosphate is a good choice for radiation biology studies, as this compound has no effect on radiosensitivity of DNA (Achey, Duryea, & Michaels, 1974). Moreover, the values of constant rate for HEPES, Tricine and Tris were represented  $5.1 \times 10^9$ ,  $1.6 \times 10^9$  and  $1.1 \times 10^9$  l.mol<sup>-1</sup>.S<sup>-1</sup> toward  $^{\circ}\text{OH}$  radicals, respectively (Hicks & Gebicki, 1986). Other investigations indicated that Tris buffer has radical scavenging capability toward  $^{\circ}\text{OH}$  radicals (Finkelstein, Rosen, & Rauckman, 1980; Saprín & Piette, 1977). Hydroxyl radicals are highly reactive in direction of interaction with macromolecules in physiological conditions (Cheeseman & Slater, 1993; Du & Francisco, 2008; Harman, 1992; Loizos, 2004; Pryor et al., 2006; Winterbourn, 1995). These radicals can damage proteins by creating protein backbone cleavages, making inter and intra molecular cross-links, generating carbonyl groups, conversion of free thiol groups (-SH) to other forms and so on (Anraku, Yamasaki, Maruyama, Kragh-Hansen, & Otagiri, 2001; Leeuwenburgh, Hansen, Shaish, Holloszy, & Heinecke, 1998; Plowman, Deb-Choudhury, Grosvenor, & Dyer, 2013). Human serum albumin includes 582 amino-acids with a molecular weight of 66KD

(Meloun, Morávek, & Kostka, 1975). The normal concentration of this protein in human plasma is ranged between 35 and 50 g/l. Albumin has several important functions in physiological conditions like transferring metals, fatty acids (Curry, Brick, & Franks, 1999), cholesterol, bile pigments, and drugs (SUDLOW, BIRKETT, & WADE, 1976; Vallner, 1977). As this protein is continuously exposed to oxidative stress, a crucial part of antioxidant power of the human body is attributed to HSA (Bourdon & Blache, 2001; Friedrichs, 1997; Taverna, Marie, Mira, & Guidet, 2013). Considering above information, this study was planned to compare °OH radical scavenger activity of Tris and phosphate buffer by measuring generated carbonyl groups on the most abundant protein in the humans blood plasma, called human serum albumin.

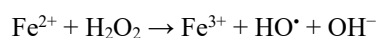
## 2. Materials and Methods

### 2.1 Materials

Human serum albumin (Sigma), Ferrous Sulfate (Merck), Hydrogen Peroxide (Merck), Tris (hydroxymethyl) (Merck), phosphate buffer (Potassium dihydrogen phosphate + Potassium hydrogen phosphate) (Merck), 2, 4 dinitrophenyl-hydrozine (DNPH), Hcl, Guanidine hydrochloride (GuHcl) (Merck), trichloroacetic acid (TCA) (Merck)

### 2.2 Fenton Reaction

Fenton reaction is a common method of generating highly reactive hydroxyl radicals as a final product in most chemical systems as follows (Thomas, Mackey, Diaz, & Cox, 2009):



In order to organize this reaction, a mixture of Ferrous Sulfate (10mM) and Hydrogen Peroxide (10mM) was incubated with human serum albumin (500µM) which have already been prepared in phosphate buffer (10mM) and Tris (10mM), pH 7, separately. An untreated sample was chosen as blank in each group. All experiments were carried out in triplicate.

### 2.3 Carbonylation Assay

This technique is a colorimetric method of measuring carbonyl groups which generate by oxidative stress (Luo & Wehr, 2009; Weber, Davies, & Grune, 2015). In this method, protein carbonyl groups are quantified as a result of interaction with 2,4-dinitrophenylhydrazine (DNPH) in order to create protein-bound 2,4-dinitrophenylhydrazones (Augustyniak et al., 2015; Luo & Wehr, 2009).

Here we describe the method of measuring DNPH content using a spectrophotometric assay proposed by Levine et al with some modifications (Levine et al., 1990). In the course of this technique, after oxidization of protein solutions, 200 µl of %0.2 DNPH (prepared in 2N HCL) was added to 200 µl of oxidized protein solution and incubated for 1hour in dark conditions, at room temperature. During that time, samples vortexed every 10 minutes. Protein pellets were sedimented by 20% TCA (ice cold) and spun at 10000rpm for 15 minutes. Washing step carried out three times using ethanol/ethyl acetate (1/1; v/v) in order to get rid of the extra of DNPH.

The acquired pellets were dissolved in 100 µl of 6M Guanidine hydrochloride and the absorbance was calculated by spectrophotometer at 370 nm against its blank (Hcl treated). The quantity of carbonyl groups in each sample and its control was measured by Beer-Lambert Law with the molar extinction coefficient for dinitrophenylhydrazine at 370nm (22000 M<sup>-1</sup> cm<sup>-1</sup>). The absorption values of DNPH treated samples were subtracted from blank and normalized to 15µM of protein. Finally, Carbonyl ratio was calculated by dividing carbonyl to protein concentration (mol/mol).

### 2.4 Data Analysis

All of statistical analyses were carried out using Microsoft office software (Excel 2007). Difference between two samples were analyzed by Student's *t*-test with the significance level at P<0.05. All results are shown as means ± SE.

## 3. Results

Solutions composed of different organic buffers can interact with °OH radicals with unequal speeds or constant rates (Hicks & Gebicki, 1986). Here, in figure 1, Carbonyl/Protein (mol/mol) ratio is illustrated following °OH radical oxidation of Human serum albumin.

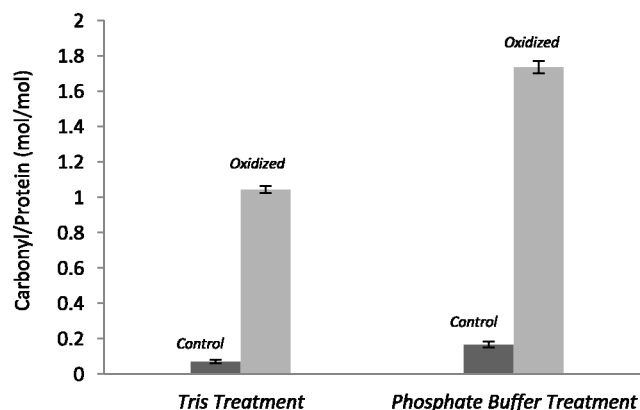


Figure 1. Carbonyl ratios of control and oxidized HSA treated with Tris and Phosphate Buffer

As can be seen in Figure 1, the ratio of carbonyl/protein is significantly increased in both Tris and phosphate buffer treated samples after treatment with Fenton reaction ( $p < 0.001$ ). Regarding the average values of carbonyl/protein in oxidized samples, these figures are  $1.73 \pm 0.03$  and  $1.04 \pm 0.02$  in phosphate buffer and Tris treated samples, respectively. Accordingly, phosphate buffer treated sample showed roughly 60% increase in carbonyl/protein ratio (mol/mol) compared to its counterpart after oxidization with  $^{\circ}\text{OH}$  radicals ( $p < 0.001$ ) (Figure 1).

Table 1. Protein carbonyl concentrations ( $\mu\text{M}$ ) in both Tris and phosphate buffer treated samples in control and oxidized samples

Type of Treatment	Protein Carbonyl Concentrations ( $\mu\text{M}$ )	
	Non-Oxidized	Oxidized
Tris Treatment	$1.06 \pm 0.02$	$15.90 \pm 1.74$
Phosphate Buffer Treatment	$2.52 \pm 0.18$	$26.46 \pm 1.5$

By comparing carbonyl concentrations in Tris and phosphate buffer treated samples after incubation with Fenton system it is clear that this figure is highly increased in phosphate buffer treated samples in comparison with that of tris samples ( $p < 0.001$ ).

#### 4. Discussion

Tris (hydroxymethyl) and phosphate buffer (Potassium dihydrogen phosphate + Potassium hydrogen phosphate) are the most extensively used buffers in biological and chemical studies, notably in preparation of protein solutions for radiation biology and chemistry experiments (Yukawa, Nagatsuka, & Nakazawa, 1983). In this fact, human serum albumin (HSA) is one of the most common proteins being investigated by scientists during recent decays (Kondakova, Ripa, & Sakharova, 1988; Maciazek-Jurczyk & Sulkowska, 2015; Sitar, Aydin, & Cakatay, 2013). A broad range of in vitro and in vivo methods have been launched to estimate radical scavenging power of buffers. Generation of formaldehydes was reported after  $^{\circ}\text{OH}$  radical oxidation of Tris (hydroxymethyl) buffer using a Fenton system (Shiraishi, Kataoka, Morita, & Umemoto, 1993). Nonetheless, formaldehyde production was revealed as a result of  $^{\circ}\text{OH}$  radical oxidation of dimethylsulfoxide (DMSO). It was suggested that the existence of formaldehyde depict the appearance of  $^{\circ}\text{OH}$  radicals in biological solutions (Klein, Cohen, & Cederbaum, 1980). Fenton system serves as a  $^{\circ}\text{OH}$  radical source in order to oxidize organic substances (Thomas et al., 2009; Zepp, Faust, & Hoigne, 1992). This reaction can generate advanced oxidation products (AOPP) and make conformational and structural changes on human serum albumin (Meucci, Mordente, & Martorana, 1991; Taverna et al., 2013). Carbonyl groups are one of the main generated end products during oxidative stress (Dalle-Donne, Rossi, Giustarini, Milzani, & Colombo, 2003). In this study, a spectrophotometric method for protein carbonyl assay was chosen to compare radical scavenger power of Tris (hydroxymethyl) versus phosphate buffer toward  $^{\circ}\text{OH}$  radicals. It is clearly demonstrated in *figure 1* that Tris buffer is a stronger radical scavenger than phosphate buffer toward  $^{\circ}\text{OH}$  radicals at  $\text{pH}=7$  in vitro. These results are in agreement with former results reported by Mark Hicks et al. They revealed that 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), Tricine (N-[Tris(hydroxymethyl)methyl]glycine) and Tris buffers are efficient radical scavenger with the rate constants

around  $10^9 \text{ M}^{-1}\cdot\text{s}^{-1}$  (Hicks & Gebicki, 1986). According to carbonyl/protein ratio in our results, it is clearly illustrated that this value in phosphate buffer is much higher than Tris buffer by a factor of 1.66 at the same concentrations (10mM). These findings confirm that under these conditions Tris buffer can prohibit  $^{\circ}\text{OH}$  radical damages more than phosphate buffer in vitro. However, there are numerous evidences that  $^{\circ}\text{OH}$  radicals might be generated heterogeneously in biological systems (Samuni, Aronovitch, Godinger, Chevion, & Czapski, 1983; Shinar, Navok, & Chevion, 1983), meaning that hydroxyl radicals could produce at some inaccessible sites to buffer substances in aqueous solutions. In conclusion, our findings suggest that the type of buffer can be considered as an interfering item with radiation biology or chemistry experiments. Complimentary investigations are needed to unravel the interaction of free radicals with buffers in detail.

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