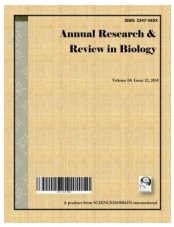

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Effects of pH Alteration on the Free Radical Scavenging Ability and Other Antioxidant Properties of Reduced Glutathione

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Authors' contributions

This work was carried out in collaboration between both authors. Author IJK designed the study. Author TO anchored the field study, gathered the initial data and performed preliminary data analysis. Author TO manage the literature searches and produced the initial draft. Both authors read and approved the final manuscript.

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ABSTRACT

Reduced glutathione (GSH), an endogenous antioxidant ubiquitously distributed in the physiological system has been widely reported to exhibit potent antioxidant properties *in vivo* and *in vitro*. However, information regarding the effects of altered pH on its widely reported antioxidant properties still remains scanty in the literature. Hence, the present study is geared towards unraveling the effect of altered pH on the antioxidant properties of GSH *in vitro*. This was done by measuring the effects of pH (4.4, 5.4, 6.4, 7.4, 8.4 and 9.4) on some antioxidant parameters including free radical scavenging properties against 2, 2 - diphenyl-1-picryl hydrazyl radicals (DPPH), ferric reducing properties, Fe^{2+}- chelating properties and inhibition of both deoxyribose

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degradation and lipid peroxidation. Results indicated that in all antioxidant indices determined, pH alteration did not have any significant effect on the antioxidant activity of GSH *in vitro*. From the foregoing, it is obvious that GSH is a potential antioxidant that could be exploited for the management of pH implicated pathological conditions such as acidosis and alkalosis.

Keywords: GSH; pH alteration; antioxidant properties; in vivo; in vitro; free radical.

1. INTRODUCTION

Glutathione (GSH), a relatively small intracellular thiol (SH) and ubiquitous tripeptide is currently one of the most studied endogenous antioxidant probably due to its central role as a water soluble reductant with vast clinical relevance [1]. Reports have indicated its roles in antioxidant defense, detoxification of electrophilic xenobiotics, modulation of redox regulated signal transduction, storage and transport of cysteine, regulation of cell proliferation, synthesis of deoxyribonucleotides, regulation of immune responses, and regulation of leukotriene and prostaglandin metabolism [2]. GSH has been reported as a metabolic regulator that can be used to measure health status in experimental animals [3]. In fact, GSH and its metabolites play a critical role in energetics and neurotransmitter biosynthesis, through several prominent metabolic pathways [4,5]. It also regulates the pro-inflammatory potential of several eicosanoids [5] and helps several tissues of the eye against photoradiation damage. Besides, GSH is so pivotal to the P_{450} detoxification enzyme system in the liver and other organs [2]. It is an essential cofactor for many sulfhydryl enzymes furnishing them with thiol reducing equivalents thereby keeping the redox-sensitive active sites on such enzymes in the reduced state due to its high electron-donating capacity and high intracellular concentration. It plays a role in the quenching of oxygen centered free radicals especially on DNA [6].

In humans, GSH depletion is linked to a number of pathological conditions [1]. Hence, in recent times, there is a growing awareness about its role in the pathophysiology of many diseases as a non-enzymatic antioxidant. Despite the wealth of information on the antioxidant properties of GSH, the effect of pH alteration on its antioxidant properties remains an enigma. Meanwhile, changes in the pH of a biochemical system/reaction can influence the structure of enzymes, their expression and activity [7].

The influence of pH on the redox state and bioavailability of some metals such as calcium, magnesium, zinc which are often essential for protein conformation cannot be overemphasized. Specifically, pH influences the bioavailability of sodium and calcium [8], which facilitates the flow of ions across neuronal membranes, thereby influencing action potential, rate of neuronal firing and ultimately fixation of memories. pH also plays a role in the absorption and bioavailability of chromium and the balance of iron as Fe II/Fe III [9,10,7,11]. Besides, low extracellular pH influences endocrine function, neural flow of oxygen, blood glucose level and metabolism, the accumulation of metabolic byproducts [12,13].

From the aforementioned, pH alteration is a common physiological phenomenon that could be triggered by several factors, including diseases. Hence, it is pertinent to unravel the effect of altered pH on the antioxidant properties of GSH as a way of measuring its capacity to offer pharmacoprotection in the face of metabolic disturbances.

2. MATERIALS AND METHODS

2.1 Chemicals

Reduced glutathione (GSH) and thiobarbituric acid (TBA) and DPPH were obtained from Sigma (St. Louis, MO). All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

2.2 Animals

Male adult Wistar rats (200–250 g) were used. Animals were kept in separate animal cages, on a 12-h light: 12-h dark cycle, at a room temperature of 22–24ºC, and with free access to food and water. The animals were used according to standard guidelines of the Committee on Care and Use of Experimental Animal Resources, Washington DC, eighth edition.

2.3 Free Radical Scavenging Ability

The free radical scavenging ability of the GSH against DPPH free radical was evaluated

according to the method of Gyamfi et al. [14]. Briefly, 600 µl of GSH (0–100 µM) was mixed with 600 µl, 0.4 mM methanolic solution containing DPPH radicals (pH 4.4, 5.4, 6.4, 7.4, 8.4 and 9.4). The reaction mixture was left in the dark at room temperature for 30 min before measuring the absorbance at 516 nm using Camspec M106 spectrophotometer from Camspec analytical Scientific Ltd. UK.

2.4 Fe^{2+} Chelating Assay

The Fe^{2+} chelating ability of GSH was determined using a modified method of Puntel et al. [15]. Freshly prepared 500 µM $FeSO_4$ (150 µl) was added to a reaction mixture containing 168µl of 0.1 M Tris–HCl (pH 4.4, 5.4, 6.4, 7.4, 8.4 and 9.4), 218 µl saline and GSH (0 –100 µM). The reaction mixture was incubated for 5 min, before the addition of 13 µl of 0.25% 1, 10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in a Camspec M106 spectrophotometer.

2.5 Reducing Property

The reducing property of the GSH was determined by assessing its ability to reduce $FeCl_3$ solution as described by Pulido et al. [16]. 250 µl of GSH (0–100 µM) was added to 250 µl, 200 mM sodium phosphate buffer (pH 4.4, 5.4, 6.4, 7.4, 8.4 and 9.4) and 250 µl of 1 g/100 ml potassium ferrocyanide. The temperature of the mixture was then raised to 50ºC for 20 min. Thereafter 250 µl, 10% trichloroacetic acid was added and the reaction mixture centrifuged at 650 rpm for 10 min. 250 µl of the supernatant was mixed with equal volume of water and 100 µl of 0.1% ferric chloride. The absorbance was later measured at 700 nm.

2.6 Deoxyribose Degradation

Deoxyribose degradation was determined as described by Halliwell et al. [17]. Deoxyribose is degraded by hydroxyl radicals with the release of thiobarbituric acid (TBA) reactive material. Deoxyribose (3 mM) was incubated at 37ºC for 30 min with 50mM potassium phosphate (pH 4.4, 5.4, 6.4, 7.4, 8.4 and 9.4) plus ferrous sulphate (0.1 mM) and/or H_2O_2 (1 mM) to induce deoxyribose degradation, and GSH at a concentration of 0 –100 µM. After incubation, 0.4 ml of TBA 0.8% and 0.8 ml of 2.8% TCA were sequentially added, and the tubes were boiled for 20 min at 100ºC. It was allowed to cool and absorbance was measured at 532 nm.

2.7 Thiobarbituric Acid Reactive Species (TBARS) Assay

Rats were decapitated under mild ether anesthesia and the brain and liver was rapidly excised placed on ice and weighed. Tissues were immediately homogenized in cold 50 mM Tris–HCl; (pH 4.4, 5.4, 6.4, 7.4, 8.4 and 9.4 (1/10, w/v). The homogenate was centrifuged for 10 min at 4000 rpm producing low-speed supernatant (S1) that was used for the assay.

An aliquot of 100µl of S1 was incubated for 1 h at 37°C in the presence of GSH (concentrations range of 0–100 µM), with and without the prooxidants; iron (final concentration (10 µM)) and sodium nitroprusside (SNP) (final concentration 30 µM). One rat brain and liver was used per experiment. Lipid peroxidation was measured by the method of Okhawa et al. [18] but the pH of the buffer for the color reaction was pH of 3.4. Colour development was done by the addition of 300 µl of 8.1% SDS to S1, followed by the addition of 500 µl acetic acid/HCl (pH 3.4) and 500 µl 0.8% of thiobarbituric acid (TBA) in sequence. This temperature of the reaction mixture was then raised to 95°C for 1 h. TBARS produced were measured at 532 nm.

2.8 Statistical Analysis

All values obtained were expressed as mean±SEM. The data were analyzed by Statistical Package for social sciences (SPSS) version 18 using appropriate ANOVA followed by Duncan's multiple range tests where appropriate and this is indicated in the text of results. The differences were considered significant when $p < 0.05$.

3. RESULTS

3.1 General Observation

Generally, the results show the effect of pH on the antioxidant properties of GSH. However, in order to explore the possible effect of pH on the antioxidant properties and mechanisms of GSH, different pHs were tested both in the acidic and basic media. In the acidic medium, pH 6.4, 5.4 and 4.4 were tested whereas in the basic medium, pH 8.4 and 9.4 were tested. However for simplicity and easy understanding, the data obtained for pH 4.4, 7.4 and 9.4 are shown whereas data obtained for pHs 5.4, and 6.4 are not shown. However, similar trend of results were obtained for pH 6.4, 5.4 and 4.4. In a similar vein, similar results were obtained for pH 8.4 and 9.4.

3.1.1 Effect of pH on the free radical scavenging activity of GSH

Fig. 1 shows the effect of pH on the ability of GSH to scavenge DPPH radicals. It shows that there was no significant difference in the free radical scavenging property of GSH both in the basic and acidic medium when compared to the neutral pH ($p < 0.05$).

3.1.2 Effect of pH on the iron chelating properties of GSH

The effect of pH on the ability of GSH to chelate Fe^{2+} is as presented in Fig. 2. Two-way ANOVA shows that GSH did not exhibit ability to chelate iron in the pH ranges tested ($p < 0.05$).

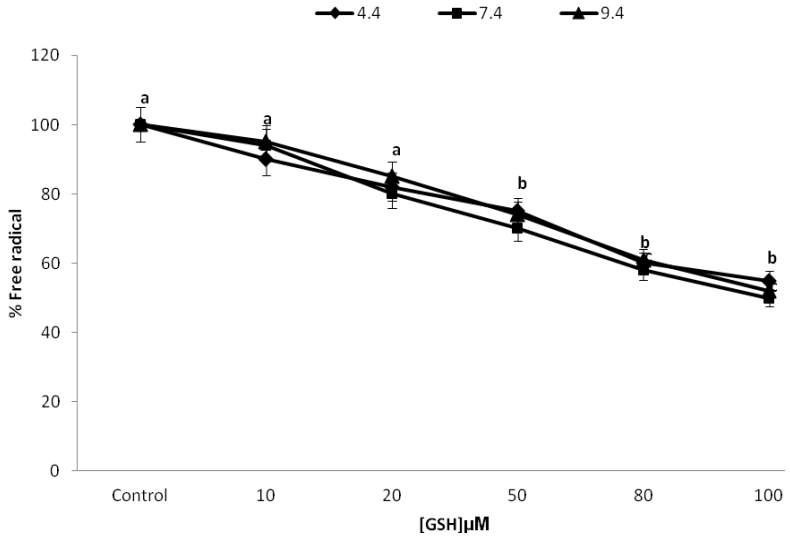

Fig. 1. Free radical scavenging property of GSH at various pHs. Data show means ± SEM values averages from 3 independent experiments performed in triplicate. 'b' indicate a significant difference from the control 'a' at $p < 0.05$

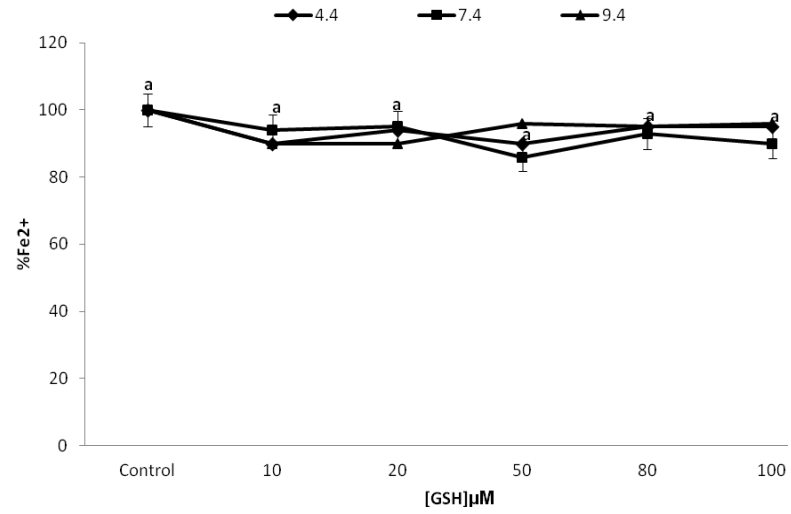

Fig. 2. Fe^{2+}-chelating property of GSH at various pHs. Data show means ± SEM values averages from 3 independent experiments performed in triplicate. 'a' indicates the control

3.1.3 Effect of pH on the reducing properties of GSH

Fig. 3 illustrates the effect of pH on the ability of GSH to reduce Fe3+ to Fe2+. The reducing property of GSH was not significantly altered in the various pH's tested. Two-way ANOVA revealed that there was no significant difference in the reducing property of GSH both in the basic and acidic medium when compared to the neutral pH ($p < 0.05$).

3.1.4 Effect of pH on the hydroxyl radical scavenging property of GSH

Fig. 4 shows the effect of pH on hydroxyl radical scavenging ability of GSH. It shows that GSH did not scavenge hydroxyl radical at all regardless of the pH.

3.1.5 Effect of pH on the inhibitory effects of GSH against lipid peroxidation

Fig. 5(a-d) shows the results of iron and sodium nitroprusside-induced oxidative assault on hepatic and cerebral lipids in the basic, neutral and acidic media. It was observed that irrespective of prooxidant or pH used, GSH exerted a significant inhibitory effect on lipid peroxidation.

4. DISCUSSION

Considering the critical role of acid-base balance in metabolic processes, an array of regulatory mechanisms must exist in the physiological system to keep the pH homeostasis of various compartments fairly constant. Any disturbance in pH balance in a normal healthy individual could trigger series of reactions that could culminate in the onset and complications of several pathological conditions. Interestingly, the physiological system is naturally endowed with buffers that help regulate and keep pH at optimal level required. However, recent reports have linked virtually all known diseases to the detrimental effect of free radicals which are produced during normal metabolism in the mitochondria [1]. To this end, there are various antioxidants that neutralize the destructive effects of these radicals. One of such is reduced glutathione which has been reported to be capable of relieving the body from oxidative stress [1]. However, the effect of altered pH homeostasis on its antioxidant properties is still largely unknown.

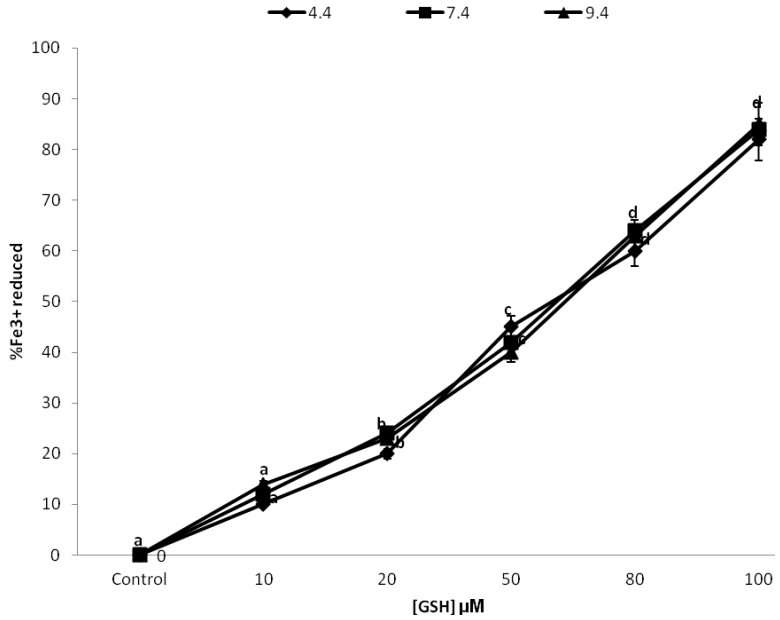

Fig. 3. Ferric reducing property of GSH at various pHs. Data show means ± SEM values averages from 3 independent experiments performed in triplicate. 'b', 'c' and 'd' indicate a significant difference from the control 'a' at $p < 0.05$

Meanwhile, the antioxidant capacity of potential antioxidant agents is routinely assessed in vitro by their ability to bleach the purple colour of DPPH turning it to a golden yellowish stable product which can be measured spectrophotometrically at 516 nm. Addition of GSH to DPPH at the various pHs tested caused a marked, concentration -dependent free radical scavenging effect at all pHs tested (Fig. 1). This observation suggests that GSH is a potent antioxidant whose radical scavenging capacity is not perturbed by alteration in the pH of the medium. This may further suggest that GSH would be a potential antioxidant candidate for the management of diseases whose complications often come along with pH alteration.

Apart from free radical scavenging, antioxidants are often identified using their ability to chelate transition metals such as (Fe^{2+}) which apart from its role in the transport of oxygen in the blood often act as prooxidant catalyzing free radical production processes. Fig. 2 showed that GSH did not chelate Fe^{2+} at any pH, suggesting that its antioxidant mechanism may exclude iron chelation, but could occur via other known antioxidant mechanisms. Hence, other antioxidant parameters were determined *in vitro*.

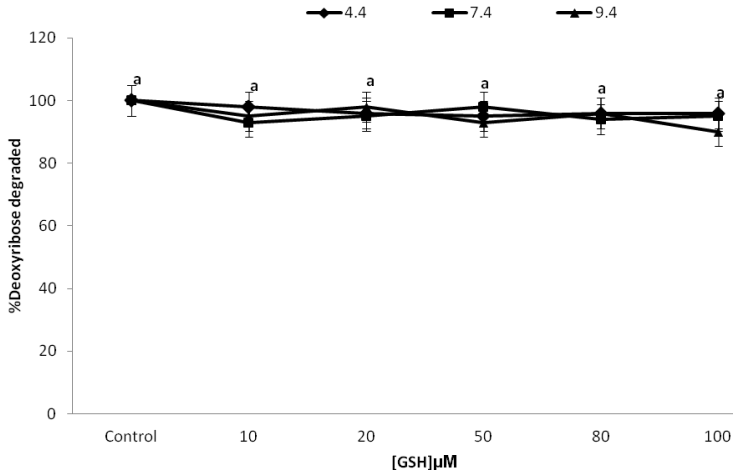

Fig. 4. Hydroxyl radical scavenging ability of GSH. Data show means ± SEM values averages from 3 independent experiments performed in triplicate. 'a' indicates the control

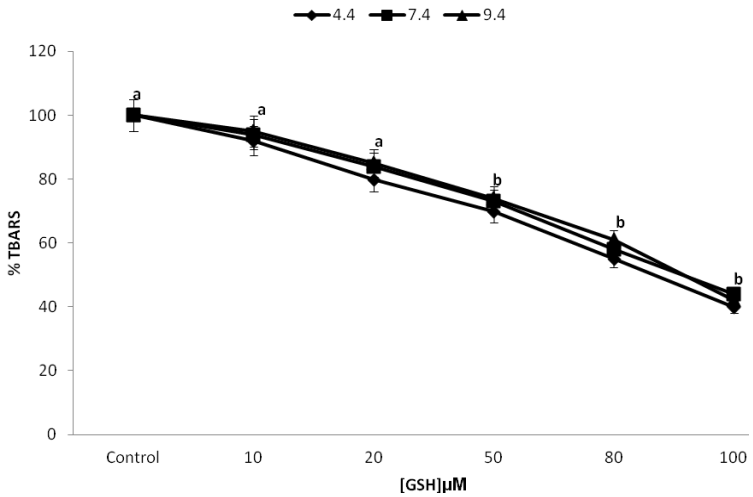

Fig. 5a. Inhibitory effect of GSH on Fe^{2+}- induced lipid peroxidation in rat liver. Data show means ± SEM values averages from 3 independent experiments performed in triplicate. 'b' indicates a significant difference from the control 'a' at $p < 0.05$

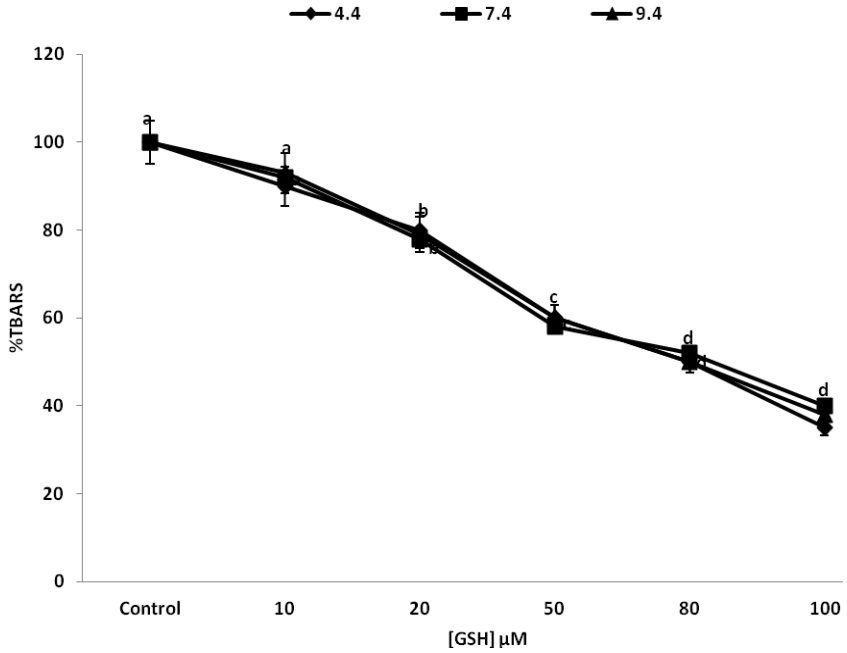

Fig. 5b. Inhibitory effect of GSH on Fe^{2+}- induced lipid peroxidation in rat brain. Data show means ± SEM values averages from 3 independent experiments performed in triplicate. 'b', 'c' and 'd' indicate a significant difference from the control 'a' at $p < 0.05$

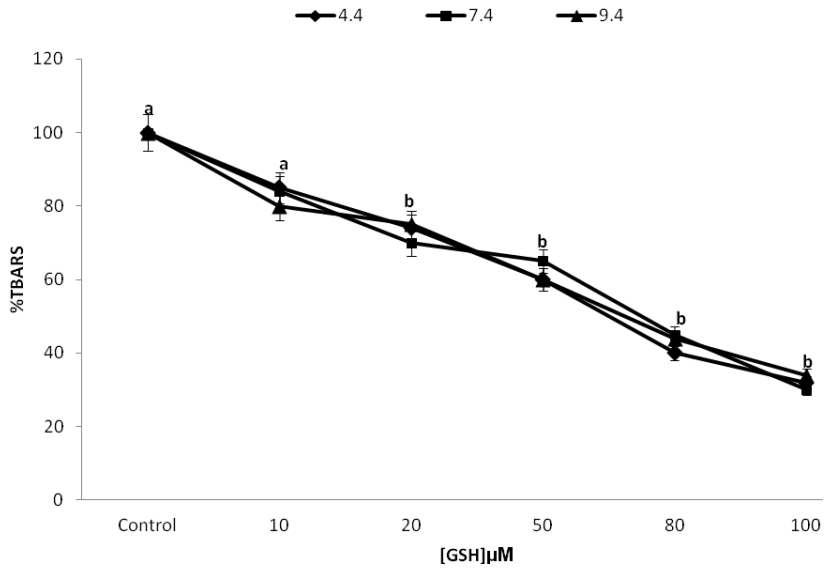

Fig. 5c. Inhibitory effect of GSH on SNP- induced lipid peroxidation in rat liver. Data show means ± SEM values averages from 3 independent experiments performed in triplicate. 'b' indicates a significant difference from the control 'a' at $p < 0.05$

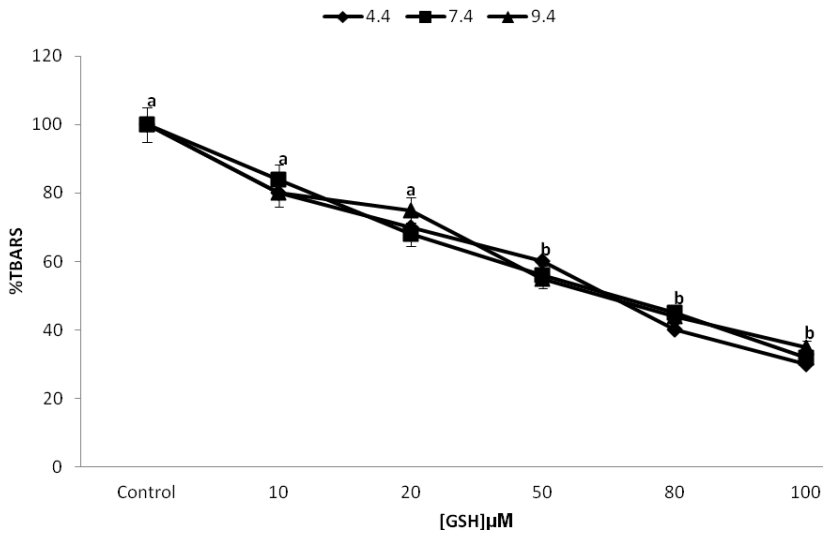

Fig. 5d. Inhibitory effect of GSH on SNP- induced lipid peroxidation in rat brain. Data show means ± SEM values averages from 3 independent experiments performed in triplicate. 'b' indicates a significant difference from the control 'a' at $p < 0.05$

The reductive capacity of any compound on Fe^{3+} causing its reduction to Fe^{2+} is thought to involve the donation of an electron and has been considered a potent antioxidant mechanism. Hence, any agent capable of reducing transition metals (in this case Fe^{3+}) is considered a potential antioxidant candidate that could be exploited to combat oxidative stress. Meanwhile, the fact that several endogenous antioxidant enzymes rely on the reducing power of GSH is enough to conclude that GSH is good reductant. Interestingly, Fig. 3 showed that GSH demonstrated a potent concentration dependent ferric reducing effect even at the least concentration used. Worthy of note is the fact that, altered pH has little or no effect on its reductive capacity as it exhibited significant concentration-dependent reducing effect at all pHs tested. This may explain why the GSH/ GSSG ratio is often used as a putative marker of the health status of an organism since the oxidation of GSH without concomitant reduction back to its reduced form could be a signal of oxidative stress.

Hydroxyl radical generated from the interaction between iron with hydrogen peroxide via Fenton chemistry are exceptionally reactive and could attack critical macromolecules especially DNA. The hydroxyl radical is the most reactive of the free radicals and in the presence of various transition metals (e.g. Fe^{3+}, Cu^{2+}) it is known to directly target cellular lipids, proteins, nucleic bases, causing DNA base modification or fragmentation [19]. Hence, any agent that can protect DNA or any of its constituents from free radicals is considered an antioxidant. Specifically, deoxyribose, which is the sugar moiety of DNA is highly susceptible to hydroxyl radical attack. Hence, a measure of the antioxidant capacity of agent is the ability to protect deoxyribose and by extension DNA from free radical attack. Unfortunately, GSH did not show any significant hydroxyl radical scavenging effect at all pHs tested showing that hydroxyl radical scavenging is not one of its antioxidant mechanisms. However, it is important to mention that, although GSH did not exhibit hydroxyl radical scavenging *in vitro*, it is required for the activity of several endogenous antioxidant enzymes including glutathione peroxidase, which is a potent scavenger of hydroxyl radicals. Since, these antioxidant enzymes would not be able to exhibit their potency in the absence of GSH, it could be considered an indirect hydroxyl radical scavenger *in vivo*.

Besides, free radicals have been found to react with macromolecules (lipids, proteins, DNA) within the cell, with one of the most frequent targets being the polyunsaturated fatty acids largely found in the cell membranes. The systematic oxidation of these polyunsaturated fatty acids is otherwise called lipid peroxidation.

Lipid peroxidation has been found to limit different aspects of muscle or cell function by decreasing the fluidity of the membrane, making it more difficult for proteins/nutrients to pass through [20]. Hence, any agent capable of inhibiting lipid peroxidation is a good antioxidant. In the light of these, two prooxidants (Fe^{2+} and SNP) were used to intentionally assault both cerebral and hepatic lipids thereby measuring the protective shield of GSH against lipid peroxidation at all pHs. Figs. 5a-d showed that GSH markedly inhibit TBARS formation in both cerebral and hepatic lipids at all pHs.

From the aforementioned, the potent antioxidant effect of GSH at various pHs may be related to its structure [21]. GSH is a tripeptide comprising of glycine, cysteine and glutamate. In solution, the various amino acids components especially glycine could act as buffers and furnish GSH with the capacity to remain unchanged irrespective of the prevailing pH. Furthermore, the presence of sulfhydryl group of the cysteinyl residue could also contribute immensely to its antioxidant capacity via its high reductive capacity which may be responsible for all the antioxidant properties of GSH *in vitro* and *in vivo*. Finally, GSH has four pKa values (pK_1 2.12, pK_2 3.53, pK_3 8.66, pK_4 9.12), two on both sides of the pH scale. This property may further enhance its stability in both acidic and alkaline environments thereby preventing alteration of its antioxidant property both in the acidic and alkaline regions. Hence, the antioxidant properties of GSH can be attributed to its component amino acids with its attendant pKa values.

5. CONCLUSION

pH alteration has no effect on the antioxidant properties of GSH. Hence, it could be exploited in the management of free radical implicated diseases associated with pH alteration.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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