

OXIDATIVE STRESS: NATURAL HISTORY AND MODULATION IN SURGERY
AND TRAUMA PATIENTS

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ABSTRACT

Oxidative stress has been associated with many disease conditions in adults and neonates based on clinical and post mortem studies. Trauma is the commonest cause of oxidative stress. However a gap in knowledge of the natural history of oxidative stress in humans was identified as most studies have been post mortem or in animals.

The aim of this research is to understand treat and oxidative stress in trauma and surgical patients. The study involved three components including: the development and evaluation of the novel oxistress assay; study of clinical trauma and oxidative stress; and clinical trial of alanyl-glutamine supplementation following major surgery. The novel oxistress assay was used on urine samples in the normal population to determine reference values and subsequently on hospital patients to determine sensitivity and specificity. The study of clinical trauma and oxidative stress evaluated plasma antioxidants (FRAP assay), red cell glutathione (Asensi's method), plasma and urine protein carbonyl (Levine's method) and total oxidants in plasma and urine (oxistress assay) over 7 day period following trauma. The clinical trial was a double blind study of 69 major surgery patients evaluating biochemical and clinical parameters over 7 day period in comparison with pre-operative status.

The novel oxistress assay proves to be a sensitive and accurate bedside diagnostic tool for oxidative stress. It can also be used in the laboratory setting. Oxidative stress is associated with increased trauma severity resulting in antioxidant depletion, strong oxidant production and protein degradation. The presence of pre-morbid medical factors also increased oxidative stress in trauma patients. Oral alanyl-glutamine supplementation (0.3 g/kg) increased plasma glutamine and antioxidant levels while decreasing urine oxidant levels. It significantly reduced hospital stay in non-cancer and higher disease complexity patients. The intervention also reduced the resource intensity weighting (RIW) score.

Oxidative stress is a clinical problem in surgery and trauma patients that can now be easily diagnosed at the bedside using the novel oxistress assay. Treatment with alanyl-glutamine is effective in reducing oxidative stress and improving clinical outcome. It is highly recommended probably at a higher dose in order to achieve optimal results.

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DEDICATION

This thesis is dedicated to my wife Aderonke who gave a lot of moral and physical support towards the completion of the study and my three children Fiyinfoluwa, Ibukunoluwa and Oluwafunmilola who also encouraged me and made huge sacrifices to ensure my completion of the PhD.

TABLE OF CONTENTS

PERMISSION TO USE	i
ABSTRACT.....	ii
ACKNOWLEDGEMENTS	iii
DEDICATION.....	iv
TABLE OF CONTENTS	v
LIST OF FIGURES.....	viii
LIST OF TABLES	x
1. INTRODUCTION	1
2. LITERATURE REVIEW.....	3
2.1. Concept and Components of Oxidative Homeostasis.....	3
2.2. Pathophysiology and Intrinsic Response to Oxidative Stress.....	4
2.2.1. Pathophysiology of Oxidative Stress.....	4
2.2.2. Intrinsic Response to Oxidative Stress	6
2.2.3. Trauma and Oxidative Stress	6
2.2.4. Neutrophils and Oxidative Stress	8
2.2.5. Sources of Antioxidants in the Body.....	10
2.3. Measuring Oxidative Homeostasis Components Applicable to the Study.....	11
2.3.1. Measuring Total Antioxidant Capacity in Plasma.....	11
2.3.2. Glutathione System	20
2.3.3. Methods of Measuring Protein Carbonyl in Biological Samples.....	22
2.3.4. Lipid Peroxide Measurements in Biological Samples	25
2.3.5. Urine Measurement of Oxidative Stress.....	27
2.3.6. Myeloperoxidase (MPO):.....	29
2.3.7. Plasma Elastase and Elastase Inhibitor:	29
2.3.8. Summary: Methods Used for the Study:.....	30
2.4. Modulation of Oxidative Stress	31
2.4.1. Elective Surgery and Oxidative Stress	32
2.4.2. Glutathione Cycle.....	32
2.4.3. Glutamine as an Intervention in Oxidative Stress	33
2.4.4. Enteral Feeding Versus Nil by Mouth.....	38
2.4.5. Clear Fluid Versus Regular Diet Post-Operatively.....	38
2.4.6. Enteral Versus Parenteral Nutrition	39
2.5. Evaluating Outcomes	41
2.5.1. Laboratory Related Outcome	41
2.5.2. Resource Intensity Weights (RIWs)	42
2.5.3. Estimation of Physiologic And Surgical Stress (E-PASS).....	43
2.5.4. Quality of Life	44
3. RATIONALE AND OBJECTIVES	46
3.1. HYPOTHESIS	46
3.2. RATIONALE.....	46
3.2.1. Reasons for Studying Oxidative Stress?.....	46
3.2.2. Reasons for Studying Trauma	46
3.2.3. Reasons for Measuring Oxidative Stress in the Clinical Setting:.....	47
3.2.4. Reason for Measuring Red Cell Glutathione	47

3.2.5.	Reasons for Measuring Plasma Antioxidants.....	48
3.2.6.	Reasons for Measuring the Total Oxidant in the Body.....	48
3.2.7.	Reasons for Measuring Plasma Protein Carbonyl:.....	49
3.2.8.	Reasons for Studying the Role of Neutrophils Activity in Oxidative Stress	50
3.2.9.	What is the Role Antioxidant Therapy after Surgical Trauma?	51
3.2.10.	Reasons for Evaluating Outcome	52
3.3.	Objectives of the Study	52
4.	METHODS	53
4.1.	Red Cell Glutathione Measurement.....	53
4.2.	The Novel Oxistress Method.....	53
4.2.1.	Principle of the Oxistress Assay.....	54
4.2.2.	Components of the Oxistress Assay.....	54
4.2.3.	Procedure for the Oxistress Assay.....	55
4.3.	Protein Carbonyl Method.....	56
4.4.	Neutrophil Count and Activity in Oxidative Stress	58
4.5.	Neutrophil Count	58
4.5.1.	Neutrophil Activity.....	58
4.6.	The Total Antioxidants in Plasma Using the FRAP Method.....	59
4.6.1.	Procedure for the FRAP Method.....	59
4.7.	Prospective Study on Antioxidant Therapy	59
4.7.1.	Study Design:.....	59
4.7.2.	Exclusion Criteria	62
4.7.3.	Potential Benefit of the Study	62
5.	THE NOVEL OXISTRESS ASSAY	63
5.1.	Principle of the Oxistress Assay	63
5.2.	The Oxistress Assay Development Process	64
5.2.1.	Standard Curve and Calibration of Assay	64
5.2.2.	Clinical Evaluation of the Oxistress Assay	66
5.2.3.	Further Evaluation of the Oxistress Assay: See appendix.....	70
5.2.4.	Discussion	71
6.	STUDY OF CLINICAL TRAUMA AND OXIDATIVE STRESS	73
6.1.	Introduction	73
6.2.	Red Cell Glutathione	74
6.2.1.	Red Cell Glutathione Measurement in Trauma Patients	74
6.2.2.	Discussion: Trauma and Red Cell Glutathione.....	80
6.3.	The Role Of Neutrophils in Trauma:	82
6.3.1.	Neutrophil Count and Trauma:.....	82
6.3.2.	Neutrophil Activity and Trauma:.....	86
6.4.	Strong Oxidants in Plasma and Urine (Oxistress Assay).	90
6.4.1.	Plasma Oxidants in Trauma	90
6.4.2.	Urine Oxidants in Trauma.....	92
6.5.	Plasma Antioxidant Reserve (FRAP Method).....	94
6.6.	Protein Carbonyl in Plasma and Urine.....	95
6.6.1.	Plasma Protein Carbonyl Analysis	95
6.6.2.	Urine Carbonyl Analysis.....	99

6.7.	Discussion: The Effect of Trauma on Plasma Oxidative Homeostasis: ...	102
6.7.1.	Plasma Antioxidants:	102
6.7.2.	Plasma Oxidants	103
6.7.3.	Plasma Protein Carbonyl:	103
6.8.	Discussion: Urine and Oxidative Homeostasis	104
6.8.1.	Urine Oxidants	105
6.8.2.	Urine Carbonyl	105
7.	THE PROSPECTIVE ENTERAL ANTIOXIDANT (ALANYL-GLUTAMINE) CLINICAL STUDY	107
7.1.	Introduction	107
7.2.	Demographics	108
7.3.	Results: The Biochemical Outcome of Glutamine Treatment	115
7.3.1.	Changes in Plasma Glutamine	115
7.3.2.	The Effect of Glutamine Treatment on Plasma Antioxidants	120
7.3.3.	The Effect of Glutamine Treatment on Red Cell Glutathione:	122
7.3.4.	The Effect of Glutamine on Strong Oxidants:	126
7.4.	Result: Clinical Outcomes of Glutamine Therapy	129
7.5.	Discussion:	137
7.5.1.	Demographics or Patient Selection or Study Population:	137
7.5.2.	Treatment Selection (Oral Alanyl-Glutamine):	137
7.5.3.	Biochemical Outcomes:	138
7.5.4.	Clinical Outcomes	141
8.	CONCLUSION	143
	REFERENCES	149
	Appendix to Chapter 5: Further Evaluation of the Oxistress Assay	173

LIST OF FIGURES

Figure 2.1: Concept of Oxidative Stress.....	3
Figure 2.2: Mechanism of Oxidative Stress in Trauma.....	7
Figure 2.3: Glutathione Cycle.....	33
Figure 4.1: Slide Showing Colour Reaction Of The Oxistress Solution With Different Concentrations Of Hydrogen Peroxide (Microtitre Plate Method).....	55
Figure 4.2: 1 Ml Cuvette Method Showing Colour Reaction Of The Oxistress Solution With Different Concentrations Of Hydrogen Peroxide	55
Figure 4.3: Levine’s Method & Modification	57
Figure 4.4: Effect of Various Methods Of Protein Analysis on Plasma Carbonyl Values.....	58
Figure 4.5: Study Protocol For Evaluating The Effect Of Enteral Antioxidant Therapy On Biochemical And Clinical Outcomes	61
Figure 5.1: Standard Curve of the Oxistress Assay	65
Figure 5.2: Oxistress Assay Colour Changes With Varied Hydrogen Peroxide Standards (8-16,000 μM).....	66
Figure 5.3: Plasma Peroxides at 10 & 100 mM Deoxyglucose Concentrations.....	67
Figure 5.4: Urine Peroxide Levels In Hospital Patients Vs. General Population.....	69
Figure 5.5: Peroxide Levels In Patients With Twice The Reference Values	70
Figure 5.6: Oxistress Assay (with EDTA) With Hydrogen Peroxide Standards.....	174
Figure 5.7: U-Curve of Oxistress Assay (with EDTA) With Various Dilutions of..... Hydrogen Peroxide Standards.....	175
Figure 5.8: Oxistress Assay (With And Without EDTA) With Hydrogen Peroxide Standards.....	176
Figures 5.9 A-D: Color Changes - Oxistress Assay (with EDTA) and 0.88 M..... Hydrogen Peroxide Standard	177
Figure 5.10: Colour Change In The Oxistress Assay (Without EDTA) Versus..... Hydrogen Peroxide Standards (Up To 2500 μM).....	177
Figure 5.11: Oxistress Assay (without EDTA) Reaction Over Time	178
Figure 5.12: The Effect Of Variable Deoxyglucose Concentrations In The Oxistress Assay.....	179
Figure 5.13: Glucose Versus Deoxyglucose In Oxistress Assay.....	181
Figure 5.14: Colour Changes With The Different Assay Storage Methods..... Storage Method Vs. EDTA-Free Oxistress Assay Reaction With Hydrogen Peroxide Standards:.....	183
Figure 5.15: Scattergram Of Various Storage Methods For EDTA-Free Oxistress Assay	183
Figure 5.16: Sustaining Color Change Of After The Reaction:.....	184
Figure 6.1. Red Blood Cell Glutathione (GSH) Levels In All Trauma Patients	77
Figure 6.2: Changes In Glutathione Levels At 6 And 24 Hr Versus Trauma Severity.....	78
Figure 6.3: Red Cell GSH Changes Versus Trauma Severity/ Preexisting Medical Problem.....	79
Figure 6.4: Red Cell GSH Changes Versus Body Region Affected By Trauma.....	80
Figure 6.5: Neutrophil Count (Mean \pm SEM) At 6hr Relative To Trauma Severity.....	84
Figure 6.6: Neutrophil Count (Mean \pm SEM) At 6 Hr In Head And Body Trauma.....	85

Figure 6.7: Neutrophil Count (Mean \pm SEM) At 6 Hr In Head And Spine Trauma	86
Figure 6.8: Plasma Neutrophil Activity Following Trauma	88
Figure 6.9: Neutrophil Activity Versus Trauma Severity	89
Figure 6.10: Changes In Neutrophil Activity In Organ Specific Trauma	90
Figure 6.11 Plasma Free Radicals Post Trauma (Oxistress Assay Method)	92
Figure 6.12: Urine Free Radicals Post Trauma (Oxistress Assay Method).....	94
Figure 6.13: Plasma Antioxidant Changes Over Time –FRAP Method	95
Figure 6.14: Plasma Carbonyl Content In All Trauma Patients	96
Figure 6.15: Plasma Carbonyl Content In Major and Minor Trauma Patients.....	97
Figure 6.16: Plasma Carbonyl Versus Trauma Severity	98
Figure 6.17: Plasma Carbonyl Versus Oxidant Levels In Trauma Patients	99
Figure 6.18: Grades Of Urine Precipitation.....	100
Figure 6.19: Appearance of Urine Precipitate With Trauma Severity Over Time	101
Figure 7.1: Age Distribution of Study Patients (Bar Chart).....	110
Figure 7.2: Age Distribution - Cancer Versus Non Cancer:.....	111
Figure 7.3: Treatment Specific Demographics - Cancer Versus Non Cancer:.....	112
Figure 7.4: Treatment Specific Demographics – Age Distribution:	113
Figure 7.5: Surgery Site - Treatment Versus Non Treatment.....	114
Figure 7.6: Changes In Plasma Glutamine Levels In All Patients.....	116
Figure 7.7: Plasma Glutamine Levels In Treatment Versus Non Treatment Groups	117
Figure 7.8: Changes In Plasma Glutamine At 24 Hr Post- Surgery.....	118
Figure 7.9: Effect of Treatment and Non Treatment at 72 hr Post-Surgery	119
Figure 7.10: Age Versus Changes In Plasma Glutamine Levels	120
Figure 7.11: Glutamine Treatment Vs Red Cell Glutathione At 24 Hr	123
Figure 7.12: Glutamine Treatment Vs Red Cell Glutathione At 72 Hr	124
Figure 7.13: Glutamine Treatment Vs Red Cell Glutathione (Cancer Versus Non Cancer At 24 Hr).....	125
Figure 7.14: Effect Of Glutamine On Changes In Plasma Strong Oxidants At 24 Hr....	127
Figure 7.15: Effect Of Glutamine On Changes In Urine Strong Oxidants At 24 Hr.....	128
Figure 7.16: Scatter-gram Of Glutamine Treatment Vs Urine Strong Oxidants at 24 Hr.	129
Figure 7.17: Complexity of Condition in Treatment Vs. Non-Treatment Groups	132
Figure 7.18: Complexity of Condition vs. Length Of Hospital Stay	132
Figure 7.19: Length of Hospital Stay Versus Type of Surgery	134
Figure 7.20: Glutamine Treatment Versus RIW Score	135

LIST OF TABLES

Table 2.1: Key Functions of Glutamine	35
Table 2.2: Equation	44
Table 6.1: Changes In Red Cell Glutathione Levels In The Patient Groups.....	75
Table 6.2: Changes in Plasma Oxidant after Trauma.....	91
Table 6.3: Changes In Urine Oxidant Levels After Trauma	93
Table 7.1: Patient Selection Process For The Alanyl – Glutamine Study.....	108
Table 7.2: Sex Distribution.....	109
Table 7.3: Age Distribution of Study Patients	109
Table 7.4: Surgery Type:	110
Table 7.5: Cancer Versus Non Cancer Patient Distribution:	111
Table 7.6: Changes In Plasma Glutamine At 24 Hr And 72 Hr Post-Surgery	119
Table 7.7: Plasma Antioxidant Capacity in Treatment and Non-Treatment Group At 0 hr And 24 hr (FRAP Method).	121
Table 7.8a: Glutamine Rx Versus Plasma Antioxidant Levels At 24hr (FRAP Method).	121
Table 7.8b: Glutamine Rx Versus Plasma Antioxidant Levels At 72 hr (FRAP..... Method)	122
Table 7.9: Preoperative Red Cell Glutathione Levels.....	122
Table 7.10: Glutamine Treatment Vs Red Cell Glutathione (Cancer Versus Non Cancer At 24 And 72 Hr).....	125
Table 7.11: Plasma Oxidant Levels at 0 hr, 24 hr And 72 hr	126
Table 7.12: Complexity of Condition Versus Plasma Glutamine Levels	131
Table 7.13: Complexity Versus Length Of Hospital Stay.....	131
Table 7.14: Effect oOf Treatment on Length of Hospital Stay in All Patients.....	133
Table 7.15: Summary Table- Effect of Glutamine Treatment in Cancer Versus Non- Cancer Patients.....	133
Table 7.16: Glutamine Treatment Or Complexity Versus RIW Score	136
Table 7.17: Glutamine Treatment And Complexity Versus RIW Score.....	136

1. INTRODUCTION

Oxidative stress is defined as a state in which the level of toxic reactive oxygen intermediates (ROI) overcomes the endogenous antioxidant defenses of the host. Toxic reactive oxygen intermediates (ROI) are produced by phagocytic cells following injury and inflammation of tissues as a mechanism to kill invading microorganism [1]. When inflammation becomes systemic as in inflammatory response syndrome there is loss of control of ROI production leading to non discriminant injury of tissues and organs in the host [1]. Oxidative stress has been shown to cause secondary damage through delayed cellular death and inflammation [2-4]. Therefore, minimizing oxidative stress may prevent cellular death, decrease inflammation, and prevent some morbidity and mortality [5].

Oxidative stress has been associated with many diseases such as diabetes, hypertension, heart failure, Parkinson's' disease [6], renal disease, epilepsy [7], Alzheimer's [8] and other neurodegenerative diseases [9] by clinical and post mortem studies. It is also implicated in acute medical and critical care evident by increased oxidant activity in the lungs of patients with acute respiratory distress syndrome [10] resulting in the development of multiple organ failure and death [11]. Oxidative stress is associated with many neonatal diseases such as retinopathy of prematurity, bronchopulmonary dysplasia, necrotizing enterocolitis and periventricular leucomalacia [12]. All newborns, particularly preterm newborns are highly susceptible to oxidative tissue damage which may be caused by strong oxidants [13, 14].

Trauma is the commonest cause of oxidative stress [15, 16]. Sheridan and associates showed that antioxidant therapy in burn patients reduced the length of time on mechanical ventilation and levels of malondialdehyde as compared to controls [17]. The consumption of endogenous antioxidants by free radicals resulting in oxidative stress may promote the development of multiple system organ failure (MSOF) after polytraumatic injury [18].

Modulation of oxidative stress involves various measures to decrease the level of ROI or increase the level of antioxidants, which has been done mostly in animal models. A clear understanding of the natural history in the human population is required in order

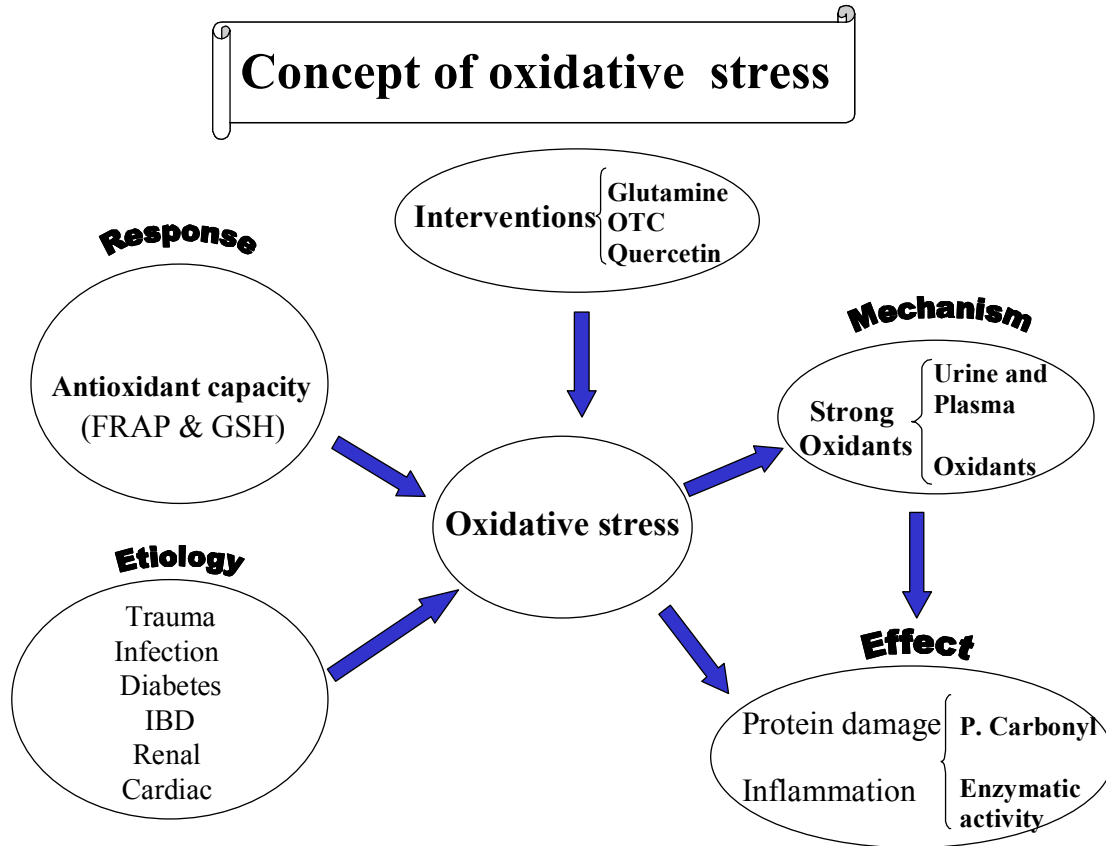
to effectively modulate oxidative stress. Trauma patients present the best study group not only because trauma is the commonest cause of oxidative stress but also because of the advantage of an identifiable onset of injury. The aim of this research is to understand the natural history and treat oxidative stress in trauma and surgical patients. This is based on the hypothesis that modulation of oxidative stress will improve patient outcome after trauma or surgery.

The study is divided into three components. The first component is the identification of clinically applicable methods for studying oxidative stress and subsequently understanding the natural history of oxidative stress after trauma. The second component is modulation of oxidative stress in surgical patients by the introduction of glutamine as a source of antioxidant during the postoperative course of the patient. The third component involves the development of new methods for measuring oxidative damage that should enable elucidation of the precise role of reactive oxygen species in a clinical setting.

2. LITERATURE REVIEW

2.1. Concept and Components of Oxidative Homeostasis.

Figure 2.1: Concept of Oxidative Stress.



Oxidative homeostasis is the way the body maintains the balance between free radical (oxidants) production and antioxidant reserve. ROI are produced as a result of normal physiological processes such as oxygenation (2%-3% of metabolized oxygen is also converted to superoxide anion free radicals, electron transfer and lipid metabolism. ROI are quickly reduced by antioxidants. Antioxidants are defined functionally as compounds that provide protection against the harmful effects of free radicals and other reactive oxidants. They may be found either in the circulation such as in plasma or red blood cells, or in organs such as the kidney and the liver. Oxidative stress is a

derangement of the normal physiological balance defined as a state in which the level of ROI overcomes the endogenous antioxidant defenses of the host [19].

Reactive oxygen species (ROS) is a collective term for radical or non-radical forms of partially reduced oxygen which include: superoxide, hydrogen peroxide, hydroxyl radical and hypochlorous acid [20-22], [23], [24]. ROS has been used interchangeably with ROI by different authors and the two terminologies are synonymous.

During illness, phagocytic cells, as a mechanism to kill invading microorganisms, produce ROI. ROI cause direct injury to cellular proteins and nucleic acids by oxidative damage, and also cause lipid peroxidation, leading to cell membrane destruction. They also act indirectly as secondary messengers in the intracellular signaling pathways of inflammatory cells. The most significant of these is through the nuclear factor κ B, which is a central transcription factor involved in the regulation of numerous pro-inflammatory genes [5, 25, 26]. When inflammation becomes systemic i.e. the inflammatory response syndrome, loss of control of ROI production may lead to nondiscriminant bystander injury in the host [1].

2.2. Pathophysiology and Intrinsic Response to Oxidative Stress.

2.2.1. Pathophysiology of Oxidative Stress.

Oxidative stress is the enhancement of the body's production of free radicals and other strong oxidants either by increased production of the radical or depletion of body stores of antioxidants. Free radicals can attack any biochemical component of the cell but lipids, proteins and nucleic acids are the most important targets. Free radicals generally react with the first structure they encounter- most frequently the lipid components of cell or organelle membranes [27].

Superoxide anions are produced by a number of cellular reactions, the most important of which is normal mitochondrial respiration, where a significant 1-3% of oxygen is partially reduced to the superoxide anion [28-30]. This proportion is increased during hypoxia and reperfusion following an ischemic insult [30]. Superoxide anion can also initiate the cascade of arachidonic acid metabolism, resulting in the formation of more superoxide and in the liberation of Fe^{2+} from ferritin stores [31]. The enzyme

superoxide dismutase (SOD) converts superoxide to hydrogen peroxide and molecular oxygen; however, superoxide can also spontaneously dismutate to form hydrogen peroxide and singlet oxygen, a strong oxidizing agent [20]. Hydrogen peroxide is a mild oxidant that readily crosses cell membranes. Hydrogen peroxide can dismutate to hydroxyl radical in the presence of transition metal ions such as Fe^{2+} [32]. The hydroxyl radical is the most powerful oxidant formed in the biological system with a diffusion ratio of 0.3 nm before it abstracts an electron from another molecule. A single hydroxyl radical and molecular oxygen can react with a poly-unsaturated fatty acid not only altering its structural and functional integrity but also generating multiple fatty acid peroxy radical which spontaneously reacts with other lipids, proteins, or nucleic acids thereby propagating a cascade of electron transfer and the consequent oxidation of these substances. The cell injury produced by lipid peroxidation of cell membranes can range from increased permeability to cell lysis. Proteins, both structural and enzymatic are also vulnerable to free radical mediated denaturation [33]. Toxic oxygen metabolites can also directly attack nucleic acids causing base hydroxylation, crosslinking or scission of DNA strands that can result in cell death and mutation [34]. The hydroxyl radical can therefore wreak havoc in cells by causing DNA strand breakage, oxidizing proteins, as well as by causing lipid peroxidation [35-37].

Lipid peroxidation is one of the major ways that ROS cause cell damage since peroxidation results in changes in fluidity, membrane permeability and may alter functions of proteins embedded in the membrane. The hydroxyl radical extracts a hydrogen atom from a methylene carbon of a polyunsaturated fatty acid forming a carbon-centered lipid radical. The lipid radical can interact with molecular oxygen to give rise to a peroxy radical. The peroxy radical is converted to a lipid hydroperoxide by extracting a hydrogen atom from a methylene carbon to form a new lipid radical, thus, initiating a propagating chain of lipid peroxidation. The lipid hydroperoxides formed are not innocuous entities since in the presence of iron complexes they decompose, forming alkoxy and peroxy radicals thus resulting in new chains of lipid peroxidation being initiated [38]. It is clear that iron in the presence of peroxides is a danger to the cell [38]. This peroxidation chain is generally ended when the lipid radicals interact with vitamin E forming a lipid alcohol and a tocopherol radical [37].

2.2.2. Intrinsic Response to Oxidative Stress

Sources of strong oxidants are hydrogen peroxide and organic peroxides. These peroxides are scavenged by glutathione peroxidase using reduced-glutathione (GSH) as the electron donor [39]. In addition, vitamins E (tocopherol) and C (ascorbic acid) play central roles in inactivating free radicals, in particular lipid radicals [40, 41]. Tocopherol inactivates the lipid peroxy radical forming a tocopherol radical and lipid peroxide. The tocopherol radical is inactivated by ascorbate resulting in the regeneration of tocopherol and the formation of ascorbate radical. The ascorbate radical dismutates forming ascorbate and dehydroascorbate, and the dehydroascorbate is subsequently reduced by GSH to form ascorbate and oxidized glutathione [42, 43].

It is clear that GSH plays a central role in the cell's defense mechanisms against oxidative stress. It is required by glutathione peroxidase to eliminate peroxides (compounds that can form powerful oxidants in the presence of transition elements). GSH is also required for the ultimate regeneration of vitamin E, a compound that plays a critical role in scavenging lipid radicals.

2.2.3. Trauma and Oxidative Stress

Injury is the commonest etiology of oxidative stress. This injury could be post-ischemic, reperfusion, heat, physical trauma, cold, excessive exercise, or exposure to toxin, radiation or infection. Trauma causes oxidative stress through three major pathways including necrosis, bacteria invasion and hypoxia. Each of the pathways results in activation of complement, polymorphonuclear cells and macrophages causing increased release of reactive oxygen intermediates (ROI). The release of ROI leads to further activation of neutrophils and macrophages. In addition hypoxia and shock increase the release of endothelin-1 and scavenging of nitric oxide (.NO) producing vasoconstriction, further hypoxia, ischemia, and subsequent release of tumour necrosis factor (TNF). These changes result in cell death or organ dysfunction [18, 44, 45]. Injury can give rise to systemic decreases in anti-oxidant capabilities beyond the tissues affected by the trauma. For example severe skin burn has been demonstrated to decrease brain GSH by 50-70% [46].

Figure 2.2: Mechanism of Oxidative Stress in Trauma

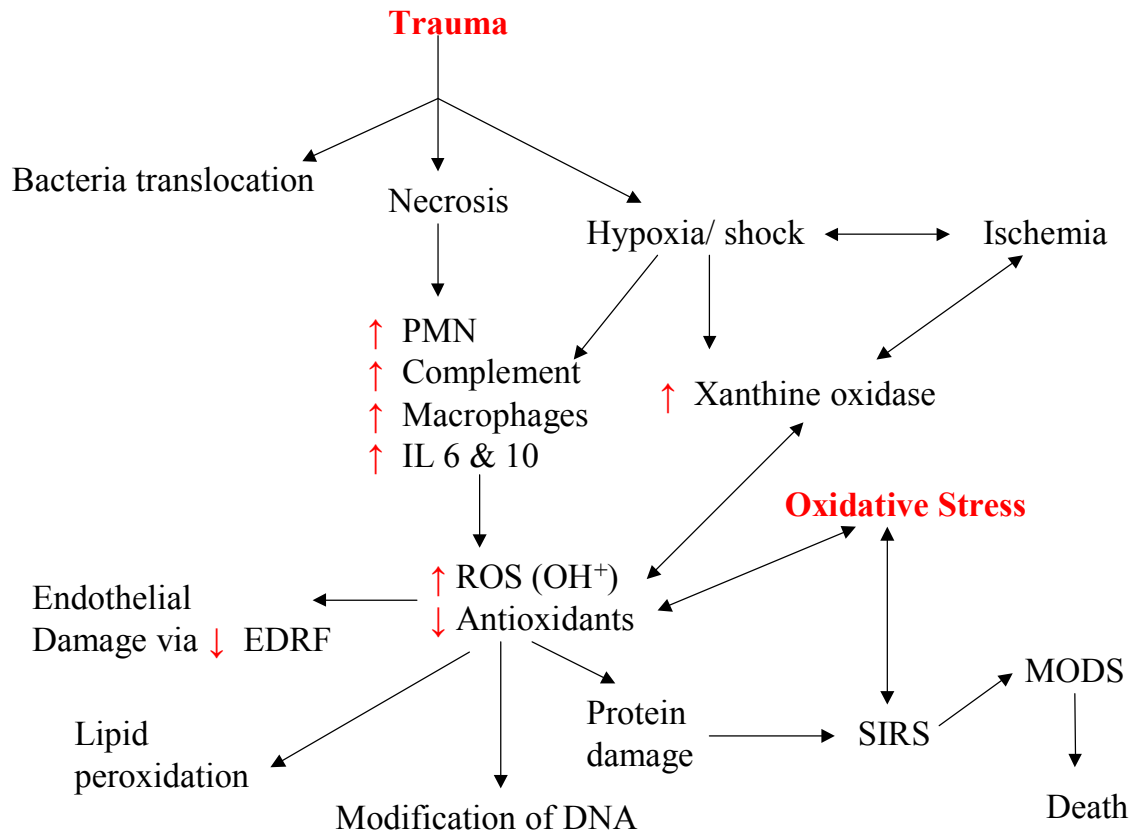


Figure 2.2: Graph outlining how trauma causes oxidative stress. PMN = polymorphonuclear cells; IL = interleukin; ROS = reactive oxygen species; SIRS= systemic inflammatory response syndrome; MODS = multiple organ dysfunction syndrome

Burns increase xanthine oxidase activity in the affected tissue and cause neutrophil activation. Animal studies have demonstrated decreased burn edema and lessening of distant organ dysfunction with the use of antioxidants, suggesting a cause and effect relationship [47]. There are clinical and experimental findings supporting the depletion of hepatic stores of glutathione by systemic stress, such as that produced by major surgery, starvation, sepsis, organ transplantation, and neurologic death [48, 49]. Sepsis, in addition to depleting glutathione also depletes the endothelial derived relaxing factor (EDRF). EDRFs are a group of substances such as .NO (nitrous oxide), prostaglandins, and

endothelium-derived hyperpolarizing factor (EDHF) produced by the endothelium to regulate the relaxation of the underlying smooth muscle layer and maintain vascular tone [50]. NO is a prominent EDRF as well as a potent antioxidant which terminates oxidant stress. It produces this effect by (i) suppressing iron-induced generation of hydroxyl radicals (.OH) via the Fenton reaction (ii) interrupting the chain reaction of lipid peroxidation, (iii) augmenting the antioxidative potency of reduced glutathione (GSH) and (iv) inhibiting cysteine proteases [51]. Sepsis causes endothelial damage from persistent and repetitive inflammation scavenging and inhibiting production of .NO [51-53]. Oxygen free radicals contribute significantly to reperfusion injury as well as to preservation injury. Severe oxidative stress by reducing hepatic GSH can lead to hepatocyte necrosis [54].

2.2.4. Neutrophils and Oxidative Stress

Neutrophils are formed in the bone marrow. The turnover of these cells is very high, approximately 10^{11} cells/day or 100 g/day [55]. Neutrophils contain four well-defined types of intracellular granules: azurophilic, specific, gelatinase (tertiary) and secretory vesicles. The azurophilic granules contain most antibacterial compounds such as myeloperoxidase, elastase, and neutral proteases. Fusion of these granules with phagocytic vesicles is important in bacteria killing. Elastase alters bacteria locomotion by hydrolysis of certain extracellular matrix components, neutral proteases damage the extracellular matrix and myeloperoxidase an oxidant combines with H_2O_2 and an oxidizable halide to form strong antimicrobial compound [55-57]. Specific granules are more readily released from the cell suggesting important function in the extracellular milieu. Some products of specific granules can activate complement cascade [58]. Specific granules contain collagenase, which may be important in hydrolyzing extracellular matrix, as well as apolactoferrin which binds iron and may exert an antibacterial effect by depriving the bacteria of iron [59-61]. Tertiary and secretory vesicles contain membrane proteins that can be rapidly upregulated to the cell surface and may play a role in the alteration of functional utility of surface proteins following stimulation [62-64].

Neutrophils also contain many lysosomes with a powerful hydrolytic and proteolytic potential. Normally, lysosomal enzymes along with oxidizing agents produced when phagocytosis is triggered, serve two main purposes: intracellular protein catabolism including the degradation of intra and extracellular endogenous substances and the degradation of phagocytized viruses and bacteria [65, 66]. Most lysosomal enzymes fulfil their physiological function in the cell with only small leaks but under certain circumstances major escapes occur [67].

Leakages could result from incomplete phagocytosis of large structures, or disintegration of phagocytes by toxins causing irreparable damage to the cell structure and function [57, 68-70]. Neutrophil rupture and the resulting leakages have been associated with severe inflammation and multiple injury [65]. A positive correlation exists between myeloperoxidase, elastase levels and the Acute Physiology and Chronic Health Evaluation 3 (APACHE3) score [71].

Neutrophils possess antioxidant mechanisms that protect against host injury by the potent products of phagocytosis. These include superoxide dismutase, glutathione peroxidase, catalase, vitamin E and ascorbic acid. They play a significant role in limiting tissue destruction caused by proteases however neutrophil oxidants are known to be the single most important direct mediators of immune injury [57].

The relevance of the absolute neutrophil count in trauma has been highly debated with two major theories. Some argue that the increased count is due to a balance between production and demargination rather than the trauma, while others argue margination and breakdown due to the trauma are responsible [72, 73]. There is however increasing evidence of a link between absolute neutrophil count and postoperative surgical stress or infection [74-78]. Inhibition of apoptosis (programmed cell death) and subsequent prolongation of inflammation have been suggested as the basis of the increase in neutrophil count [73, 79-82]. Mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF- κ B) activation have been identified as proximal events signalling the inhibition of apoptosis [83, 84].

2.2.5. Sources of Antioxidants in the Body

The main feature of living organisms is their capability to protect themselves against uncontrolled oxidation. All organisms are subject to the permanent influence of oxygen and other oxidatively active causes such as Ultraviolet (UV) sun radiation, atmospheric noxae (e.g. cigarette smoke, pollutants e.g ozone, ionizing radiation and xenobiotics i.e drugs such as paracetamol, bleomycin, anthracycline etc) [85-88]. Ultraviolet radiation is known to decrease the levels of antioxidants, inactivate the antioxidant enzymes and increase lipid peroxidation in the skin [85, 89, 90]. Atmospheric noxae deplete antioxidants and increase free radical formation especiall in the respiratory system [88]. Antioxidants play a critical role in health maintenance and the inhibition of oxidative damage. Therefore antioxidant activity could be defined as protection against oxidative damage and this is measured in terms of the antioxidative reserve or capacity in a biological substrate.

Antioxidants in plasma can be classified into two groups namely the water-soluble and lipid soluble. The water-soluble antioxidants include ascorbic acid or vitamin C, uric acid, protein thiols, and bilirubin. Plasma also contains very low levels of glutathione, which is a major intracellular antioxidant. The lipid-soluble antioxidants comprise of α and γ tocopherol, ubiquinol, lycopene, β carotene and some other carotenoids and oxycarotenoids [91].

The mechanisms of antioxidant protection can be classified into four categories: compartmentation, detoxification, repair and utilization. Compartmentation is both the spatial separation of potentially harmful but essential substrates (e.g. storage of iron in ferritin) and cell and tissue distribution of antioxidative compounds and serves the prevention of uncontrolled oxidation [87]. The most significant mechanism from the therapeutic point of view is detoxification because it results in the breakdown of free radicals to non-toxic substances (oxygen, water), thus protecting from many diseases. Detoxification of oxidative molecules i.e. radicals and peroxides, is ensured by enzymatic and non-enzymatic substances. The detoxification enzymes are present intra- and extra-cellularly and protect cells from the destructive side effects of free radical chain reaction [87]. Antioxidants repair by reverting the original changes due to free radicals when they are still reversible [40, 87, 92]. Utilization is considered as a secondary antioxidant

protection mechanism whereby further degradation of denatured and peroxidized potentially toxic proteins and lipids occurs [87].

Total antioxidant capacity is therefore the total ability of the body to protect itself from destructive side effects of physiological metabolism. The human plasma is often used for studying free radical induced damage because it contains critical targets of oxidative damage such as lipoproteins and many important antioxidants in the same physiological environment.

2.3. Measuring Oxidative Homeostasis Components Applicable to the Study

2.3.1. Measuring Total Antioxidant Capacity in Plasma

The concept of total antioxidant capacity of biological fluids is important because it determines the capacity of biological fluids to withstand oxidative stress. Most of the methods used for the measurement of antioxidant activity are inhibition methods except for the ferric reducing/ antioxidant power (FRAP) and total serum reductive capacity, which measure the reductive capacity of antioxidants. The inhibition methods involve two phases, the first in which there is free radical generation with an endpoint indicating the presence of the free radical. In the second phase the antioxidant activity of the added sample inhibits this endpoint by scavenging the free radical. The methods vary greatly with respect to the free radical that is generated, the reproducibility of the generation process and the end point used. The reductive methods do not generate free radical but measure serum ability to act against an oxidizing agent which was Fe^{3+} for the FRAP assay and phospholipase A_2 (PLA_2) for TORC. This literature review will focus on three inhibition methods and two reduction methods. The unit of antioxidant capacity is the trolox which is defined as the millimolar concentration of a substance that has the same antioxidant capacity as a millimolar of Trolox i.e. Trolox equivalent activity (TEAC) [93].

2.3.1.1. Total Radical Trapping Potential (TRAP) Method:

One of the most frequently employed procedures to evaluate the antioxidant status is the total radical trapping potential (TRAP) method developed by Wayner [94]. TRAP is defined as the number of molecules of peroxy radicals trapped per litre of fluid. Total antioxidant activity (TAR) describes the ability of an antioxidant to instantaneously

reduce chemiluminescence in the TRAP assay. It is based on the measurement of induction times in the oxidation of a lipid dispersion exposed to a free radical source with constant and known rate of free radical production under aerobic conditions [94]; [95]. This method was based on the discovery that the thermal decomposition of the water-soluble azo compound 2,2'-azobis (2- amidopropane) hydrochloride (ABAP) yields peroxy radicals at a known and constant rate. Each molecule of Trolox (Trolox™, Hoffman-LaRoche, Basel, Switzerland) α -tocopherol and or phenolic antioxidant traps two peroxy radicals giving the Trolox a stoichiometric factor of 2.0, in the TRAP assay. A serum sample is mixed with linoleic acid to prevent the termination reaction of two peroxy radicals occurring, and an aliquot (25-50 μ L) is added to the air-saturated buffer in the chamber of an oxygen electrode (Clark-Collip rate amperometric electrode). The reaction is initiated by the addition of ABAP and it is monitored until oxygen uptake is maximal. After 50% of the oxygen has been taken up, Trolox is added to calibrate the system and the oxygen uptake is further monitored. The total assay time for a single sample varies according to its antioxidant capacity, but that for serum takes approximately 90 min, the reference interval is 571-1284 μ M of TEAC [94].

A major problem with the original TRAP assay method lies in the oxygen electrode end point. An oxygen electrode will not maintain its stability over the period of time required (up to 2 hr) per sample. Such instability is more pronounced in plasma samples than with pure solutions. Hence a high degree of imprecision is inherent [95]. Other problems with the assay are that it is too lengthy to carry out precision monitoring or to permit analysis of large numbers of samples [95, 96].

There have been a few modifications to the original TRAP technique: the first involves the use of chemiluminescence, which lends itself to automation and is more precise. The process involves peroxy radical enhanced chemilinescent reaction. The addition of an antioxidant extinguishes the chemiluminescence. The duration of the chemiluminescence is directly proportional to the radical trapping ability of the antioxidant sample. A further modification is the introduction of an assay for the lipid phase using 2,2'-azobis (2,4-dimethylvaleronitrile) (AMVN) TRAP_{LDL} as the source of peroxy radicals [95, 97].

TRAP measures the levels of the following antioxidants: Uric acid (urate), Protein-sulphydryl groups (-SH), α -Tocopherol, Ubiquinol-10 [98, 99]. The chemiluminescence enhanced TRAP method has been proven to be reliable in evaluating the total activity of chain-breaking antioxidants in biological fluids. The chemiluminescence method is also less laborious because up to 24 samples can be processed in 8 hr using an LKP Wallac luminometer [98].

2.3.1.1.1. Pitfalls of the TRAP Method

There are numerous potential problems with the TRAP method:

- i. Uric acid gives the strongest contribution in the TRAP assay because of its high plasma concentration (120-350 μ M in healthy adults). In patients with hyperuricemia (e.g. goiterous state and non-insulin dependent diabetes mellitus) changes in the levels of other antioxidants may not be reflected in the TRAP value due to the high concentration of uric acid.
- ii. Plasma TRAP may not be the tool of choice for measuring oxidative stress because free radical production would probably have to be very extensive to disturb the systemic steady-state level of antioxidants. Antioxidant systems are also highly compartmentalized in their actions such as on the cell membrane (e.g. glutathione, superoxide dismutase, catalase) or in extracellular fluids (e.g. ascorbic acid, uric acid, albumin). Since the TRAP method like others does not measure all of these antioxidants it will not measure the total antioxidant capacity in systemic oxidative stress [98].
- iii. The rate of lipid peroxidation as induced by azo inhibitors has been shown to be dependent on the pH of the buffer used [99-101]. It is therefore important to tightly control the pH of the TRAP buffer in order to control the amount of peroxy radicals produced [98].

2.3.1.1.2. Clinical Utility of the TRAP method

The TRAP method has been used extensively in previous clinical studies.

- i. TRAP, TRAP_{LDL} and their main components have been used to study the effects of acute infection, diabetes, immobilization, Alzheimer's disease, coronary heart

disease, and cancer [98]. It was observed that plasma antioxidant defenses seemed to respond to the basic metabolic rate and the challenges caused by physiological or pathological stress.

- ii. During acute infection and immobilization, levels of ascorbic acid and α -tocopherol remained unchanged whereas the amount of TRAP_{UNID} in TRAP declined sharply [102, 103]. The plasma of NIDDM (non-insulin dependent diabetes mellitus) patients with CHD (coronary heart disease) had a significantly higher value of unidentified antioxidative potential than that of patients without CHD [104]. This relation was strongly dependent upon smoking, but plasma TRAP was not changed in diabetes and but reduced in patients with Alzheimer's disease [104, 105].
- iii. In lung cancer patients with a poor response to treatment, TRAP was reduced [106, 107]
- iv. The enhanced chemiluminescence assay demonstrated reduced serum antioxidant capacity in centenarians. The importance of this intriguing observation with regards to the aging process is yet to be determined [108, 109].

The chemiluminescence-enhanced TRAP has revealed important information in evaluating the antioxidant status of human plasma [98]. The total antioxidative potential cannot be evaluated reliably by measuring only TRAP because of its dependence on uric acid. TRAP combined with its main components is likely to give more valid information in determining the total antioxidant status [98].

2.3.1.2. The 2-2'-Azinobis-(3 Ethylbenzothiazoline-Sulfonic Acid (ABTS)

Method

The 2-2'-azinobis-(3 ethylbenzothiazoline-sulfonic acid (ABTS) method is a spectrophotometric method for measuring antioxidant capacity which is based on the inhibition of the absorbance of the radical cation of ABTS by antioxidants [95]. Antioxidant compounds suppress the absorbance of the ABTS radical cation to an extent and on a time scale dependent on the antioxidant capacity of the substance under investigation [93, 110]. The ABTS radical cation can be generated by the interaction of ABTS (150 μ M) with the ferrymyoglobin radical species, which is a product of activation of metmyoglobin (2.5 μ M) by H₂O₂ (75 μ M).

The ABTS detects antioxidants such as bilirubin, urate, ascorbate, α -tocopherol, albumin and glutathione [95, 96, 111]. The different analytical strategies for assay design are: decolorization assay, inhibition assay (fixed time point), inhibition assay (reaction rate), and lag phase measurement [95].

2.3.1.2.1. Advantages of the ABTS Method

A number of advantages of the ABTS method have become evident:

- i. It can be done either as an automated or as a manual assay [93, 96].
- ii. Analytical parameters of the assay i.e. intra assay and inter assay Coefficients of Variation (CV) are quite good. The CV interval ranges from 0.54%-1.59% in the intra assay and 3.6%-6.1% in the inter assay [95].

2.3.1.2.2. Disadvantages and Pitfalls of the ABTS Method

Unfortunately a number of disadvantages of this method also exist:

- i. It has a lag phase [112].
- ii. It requires fixed times in reading the reaction.
- iii. It is evident from the performance of the kit that even slight changes in reaction conditions have marked effects on the apparent contributions of individual antioxidants, notably albumin, thus any inference regarding contributions to total antioxidant activity in vivo must be done with even greater caution [96].
- iv. The ABTS method is strongly influenced by temperature [113]. It is necessary to define a precise analytical protocol that merits valid comparison of results between laboratories [96].
- v. The three key antioxidants of plasma (albumin, urate and ascorbate) inhibit the production of ABTS by differing mechanisms because the kinetics of chromophore generation may vary markedly. It is an essential requirement of any technique that proposes to measure total antioxidant capacity that all contributing antioxidants behave in an analogous manner. This requirement is clearly not met as previously discussed thus the result for the assay can only be regarded as semiquantitative.

- vi. ABTS is also affected by increased levels of urate production and the levels fall after dialysis [114]. This will therefore lead to false- positive results in hyperuricemia and false-negative results in hypouricemia.
- vii. It is dependent on the solubility of the substrates.

2.3.1.2.3. Clinical Utility of ABTS Method

- i. There is evidence using the ABTS method that preterms have significantly lower plasma antioxidant activity than term babies. The study showed that preterm babies required five days to stabilize their antioxidant levels to adult levels [93].
- ii. There is also evidence that mothers of preterm babies less than 32 weeks have reduced antioxidant capacity when compared with mothers of term babies [115].
- iii. There is some evidence of significant decreased total antioxidant capacity in HIV patients [116, 117].
- iv. There is some evidence that total antioxidant capacity is reduced in critically ill patients compared to controls, and it is also reduced in hemodialysis patients [118].

2.3.1.3. Oxygen-Radical Absorbance Capacity Assay (ORAC)

A relatively simple but sensitive and reliable method of quantifying the oxygen-radical absorbing capacity (ORAC) of antioxidants in serum using only a few microliters is described. In this assay system, beta-phycoerythrin (beta-PE) is used as an indicator protein, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) as a peroxy radical generator, and 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble vitamin E analogue) as a control standard. Results are expressed as ORAC units, where 1 ORAC unit equals the net protection produced by 1 microM Trolox. The uniqueness of this assay is that total antioxidant capacity of a sample is estimated by taking the oxidation reaction to completion. All of the non-protein antioxidants alpha-tocopherol, vitamin C, beta-carotene, uric acid, and bilirubin and most of the albumin in the sample are oxidized by the peroxy radical. Results are quantified by measuring the protection produced by antioxidants. This solves many problems associated with kinetics or lag-time measurements. There is linear correlation between the ORAC value and concentration of serum Trolox, vitamin C, uric acid, and bovine albumin. The coefficient

of variation within a run is found to be about 2% and from run to run about 5%. Trolox, alpha-tocopherol, vitamin C, beta-carotene, uric acid, and bilirubin completely protect beta-PE from oxidation, while bovine albumin protects beta-PE only partially. On a molar basis, the relative peroxy radical absorbance capacity of Trolox, alpha-tocopherol acid succinate, uric acid, bilirubin, and vitamin C is at a ratio (1: 1: 0.92: 0.84: 0.52.). Bovine albumin per unit weight has a lower peroxy absorbing capacity than these antioxidants [119, 120]. The ORAC assay requires more than 60 min to quantify results [121].

2.3.1.3.1. Clinical Utility of Oxygen-Radical Absorbance Capacity Assay

The study comparing nephrotic syndrome patients with controls demonstrates decreased free-radical trapping capacity of plasma that is inversely correlated with hypoalbuminaemia in the patients. This decreased total plasma antioxidant potential in combination with hyperlipidaemia may contribute to the increased risk of cardiovascular disease seen in nephrotic syndrome [122].

In diabetes the ORAC has been used to provide evidence of oxidative stress in patients showing a decrease in the antioxidant capacity as compared with the controls [123]. Other studies by Pieri and Testa [124] showed an involvement of oxidative stress in the glycation of haemoglobin especially in old diabetic patients, and provide support for the potential use of an antioxidant therapy in these patients, irrespective of their glycaemic control. Wang [125] and Cao [121] demonstrated using ORAC method that increased consumption of fruit and vegetables can increase the plasma antioxidant capacity in humans.

2.3.1.4. The Ferric Reducing / Antioxidant Power (FRAP) Method

The Ferric Reducing/Antioxidant Power (FRAP) assay is a direct test of “total antioxidant power” [112, 126]. The FRAP assay an assay that measures the ferric reducing ability of the sample [121]. It uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant present in the stoichiometric process. The FRAP assay measures the *total reducing power* of the antioxidant based on the fact that electron-donating antioxidants can be described as reductants. These

reductants inactivate the oxidants in a redox reaction: one of the species is reduced while the other is oxidized, and it is this total reducing power that is referred to as “total antioxidant power” [127].

At low pH, reduction of a ferric tripyridyltriazine (Fe^{III} - TPTZ) complex to a ferrous form results in the production of an intense blue color, and thus can be monitored by measuring the change in absorbance at 593 nm. The reaction is nonspecific, in that any half-reaction that has a lower redox potential, under reaction conditions, than that of ferric/ferrous half-reaction will drive the ferric (Fe^{III}) to ferrous (Fe^{II}) reaction. The change in absorbance therefore is directly related to the combined or “total” reducing of the electron-donating antioxidants present in the reaction mixture [112].

The ferric reducing/ antioxidant power and ascorbic concentration (FRASC) is a modification of the FRAP that quantifies three indices of antioxidant status: the total reducing (antioxidant) power, the absolute concentration of ascorbic acid, and the relative contribution of ascorbic acid to the total antioxidant power of the sample virtually simultaneously [112, 126]. This makes the FRASC more clinically relevant, however, this study employed the FRAP method because it was not deemed necessary to quantify ascorbic acid levels in plasma.

2.3.1.4.1. Advantages of the FRAP Method

The FRAP method as a measure of total antioxidants has certain advantages:

- i. It is a direct test of total antioxidant power unlike the previous methods which were all indirect tests [97, 99, 112, 119, 120, 128, 129].
- ii. It does not use lag phase type of measurement [127].
- iii. In FRAP assay, sample pre-treatment is not required, stoichiometric factors are constant, linearity is maintained over a wide range, reproducibility is excellent, and sensitivity is high [127].
- iv. FRAP can be performed automated, semi-automated, and manually [127].
- v. It does not require specialized equipment, skills, or critical control of timing and reaction conditions [127].

- vi. Uric acid accounts for most of the antioxidant capacity measured by all methods and this could affect the accuracy of the obtained results. The FRAP and its modification, FRASC have corrected for the high (60%) uric acid contribution.
- vii. The ferric reducing/ antioxidant power and ascorbic acid concentration (FRASC) supply three indices of antioxidant status: the total reducing (antioxidant) power, the absolute concentration of ascorbic acid, and the relative contribution of ascorbic acid to the total antioxidant power of the sample virtually simultaneously [126, 127].
- viii. It can be used for a wide range of biological fluids [127].
- ix. Analytic performance of FRAP was quite satisfactory with the coefficient of variation of less than 1% within runs and less than 3% between runs. The FRASC was very good and the results were consistent [127].

2.3.1.4.2. Disadvantages of the FRAP Method

The use of ferric ion (Fe^{2+}) as final product in the FRAP assay may cause problems when an analyzed antioxidant such as ascorbic acid, not only reduces Fe^{3+} to Fe^{2+} but can react with Fe^{2+} to generate additional free radical [121]. FRAP does not also measure the SH-group-containing antioxidants such as plasma proteins, which could potentially limit its clinical utility [121].

2.3.1.5. The Total Reductive Capacity (TORC) Assay

This is a simple fast substrate based assay developed by Mayer et al (2001) to measure the antioxidants in serum [130]. The assay uses a thiol-labelled arachidonic acid as substrate for the reductive action of tripeptides such as glutathione. The thiol-labelled arachidonic acid is suspended in a buffer containing hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), glycerol and Triton-X-100. The substrate (100 μl) and equal volume of the standard or serum are added to a microtitre plate and incubated at room temperature for 15 minutes. The standard is bee venom phospholipase A_2 (PLA_2). When coupled with monochrombimone serum free thiols (glutathione etc) produce fluorescence measured using a regular fluorometer and compared with a standard curve. The assay has >7% interassay variation and a 97% retrieval rate. The normal levels in

healthy population range from 100-120 ng/ml. This method was found to of predictive value in evaluating patient outcome post-trauma.

2.3.2. Glutathione System

Glutathione (L- γ -glutamyl-cysteinyl-glycine; GSH) is an α -amino acid as well as tripeptide, it is formed from glutamine, cysteine and glycine [131]. This compound is widespread in animal tissue, plants and microorganisms; it is therefore the most prevalent cellular thiol and most abundant low molecular weight peptide. There is a wide variation in the concentration of glutathione in cells and organs. Red blood cells have \sim 1 mM [132]; lens has 10 mM [133]; liver has 5-7 mM [134]; kidney, heart and lung have 2-3 mM [134]; the brain and the spinal cord have concentration as low as 1-2 μ Mol/g [135] (\approx 1-2 mM), plasma 23 μ M [136].

Glutathione has a number of functions including metabolism, catalysis, transport and protection of cells against oxidation [132, 137-139]. Glutathione reduces disulfide linkages of proteins and other molecules in the synthesis of the deoxyribonucleotide precursors of DNA. It also plays a role in the inactivation of a number of drugs and in the metabolic processing of certain endogenous compounds such as estrogens, prostaglandins, and leukotrienes [137-139]. Glutathione acts as a coenzyme for several enzymes (catalysis). Glutathione is involved in transmembrane transport of amino acids particularly cysteine (and glutamine) which is regarded as the limiting amino acid for glutathione metabolism and for protein synthesis [137, 140]. Glutathione protects cells against the effects of free radicals and ROS (e.g peroxides) formed during metabolism [18, 137, 139, 141].

In this study changes in red cell glutathione was measured based on the hypothesis that trauma results in depletion of the red cell glutathione defense system. In addition, the red cell requires significant extracellular oxidative stress to produce depletion in its glutathione level thus making red cell glutathione a reasonable index of oxidative stress. The red cell also transports glutathione from organs such as liver and kidney to areas of need [142].

The red cell has concentration of glutathione ranging from 0.6 mM to about 2 mM [131, 143]. This concentration is about 100 times the concentration found in plasma. The

red cell only requires about 30%- 40% of the activity of the glutathione reductase to maintain normal levels of glutathione. Trauma causes the release of free radicals in the body leading to consumption of antioxidants in extracellular fluid and subsequent depletion of red cell glutathione [42, 132, 144]. This results in increased production of oxidized glutathione, which is reconverted to reduced-glutathione in the presence of NADPH and glutathione reductase.

When there is severe oxidative stress the rate of depletion of reduced glutathione is faster than the production resulting in an efflux of oxidized glutathione from the cell into the plasma [145, 146]. This is measurable as a reduction in the total red cell glutathione and has been associated with multiple organ failure and death [145].

2.3.2.1. Enzymatic Methods of Measuring Reduced Glutathione

There are several ways of measuring glutathione in the red cell. Some involve calculating the difference in the ratio of the reduced-and-oxidized glutathione to reduced glutathione using the Ellman's reagent (DTNB) from fresh blood and this could be read using a spectrometer at a wave length of 320 nm [147, 148]. Reed [149] measured GSH, GSSG and other thiols by making derivatives of free thiols with iodoacetic acid followed by conversion of free amino groups to 2,4-dinitro phenyl derivatives and HPLC separation and measurement.

The Asensi's modification of Brigelius's method used in the study is a colorimetric method based on the conjugation of chlorobenzidine with glutathione and is catalyzed by glutathione-S-transferase. The adduct formed, S-(2,4-dinitrophenyl)glutathione exhibits its maximum absorbance at 340 nm. The specimen is pre-treated with Trichloacetic acid (TCA) and the supernatant is stable for 1 wk when stored at -20⁰C [150, 151]. The Asensi's modification of Brigelius's method provides an end point reading that can measure as low as 0.2 nm in the cuvette which corresponds to 0.3 μ M GSH [152].

Brown and Armstrong [153] modified of the Beutler [154] method of measuring reduced glutathione. This involves the addition of meta-phosphoric acid / EDTA and sodium chloride as a stabilizing solution to fresh blood samples. The mixture is centrifuged and the supernatant can be stored at -80⁰C. It is stable at this temperature for

one year and the glutathione content is measured using HPLC. This method was used by Richie et al [132] in a large-scale study with an absolute limit of 5 pmol and CV interval of 2.3%. Other methods using the HPLC include the N-(1-Pyrenyl) maleimide (NPM) assay which is a rapid simple method for analyzing both oxidized and reduced glutathione as well as other thiols including cysteine, γ -glutamyl-cysteine, homocysteine, cysteinylglycine and N-acetylcysteine. The assay provides a sensitive and accurate means of correlating thiol status with alteration in metabolism, signal transduction and gene expression.

2.3.3. Methods of Measuring Protein Carbonyl in Biological Samples

All proteins are subject to modification by Hydroxyl radicals ($\cdot\text{OH}$) or Hydroxyl and Oxygen radicals ($\cdot\text{OH} + \text{O}_2^-$). The modifications includes alteration in molecular weight and net electrical charges [155] of which protein carbonyl is a product. Carbonyl formation is believed to be a result of Fe (II) binding to a metal binding site on the protein. The formed protein-Fe (II) complex reacts with H_2O_2 to generate an activated oxygen species in-situ an activated oxygen species which in turn reacts with the side chain of amino acid residues at metal binding sites resulting in its' conversion to carbonyl derivatives [156, 157]. The carbonyl formation can either be primary or secondary. Primary formation results from protein exposure to reactive oxygen compounds leading to the modification and interconversion of amino acid side chains [155, 156, 158, 159]. Two mechanisms have been associated with secondary formation. The first is the oxidation of other modified proteins [160], while the other is amino acid reaction with lipid peroxidation products or reducing sugars [161-163]. Both primary and secondary reactions result in the α -amino acid yielding a Schiff base possessing carbonyl function. Schiff bases are produced by the condensation of an amine with the carbonyl group of an aldehyde or ketone in a reversible covalent reaction [164]. Carbonyl formation is not just a marker of oxidative damage but also part of the pathophysiology [161, 165]. A typical value of carbonyl content in a healthy animal is 2 nmol/mg protein [166].

Protein carbonyls can be measured in any organ or tissue with protein. The tissues most commonly analyzed are the brain, liver, kidneys, muscles and plasma. All of these have been used for most clinical studies. Jones et al., (1956) were the first to identify

specific protein carbonyl using infrared and ultraviolet wavelengths. Their work enhanced easy identification of protein carbonyls. There are several highly sensitive methods for analyzing carbonyl groups. Some of these including colorimetric, ELISA, Western blot with its modification (Oxyblot), histochemical and urine methods will be discussed in the subsequent paragraphs.

2.3.3.1. Colorimetric Method

This was originally developed by Levine and Garland [167] and has undergone some modifications as documented by Evans et al [168]. It involves the formation of the protein-bound hydrazone from 2,4 dinitrophenylhydrazine which is read spectrometrically. The supernatant of the specimen or plasma is mixed with dinitrophenylhydrazine in hydrochloric acid at a ratio of 1:4 volume parts but the control excludes dinitrophenylhydrazine. Both sample and control are put on a rotator at room temperature for 20 minutes and the protein is then precipitated with trichloroacetic acid (TCA). The precipitate is washed thrice with ethyl acetate/ethanol mixture and redissolved in guanidine hydrochloride. The supernatant is read at 370 nm for evaluation of the carbonyl content. The concentration of carbonyl is determined by multiplying the absorbance by 45.45 nmol/ml using a microtitre plate reader with a path length check. The path length check makes the result of the absorption equivalent to a 1cm path. The protein content can either be evaluated using the bicinchoninic acid (BCA) method: 20 µl of plasma and albumin standard are added to 200 µl of the BCA solution which is read at 562 nm [169]; or reading the blank supernatant at wavelengths 200 nm and 280 nm and using the difference to estimate the quantity of protein [170]. Plasma samples are compared with albumin standard at the same wavelength and the final result is carbonyl concentration divided by the amount of protein in mg/ml (the unit is nanomoles carbonyl/mg of protein). Maximal plasma levels are about 12.5 nmol carbonyl /mg of protein with albumin constituting 6.7 nmol/mg of protein [171].

2.3.3.2. Western Blot

Oxidative changes in plasma can be measured by the western blot assay because it can identify individual oxidized protein in whole tissue. Protein oxidation results in the

formation of carbonyl groups (aldehydes and ketones) on some amino acids and these can be derivatized with 2,4 –dinitrophenyl-hydrazine (DNPH). The derivatized protein are separated by SDS- PAGE and then analyzed for carbonyl contents by immunoassay with anti-DNP antibodies [157]. This process has been modified in the Oxyblot assay (Intergen) and provides reagent for a simple and sensitive immunodetection of carbonyl groups. The kit uses a membrane filter and provides primary and secondary antibodies as well as goat and anti rabbit IgG. These are treated with a chemiluminescent reagent. The protein is transferred to the membrane filter, which is treated with antibodies and the chemiluminescent reagent. The luminol is converted to a light emitting form at wavelength 428 nm by the antigen-primary antibody-secondary-peroxidase complex in an H₂O₂ catalyzed oxidation reaction. The light is detected by short exposure to blue-light sensitive films. With the kit as little as 5 femtomoles of carbonyl can be detected. It is interesting to note that fibrinogen is about the most sensitive plasma protein to oxidative damage. It is 20 times more reactive than other proteins to oxidative damage (fibrinogen \approx 9.7molcarbonyl/mol of protein: albumin \approx 0.5 mol carbonyl/mol of protein) [171]. Fibrinogen damage from free radicals may be responsible for bleeding disorders associated with multiple organ failure [172, 173].

2.3.3.3. Enzyme-Linked Immunosorbent Assay (Elisa Assay)

The ELISA technique is a method of measuring protein carbonyl involving several reactions based on the recognition of protein bound DNP with an anti-DNP antibody. The protein from any sample is nonspecifically adsorbed to the ELISA plate. A DNP probe is inserted into the ELISA plate followed by the addition of a commercial biotinylated anti-DNP antibody and streptavidin-linked horseradish peroxidase. 200 μ l of a solution containing *o*-phenylenediamine (0.6 mg/ml), H₂O₂ (1:2500) in 50 mM NA₂HPO₄ and 24 mM citric acid is added producing in a colour change. This is stopped at 25 minutes by the addition of 100 μ l of 2.5M sulfuric acid. The absorbances are read with a 490 nm filter using a microplate reader. This technique enables quantifying of the carbonyl produced and also has comparable sensitivity to the Western blot [174]. However there is anecdotal evidence that it is more sensitive and more quantitative than a Western blot. This technique was used by Buss et al [174] for concentrations

ranging from 0-2.5 nmol/mg of protein without the requirement of large sample quantities in a study. They concluded that the ELISA method was more sensitive and discriminatory than the colorimetric method [174].

2.3.3.4. Urine Assays: Protein Carbonyl

There are several methods of measuring the carbonyl in urine all of which use dinitrophenyl hydrazine (DNPH). The clinical method involves mixing equal quantities of filtered urine and DNPH. The production of a yellowish-white to yellowish precipitate is indicative of high keto acid in the urine. Acetone a source of interference could be excluded by boiling the urine to 100 degrees before doing the test [175]. This assay is also used in the diagnosis of maple syrup urine disease confirmed by Nuclear Magnetic Resonance Spectroscopy (NMR) [176]. The HPLC method involves acid hydrolysis of urine sample and a derivatization step of 2,5-hexanedione with 2,4 DNPH at 70°C for 20 minutes. A mixture of phosphate buffer (pH 3.3) and acetonitrile (50:50) is used as eluent and UV detection is at 334 nm. A linear response is obtained in the range of 0.19-20 mg/l of carbonyl [177]. Kim and Gallaher [178] evaluated the role of urine analysis as a mode of measuring oxidative stress using the HPLC method on urine samples to review 14 non-polar and 11 polar aldehydes in determining carbonyl content. They concluded that urinalysis was not a reliable method for assessing oxidative stress.

Gas chromatography/ mass spectrometry have also been used to measure urine carbonyl but the results were inconclusive [179]. We decided to review urine carbonyl levels in trauma as a visual marker of plasma carbonyl and subsequently quantified the precipitate in a neonatal study.

2.3.4. Lipid Peroxide Measurements in Biological Samples

Lipid peroxides are formed as a consequence of free radical attack on polyunsaturated fatty acids. The polyunsaturated fatty acids break down in the presence of iron or other metals forming aldehydes e.g. malondialdehyde (MDA) via the formation of cyclic peroxides and endoperoxides (precursors of prostaglandin). There are many ways of measuring lipid peroxides. The most common involves thiobarbituric acid (TBA)

reaction either directly in plasma or following the hydrolysis at acid pH producing MDA from the breakdown of lipid peroxides. It was applied first by Kohn and Liversedge in 1944 who observed that brain incubated aerobically produced a color change with 2-thiobarbituric acid. The color change is said to result from the formation of a complex between TBA and unsaturated fatty acid.

The normal range of lipid peroxides in the plasma from 4 studies is 0.41- 1.29 $\mu\text{mol/l}$ in men and 0.33- 1.22 μM in women [180]. It could be measured using HPLC [181, 182] while fluorimetric methods [183] are also available as a kit [184]. Other methods of measuring lipid peroxide involve the measurement of cholesterol oxidation products. The major product of cholesterol oxidation is cholesterol-3-5-diene-7-one while other products include cholesterol α - and β - epoxide, 7- ketocholesterol and 25-hydroxy cholesterol. The method for identifying these products was developed by Addis et al [185] and Subbiah et al [186], and subsequently modified by [187]. A commercial kit has been introduced which measures plasma lipid peroxides by utilizing hemoglobin catalysis of the reaction between hydroperoxides and 10-N-methylcarbamoyl-3-7-dimethylamino-10 H-phenothiazine to produce methylene blue [180].

2.3.4.1. D-Roms Test

This method developed by Cesarone, Belcaro et al. (1999) measures free radical-derived products in plasma based on the ability of transition metals to catalyze the breakdown of peroxides to free radicals which are trapped by an alchilamine [188]. Iron ions bound to serum proteins catalyze in vitro the breakdown of hydroperoxides to alkoxy and peroxy radicals. Iron ions bound to serum proteins catalyze in vitro the breakdown of hydroperoxides to alkoxy and peroxy radicals. The alchilamine reacts, forming a colored radical detectable at 505 nm through a kinetic reaction. The measurement of free radicals requires the addition of 10 μl of heamolysis-free serum to 1 ml buffer (R2) at pH 4.8. The mixture is added to the chromogen R1 (alchylamine) and incubated for 1 minute at 37⁰C. A color change is read initially and subsequently after one minute and the difference is multiplied by a K factor (9000) to give the concentration of free radicals in the plasma [188]. This method was effective in assessing the

bioavailability of antioxidants in food supplements [189] and the effect of antihypertensive drug on oxidative stress [190].

2.3.5. Urine Measurement of Oxidative Stress

There has been an increasing need to develop biomarkers of oxidative stress applicable to humans. Some markers found in urine, breath and blood could be used as indices of oxidative stress. Urine analysis provides a non-invasive evaluation of metabolites like F₂-isoprostane, urinary aldehydes, hydrogen peroxide and total oxidants including, lipid peroxides and other hydroxyl products of oxidative damage. It can therefore be a useful tool for measuring oxidative damage in the clinical setting.

F₂- Isoprostane is formed as a result of direct attack of reactive oxygen species on arachidonic acid resulting in the formation of 64 different isoprostanes of which 8-isoprostaglandin F₂α (8-iso-PGF₂α) is the most stable product. F₂-isoprostane and has been shown to be a reliable indicator of lipid peroxidation that may be related to in vivo free radical generation, oxidative damage, and antioxidant deficiency [191] but it has a short half-life of 20 min in blood. The urine method has the advantage of circumventing this short half life even though it has the potential of contribution from the kidney [192]. The urine assay of 8-iso-PGF₂α has been used to monitor treatment of patients on antioxidant therapy [193] and as a mode of assessing oxidative stress in asthmatic patients [194]. To measure 8-iso-PGF₂α mass spectrometry and ELISA methods have been employed [195], but the latter method has shown some inconsistencies when compared with the former or gas chromatographic methods [196].

2.3.5.1. Peroxide Measurement Using the Oxoglutarate Decarboxylation Assay

Long et al. (1999) reported urine peroxide levels ranging from 22- 173 μM (CI=38.5-46.3 μM) in healthy controls using the oxoglutarate decarboxylase assay method. Both male and female subjects were studied and the average concentration of H₂O₂ was approximately 100 +/- 60 μM [197]. Peroxide excretion was found to be unexpectedly high and might thus be useful for clinical diagnosis and therapy in diseases purported to be related to oxidative stress [197, 198].

This is a new radio-isotopic method for determination of peroxide in subnanomolar concentrations based on the reactivity of peroxide with alpha-ketoglutaric acid containing [I-14C]-alpha-ketoglutaric acid, and measurement of the resulting 1-14CO₂ by radioactivity[199]. The measurement of standard peroxide, alone or mixed with some biologically derived samples, using this technique was found to be 97 +/- 2.7% [199]. The use of radioactivity enabled demonstration of H₂O₂ in human urine. Urine samples were incubated with alpha ketoglutarate pulsed with iwts 1-14C-analogue, and CO₂ formed by decarboxylation was determined by radioactivity measurements. Blanks were prepared by pre-incubation of the samples with catalase.

2.3.5.2. 8-Oxoguanine Assay

Free radicals may react with DNA causing reversible and irreversible damage, leading to mutation, carcinogenesis or cell death [200, 201]. Free radical damage to DNA leads to the formation of 8-oxoguanine, a sensitive and specific indicator of oxidative DNA damage. A highly sensitive and selective method for determining 8-oxoguanine in plasma and urine was developed by high-performance liquid chromatography with electrochemical detection. The compound was separated by gradient elution on a C-18 reversed-phase column with a mobile phase of acetonitrile and 0.1 M sodium acetate at pH5.2. The internal standard was 8-hydroxy-2'-deoxyguanosine and 8-oxoguanine was detected electrochemically by setting the potential to +300 mV vs. Pd reference (Potential difference reference). The sensitivity of the assay was 22 ng/ml with a signal- to-noise ratio of 7:1. The within-day relative standard deviations for 8-oxoguanine quality control samples with concentrations of 3340, 1340 and 84 ng/ml were 3.6, 4.3 and 5.7% for plasma, and 4.1, 4.6 and 6.2% for urine respectively. The day-to-day relative standard deviations for the same samples were 3.8, 6.8 and 7.1% for plasma, and 3.9, 7.0 and 7.9% for urine respectively [197].

2.3.5.3. The Novel Oxistress Assay

The novel oxistress assay was developed based on the principle that glucose has a very rapid reaction to the presence of hydroxyl ion. The assay measures the quantity of hydrogen peroxide equivalents in urine and plasma that creates a color change in the

presence of thiobarbituric acid. This is then quantified using a spectrophotometer at a wavelength of 532 nm. The details of the assay and the results are further discussed in Chapters 4 and 5.

2.3.6. Myeloperoxidase (MPO):

Myeloperoxidase is a product of a single gene on chromosome 17 whose primary translational product is an 80kD protein that undergoes two cleavages to form the mature MPO. The mature MPO is 150 kD, consisting of a pair of heavy-light protomers whose heavy subunits are linked by disulfide bonds. They are made up of both carbohydrate (4% mannose rich) and two hemes per molecule that are covalently linked to the heavy subunits [202, 203]. Myeloperoxidase is in high concentration in azurophilic granules and is released into the phagosomes during the granule-phagosome fusion. Myeloperoxidase, H₂O₂ generated during phagocytosis, and a halide constitute a potent antimicrobial system which are effective against bacteria, fungi, viruses, mycoplasma and cancer [204-206]. MPO is also released to the outside of the cell by leakage prior to the closure of phagosome, and finally by secretion as when neutrophil binds to a complement and or antibody coated nonphagotomable surface or by neutrophil lysis [207].

Patients with hereditary MPO deficiency have their fungicidal capacity greatly depressed and the bacterial killing is characterized by a lag period following which the bacteria is killed [207]. Hydrogen peroxide is a weak antimicrobial however the MPO and the halide augment it's antimicrobial effect [205, 208]. There is an association between severity of illness and myeloperoxidase levels as in Chronic Myeloid Leukeamia (CML) [56] and sepsis [209, 210].

2.3.7. Plasma Elastase and Elastase Inhibitor:

Neutrophil elastase (NE) is a 30 kd neutral proteinase consisting of 218 amino acids present in the azurophilic granules of polymorphonuclear cells [211]. Elastase is produced in the azurophilic granules of polymorphonuclear leucocytes and is active at a neutral pH. It is thought to be an important mediator of connective tissue destruction [212, 213]. It can degrade almost all components of the extracellular matrix. It also cleaves plasma proteins and attacks intact cells [214]. There is considerable variation in

elastase activity between individuals which may be genetically determined [215, 216]. The suggested physiologic action of elastase is in the destruction and the digestion of ingested microorganism [214]. In support of this premise the plasma and interstitial fluids contain a series of powerful antiproteinases that can effectively regulate extracellular neutrophil elastase and prevent the enzyme from attacking extracellular substrates [214]. The presence of active proteinases suggested that the antiproteinase shield had been subverted and the exudate fluid had lost the ability to inhibit exogenous proteinases, which is evidence that activated neutrophil can circumvent the entire antiproteinase shield and attack host tissue [57].

The activity of the elastase is increased by the lipopolysaccharides, the activity of other free proteases and the oxidant load [217, 218]. Increased elastase levels have been associated with septicemia head injury, pancreatic shock and acute inflammation [65, 219]. This is a result of acute response of neutrophils to injury and increased endothelial permeability resulting in the release of elastase from the primary granules and a leak to the surrounding tissue. In patients with multiple injury and blood transfusion, the elastase levels rise from 5 to 10 fold within 8 to 14 hr after injury depending on severity [65]. Low serum elastase activity and high elastase inhibitor levels are associated with increased carotid plaque occurrence [220]. Elastase is also seen as an independent marker of the severity of chronic inflammatory bowel disease [211].

2.3.8. Summary: Methods Used for the Study:

In order to understand the natural history of oxidative stress in trauma patients clinically applicable methods are required to diagnose the condition. The methods need to be easy, simple, sensitive, accurate, and provide results that have potential benefit to the clinical settings. In the preceding sections various methods of evaluating oxidative stress have been discussed but only four of these were employed for this study based on the above criteria.

The FRAP method was used to estimate total antioxidant capacity in plasma because it is simple, cheap and the results were consistent irrespective of the dilution factor. None of the available techniques that claim to measure total body antioxidant capacity (FRAP, TRAP, ABTS, ORAC and TORC) do so individually but the FRAP

method has the advantage of a shorter reactive time. However, the FRAP does not measure serum protein (including lipoic acid and amino acids like glutathione) but it was the best option in the group.

The Asensi modification of Brigelius' method was chosen for red cell glutathione measurement because it is easy, does not require incubation and can be measured using a spectrometer unlike the other methods that require the HPLC machine.

Protein carbonyl was evaluated with the colorimetric method because it is an easy method. It does not require antibodies unlike the Western blot and ELISA, and can be measured using a spectrometer.

The novel oxistress assay (Chapters 4& 5) measures antioxidant status, which is a summation of the reaction between antioxidants and free radicals produced. This assay therefore allows an evaluation of the circulating free radicals thereby providing a total picture of the oxidative status of the patient. It is therefore an assay that be singularly used to diagnose oxidative stress in a clinical setting. In addition it is fast (5 min), inexpensive and produces a visible colour change at 37⁰C.

Neutrophil activity was evaluated with the Myeloperoxidase assay, which though not fast provides accurate and reliable results[221, 222]. Neutrophil count was done by the hospital laboratory.

2.4. Modulation of Oxidative Stress

Glutamine is classified as an intervention aimed at reducing oxidative stress irrespective of the aetiology. The two-fold mechanism via glutamate include increasing glutathione by alleviating the GSH inhibition of GCS and regulating uptake of cystine [223]. Trauma is the commonest cause of oxidative stress [15, 16]. Sheridan and associates showed that antioxidant therapy in burn patients reduced the length of time on mechanical ventilation and the level of malondialdehyde as compared to controls [17]. The consumption of endogenous antioxidants by free radicals resulting in oxidative stress may promote the development of multi system organ failure (MSOF) after polytraumatic injury [18]. Our unpublished preliminary results reflect a correlation between the severity of trauma and oxidative stress.

2.4.1. Elective Surgery and Oxidative Stress

Lou et al., (1996) reported 40% depletion in skeletal muscle glutathione and 20% depletion in plasma glutathione in elective abdominal surgery compared with pre-operative levels [224]. In another study Morris et. al.(2000) observed a depletion in the total antioxidant levels in plasma 6 hr post-op compared with pre-operative levels and a return to the basal levels after 12 hr [225]. It is important to note that most of their patients had mastectomy and lumpectomy because these are regarded as mild to moderate surgery. Maximum decrease in glutathione levels occurs at 24 hr after surgery [226].

2.4.2. Glutathione Cycle

Glutathione is an α -amino acid as well as tripeptide; it is formed from glutamine, cysteine and glycine (L- γ -glutamyl-cysteinylglycine; GSH) [131]. Glutamate and cysteine are direct precursors of glutathione production. Animal studies have shown that glutathione depleted rats benefited from glutamine supplements [227], [228]. Glutamate has two-fold way of increasing glutathione by alleviating the GSH inhibition of GCS, and by regulating uptake of cystine (Figure 2.3) [223].

Figure 2.3: Glutathione Cycle

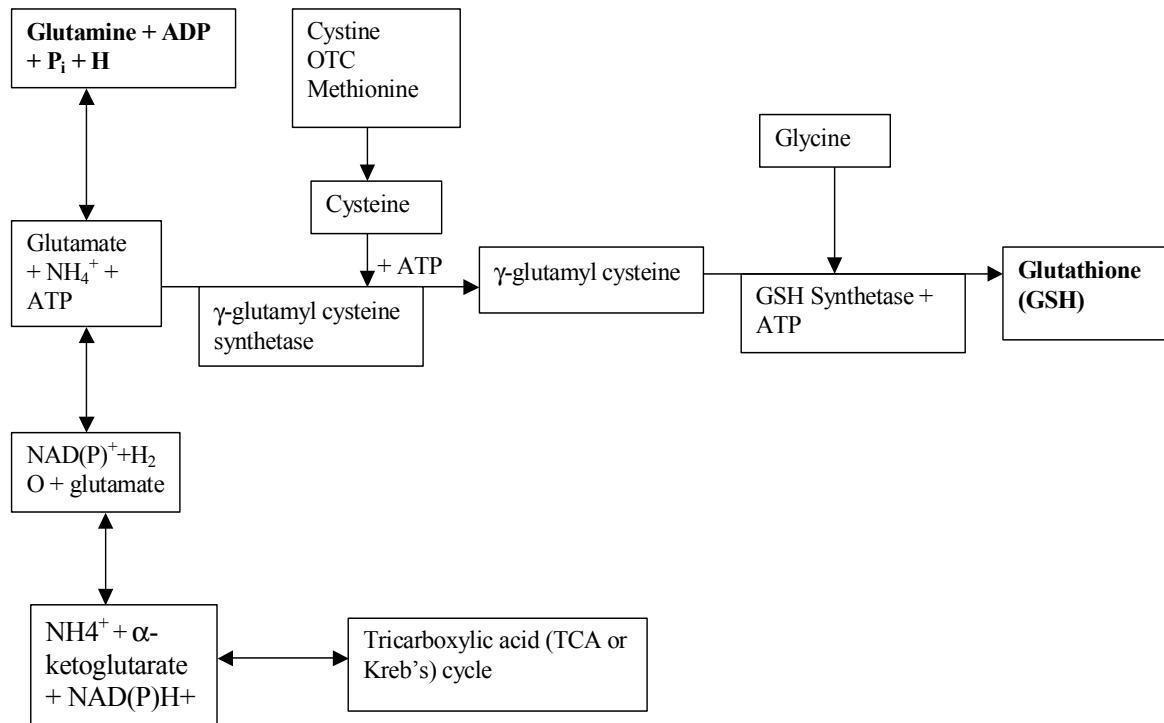


Figure 2.3: Flow chart showing the role of glutamine and other substrates in glutathione synthesis

2.4.3. Glutamine as an Intervention in Oxidative Stress

Glutamine is the most abundant non-essential amino acid in plasma (Darmaun, Matthews et al. 1986) that can be reclassified as a conditionally essential amino acid because of the body's inability to synthesize glutamine in sufficient quantities under certain circumstances such as major surgery, shock, traumatic injury and severe sepsis [229], [230].

Although glutamine constitutes >50% of the unbound amino acid pool in human skeletal muscle, rapid reduction in blood and tissue glutamine have been noted following catabolic events such as major surgery [231], trauma [232] and sepsis [233]. A summary of the functions of glutamine is presented in Table 2.1.

Glutamine exhibits extremely rapid cellular turnover rates and is a source of oxidative energy. It also serves as a metabolic precursor in the biosynthesis of nucleotides, glucose, amino sugar, as well as in glutathione homeostasis and protein

synthesis. It is a key link between carbon metabolism of carbohydrates and proteins in mammalian cells playing an important role in the growth of fibroblasts, lymphocytes and enterocytes [234]; [235]. Glutamine synthesis occurs in skeletal muscle and liver when the plasma level is insufficient to satisfy the body's requirement. Decreased glutamine availability for macrophages and lymphocytes correlated with low plasma glutamine and citrulline levels [236]. Glutamine acts as the preferred respiratory fuel for lymphocytes, hepatocytes and intestinal mucosal cells and is metabolized in the gut to citrulline, ammonium and other amino acids [237]. Reduced arginine levels observed after trauma, can be restored to physiological levels using glutamine supplementation whereas, the physiological levels of glutamine (650 μ M) are only partly restored [238]. Hepatic and renal nitrogen metabolism are linked by an interorgan glutamine flux, which couples both renal ammoniogenesis and hepatic ureogenesis to systemic acid-base regulation [239].

Glutamine via glutamate is converted to α -ketoglutarate, an integral component of the citric acid cycle. Glutamate is a component of the antioxidant glutathione and of the polyglutamated folic acid. The cyclization of glutamate produces proline, an amino acid important for synthesis of collagen and connective tissue.

Glutamine and cysteine are direct precursors in the production of glutathione. Animal studies have shown that glutathione depleted rats benefited from glutamine supplements [227], [228]. Glutamate has a two-fold way of increasing glutathione by alleviating the GSH inhibition of GCS, and by regulating uptake of cystine [223].

2.4.3.1.1. Glutamine Metabolism

In a cellular milieu, glutamine is transferred from extracellular medium to the cytoplasm and may be transported to the mitochondria via a transport system specific for L-glutamine and asparagine. This system is inhibited by thiol reagents and by L-glutamate- γ -hydroxamate a glutamine analog [240]; [241] and [242]. The two most important enzymes in glutamine metabolism are glutamine synthetase and glutaminase. Glutamine synthetase has high concentrations in muscle, liver and lungs. It catalyzes the reaction in which glutamine is formed from glutamate adding an amide group. Glutaminase is most abundant in tissues that consume glutamine and it converts glutamine to glutamate and ammonia [243].

Table 2.1: Key Functions of Glutamine

Glutamine function	Mechanism
Function in metabolism	Nitrogen shuttle: urea and ammonia clearance. <ul style="list-style-type: none"> ▪ Takes up excess ammonia and forms urea [244]. ▪ Nitrogen from ammonia is transferred to alanine via transamination with glutamate formed by reductive amination of alpha-ketoglutarate[245].
	Direct source of cell energy
Anabolism: anticatabolism	Decreases protein breakdown
	Rate-limiting factor or muscle growth
	Stimulates release of human growth hormone
Effect on wound healing	Direct fuel for fibroblast and macrophages
	Indirectly by preserving lean body mass
Preserves gut integrity	Primary fuel for gut enterocytes via glutamate via glutathione antioxidant action.
Immune function	Improves neutrophil bacterial killing and is a lymphocyte fuel
Antioxidant	Substrate for the key cellular and plasma glutathione via glutamate.

Table 2.1: Adapted from Robert H. Demling, M.D., Patrick Seigne, M.D., World J. Surg. 24, 673-680, 2000.

2.4.3.1.2. Clinical Evidence for the use of Glutamine

There have been several studies showing the beneficial effect of glutamine on complications and clinical outcome. Some of such studies were done on trauma, burns, abdominal surgery and sepsis patients. Demling et al., (1998) demonstrated that glutamine therapy reduced hospital stay and improved patient outcome [246] In a randomized trial of sixty patients grouped to either a standard enteral feeding or one containing 14.2 g glutamine/l within 48 hr of admission, Houdijk et al., (1998) observed a significant reduction in the infection rate in the supplement group - 17% vs. 45% ($p < 0.02$) [247]. A randomised prospective study using glutamine dipeptide as total parenteral nutrition concluded that the supplemented group had shorter hospital stay, improved immune status and nitrogen after abdominal surgery [248]. In another double blind randomised controlled trial in 168 patients glutamine therapy was significantly associated with a reduction in hospital stay among surgical patients [249] [250].

2.4.3.1.3. Glutamine Administration in the Elective Surgery Patient

A mild catabolic response has been well documented in patients following elective surgery. By standardizing the operation and anesthesia provided and by excluding patients with associated disease (such as diabetes mellitus or those requiring steroids), the effects of manipulations such as glutamine administration on postoperative catabolic response have been investigated.

No outcome studies are available in which to evaluate the effect of postoperative enteral nutrition with and without glutamine [251]. However, Dechelotte et al. (1998) studied protein turnover in 16 patients receiving tube feeding after esophagectomy with enteral glutamine supplementation. The endogenous de novo synthesis of glutamine was reduced by 32% ($p < 0.03$) and phenylalanine oxidation was reduced by 26% ($p > 0.05$). Protein turnover was similar in both groups. Aosasa et al. (1999) gave oral glutamine to patients receiving preoperative parenteral nutrition and compared their findings with nonsupplemented individuals. After surgery, they harvested blood mononuclear cells and stimulated production of tumor necrosis factor and interleukin-10. In patients receiving standard parenteral nutrition, there was an increase in cytokine production; this was

greatly attenuated in the glutamine group, supporting the concept that glutamine may modulate the proinflammatory cytokine response.

Fish et al. (1997) compared the effects glutamine-enriched parenteral nutrition with enteral feedings of similar composition administered to patients after gastric or pancreatic resections. Nitrogen balance and plasma protein concentrations were comparable between the two groups. Plasma concentrations of glutamine did not differ significantly between the two groups although these data tended to suggest that glutamine levels, which fell postoperatively, recovered more slowly in the enterally fed group. It should be noted, however, that nutrient delivery was increased only gradually to 100% of recommendations in the postoperative period in both groups. This gradual increase in the intravenous solution is inconsistent with clinical practice, and administering the usual quantity of total parenteral nutrition on the first postoperative day may have resulted in higher glutamine concentrations by day 5 post-surgery when measurements were made.

2.4.3.1.4. Glutamine and the Immune System

A recent *in vitro* study by Furukawa, Saito et al., (2000) evaluating the effect of glutamine supplements on phagocytosis and reactive oxygen intermediates (ROI) production by neutrophils and monocytes in postoperative patients concluded that glutamine enhances both phagocytosis and ROI production by neutrophils [252]. They observed a dose dependent response most effective at concentrations >2000 μM . This is in keeping with the findings of Wilmore, et al., (1998) who conclusion that immune function will be jeopardized at plasma glutamine levels <400 μM in post-op surgical patients [253].

2.4.3.1.5. Recommend Doses and Equivalent Supplements of Glutamine

The estimated recommended typical post-operative dose is about 0.1-0.3 g/kg/d (\approx 20 g/day), however in severely stressed patients such as burns, up to 40 g/day may be required to correct the deficit [254, 255]. This is equivalent to 3.8 g of total nitrogen [250].

2.4.3.1.6. Glutamine and Liver Disease

Much debate over the use of glutamine in the setting of liver disease has prevented general clinical use [256]. However, recent animal studies have shown that glutamine is protective against oxidative stress and has no effect on the liver oxidative metabolism [257].

2.4.4. Enteral Feeding Versus Nil by Mouth

The practice of nil by mouth (no oral feeds) post-gastrointestinal surgery during which an intestinal anastomosis has been formed is common. The rationale of nil by mouth is to prevent postoperative nausea, vomiting and protect the anastomosis. However clinical and animals studies suggests that initiating feeding early is advantageous [258]. Catchpole (1989) showed that postoperative dysmotility affects stomach and colon while the small bowel function returns to normal within 4-8 hr [259]. Feeding within 24 hr after laparotomy is tolerated and feed is absorbed [260] [261]. A large meta-analysis reviewing 11 controlled trials by Lewis et al [258], concluded that there was no advantage in keeping patients nil by mouth after gastrointestinal surgery. There was a reduction in the septic complication and length of stay in patients receiving enteral feeds. The incidence of anastomotic dehiscence, wound infection, pneumonia, intra abdominal abscess and mortality were reduced although not statistically significant ($p > 0.10$). The increase on the vomiting with early feeds (within 24 hr) had borderline significance ($p = 0.046$).

2.4.5. Clear Fluid Versus Regular Diet Post-Operatively

A large scale prospective study of 241 patients by Jeffery et al. (1996) comparing increased dietary intolerance in post-op patients receiving clear fluids vs. regular diet as the first oral intake concluded that there was no difference in both groups ($p < 0.5$) [262]. Gonzalez Ojeda A et al, also showed regular diet reduced length of hospital stay by 2.6 +/- 2.0 days compared with 3.4 +/- 2.6 days in controls ($p < 0.005$) in post-op patients. The cost of hospital stay after commencing oral intake was 2726 +/- 2107 pesos in the treatment group compared with 3547 +/- 2690 in controls ($p < 0.005$). The caloric and protein intake were 1307 +/- 523 Kcals and 55.9 +/- 23.2 grams of proteins in the

treatment group; and 651 +/- 204 Kcals and 0 grams of proteins in controls ($p < 0.00001$) [263].

The results suggest that the routine use of clear fluids as initial postoperative diet may be unnecessary and nutritionally sub-optimal compared to regular diet [262-265]. It is therefore safe to give enteral glutamine as the first oral intake after surgery. We intend to give oral glutamine as an elemental supplement because it is easily absorbed without a need for digestion.

2.4.6. Enteral Versus Parenteral Nutrition

Parenteral nutrition is designed to provide nutrition to patients who cannot be nourished adequately by enteral nutrition for critical periods. Enteral nutrition could either be tube feeding or standard care. Tube feeding is defined as either surgical or non-surgical placement of a small flexible tube into the gastrointestinal tract to provide required nutrients. Standard care is defined as the gradual reintroduction of oral diets where tolerated interruption was caused by a disease or a surgical procedure that resulted in several days of inadequate nutrient intake and the use of intravenous dextrose or fluids for hydration [266].

There is overwhelming evidence that the enteral route for nutrient delivery is by far superior to the parenteral route [267-269]. The results from a prospective trial by Kalfarentzos et al (1997) on enteral nutrition in acute pancreatitis showed that enteral feeding was well tolerated without adverse effect on the course of the disease [270]. The study concluded that enteral nutrition should be used preferentially in patients with severe acute pancreatitis [270].

2.4.6.1. Advantages of Enteral Nutrition

There are many purported advantages of the enteral route of nutrition.

- i. Enteral nutrients more effectively preserve gut barrier function and lead to modification of the intestinal hormonal environment by as yet undefined mechanism.
- ii. Gut absorption of peptides and amino acids from protein hydrolysis leads to much higher nitrogen retention than does the delivery of parenteral amino acids [271].
- iii. The average cost of TPN is as much as five times that of total enteral nutrition.

- iv. All forms of enteral nutrition have a lower infection rate in all classes of patients when compared to parenteral nutrition [266, 272]. Well nourished patients had a lower complication rate on enteral nutrition, however, there was no difference in the complication rate or mortality in the high risk malnourished surgical patient [266, 272, 273].
- v. Easy access and catheter infection associated with total parenteral nutrition is avoided.
- vi. Early enteral feeding in particular has been shown to enhance enteral nutrition [274].

2.4.6.2. Complications of Enteral Nutrition

Enteral feeds have diverse side effects such as metabolic disturbance, fluid and electrolyte imbalance and gastrointestinal side effects such as cramps, diarrhoea, nausea, vomiting, flatulence and heartburn [275]. Several studies have shown that complications were worse in malnourished and high-risk patients [266, 273]. Diarrhoea is the commonest side effect of enteral nutrition [276] which could be as high as 40 % in patients who are critically ill to as low as 3% in patients admitted for general treatment [277, 278].

2.4.6.3. Complications of Tube Feeding

Tube feeding has been shown to be generally safe and effective however there have been several associated complications such as nutrients pneumonitis, pneumothorax and pulmonary haemorrhage [279, 280]. The results of a prospective study comparing the blind to the fluoroscopic guided technique for placement of naso-enteric (Jejunal) tubes revealed most complications occurred in the blind group including aspiration pneumonia, repeat insertions [281]. There is however less complication with jejunal tubes than gastric tubes.

2.4.6.4. Elemental Diet

Elemental chemically defined formulations are designed for use in patients with “limited” digestive capacity [282] Elemental diets are traditionally used for nutritional support in

patients with diseases that affect absorption and digestion. An elemental diet has been recommended for initial enteral feeding during parenteral nutrition [283]. It is particularly useful in the transition period while the patient completely recovers bowel function as in cases of peritoneal sepsis, prolonged ileus and major surgery [284-286].

2.5. Evaluating Outcomes

Outcomes may be defined as changes either favorable or adverse that can be attributed to prior or concurrent care [287]. Outcome studies are necessary to provide objective evaluation of what might be labeled as “habitual practices in the delivery of health care”. These studies also validate the effectiveness of interventions and add to the body of evidence-based practices [288]. Outcome parameters not only include death, disability, dissatisfaction, disease and discomfort, but they also relate to health status, functional status and quality of life of patients [289].

Outcomes could be classified as patient focused, provider focused or organization focused. The patient focused outcomes can be defined as measures of the physical, physiologic, psychosocial and functional consequence of an individual’s experiences with health and illness [290]. Our study will focus on physiologic quantifiable outcome. Comparison of changes in the lean body mass of glutamine treated surgical patients with the non-glutamine treated surgical patients. A comparison of disease specific outcome of each group of surgical patients will be undertaken.

Provider focused outcome involves the cost, length of stay, complications and readmissions, but it fails to address the patient’s health status. Our study will take this into consideration by the measuring the Resource Intensity Weighting score for each patient and the length of hospital stay.

2.5.1. Laboratory Related Outcome

The role of the laboratory test in assessing clinical outcome has been controversial. This is because outcomes are viewed either in terms of health or cost. Laboratory tests are more useful for prognostic accuracy, which is the ability of a test to predict mortality, morbidity and risk stratification. Outcome studies on the other hand address questions that relate the use of a test to patient outcome. An ideal outcome study

can answer the question whether use of the studied intervention produced the anticipated outcome. A randomized controlled trial is a powerful tool for such studies [291]. Our study is a randomized controlled trial aimed at evaluating the effect of glutamine on outcome in the surgical patient, and at the same time evaluating the outcome benefit of oxidative stress assays in patient care. The other challenge to the use of laboratory related outcome is the remoteness of the many potential outcomes to the testing. This is the case with the fecal occult blood test in which the response to the result varies between physicians and patients [292]. The recommended way to overcome this problem is for both patients and physicians to specify a course of action. In our study both the intervention and the laboratory test are not routine therefore no course of action is required in response to the biochemical result [291]. The dual-energy x-ray absorptiometry (DEXA) machine will be used to evaluate lean body mass changes after surgery as it has been successfully used in previous human studies evaluating chronic conditions or the effect of treatment [293-296].

2.5.2. Resource Intensity Weights (RIWs)

RIWs define the relation between the medical and financial dimensions of hospital cases for use in planning and management ([297]). Ontario and Alberta are the first provinces to use them to adjust hospital funding ([297]). RIW ratios show the relative use of hospital resources for a typical case (successful course of treatment in an acute care hospital and discharge when the patient no longer requires the hospital's services) and atypical cases (death, transfer, sign-out and substantially longer than average stay) [297]. Different types of inpatients "consume" differing amounts of hospital resources, and it is important to be able to measure these differences in resource consumption. An equitable methodology for funding hospitals must take into account differences in case mix between facilities. An ability to examine these differences in case mix and resource utilization allows hospitals to focus efforts to improve efficiency. A standardized methodology for measuring resource consumption is critical to funding and resource allocation both at a global and organizational level.

A study was conducted on inpatient cases at the Greater Victoria Hospital Society (GVHS), British Columbia, to identify the difference between the RIWs and GVHS cost

weights. A regression analysis was performed on the more than 30,000 inpatient cost profile records from the GVHS 1995-96 cost and patient activity data. The scope of the analysis was restricted to the 424 Case Mix Groups (CMGs) that had a minimum composition of five patient profiles. Comparisons of GVHS cost weights to CIHI- RIWs yielded mostly positive results, with the noted exception of about 20 CMGs, there was a high correlation between the CIHI-RIW and the GVHS actual cost weights [298]. The use of RIWs* for equity funding and utilization management assumes validity of the cost estimates, reliability of the patient categorization scheme, equivalence of the bases for cost comparison, and equity of the subsequent resource distribution. Chu (1994) examined these assumptions, and concluded that caution must be taken when using the current RIWs and Case Mix Groups (CMGs*) for resource allocation and performance evaluation purposes [299]. RIW has represented a milestone in the history of Canadian health care product costing and management. It would be prudent for health care professionals at the operational level to provide structured and continuing feedback that can contribute to the validation and refinement of these valuable management tools. A clinical application of the RIW score in a study by Brackstone et al., (2002) concluded that RIW underrepresented trauma case cost by a factor of 3.5, which could result in underfunding and potential fiscal difficulties for a leading trauma hospital [300, 301].

2.5.3. Estimation of Physiologic And Surgical Stress (E-PASS)

The E-PASS is a way of quantifying postoperative risk by quantification of the patient's reserve and surgical stress. This estimation is based on the principle that surgical insults induce the production of proinflammatory responses [302], which are considered beneficial in augmenting immune functions and facilitating tissue repair [303]. When the surgical stress exceeds the patient's reserve capacity, homeostasis cannot be maintained and various complications result. Surgical insults prime neutrophils, which in turn attack vital organs leading to dysfunction [302]. The E-PASS scoring system is generated based on quantifying the patients' preoperative risk (PRS) and surgical stress. The preoperative risk is calculated from factors such as age, sex, performance index and the American society for anesthesiologist (ASA) classification. Based on the preoperative risk evaluation the referring physician will be able to estimate surgical stress in a patient. It is

estimated from the effect of blood loss/kg body weight, skin incision, operating time and the preoperative risk. The postoperative values i.e comprehensive risk score (CRS) for individual patients are estimated by calculating the patient's real surgical stress (SSS- Table 2.2) with the comprehensive risk score (CRS). The formula for comprehensive risk score (CRS) = 0.328 + 0.936 (PRS) + 0.976 (SSS) [304].

Table 2.2: Equation

Table 2. Equations for E-PASS scores: preoperative risk score (PRS), surgical stress score (SSS), and comprehensive risk score (CRS) (from Haga et al.)

1. $PRS = -0.0686 + 0.00345X_1 + 0.323X_2 + 0.205X_3 + 0.153X_4 + 0.148X_5 + 0.0666X_6$
X_1 : age; X_2 : presence (1) or absence (0) of severe heart disease; X_3 : presence (1) or absence (0) of severe pulmonary disease; X_4 : presence (1) or absence (0) of diabetes mellitus; X_5 : performance status index (0-4); X_6 : American Society of Anesthesiologists physiological status classification (1-5)
Severe heart disease is defined as heart failure of New York Heart Association Class III or IV, or severe arrhythmia requiring mechanical support. Severe pulmonary disease is defined as any condition with a % VC less than 60% and/or a FEV _{1.0%} less than 50%. Diabetes mellitus is defined according to the WHO criteria. Performance status index is defined by the Japanese Society for Cancer Therapy
2. $SSS = -0.342 + 0.0139X_1 + 0.092X_2 + 0.352X_3$
X_1 : blood loss/body weight (g/kg); X_2 : operation time (h); X_3 : extent of skin incision (0, minor incision for laparoscopic or thoracoscopic surgery including scope-assisted surgery; 1, laparotomy or thoracotomy alone; 2, both laparotomy and thoracotomy)
3. $CRS = -0.328 + 0.936 (PRS) + 0.976 (SSS)$

Source: Haga et al., 2001

The result of the initial E-PASS study showed a correlation between the CRS, SSS and the postoperative inflammatory parameters [305]. The E-PASS could be useful in our study for evaluating the effect of glutamine as an indirect precursor of glutathione (via glutamate) on the inflammatory parameters such as antioxidant levels and free radical production after surgery leading to oxidative stress. Table 2.2 shows how to calculate for the various components of the E-PASS. In a multicenter trial the E-PASS has been shown to be useful not only for predicting risk but also for estimating surgical quality and medical expense [304]. Our study will also evaluate the effects of the introduction of glutamine on the overall medical expense of the surgery.

2.5.4. Quality of Life

Quality of life in surgery could be broadly defined as the impact of the disease process on physical, psychologic and social aspects of a person's life and feeling of wellbeing. The objective dimension is the functional assessment of health status [306]

while the subjective assessment is based on the patient's perception. Quality of life instruments have a number of applications broadly categorized as: screening and monitoring for psychosocial problems, population surveys, medical audit, outcome measures (which are relevant to the glutamine study), clinical trials and cost utility analyses. These instruments could be discriminative, predictive or evaluative. Discriminative implies they can be used for separating groups of patients based on their quality of life e.g. patient satisfaction [307]. Predictive implies they can predict outcomes such as return to normal function [308], and evaluative when they can be used to measure the magnitude of change in an individual or group of patients over time [309]. The essential characteristics of quality of life instruments include reliability, validity, sensitivity to change, appropriateness and practicality [309, 310]. Although quality of life data provides pertinent information on patient outcome, it will not be evaluated in our study, which focuses on the acute effect of antioxidant therapy after surgery.

3. RATIONALE AND OBJECTIVES

3.1. HYPOTHESIS

The study is based on the following hypothesis:

1. Trauma results in oxidative stress
2. Measuring oxidative stress in the clinical setting results in early diagnosis
3. Antioxidant therapy can ameliorate oxidative stress in surgical patients (controlled trauma)

3.2. RATIONALE

3.2.1. Reasons for Studying Oxidative Stress?

Based on increasing awareness of the importance of oxidative stress in diseases and the limited clinical knowledge of the pathophysiology, this research was conducted to understand and modulate oxidative stress in the clinical setting. This is based on an assumption that early prevention and treatment of oxidative stress will prevent the development of the associated morbidity and mortality.

Our study focuses on understanding the natural history of oxidative stress in humans using the trauma model as most of the previous studies on the subject were conducted on animal models. There have been many methods of monitoring the effect of oxidative stress however most of these are applicable to tissue culture, organ injuries and animal studies. Our study focuses on identifying clinically applicable modes of evaluating oxidative stress therefore making the concept more clinically relevant.

3.2.2. Reasons for Studying Trauma

Trauma is relevant since the onset time can be reliably determined in the normal population, and there is evidence that trauma is the commonest cause of oxidative stress [15]. The injury could be post-ischemic, reperfusion, heat, physical, cold, excessive exercise, or exposure to toxin, radiation or infection. Injury can give rise to systemic decreases in anti-oxidant capabilities not just in tissues affected directly by the trauma e.g. severe skin burn has been demonstrated to decrease brain GSH by 50-70% [46]. Burns increase xanthine oxidase activity in affected tissue and cause neutrophil activation. Animal studies have also demonstrated a decreased burn edema and a

lessening of distant organ dysfunction with the use of antioxidants, suggesting a cause and effect relationship [47].

3.2.3. Reasons for Measuring Oxidative Stress in the Clinical Setting:

Oxidative stress is a preventable etiological factor in several diseases however it is neither treated nor prevented in the clinical setting because there are currently no tests to assess a patients' oxidative status. Most of the therapeutic approaches are aimed at the complications rather than prevention of the oxidative stress induced damage. The presence of bedside evaluation of oxidative stress will therefore make early identification, prevention and institution of treatment possible [311, 312]. Can oxidative stress be measured in a clinical setting? Yes, based on the various methods explored in our study.

3.2.4. Reason for Measuring Red Cell Glutathione

The erythrocyte was an early model for studies of oxidative stress because of its' relatively high oxygen tension, the presence of hemoglobin, and a plasma membrane rich in polyunsaturated lipids, and as a result, should be prone to oxidative stress. Erythrocytes have many scavenger systems, and can be used to examine the balance between pro-oxidants and antioxidants since they are representative cells where superoxide radicals are being continuously generated by auto-oxidation of hemoglobin [313].

The oxidative status of intact erythrocytes measures both the concentration and redox state of intracellular glutathione. The novel findings of a study of preeclamptic patients and normal pregnant women suggests that some patients may be unusually susceptible to erythrocyte glutathione oxidation, possibly leading to general cellular damage, in particular the HELLP Syndrome (Hemolysis Elevated Liver Enzymes Low Platelets) [314]. When there is severe oxidative stress the rate of depletion of reduced glutathione is faster than production resulting in an efflux of oxidized glutathione from the cell into the plasma [145, 146]. A depletion of reduced intracellular glutathione causes changes in the physicochemical state of the erythrocyte membrane and the accumulation of lipid peroxidation products [315]. The red cell has the capacity to replenish plasma antioxidants because it has 100 times the concentration of plasma GSH

[42, 316]. The normal range of erythrocyte GSH in the population is 0.6-1.4 $\mu\text{mol/L}$ [317, 318]. Erythrocyte GSH changes were measured in our study using Asensi's modification [150] of Brigelius' method [151]. Changes in GSH levels are indicative of the severity of oxidative stress and the antioxidant reserve in the red cell.

3.2.5. Reasons for Measuring Plasma Antioxidants

An antioxidant significantly delays or inhibits the oxidation of an oxidizable substance when present in lower concentration than that substance [319]. The physiological role of antioxidants, is to prevent damage to cellular components arising as a consequence of chemical reaction involving free radicals [88]. Human plasma is often used in studying damage induced by free radicals because it contains critical targets of oxidative damage such as lipoproteins and many important antioxidants in the same physiological environment. Measurement of antioxidant defenses has consistently demonstrated depressed plasma levels of vitamin E and C in patients with sepsis and adult respiratory distress syndrome (ARDS) [10, 113, 320]. Low plasma Vitamin C levels have been shown to be predictive of the development of multiple organ failure syndrome in populations at risk [11]. Plasma glutathione levels are depressed in patients with hepatic failure, polytrauma and critically ill patients [18, 321]. The plasma antioxidant capacity which is indicative of the ability of antioxidants in circulation to respond oxidant release was measured using the FRAP method [126].

3.2.6. Reasons for Measuring the Total Oxidant in the Body

A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital [322]. Oxidant generation is part of normal human metabolism and host defense mechanism, however when produced in excess oxidants cause tissue injury. Tissue injury can itself cause more oxidant generation which may further worsen the injury as exemplified in crush injury [323, 324] and ischemia/ reperfusion injuries [325, 326]. Many free radicals are highly reactive and can either donate an electron to or extract an electron from other molecules therefore behaving as oxidants or reductants. Many radicals have very short half-life (10^6 seconds) however the most important free radicals in many diseases are oxygen

derivatives particularly superoxide anion and the hydroxyl radical. The hydroxyl radical or a closely related species, is probably the final mediator of most free radical induced tissue damage [327].

The choice of plasma and urine analysis is because an evaluation of biomarkers in the circulation fluid (plasma) and in the elimination fluid (urine) will provide a comprehensive picture of the oxidant status in the organism [328]. The total oxidant levels were measured using a novel oxidant assay developed in the early phase of the study. The assay focuses on measuring the concentration of hydroxyl radical and other free radical chain reactions stimulated by the OH[•] radical effect in biological fluids. This assay measured both the total oxidant level in plasma and the patients' oxidative status in the urine.

3.2.7. Reasons for Measuring Plasma Protein Carbonyl:

Another means of estimating the activity of antioxidant in vivo is to assess their effects on markers of oxidative damage to lipid, protein and DNA. Protein carbonyl formation is the effect of oxidative damage on protein [161, 165]. All proteins are subject to modification by ·OH (Hydroxyl radicals) or ·OH+ O₂⁻ (Hydroxyl and Oxygen radicals) which includes alteration in molecular weight and net electrical charges [158] resulting in protein carbonyl formation. Protein carbonyl formation could either be primary or secondary, occurring in the blood, liver, brain, spinal cord and other organs. Primary formation results from protein exposure to reactive oxygen compounds leading to the modification and inter-conversion of amino acid side chains [155, 156, 158, 159]. The presence of protein carbonyl has become a widely accepted measure of oxidative damage under conditions of oxidative stress, aging and disease [329]. Measuring protein carbonyls in our study may provide a clinical index of the severity of oxidative stress in trauma. Protein carbonyl levels were measured using our modification of the colorimetric method originally described by Levine [167].

3.2.8. Reasons for Studying the Role of Neutrophils Activity in Oxidative Stress

Neutrophils are thought to play a key role in tissue injury [330] and are also known to play a unique role in the etiology of oxidative stress because they release a lot of active substances such as elastase, toxic oxygen radicals and vasoactive substances at the site of injury or inflammation [73, 331-333]. Activation of the neutrophil enhances its adhesiveness of neutrophil to endothelium, associated with the release of reactive oxygen intermediates (ROIs) and degranulation with release of proteases [334]. A study by Tanaka [332] suggests that neutrophil O_2^- production increases acutely in trauma. Tumor necrosis factor may mediate this O_2^- and GE production by neutrophils through increased vascular permeability and induction of endothelial adhesion molecules thereby exposing surrounding tissue to free radicals [335, 336]. The level of granulocyte elastase at 3 months after Percutaneous Transluminal Coronary Angioplasty (PTCA) correlated significantly with the percent luminal stenosis at the angioplasty site ($p < 0.05$) and the authors suggests granulocyte activation may be involved in restenosis after PTCA [337]. The enzyme elastase is released from stimulated neutrophils at the site of injury, infection or inflammation and can cause tissue damage and subsequent organ dysfunction. By measuring the complexes of elastase with its' inhibitor α_1 -proteinase (elastase α_1 -proteinase inhibitor complex) in plasma, the degree of neutrophil activation can be quantified [332, 338]. Plasma elastase concentration on admission had the highest sensitivity (84%) but the lowest specificity (39%) in predicting multiple organ dysfunction (MODS) after trauma [331]. Plasma elastase concentration above 500 ng/ml on day 3 post-trauma was predictive of subsequent severe MODS with 95% specificity and 69% sensitivity. The negative predictive value (the probability that the patient will not have the disease when restricted to all patients who test negative) was 93% [331]. The present evidence suggests therefore that elastase is an important marker of polymorphonuclear cell activity and possibly a predictor of ARDS and MODS [331].

In addition to releasing large amount of oxidants, activated neutrophils also release myeloperoxidase, the enzyme that generates the most potent neutrophil oxidant, hypochlorous acid [209]. MPO activity is correlated to the absolute number of neutrophils and therefore constitutes a specific and sensitive marker to quantify its

infiltration into the injured tissue [222, 339]. MPO activity assay has been used to study Leukocyte infiltration and accumulation after traumatic brain injury in rat [221, 339]. It has also been used to predict early risk of myocardial infarction, as well as the risk of major adverse cardiac events in the ensuing 30-day and 6-month periods. [340]. Given its proinflammatory properties, MPO may serve as both a marker and mediator of vascular inflammation and further points toward the significance of PMN activation in the pathophysiology of ACS (Acute Coronary Syndrome) [341]. Myeloperoxidase activity was significantly higher in the plasma of the sepsis patients who subsequently died. [209]. In order to determine the relationship of neutrophils to the development of oxidative stress after trauma we examined both neutrophil count and activity with the Myeloperoxidase assay.

3.2.9. What is the Role Antioxidant Therapy after Surgical Trauma?

There is anecdotal evidence in clinical and animal studies [342] [343] [135] indicating that the introduction of antioxidant therapy reduces oxidative stress and its complications. In a study by Marzi et al (1993) recombinant human superoxide dismutase (rhSOD) therapy after polytrauma attenuated multiple organ failure [342]. This effect was most evident in cardiovascular and pulmonary function. The authors speculate that earlier administration of rhSOD (such as at the onset of resuscitation) may provide even better results than they observed because therapy was commenced late in their study (about the end of the first day after trauma). A recent study by Tardiff (1997) concluded that the antioxidant probucol was effective in reducing the rate of restenosis after balloon coronary angioplasty [343]. Sheridan and associates (2000) showed that antioxidant therapy in burn patients reduced the length of time on mechanical ventilation and the level of malondialdehyde as compared to controls [17]. The consumption of endogenous antioxidants by free radicals resulting in oxidative stress may promote the development of MOF after polytraumatic injury [18]. Our prospective study looking at the role antioxidant therapy on oxidative stress and patient outcome will give an opportunity to assess the benefit of antioxidant therapy in the surgical setting.

3.2.10. Reasons for Evaluating Outcome

Outcome studies are usually used to assess large cohorts of patients often using administrative databases [344]. Outcomes studies often use patient based or patient derived evaluation of care. Our outcome study is aimed at providing information on two related questions about the intervention. The first question is to determine if enteral glutamine will raise erythrocyte glutathione levels, maintaining plasma antioxidants and reduce free radical production. The second question is to assess the impact of these changes on clinical outcome using objective measures such as the RIW score or the E-PASS method. In this era of evidence based medicine there is a need for more objective measures than mortality, morbidity and length of stay to assess the impact of the antioxidant therapy [299, 345, 346] (Avery-jones 1964). Our study is a randomized clinical trial, differences in the outcome will be used to make inferences about the treatment effectiveness [347].

3.3. Objectives of the Study

- To understand the natural history of oxidative stress in humans.
- To identify clinically applicable modes of evaluating oxidative stress.
- To examine the relationship of neutrophil count and activity to the development of oxidative stress in trauma and surgical patients.
- To develop and carry out a randomized prospective study to define the role antioxidant therapy on oxidative stress and the outcome of surgical patient. This will provide an opportunity to assess the benefit of antioxidant therapy in the surgical setting.

4. METHODS

4.1. Red Cell Glutathione Measurement

Red cell glutathione was measured using Asensi's (1999) modification [150] of the method by Brigelius et al (1983) [151]. The measurement is based on the conjugation of chlorobenzene with GSH in a reaction catalyzed by glutathione transferase forming an adduct of s- 2,4 dinitrophenyl glutathione which is read at 340 nm wavelength. This method measures reduced-glutathione (GSH) levels in the red cell. It is an end point reading that can measure as low as 0.2 nm in the cuvette which corresponds to 0.3 μ M GSH [152]. The following describes the procedure:

1. Take the whole blood and centrifuge at 300 rpm for 5 minutes.
2. Remove the plasma – it can be used for frap or oxidant studies.
3. Add equal volumes of TCA (trichloroacetate) to the RBC.
 - a. 0.5 ml of ice cold 30% trichloroacetic acid (TCA) to 0.5 ml of RBC.
4. Centrifuge mixture at 15,000 g for 5 min.
5. Take the supernatant – use immediately or this can be stored at -20⁰C (stable for 1 week).
6. Measure GSH at 340 nm wavelength after 5 minutes.
7. Phosphate buffer pH = 7.2.

Control	Test sample
<ul style="list-style-type: none">• 25 μL of supernatant• 10 μL of CDNB (2 mg/ml ethanol)• 825 μL of K₂PO₄ buffer *	<ul style="list-style-type: none">• 25 μL of supernatant• 10 μL of CDNB• 825 μL of K₂PO₄ buffer• 10 μL of 500 U/ ml of GST

4.2. The Novel Oxistress Method

This is used for measuring oxidants in plasma and urine. The concept of oxidative stress has been a well-known clinical problem in many disease states such as adult respiratory distress syndrome, diabetes mellitus, neurodegenerative diseases and many

other diseases. However the ability to make such diagnosis has been limited to either post mortem studies or research laboratories because the available methods are cumbersome and technically involved. Therefore the concept of the Oxistress assay is aimed at making the measurement of oxidative stress rapid, easy, accurate and without the need for technical support. The assay development process and clinical application is fully discussed in chapter 5 and the following is a summary of the method for the final assay component.

4.2.1. Principle of the Oxistress Assay

The principle of the assay is based on the rapid reaction of deoxyglucose in the presence of hydroxyl ion or peroxy radical to produce a color change in thiobarbituric acid.

4.2.2. Components of the Oxistress Assay

- Deoxyglucose reagent (100 mM)
- Thiobarbituric acid (TBA) (50 mM)
- Ferrous sulfate (1 mM)

Hydrogen peroxide standard with concentrations of 50, 100, 250, 500, 1000, 2500, 5000, 10,000 & 20,000 μM were used. The reagent is stable for over four weeks if divided into two components:

- ◆ Reagent 1 (R1) is a mixture of Thiobarbituric acid (TBA) (50 mM) & Ferrous sulfate (1 mM).
- ◆ Reagent 2 (R2) is the Deoxyglucose reagent (100 mM).

Equal volumes of R1 and R2 are mixed prior to use but if using freshly prepared all the components can be added at the same time.

Figure 4.1: Slide Showing Colour Reaction Of The Oxistress Solution With Different Concentrations Of Hydrogen Peroxide (Microtitre Plate Method)

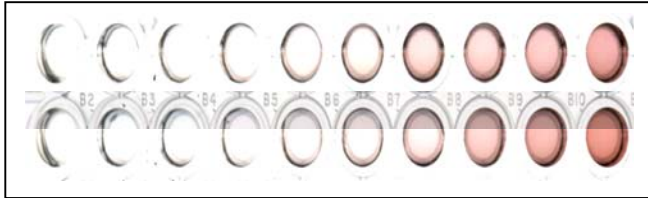
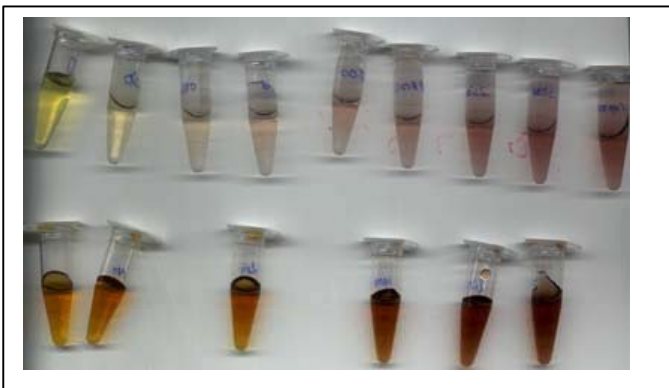


Figure 4.2: 1 ml Cuvette Method Showing Colour Reaction Of The Oxistress Solution With Different Concentrations Of Hydrogen Peroxide



4.2.3. Procedure for the Oxistress Assay

4.2.3.1. 1 ml Cuvette Method for Urine

1. Add 200 μ l of urine specimen to 800 μ l of Oxistress reagent.
2. Read color reaction after 5 minutes using a spectrophotometer at a wavelength of 532 nm.
3. Calculate the value against a hydrogen peroxide standard.

The evaluation of plasma is similar however the plasma is mixed at a one in ten ratio to the reagent (100 μ l of plasma to 900 μ l of Oxistress reagent).

4.2.3.2. Deoxyglucose Microtitre Method for Plasma

1. Make blank and peroxide standard
2. Add 100µl of blank and peroxide standard concentrations 10, 25, 50, 80, 100 µmol/l
3. Add 10 µl of plasma sample to 90 µl of buffer solution
4. Add 100 µl of deoxyglucose reagent
5. Read at 540 nm wavelength for fresh samples or incubate for 10 minutes at 37⁰C for frozen samples before reading.

4.2.3.3. Deoxyglucose Microtitre Method for Urine

1. Make blank and peroxide standard
2. Add blank and peroxide standard concentrations 10, 25, 50, 80, 100 µmol/l
3. Add 20 µl of urine sample to 80 µl of buffer solution
4. Add 100 µl of deoxyglucose reagent
5. Read at 540 nm wavelength for fresh samples or incubate for 10 minutes at 37⁰C for frozen samples before reading.

4.3. Protein Carbonyl Method

This is done using a modification of the colorimetric method (Figure 4.3) originally developed by Levine [160, 167]:

1. Add 100 µl of serum to 300 µl of 0.4 g/100 ml 2,4- Dinitrophenylhydrazine (DNPH) in 1.25 M hydrochloric acid and prepare blanks using hydrochloric acid only.
2. Vortex and allow 20 min for the reaction to take place.
3. Precipitate the proteins using 400 µl of trichloroacetic acid (TCA).
4. Centrifuge the samples for 5 minutes at 11,000 g, discarded the supernatant wash the pellets with ethanol: ethyl acetate (1:1) three times to remove the excess 2,4-DNPH.
5. Dissolve the pellets in 1ml of 6 M guanidine HCl in 20 mM potassium phosphate buffer (pH 2.3).
6. Re-centrifuge samples for 5 min to remove insoluble materials and supernatant.
7. Read with a spectrometer at 370 nm wavelength.

8. Calculate protein carbonyl levels using the molar absorbance coefficient of 22,000 $M^{-1}cm^{-1}$.
9. Evaluated total protein concentrations were using the bicinchoninic acid (BCA) assay [348] compared with bovine serum albumen standards both before and after washing the blanks with ethanol: ethyl acetate. A difference was found in the protein concentration in samples before diluting with hydrochloric acid, and before and after the washing process. There was a reduction of 2.73 ± 0.35 mg protein/ml (23%) in the samples diluted with hydrochloric ($p=0.02$). There was also a difference of 3.59 ± 0.52 mg protein/ml (39%) before and after washing ($p < 0.001$). This difference greatly influenced the calculated carbonyl content (Figure 4.4).

Figure 4.3: Levine's Method & Modification

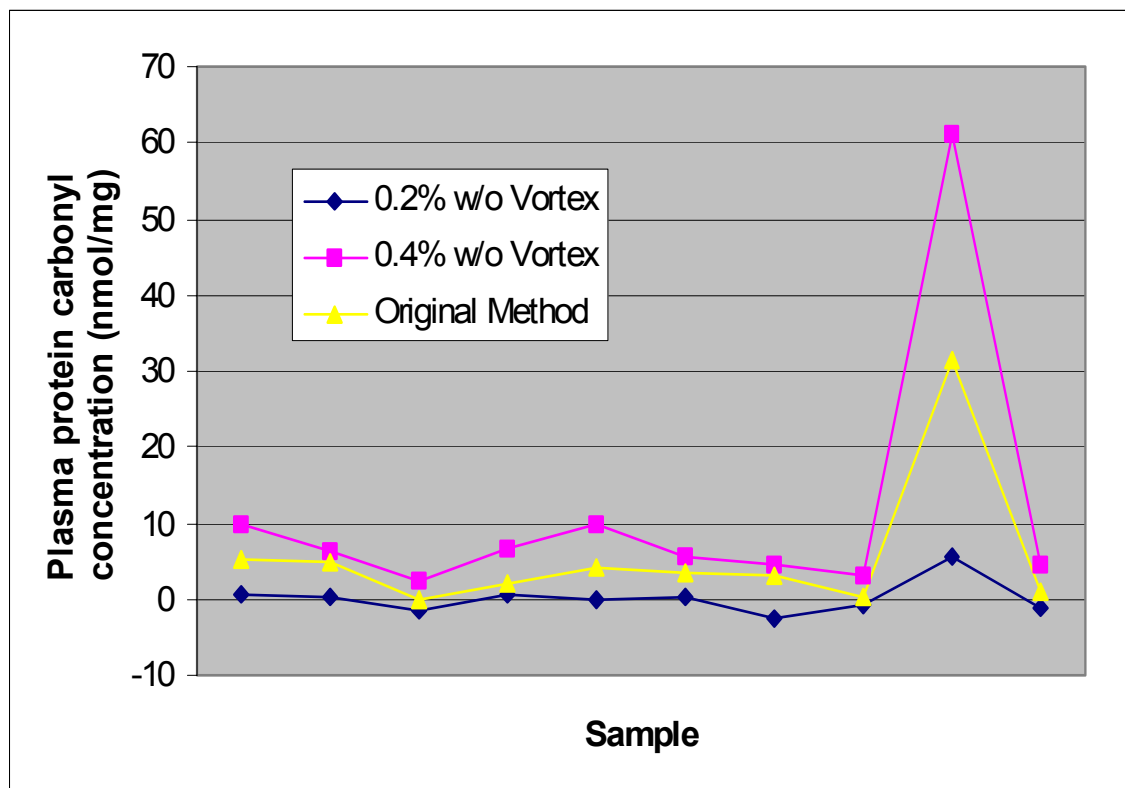


Figure 4.3: 0.4% DNPH (our modification) without vortexing measured higher carbonyl levels in the same sample implying better accuracy.

Figure 4.4: Effect of Various Methods Of Protein Analysis on Plasma Carbonyl Values

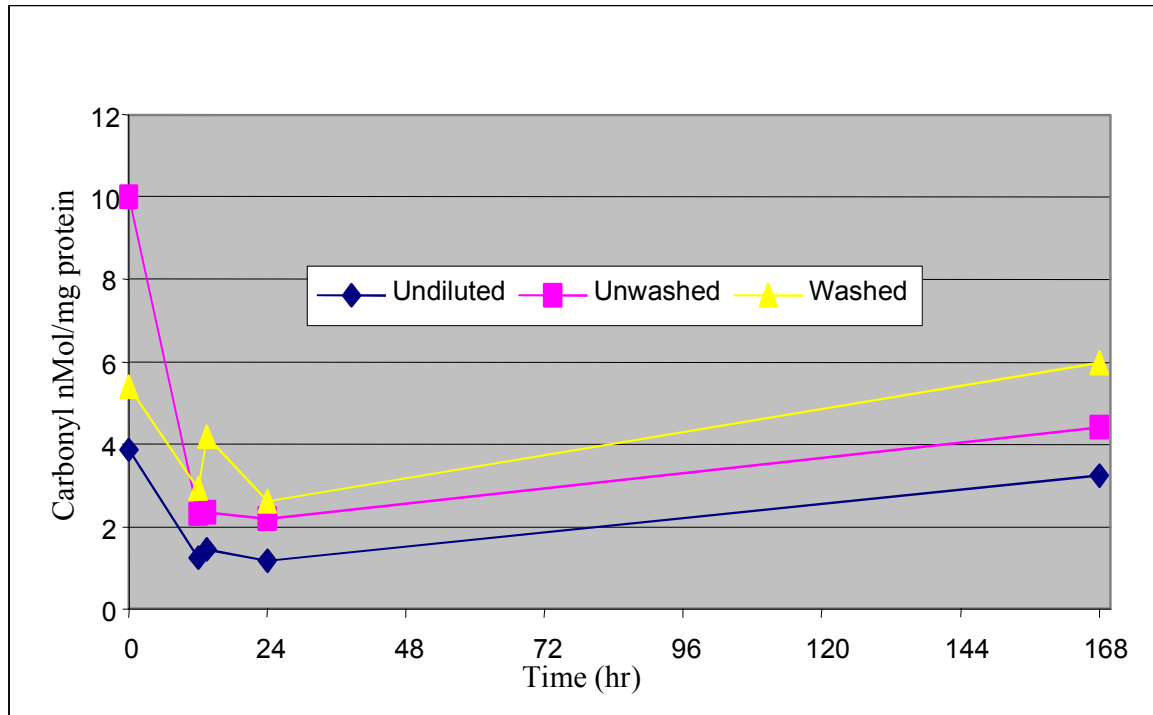


Figure 4.4: Washing reduces plasma protein levels by up to 39% thus yielding higher protein carbonyl levels.

4.4. Neutrophil Count and Activity in Oxidative Stress

In order to determine the role of neutrophils in the development of oxidative stress in trauma and elective surgery patients we examined both neutrophil count and activity.

4.5. Neutrophil Count

Neutrophil count was done as part of routine blood investigations at the Royal University Hospital.

4.5.1. Neutrophil Activity

Neutrophil activity was measured with the Myeloperoxidase assay [221, 222] The procedure is as follows:

1. Mix plasma with Solution A [0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM Potassium phosphate buffer (pH 6.0)].

2. Centrifuge at 4⁰C for 15 min.
3. Mix 6.7 ml of the supernatant with 200 µl of Solution B [50 ml of 50 mM potassium phosphate buffer (pH 6.0) mixed with 8.35 mg of o-dianisidine dihydrochloride and 25 µl of hydrogen peroxide] in a microtitre plate.
4. Read at 460 nM as a kinetic assay every 11 sec for 5 min.

4.6. The Total Antioxidants in Plasma Using the FRAP Method

The FRAP assay measures the total reducing power of the antioxidant [112]. At low pH, ferric tripyridyltriazine (Fe^{III}-TPTZ) complex is reduced to a ferrous form producing an intense blue color, which can be monitored by measuring the change in absorbtion at 593 nm. The working solution is made up of a mixture of 25 ml of acetate buffer to 2.5 ml of TPTZ solution and 2.5 ml of FeCL.6H₂0 (ratio 10:1:1), it should be kept at 37⁰C and the sample must be fully thawed. A fresh working solution must be made every day because it is stable for only 24 hr.

4.6.1. Procedure for the FRAP Method

The FRAP method was modified in this study using the micotitre plate in the following steps:

1. Add 5 µl of sample to the microtite plate
2. 150 µl of reagent (working solution)
3. Add 15 µl of distilled water
4. Stir with the pipette tip
5. Read at 593 or 600 nm wavelength.

4.7. Prospective Study on Antioxidant Therapy

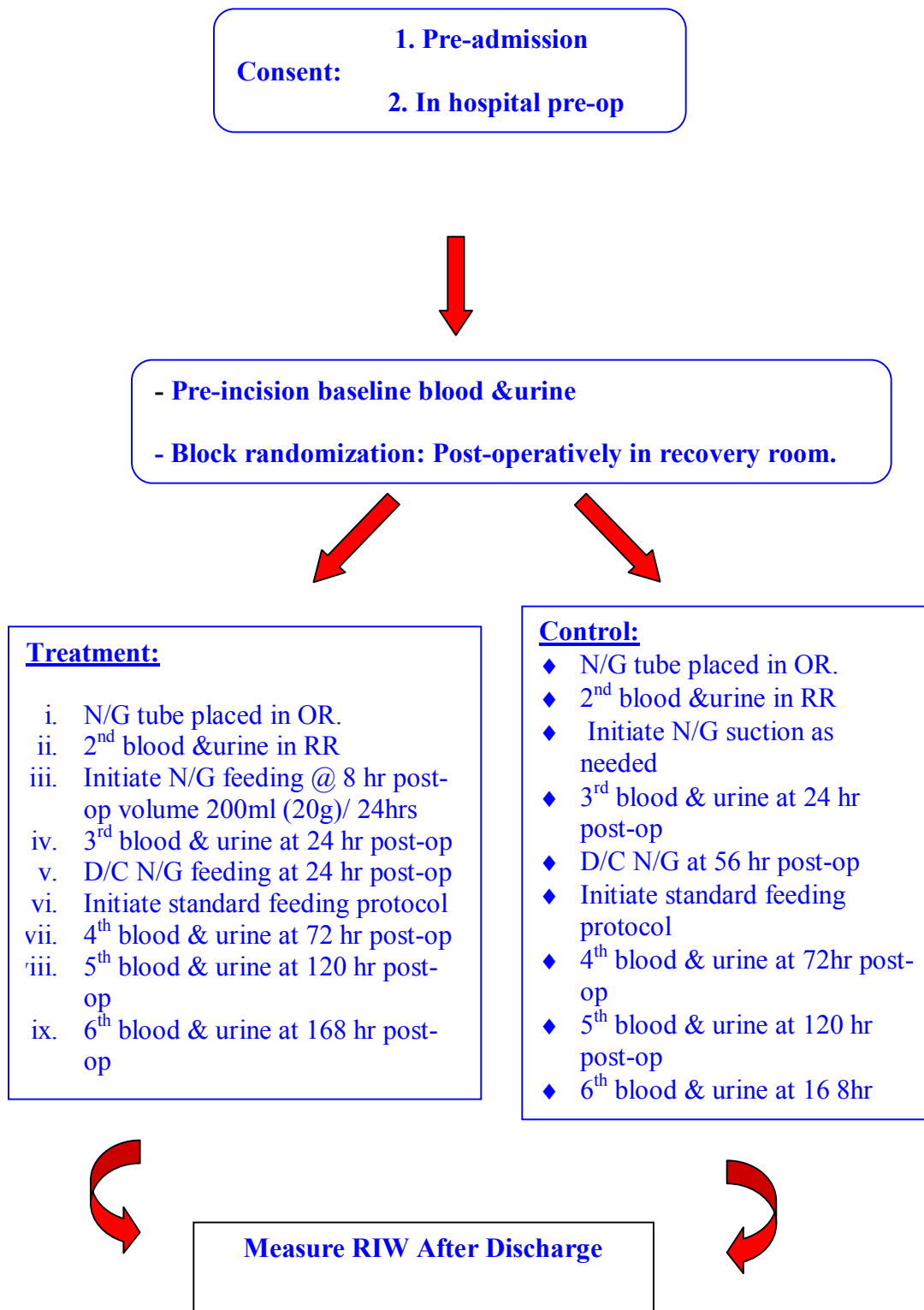
4.7.1. Study Design:

This is a prospective study of surgical patients at the Royal University Hospital. Ethics approval will be sought from the University and the Saskatoon Health Region. The study will be randomly divided into two arms (treatment and non- treatment). There will be about 60 patients in each of the arms and they will be recruited pre-operatively from general surgery, neurological surgery, plastic surgery and anterior spinal fixation patients.

Informed consent will be obtained but the patients will not however know their group allocation in the study.

The magnitude of the surgery will be evaluated from the pre and post operative notes and from the neutrophil counts which have been shown to be an index of severity of trauma [349, 350], (Obayan, unpublished observation). The degree of oxidative stress will be evaluated by measuring changes in red cell glutathione [150, 151] and strong oxidants in urine (Obayan, unpublished method). The outcome of glutamine therapy will be evaluated both clinically and biochemically. Some of the clinical outcomes will include length of hospital stay, wound complication rate, the resource intensity weight information (RIW), Karnosky score [351] and Glasgow outcome score. The biochemical index will evaluate the degree to which glutamine inhibits oxidative stress after elective surgical trauma.

Figure 4.5: Study Protocol For Evaluating The Effect Of Enteral Antioxidant Therapy On Biochemical And Clinical Outcomes



4.7.2. Exclusion Criteria

1. Patients with urea nitrogen concentration ≥ 25 mmol/l.
2. Patients with total bilirubin concentration ≥ 175 mmol/L (Ziegler et al 1992).

4.7.3. Potential Benefit of the Study

Antioxidant therapy in a clinical setting will not only improve biochemical outcome but may also improve patient outcome.

5. THE NOVEL OXISTRESS ASSAY

Oxidative stress has been implicated as a factor in many disease and injury states such as adult respiratory distress syndrome, diabetes mellitus, neuro-degenerative diseases. The extent to which oxidative stress could be predictive of clinical outcome has been largely unknown and a satisfactory method for rapidly evaluating total oxidative stress in a human subject has been unavailable. The ability to make such diagnosis has been limited to either post mortem studies or research laboratories because the available methods are cumbersome and technically involved. As well, these methods usually measure a single oxidized compound, or a narrow class of compounds, as indicators of oxidative activity making them unreliable in assessing overall oxidative stress (or status). Some of these methods have been discussed in Chapter 2.

A measure of oxidative stress in body fluids may be predictive of clinical outcome and the need of a rapid clinically applicable assay that has the ability to assess overall oxidative stress (or status) was identified at the preliminary stage of this study. One of the ways of assessing oxidative stress is by the measurement of peroxide levels in biological fluids [182, 183, 197]. Hydrogen peroxide (H_2O_2) has been widely used in the standardization of the various methods [182, 183, 188]. The Novel Oxistress Assay is an invention that aims at an easier and rapid clinically applicable method of measuring strong oxidants (H_2O_2 equivalents) in biological fluids such as plasma and urine. The ability to measure all strong oxidants should make this assay better than others.

5.1. Principle of the Oxistress Assay

The principle of the assay is based on the rapid reaction of glucose in the presence of hydroxyl ion or other peroxy radicals to produce a colour change. The procedure requires the oxidation of 2-deoxysugars to dialdehydes by strong oxidants derived from peroxide; the dialdehydes subsequently form a coloured adduct with thiobarbituric acid (TBA). Ferrous Sulphate in the reagent provides the ferrous ions that convert the peroxides to strong oxidants.

5.2. The Oxistress Assay Development Process

The process involved the evaluation of the reaction of hydrogen peroxide with certain chemicals at different concentrations and conditions to determine the optimal composition of the assay. The objective was to measure strong oxidants in biological samples known to have hydrogen peroxide concentrations less than 200 μl [197]. The assay was pink in colour and stable at room temperature with the following components:

- EDTA (1.4 mM), a chelating agent
- Deoxyglucose reagent (100 mM), a 2- deoxysugar
- Thiobarbituric acid (TBA) (50 mM)
- Ferrous sulfate (1 mM)
- Freshly prepared Hydrogen peroxide standard in concentrations of 50 μM , 100 μM , 250 μM .

5.2.1. Standard Curve and Calibration of Assay

500 μl of various concentrations of H_2O_2 from 1 μM to 250 μM was added to 500 μl of the reagent to develop a standard curve for the experiment. A linear relationship was observed between H_2O_2 concentration and TBA Reaction product absorbance at wavelength 532 nM (Figure 5.1). The reaction occurred within five minutes producing visible colour change from pink to red at 100 μM – 250 μM hydrogen peroxide levels. At higher peroxide levels (>1000 μM) orange colour change was observed (Figure 5.2).

Figure 5.1: Standard Curve of the Oxistress Assay

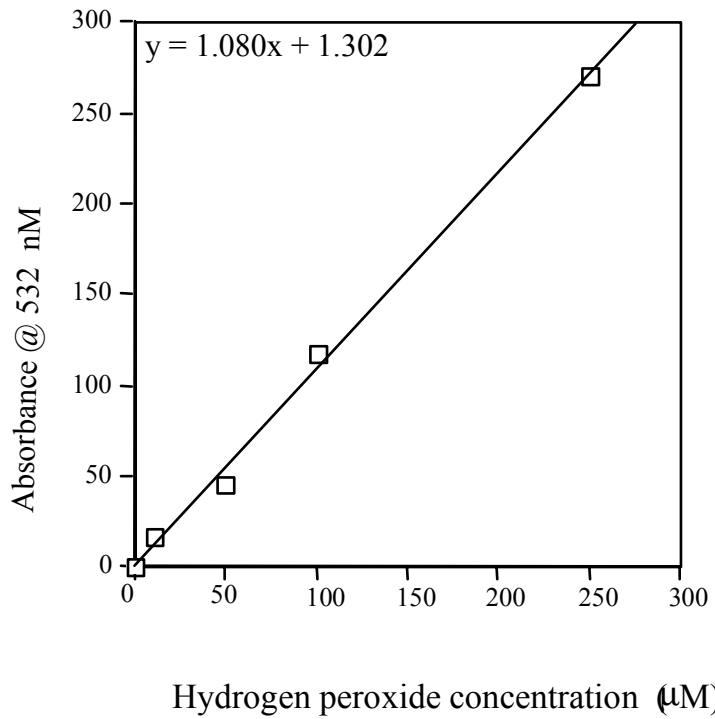


Figure 5.1: Oxistress assay produces a linear curve with different concentrations of hydrogen peroxide standards

Figure 5.2: Oxistress Assay Colour Changes With Varied Hydrogen Peroxide Standards (8-16,000 μM)

Figure 5.2A: Oxistress Assay (Acid EDTA) with Varied Concentrations of Hydrogen Standards

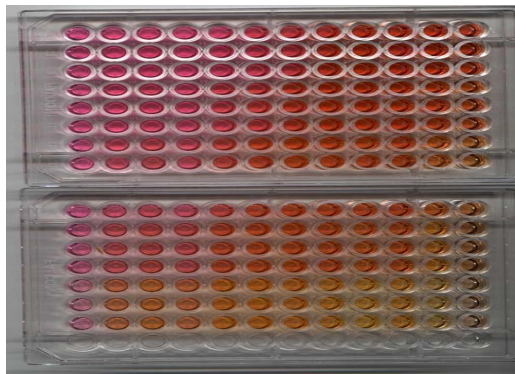
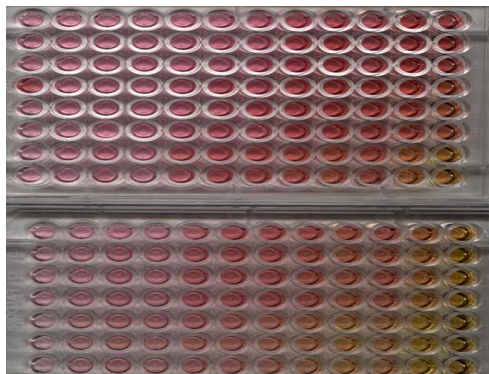


Figure 5.2B: Oxistress Assay (Sodium EDTA) with Varied Concentrations of Hydrogen Standards



5.2.2. Clinical Evaluation of the Oxistress Assay

The assay was also used for biological samples such as urine specimens from normal volunteers (general population) and hospital samples, as well as whole blood samples from the blood bank of the Royal University Hospital, Saskatoon. 100 μl of plasma (from blood bank blood) or urine samples was added to 900 μl of the reagent at 37⁰C and measured at wavelength of 532 nm. The results were similar to those of other methods such as the Xylenol Orange method [197, 352].

5.2.2.1. Measuring Strong Oxidants in Plasma with the Oxistress Assay

Plasma from blood bank blood was used as a standard to determine the optimal concentration of the deoxyglucose component by evaluating various concentrations (10 mM – 100 mM) and plasma/ assay dilution ratios of 1/3 -1/20 (Figure 5.3). A linear curve was observed at 100 mM deoxyglucose concentration while a flat curve was observed at 10 mM implying that the latter concentration is not suitable for plasma peroxide measurement. The use of 100 mM of deoxyglucose as the 2-deoxysugar in the reagent was based on this finding.

Figure 5.3: Plasma Peroxides at 10 & 100 mM Deoxyglucose Concentrations

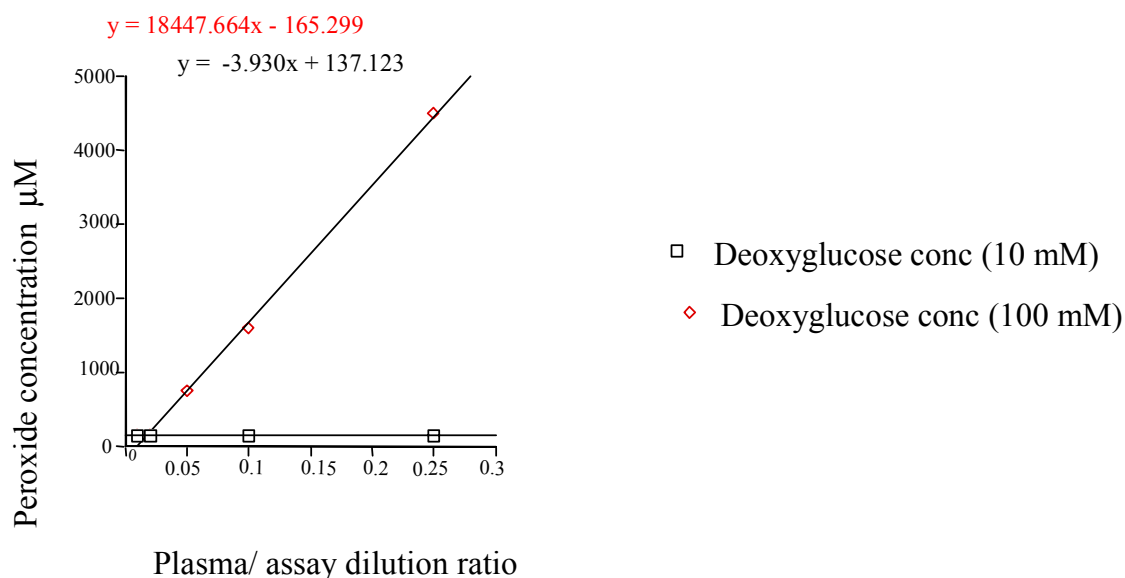


Figure 5.3: 100 mM deoxyglucose produced a linear curve with various dilutions of blood bank blood while 10 mM produce a flat graph.

5.2.2.2. Measuring Strong Oxidants in Urine with the Oxistress Assay

The analysis of 60 urine samples of hospital patients' "Stat urinalysis" from the Dept. of Chemical Pathology, Royal University Hospital, Saskatoon, in comparison with 50 samples from the general population was undertaken. Patient diagnosis was unknown prior to sample analysis and no medical history was obtained from the general population, hence the assumption of a healthy group. The following results illustrated in Figure 5.4 were obtained:

1. The values for the hospital patients ranged from 0 µM to 1060 µM with a median (inter-quartile range) of 102 µM and mean 168 µM (95 %CI range 119-217 µM).
2. The values for the general population ranged from 0 µM to 231 µM with a median (inter-quartile range) of 36 µM and a mean of 49 µM.

3. The Mann-Whitney test for non-parametric analysis showed an extremely significant difference between the median values of the two groups, two-tailed $p=0.0001$.
4. The estimated reference value range for the normal population range is $34 \mu\text{M}$ – $65 \mu\text{M}$.

An analysis of patients with twice the estimated normal reference value of urine peroxide was also done (Figure 5.5). The diagnosis in most of these patients has been associated with oxidative stress making the Oxistress assay a clinically applicable diagnostic tool.

Figure 5.4: Urine Peroxide Levels In Hospital Patients Vs. General Population

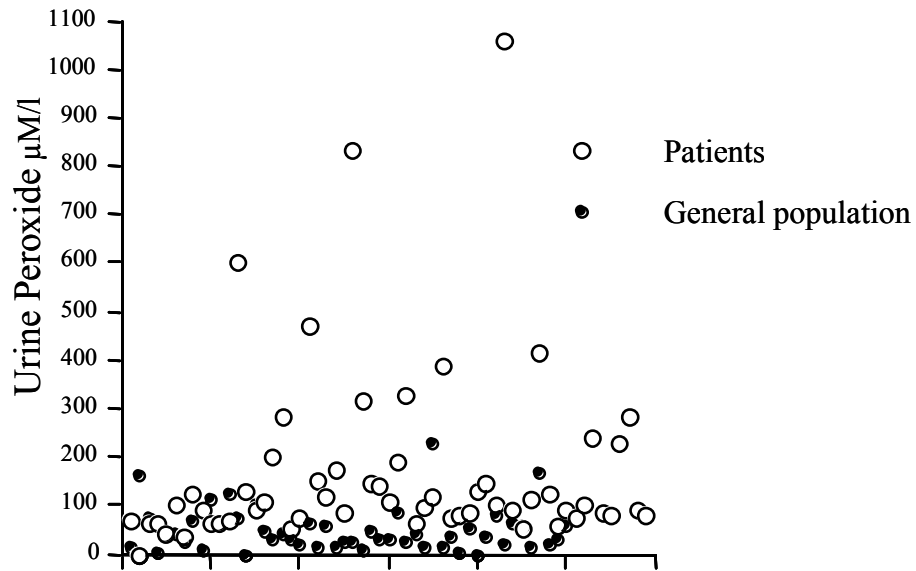


Figure 5.4: Patients had higher urine peroxide levels than the general population ($p=0.0001$)

Figure 5.5: Peroxide Levels In Patients With Twice The Reference Values

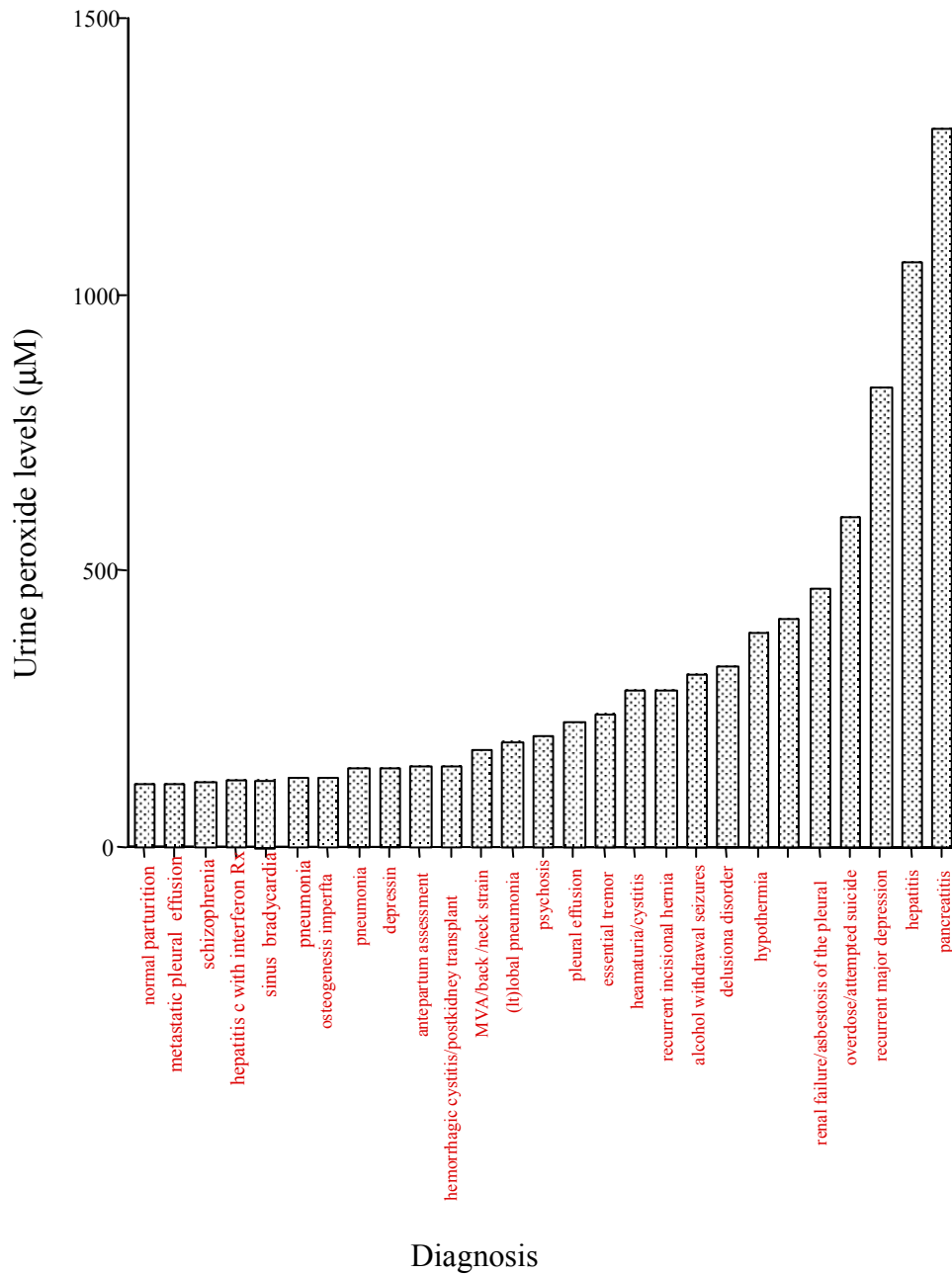


Figure 5.5: Diagnosis in patients with twice the reference values that has been associated with oxidative stress.

5.2.3. Further Evaluation of the Oxistress Assay: See appendix

The following were the aims and objectives of this phase of the evaluation:

- ◆ To better evaluate the role of EDTA

- ◆ To better evaluate the role of deoxyglucose (or glucose) in the assay.
- ◆ To evaluate the stability of the assay and the type of color change with storage and reactions with hydrogen peroxide standards over time.

5.2.4. Discussion

This assay was developed because of the need for a less cumbersome or simple reliable method that can measure a wide range of peroxide levels in biological fluids thus making bedside clinical diagnosis of oxidative stress a reality. The assay components were determined based on their individual properties and potential to produce a colour change in a reaction with hydrogen peroxide. The Oxistress assay (with the EDTA component) was used on hydrogen peroxide standards as well as biological samples from the general and hospital populations. The microtitre plate and 1 ml cuvette methods produced similar results and the dipstick method presently in the developmental phase is also expected to produce similar results as these two methods.

Our study demonstrated that EDTA has an inhibitory effect on the color reaction produced by the assay at higher hydrogen peroxide concentrations ($>800 \mu\text{M}$). Kikugawa et al. (1992) also reported partial inhibitory effect of EDTA on the ability of thiobarbituric acid to form a color complex in rat brain samples [353]. The removal of EDTA from the assay resulted in a linear curve with hydrogen peroxide standards at all concentrations. This limitation with EDTA was not observed in the biological samples because with sample dilutions up to $1500 \mu\text{M}$ peroxide levels were measured. The observed limitation with EDTA however suggests it may not be an ideal component of a bedside or dipstick Oxistress assay measurement tool even though the dilution method makes it a very easy and reliable laboratory tool.

Deoxyglucose and glucose were separately evaluated to determine the ideal sugar for optimal assay function. Glucose required higher concentration of hydrogen peroxide to produce a visible color change and deoxyglucose produced a deeper color change making it the preferred assay sugar. The optimal deoxyglucose concentration was also determined to be 100 mM .

Reference ranges were established with urine samples from the general and subsequently applied hospital population. The Oxistress Assay urine study has the largest

reported population (N=110) and a statistically significant difference was established between hospital patients and the general population. The Oxistress Assay was both sensitive and specific in disease states associated with oxidative stress. Previous studies had indicated very low levels of H₂O₂ (<100 μM) in human urine samples, however our study has established measurable high peroxide equivalents in both general and hospital populations. These findings agree with those of Long et al (1999) [197], who reported much higher levels than previously published (>100 μM) using 3 different methods of measuring H₂O₂ in urine. The Oxistress Assay was also successfully used in human plasma samples. We measured a consistent concentration of H₂O₂ equivalent in plasma irrespective of the plasma/ assay dilution ratio.

The novel Oxistress Assay has proven to be a reliable clinical diagnostic tool for Oxidative Stress. In it's current form the assay can be used on plasma urine samples for rapid (5 min) patient evaluation. The Oxistress assay dipstick being developed will provide a bedside test making diagnosis easier. The EDTA-free assay is also very good laboratory tool for measuring high levels of hydrogen peroxide making it useful in both human and non-human samples. The novel Oxistress assay has received full patent rights from the United States Patent Office in June 2004.

6. STUDY OF CLINICAL TRAUMA AND OXIDATIVE STRESS

6.1. Introduction

This is a study of the natural history of Oxidative Stress in Trauma since the onset time can be reliably determined in the normal population, and there is evidence that trauma is the commonest cause of oxidative stress [15, 16]. Trauma patients also form a good cohort for understanding the natural history since most are not known to have prior oxidative stress. Animal studies suggest strong oxidants and neutrophils play important roles in the pathophysiology of oxidative stress after trauma, shock and burns. The consumption of endogenous antioxidants by free radicals resulting in oxidative stress may promote the development of multiple organ organ failure (MOF) after polytraumatic injury [18] which would result in death. Therefore the need to make an early diagnosis of oxidative stress based on a clear understanding of the pathophysiology cannot be overemphasized.

A prospective observational study of the natural history was conducted on all multiply injured patients over 15 yr in age (N= 120) admitted to a regional trauma unit (Royal University Hospital, Saskatoon) between April and September 2000 were entered into the study. Patients were subsequently divided into four groups, the first three based on the injury severity score (ISS) and the fourth based on pre-morbid medical factors. The ISS classifies patients into two broad groups i.e. major (or severe = $ISS \geq 16$) and minor trauma ($ISS \leq 15$). The minor group could be sub-classified into moderate ($ISS 8-15$) and mild trauma ($ISS \leq 7$) [354-356]. Further analysis was done based on the body region affected by trauma.

The aim of the study was to understand the effect of trauma on oxidative stress by measuring the production of strong oxidants in plasma and urine, strong oxidant protein damage (protein carbonyl) and changes in antioxidant reserves in blood (red cell and plasma). The parameters studied over a 7 day period were red cell glutathione levels, total antioxidants in plasma, free radicals in plasma and urine, and neutrophil count & activity. The methods were discussed in Chapter 4 and the results are presented in this chapter. Glutathione (GSH) an antioxidant abundant in erythrocytes was measured using a modification of the Brigelius' method in packed red cell samples taken at 0, 6, 12, 24, 72 hr and on day 7. Changes in plasma antioxidant reserve were measured with the ferric

reducing ability (FRAP) assay, changes in strong oxidants with the novel Oxistress assay and changes in protein carbonyl with a modification of Levine's method. This study formed the basis of patient selection for the evaluation of prophylactic antioxidant in therapy elective surgery patients.

6.2. Red Cell Glutathione

6.2.1. Red Cell Glutathione Measurement in Trauma Patients

Changes in red cell Glutathione levels for each trauma patient group is summarized in Table 6.1. The effect of trauma on the red cell glutathione defense system over a 7 day period for all trauma patients is also shown in Figure 6.1. In all trauma groups combined there is no significant change in the red cell glutathione within the first 6 hr ($p = 0.09$) however between 6-18 hr an increment of 20%-30% ($p = 0.004$) followed by a decline at 24-30 hr is observed. The maximum depletion in the red cell glutathione was at 24-30 hr ($p = 0.037$). A gradual increase to the 12 hr levels occurs at 72 hr ($p = 0.16$) and the upward trend continues until 168 hr (day 7).

Table 6.1: Changes In Red Cell Glutathione Levels In The Patient Groups

Patient group	GSH Levels (μM)	0-6 hr (μM)	6-12 hr (μM)	12-18 hr (μM)	18-24 hr (μM)	72 hr (μM)	168 hr (μM)
All trauma (N=120)	Mean	714	880	860	656	877	1018
	SEM	43.8	71.7	71.0	51.3	96.5	193.3
Severe trauma (ISS score >16) (N =39)	Mean	819	1054	902.5	546	930	1165
	SEM	66.5	149.7	110.4	52.0	127.9	237.5
Moderate trauma (ISS score 9-15) (N =39)	Mean	635	797	789	698	760	757
	SEM	67.1	55.4	112.4	83.9	103.4	177.1
Mild trauma (ISS score 1-8) (N =22)	Mean	669	725	869	841	973	973
	SEM	99.0	104.9	149.2	160.2	322.7	170.0
Preexisting Medical Condition (N =10)	Mean	632	992	840	550	808	1217
	SEM	49.3	198.5	133.4	109.9	130.4	427.3
Head injury (N =11)	Mean	857	815	700	496	750	707
	SEM	91.0	15.0	85.4	89.4	145.9	154.3
Body injury (N =30)	Mean	608	968	903	762	798	773
	SEM	97	122.7	129.6	130.9	83.3	31.8
Combined head/body injury (N=12)	Mean	891	906	993	566	932	730
	SEM	73.5	88.9	298.7	73.3	217.2	111.6
Spine trauma (N=9)	Mean	790	740	970	808	1080	990
	SEM	10	140	319	308	480	0

The natural history of the effect of trauma on red cell glutathione in the different patient groups at different time points is summarized in Figure 6.3. Severe trauma patients had the greatest depletion at 24 hr ($p = 0.0024$), followed by patients with preexisting medical problems ($p=0.0907$) and moderately injured patients ($p = 0.1759$), while minimally injured patients had an increase in the red cell glutathione levels at 24 hr ($p = 0.09$). Patients with pre-existing medical problems had lower baseline red cell glutathione levels than the other three patient groups ($p = 0.1$). Unlike the other groups, similar red cell glutathione response was observed in patients with pre-existing medical problems irrespective of their trauma severity score. There was a rise in red cell glutathione levels between 6 and 12 hr followed by depletion similar to the severe trauma

patients at 24 hr ($p = 0.09$) and subsequently a gradual rise until day 7 when the levels were comparable with the severe trauma group.

Patients with head injury had a continual decline in the red cell glutathione levels from admission till 24 hr ($p = 0.041$) followed by a sustained rise from 72 hr till day 7 ($p = 0.001$), by which time they still failed to reach the initial level (Figure 6.4). Combined head and body injury patients had a rise in glutathione levels between 6 and 18 hr followed by a sharp decline at 24 hr ($p = 0.0087$) and although there was a significant rise at 72 hr the initial values were also not attained by this group. Patients with severe body injury demonstrated a pattern similar to the combined head and body injury except that the decline at 24 hr was not as sharp ($p = 0.127$) and achieved higher than initial red cell glutathione levels by the seventh day. Isolated spinal injury patients demonstrated and maintained a continual rise in red cell glutathione levels throughout the study period except for the slight decline at 24 hr ($p = 0.8582$) (Figure 6.4). The severity of trauma, a pre-existing medical problem and organ affected by the trauma appear to be factors most responsible for the observed decline in the red cell glutathione defense system at 24 -30 hr.

Figure 6.1. Red Blood Cell Glutathione (GSH) Levels In All Trauma Patients

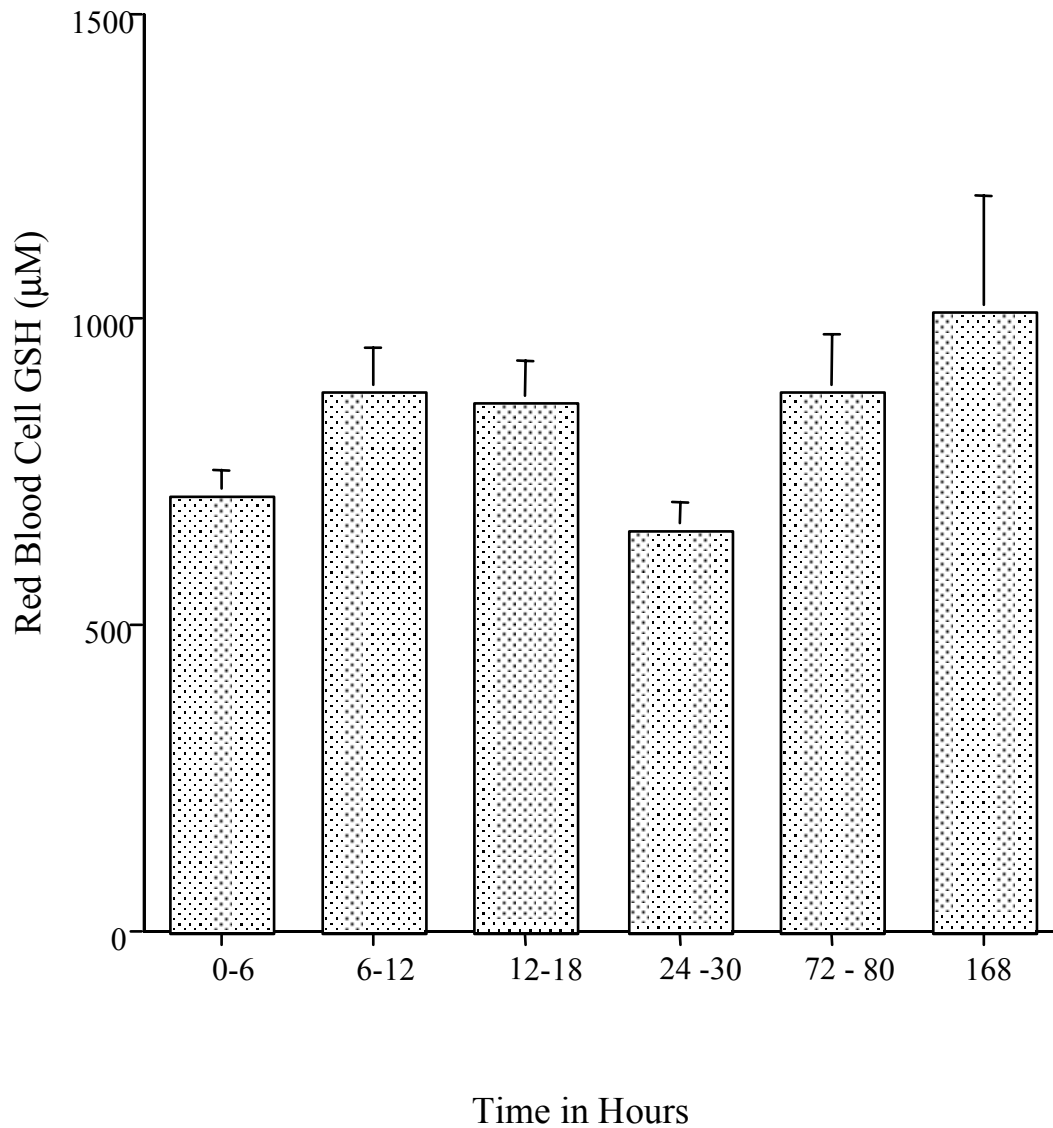


Figure 6.1: Red blood cell glutathione (GSH) levels (mean \pm SEM) in all trauma patients (n=120) seen between 0–168 hr. There is an increment of 20-30% ($p = 0.004$) between 6-18 hr and maximum depletion at 24-30 hr ($p=0.037$) in the red cell glutathione.

Figure 6.2: Changes In Glutathione Levels At 6 And 24 Hr Versus Trauma Severity

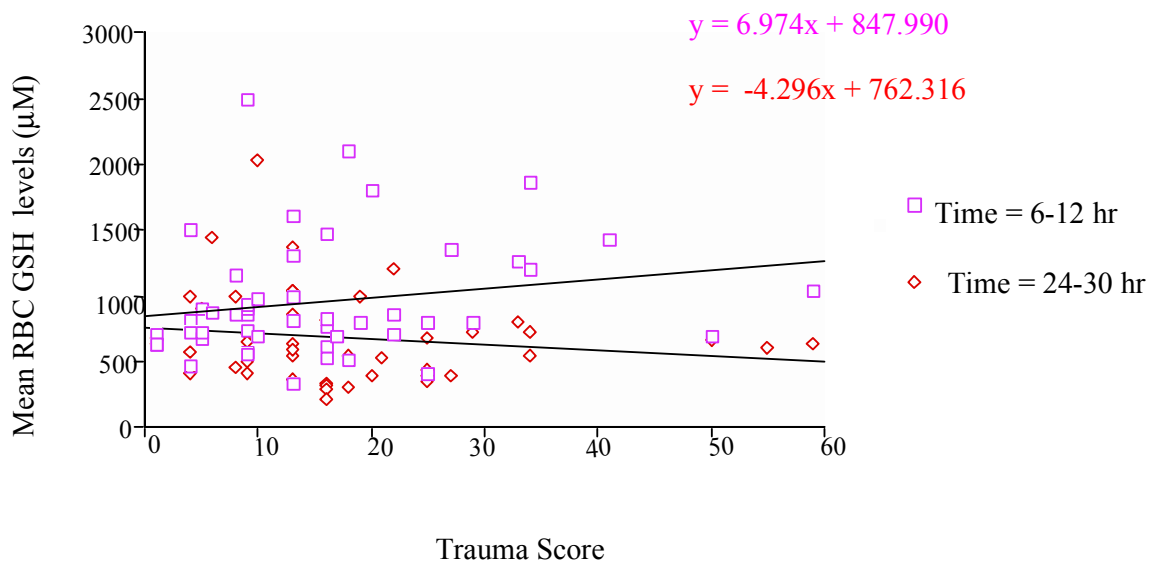


Figure 6.2: Scattergram showing the distribution of red cell GSH in relation to trauma severity. The higher the trauma score or severity the greater the depletion in red cell GSH ($p = 0.0024$) at 24 hr.

Figure 6.3: Red Cell GSH Changes Versus Trauma Severity/ Preexisting Medical Problem

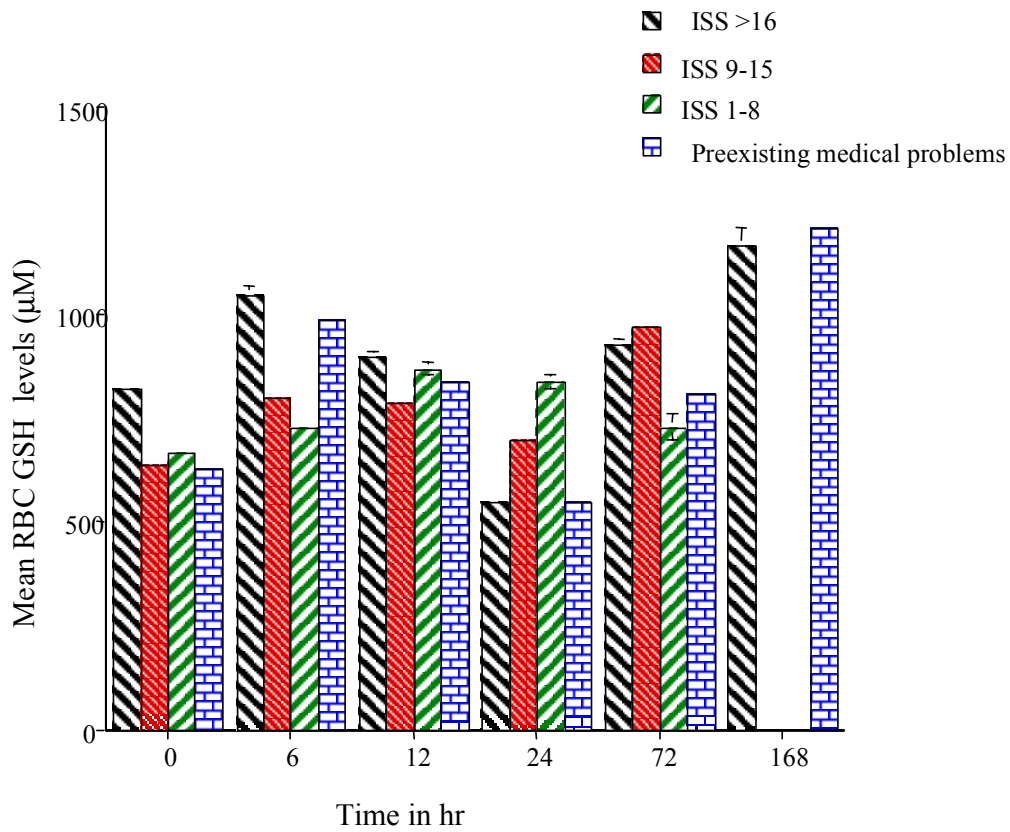


Figure 6.3: The relationship between trauma severity (ISS score), pre-existing medical problems and changes in red cell glutathione (mean \pm SEM) content is demonstrated above. The SEM of some sample sets are so small they do not appear on the graph. All four groups except ISS score < 8 had maximal depletion at 24 hr. Severe trauma patients' (ISS score >16) had the greatest depletion at 24 hr ($p=0.0024$), moderately injured patients (ISS score 9-15) had less depletion ($p=0.042$), while minimally injured patients (ISS score 1-8) had an increase in the red cell glutathione levels at 24 hr ($p=0.09$). Patients with pre-existing medical problems had depletion similar to severe trauma patients ($p=0.09$) and lower baseline red cell glutathione levels than the other three patient groups ($p=0.1$). (ISS 1-8 = mild trauma; ISS 9-15 = moderate trauma and ISS >15 = severe trauma).

Figure 6.4: Red Cell GSH Changes Versus Body Region Affected By Trauma.

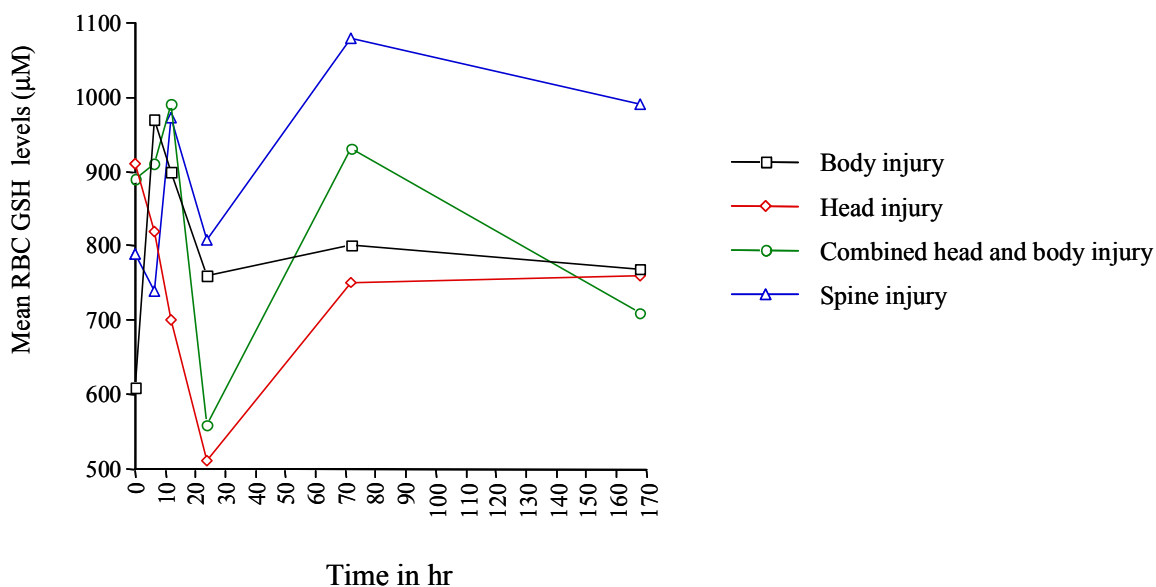


Figure 6.4: In comparison with admission baselines head jury caused the greatest depletion at 24 hr ($p=0.041$) followed by combined head and body injury ($p=0.0087$), and severe body injury ($p=0.127$) with the least depletion in spinal injury ($p=0.8582$).

6.2.2. Discussion: Trauma and Red Cell Glutathione

Studies have shown a clear association between severe trauma and the release of reactive oxygen species [44] which results in the consumption of antioxidants and subsequently oxidative stress [357]. The red blood cell has been shown to have high concentrations of GSH [131] and about 95% of it's GSH is in the reduced form [18]. The red cell also has the ability to regenerate about 35 $\mu\text{mol/l}$ of ascorbic acid every 3 min from ascorbate radical [42]. Significant extra-cellular oxidative stress is required to deplete the GSH level in the red cell [316]. As reported in the previous section, an initial increase in the red cell GSH between the 6 and 18 hr (Figures 6.1 - 6.3) was observed. The mechanism of this increase could be red cell transportation of GSH produced in the liver [142, 358], however, other factors such as increased concentration of red cell glutamic acid, glycine and the maintenance of body temperature may play a role in the

rapid production of GSH in the red cell during this period of oxidative stress [131, 143, 359]. Maintaining the body temperature is very important for GSH metabolism because heat stress not only produces oxidants but it impairs the enzyme systems necessary for detoxification [143]. The possible role of fresh blood transfusion in increasing red cell GSH can also not be excluded. Another possibility is an increase in red cell release from the bone marrow and other stores in response to hypoxia and hemodynamic instability.

The maximal depletion observed in red cell GSH at 24- 30 hr in all patients was more in those with elevated neutrophil activity [76, 360], shock or multi-organ damage [361]. The groups most affected were the severely injured, head injured and patients with pre-existing medical problems (Figures 6.1-6.4). This observed depletion in red cell GSH may be due to peak strong oxidant release in plasma and subsequent consumption of plasma antioxidants. Other researchers have also reported decreased plasma GSH associated with severe multiple trauma thus validating our findings [18, 130].

Patients with pre-existing medical problems such as liver cirrhosis, diabetes and chronic obstructive lung disease form a special group in trauma because outcome is more dependent on these medical co-morbidities than the trauma severity based on injury scoring system [362]. This finding was demonstrated in an earlier study by Mackenzie, which recommends that all patients with pre-existing medical problems be treated in a trauma center irrespective of their trauma severity [363]. Our study results show that patients with pre-existing medical problems have lower baseline GSH than the other groups and were as depleted as the severely injured patients at 24 hr irrespective of their trauma severity (Figure 6.3). These findings agree with a study on insulin-dependent diabetic patients who had lower basal levels of antioxidants and demonstrated lower plasma and erythrocyte antioxidant capacity when confronted with oxidative stress [364]. The researchers suggest their findings may be due to lower erythrocyte glutathione reductase (46%), glutathione peroxidase level (30%) and glutathione -s transferase (26%) activities when compared to the controls [364].

Animal studies have shown that the cytotoxic process triggered by head injury causes the release of reactive oxygen species leading to the development of secondary injury [365], and systemic depletion of low molecular weight antioxidants such as GSH [360, 366], maximal at 24 hr. These findings were supported by our study, which

revealed the maximum depletion of red cell GSH was at 24-30 hr after head injury. The return to baseline levels had not yet occurred by day 7 (Figure 6.4), a finding not previously reported in human studies. The depletion was worse in head and multiple system compared with severe single system injured patients (without head injury). Spinal cord trauma resulted in less red cell GSH depletion than in other organ systems at 24 hr when compared with the levels at 6 hr. This is not surprising because spinal injuries are usually minimal injuries, but there was an expectation of greater systemic impact on the red cell GSH because of the CNS involvement.

6.3. The Role Of Neutrophils in Trauma:

6.3.1. Neutrophil Count and Trauma:

A direct relationship between quantitative neutrophil count and trauma severity within 6-12 hr of trauma was observed (Figure 6.5). The more severe the trauma the higher the neutrophil count between patient groups ($p = 0.019$). The effect of organ specific damage on neutrophil count in the first 6-12 hr revealed higher counts in head injury (similar to the animal head injury models) [360] than in isolated body injury patients although this difference was not statistically significant ($p = 0.38$) (Figure 6.6). Head injury patients also had higher neutrophil count ($15 \times 10^9/l$) than spine injury ($8 \times 10^9/l$).

Debate exists about the cause and the clinical value of changes in the absolute neutrophil count in trauma. Some suggest these changes are due to an imbalance between production and demargination while others feel margination and breakdown are responsible [72, 73]. Recent studies have linked changes in the absolute neutrophil count with post-operative surgical stress [74-78]. Most of these studies were related to surgical stress or infection and the earliest changes were observed after 24 hr [74].

Our results however depicted an increase in the absolute neutrophil count, which correlated with trauma severity based on the injury severity score ($p = 0.019$) as early as 6 hr (Figure 6.5). One possible reason for the increased neutrophil count is the inhibition of apoptosis (programmed cell death) and subsequent prolongation of inflammation [73, 79-82]. Trauma not only reduces the rate of apoptosis but increases the functional longevity of neutrophils [73]. Recent studies have shown that mitogen activated protein

kinase (MAPK) and nuclear factor kappa B (NF- κ B) activation by oxidative stress appear to be responsible for the reduction in apoptosis [83, 84]. Neutrophil count appears to be associated with the degree of the trauma and may be predictive of the eventual outcome of the patient.

Neutrophil accumulation contributes to host defense via their bactericidal and phagocytic properties. However this could potentially exacerbate injury by obstructing brain microvasculature and aggravating the ischemic process secondary to trauma. Animal studies have shown that traumatic brain injury results in neutrophil infiltration of the brain as well as systemic increase within the first few hours [360, 365-368] but previous human studies have not reported the significance of systemic neutrophil count in head injury. Our results suggest that neutrophil count may be predictive of oxidative stress in head injury patients contrary to previous reports. Head injured patients in our study had higher absolute neutrophil count than spineally injured patients and this finding suggests a direct relationship between injury severity and post injury neutrophil count. This suggestion is supported Malone et al (2001) who concluded that neutrophil count on admission is an independent marker predicting outcome in trauma [369]. We observed an increase in the systemic neutrophil count in our head injury patients highest at 6 hr and similar to the animal head injury models.

Figure 6.5: Neutrophil Count (Mean \pm SEM) At 6hr Relative to Trauma Severity.

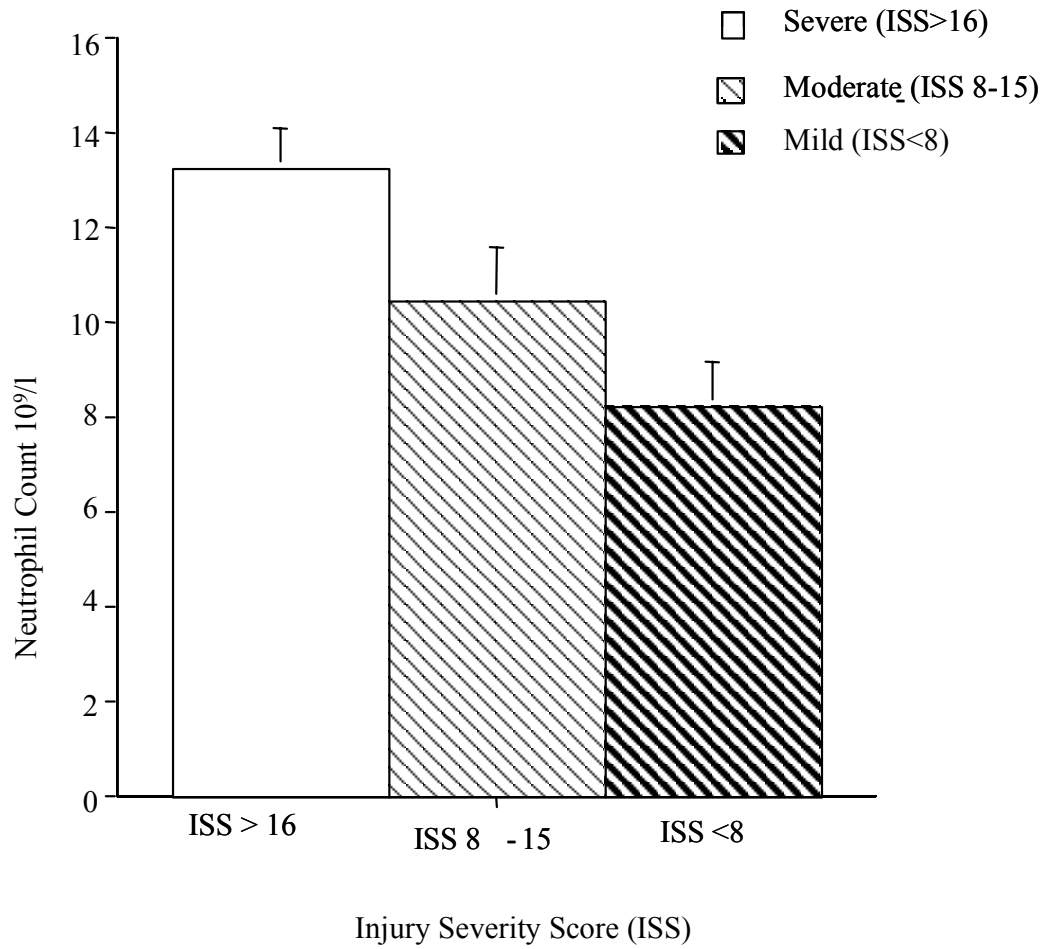


Figure 6.5: A significant variation in neutrophil count based on trauma severity ($p= 0.019$) is observed.

Figure 6.6: Neutrophil Count (Mean \pm SEM) At 6 Hr In Head And Body Trauma

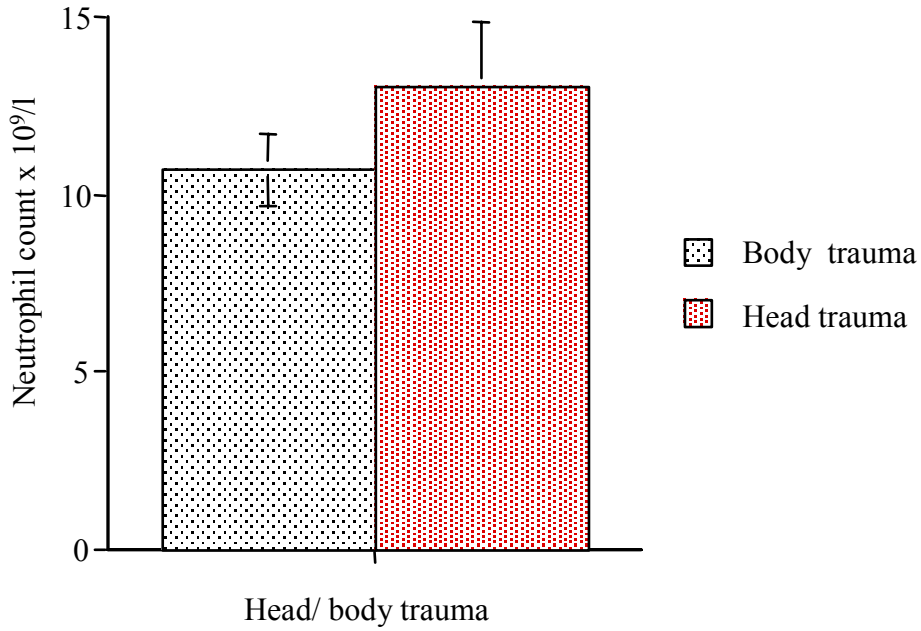


Figure 6.6: Graph comparing neutrophil count in isolated head trauma with body trauma. Neutrophil count was higher in head trauma than in body trauma at 6 hr ($p= 0.38$).

Figure 6.7: Neutrophil Count (Mean \pm SEM) At 6 Hr In Head And Spine Trauma

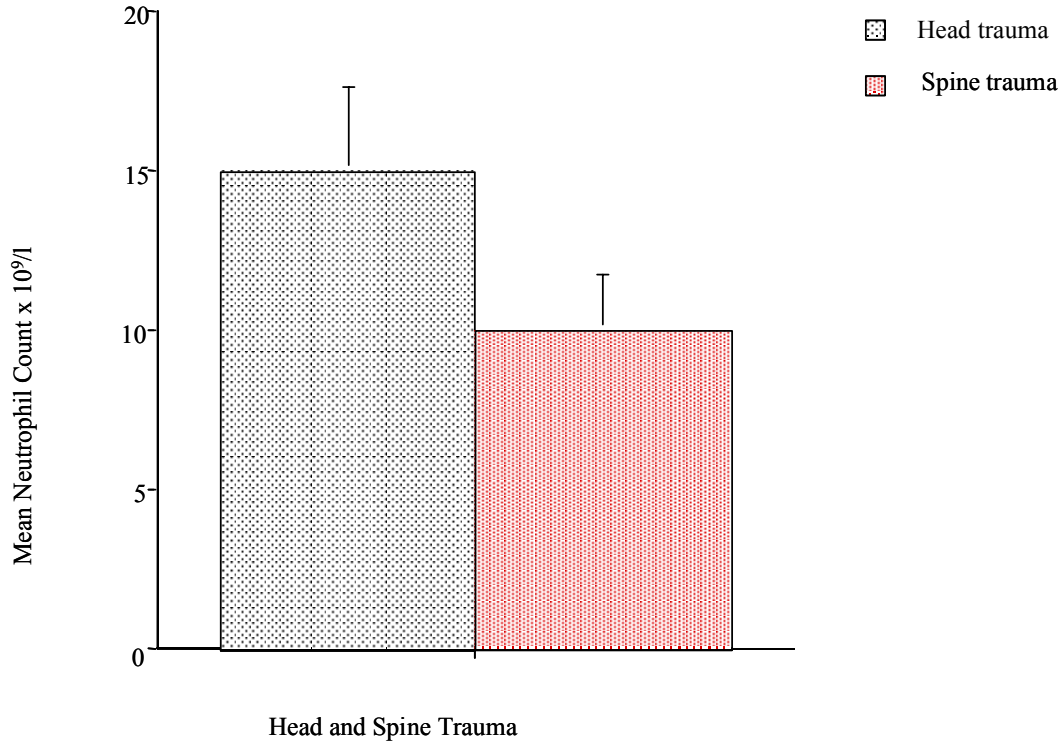


Figure 6.7: Graph comparing neutrophil count in head versus spine trauma. Neutrophil count in head trauma was approximately double that in spine trauma.

Discussion: Neutrophil Activity and Trauma:

Activated PMN cells have the capacity to generate and release substantial amounts of reactive oxygen species after trauma. A study by Tanaka suggests that neutrophil O_2^- production increases acutely in trauma [370]. They reported highest sensitivity (84%) but low specificity (39%) in predicting multiple organ dysfunction (MODS) after trauma with admission plasma elastase levels. However another study reported concentrations above 500 ng/ml on day 3 post-trauma was predictive of subsequent severe MODS with 95% specificity and 69% sensitivity [331]. In our study population we observed two neutrophil activity peaks irrespective of the injury score, the first between 7 hr and 10 hr and the second smaller peak between 13 hr and 16 hr (Figure 6.8). We also observed a tendency towards increased neutrophil activity with increase trauma severity (Figure 6.9), however this trend was not statistically significant.

Our study also showed minimal changes in systemic neutrophil activity in previously healthy spinally injured patients however the presence of previous medical morbidity had priming effect on the neutrophils thus increasing their activity (Figure 6.10). Head injury increased systemic neutrophil activity in all patients and this might be responsible for the severity of antioxidant depletion in head injured as compared to spinally injured patients. The observed increased in activity may also be due to the synchronous effect of increased neutrophil count and up regulation of neutrophil activity, which is associated with trauma.

Figure 6.8: Plasma Neutrophil Activity Following Trauma

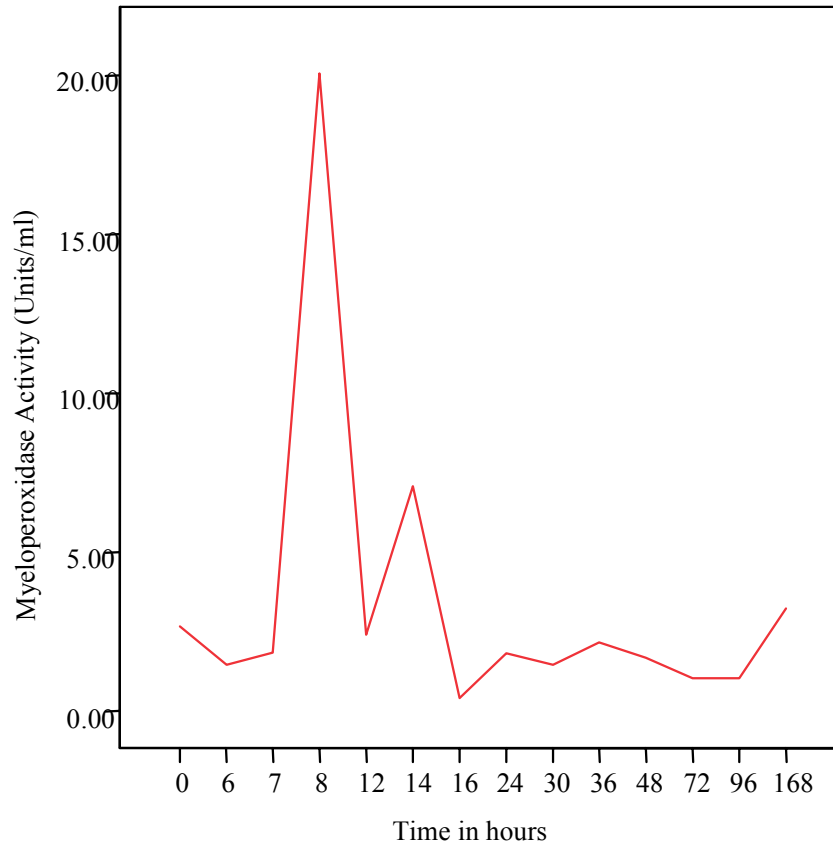


Figure 6.8: Graph showing changes in Neutrophil activity over 7-day period. Two activity peaks were observed following trauma – 1st between 7 and 10 hr and second smaller peak between 13 and 16 hr.

Figure 6.9: Neutrophil Activity Versus Trauma Severity

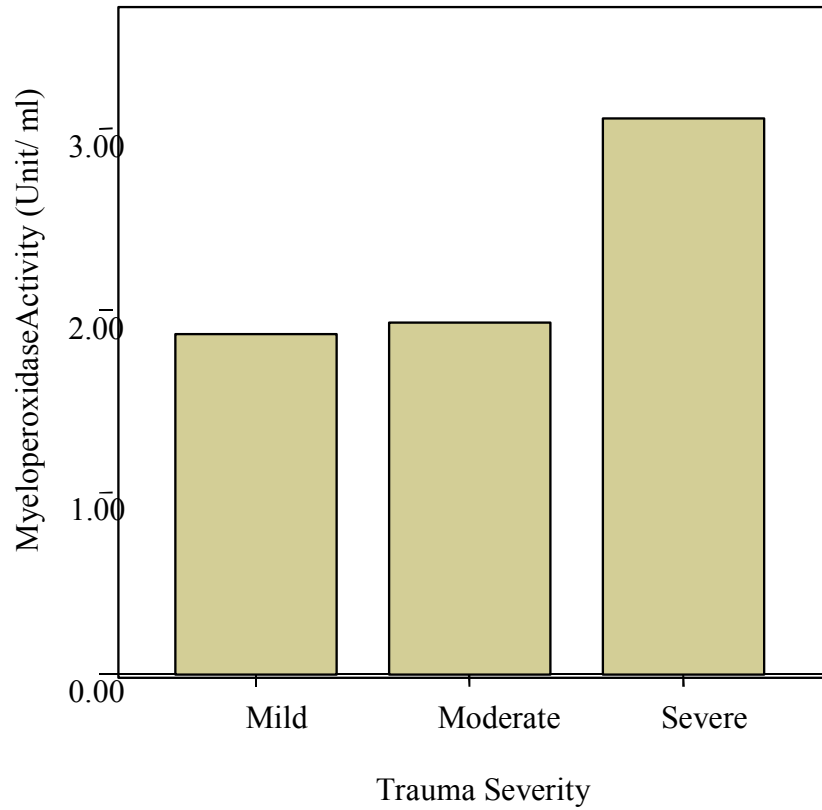


Figure 6.9: Graph showing the relationship between trauma severity and neutrophil activity. There is increased neutrophil activity with trauma severity, however this is not significant ($p=0.349$)

Figure 6.10: Changes In Neutrophil Activity In Organ Specific Trauma

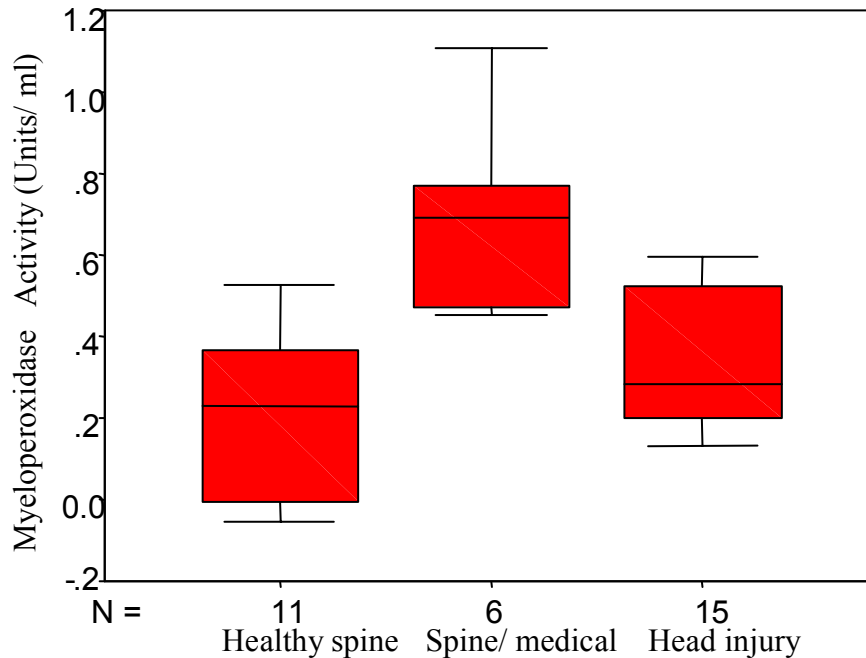


Figure 6.10: Graph comparing myeloperoxidase activity in healthy spine injury, spine/ medical injury and head injury. Neutrophil activity is highest in spinal/ medical injury patients.

6.4. Strong Oxidants in Plasma and Urine (Oxistress Assay).

Plasma or urine oxidant level is the quantity of strong oxidants generated after trauma. Similar pattern of urine and plasma oxidant levels were observed in all trauma patients (Tables 6.2; 6.3). Peaks were noticed at 6 hr and 24 hr and depletion at 12 hr and 72 hr. It is important to note that the oxidant levels were higher (about five-fold) and the changes were more profound in plasma than in urine.

6.4.1. Plasma Oxidants in Trauma

Our results revealed fluctuating plasma oxidant levels post trauma - levels were highest within the first 6 hours with a 25% decrease between 6 and 12 hr ($p= 0.0205$). Subsequently a 30% increase between 12-18 hr ($p= 0.0047$) followed by a steady decline

over the next 48 hr ($p= 0.0104$) was observed. A 10% increase between 72 hr and day 7 ($p=0.2$) likely due to complications in patients following trauma was noticed subsequent to the previously observed decline (Figure 6.11). There were significant plasma oxidant changes resulting from physical trauma ($p =0.041$) and a similar trend was observed in relation to trauma severity.

Table 6.2: Changes in Plasma Oxidant after Trauma

Time in hr	0-6 hr	6-12 hr	12-18 hr	24-30 hr	72 hr	168 hr
Mean oxidants (peroxide equivalent)	755.71	471.5	797.5	649.4	532.12	639.3
SEM	55.55	79.58	46.01	60.47	75.23	90.27
95% CI	619 -891	283 -659	688.7 - 906.3	512.6 - 786.2	354.2 - 710	407.2 - 871

Table 6.2: Mean plasma oxidant for all patients revealed fluctuating levels: high on admission (0-6 hr) and highest at 12- 18 hr. Decline between 6- 12 hr was significant ($p= 0.02$). This trend occurred irrespective of trauma severity.

Figure 6:11 Plasma Free Radicals Post Trauma (Oxistress Assay Method)

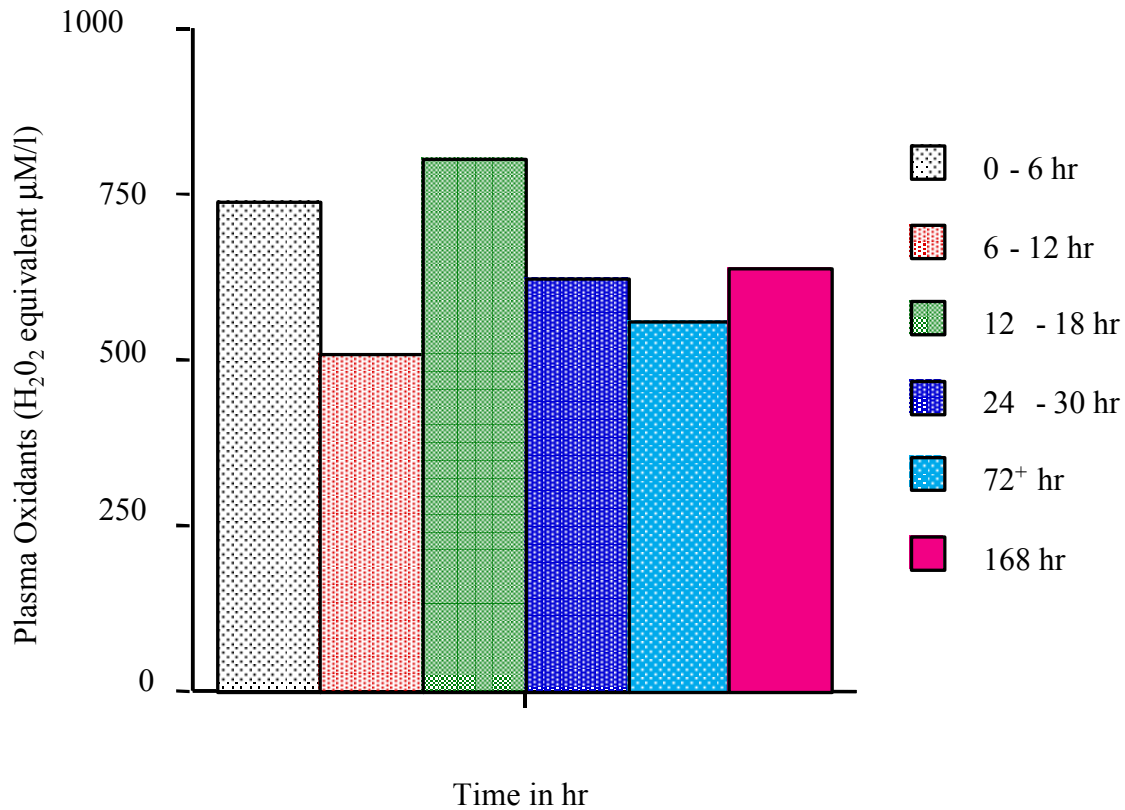


Figure 6.11: Mean plasma oxidant for all patients revealed fluctuating levels: high on admission (0-6 hr) and highest at 12- 18 hr. Decline between 6- 12 hr was significant ($p= 0.02$). This trend occurred irrespective of trauma severity.

6.4.2. Urine Oxidants in Trauma

The prospective study revealed trauma results in more than two-fold the normal urine excretion of oxidants. Changes in urine were similar to plasma however the highest oxidant levels were observed between 24-30 hr as compared to the finding in plasma. There is a high urine oxidant level followed by a significant decline between 6 and 12 hr ($p=0.02$). An increase between 12-18 hr post injury ($p=0.1$) and a further increase in the 24-30 hr period ($p=0.2$) were observed. Trauma results in a significant increase in excretion of urine oxidants between 12 hr and 24 hr ($p=0.012$). This increase was followed by a significant decline at 72 hr ($p= 0.01$).

Table 6.3: Changes in Urine Oxidant Levels After Trauma

Time in hr	0-6 hr	6-12 hr	12-18 hr	24-30 hr	72 hr	168 hr
Mean oxidants (peroxide equivalent)	157.2	127.4	153.88	164	129.5	142.5
SEM	4.14	8.9	6.2	4.9	9.9	11.2
95% CI	148- 166	107- 147	139 -168	152-175	106 - 152	115.8 -169

Table 6.3: Mean urine oxidant levels for all patients after trauma were high on admission (0- 6 hr) but peaked at 24- 30 hr. This trend also occurred in all groups irrespective of trauma severity.

Figure 6.12: Urine Free Radicals Post Trauma (Oxistress Assay Method)

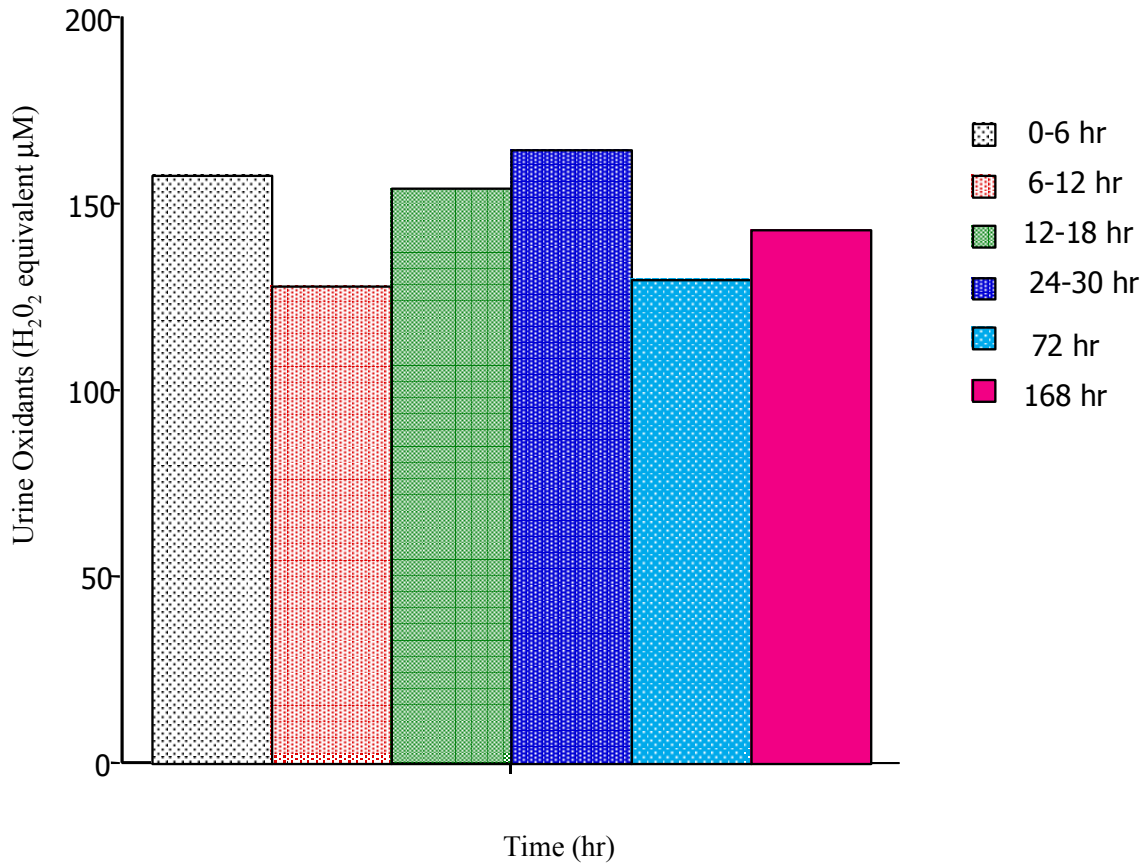


Figure 6.12: Mean urine oxidant levels for all patients after trauma were high on admission (0- 6 hr) but peaked at 24- 30 hr. This trend also occurred in all groups irrespective of trauma severity.

6.5. Plasma Antioxidant Reserve (FRAP Method)

The highest concentration of plasma antioxidant reserve (total antioxidants in plasma) was 1000 μM ferrous sulphate equivalent in all patients and this was observed on admission irrespective of trauma score. There was however a 30% drop within 6 hr followed by a further 15% depletion in the first 24 hr of trauma. This depletion in plasma antioxidants was significant ($p = 0.017$). There was slight recovery from 72 hr but only 15% increase from the lowest level (at 24 hr) was observed by day 7 (Figure 6.13). The admission levels were therefore not regained by day 7 in all patients. Trauma severity and pre-morbid medical conditions had similar trend as observed in all patients combined.

Figure 6.13: Plasma Antioxidant Changes Over Time –FRAP Method

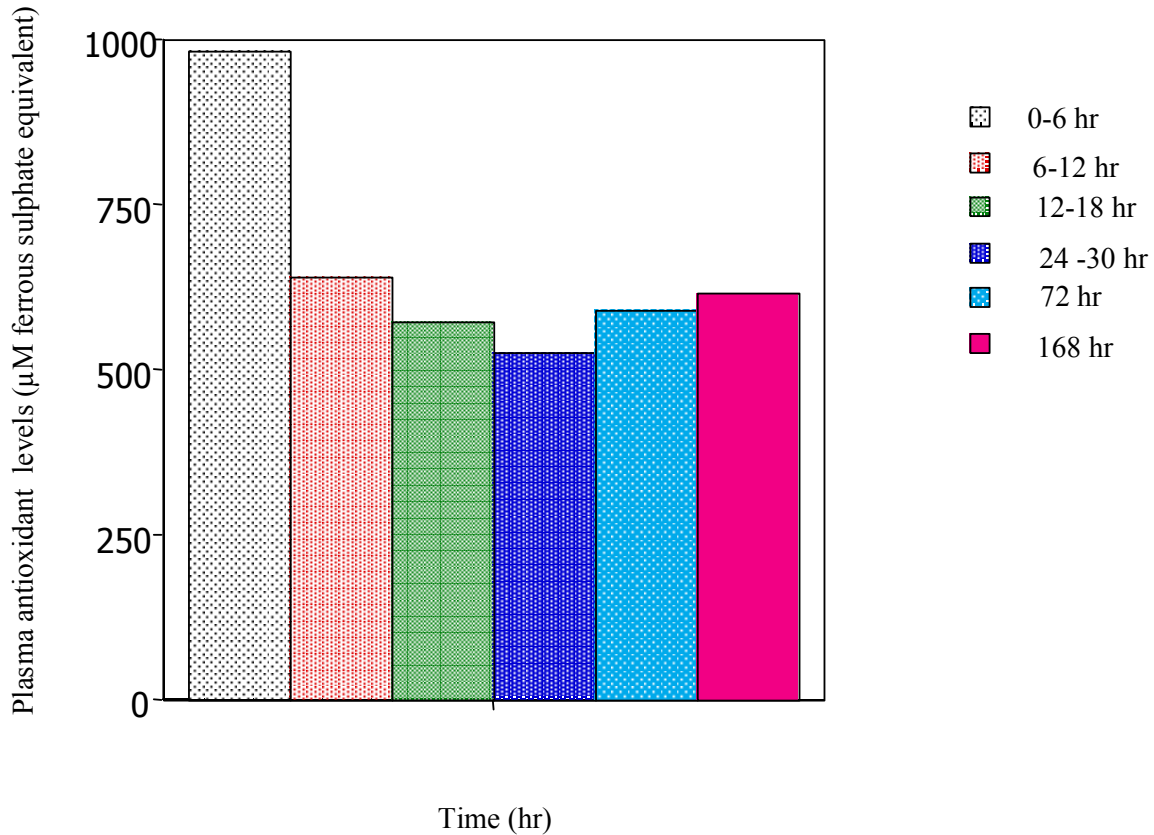


Figure 6.13: Trauma results in significant depletion in antioxidant reserve in all patients ($p = 0.017$). Highest level was on admission and greatest depletion was at 24 hr. Baseline levels were never regained and similar trend was observed irrespective of trauma severity.

6.6. Protein Carbonyl in Plasma and Urine

6.6.1. Plasma Protein Carbonyl Analysis

The average age of patients in the plasma protein carbonyl group was 40.7 ± 5.6 yr (range 17–81 yr, $n=15$). The average carbonyl content across all samples over the study period was 1.49 nmol/mg protein. There were two peaks, first on admission and slightly higher peak on day 7 (168 hr) and declines were observed within 6-12 hr and 24 hr (Figure 6.14).

The two main ISS groups (minor and major trauma) were equally matched in age and sex. There were five patients in Group 1 (minor trauma). The mean ISS-90 was 9.4 ± 1.91 (Range 4-16, $n = 5$). The average carbonyl content on admission to hospital was

0.87 ± 0.2 nmol carbonyl/mg total protein (range 0.45-1.61 nmol/mg, n=5) and 0.93 nmol/mg over the study period. The rise in carbonyl content varied in time between patients. Group 2 (major trauma) consisted of ten patients. The mean ISS-90 was 25.3 ± 1.86 (range 17-34, n=10). The average carbonyl content on admission to hospital was 1.95 ± 0.38 nmol carbonyl/ mg total protein (range 0.03- 3.87, n=10.) and 1.68 nmol/mg over the study period. The individual peak carbonyl levels occurred at the time of hospital admission and on day 7. Day 7 levels were higher than admission levels in the 2 groups although more marked in severe trauma. The carbonyl content was significantly higher than in minor trauma throughout the study period (p<0.001). The same trend was noticed on admission and the difference between the two groups was also significant (p=0.025) (Figure 6.15).

Grouping the patients into ISS-90 scores of 0-9, 10-19, 20-29, and >30 (Figure 6.16) produced similar results as grouping into minor and major trauma (Figure 6.15) but the difference between these four sub-groups was not statistically significant (p=NS). Comparing plasma carbonyl with oxidant levels in the same patient revealed a linear relationship (p= 0.031) between the two groups (Figure 6.17).

Figure 6.14: Plasma Carbonyl Content in All Trauma Patients

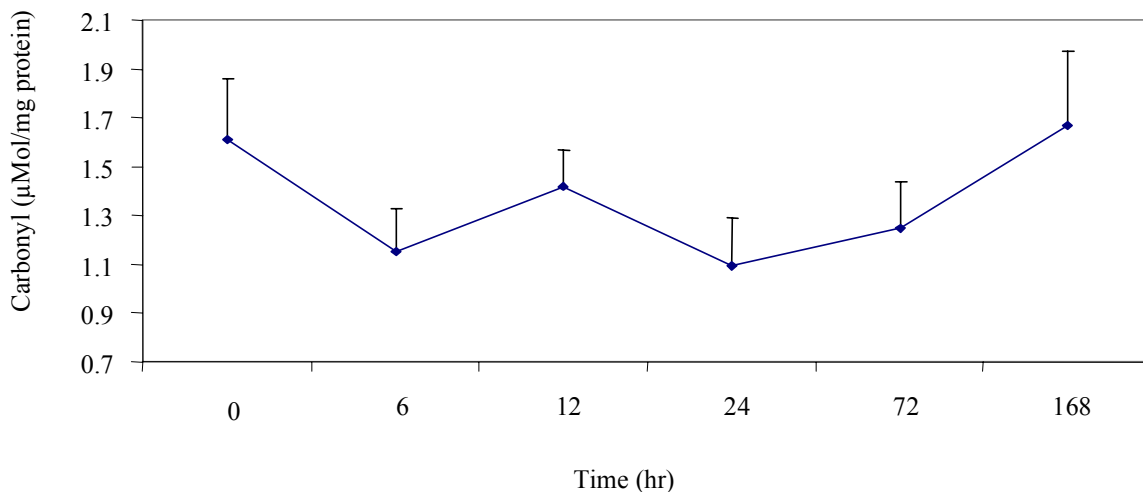


Figure 6.14: Changes in plasma carbonyl in all trauma patients revealed 2 peaks on admission and 168 hr. Declines occurred at 6-12 hr and 24 hr. Average carbonyl content over the study period was 1.49 nmol/mg protein.

Figure 6.15: Plasma Carbonyl Content in Major and Minor Trauma Patients

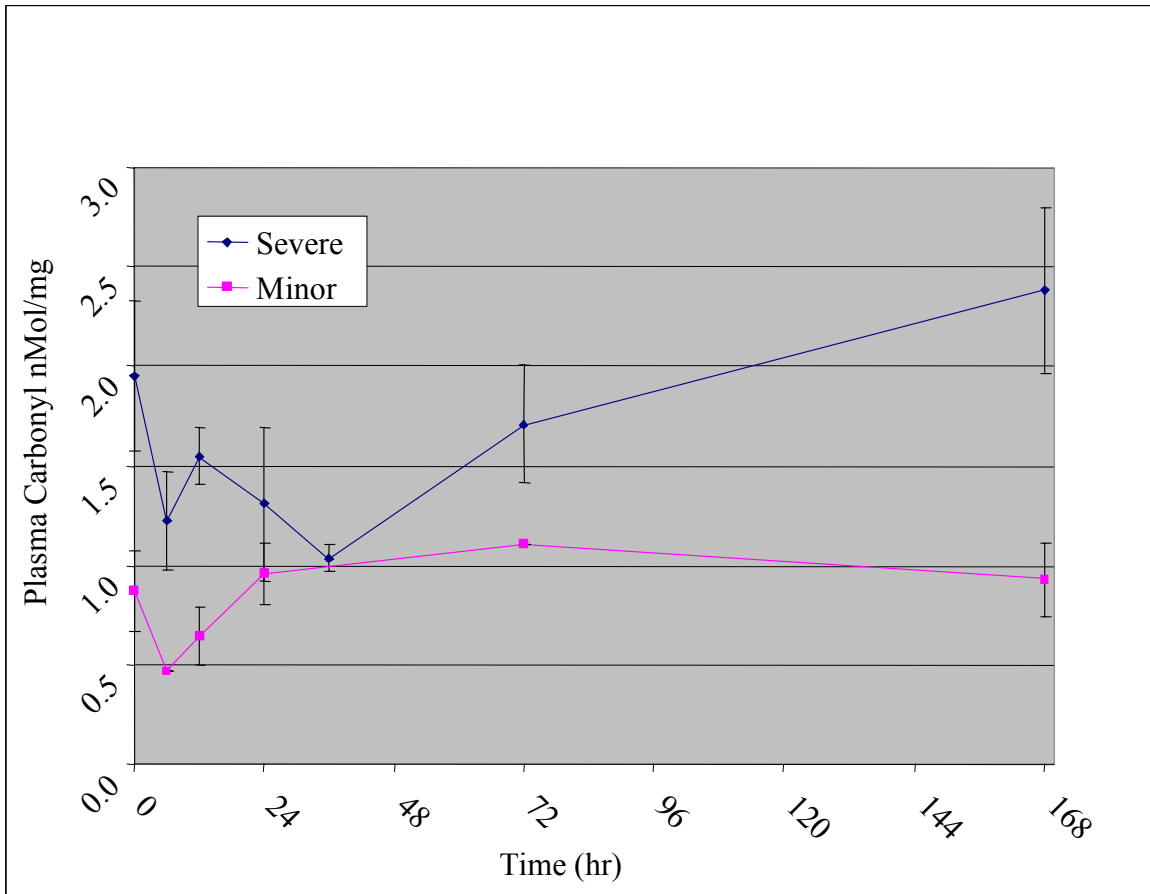


Figure 6.15: Carbonyl content in severe trauma was significantly higher in severe trauma than in minor trauma on admission ($p= 0.025$) and throughout the study period ($p<0.001$). Day 7 levels were higher than admission levels in the 2 groups although more marked in severe trauma.

Figure 6.16: Plasma Carbonyl versus Trauma Severity

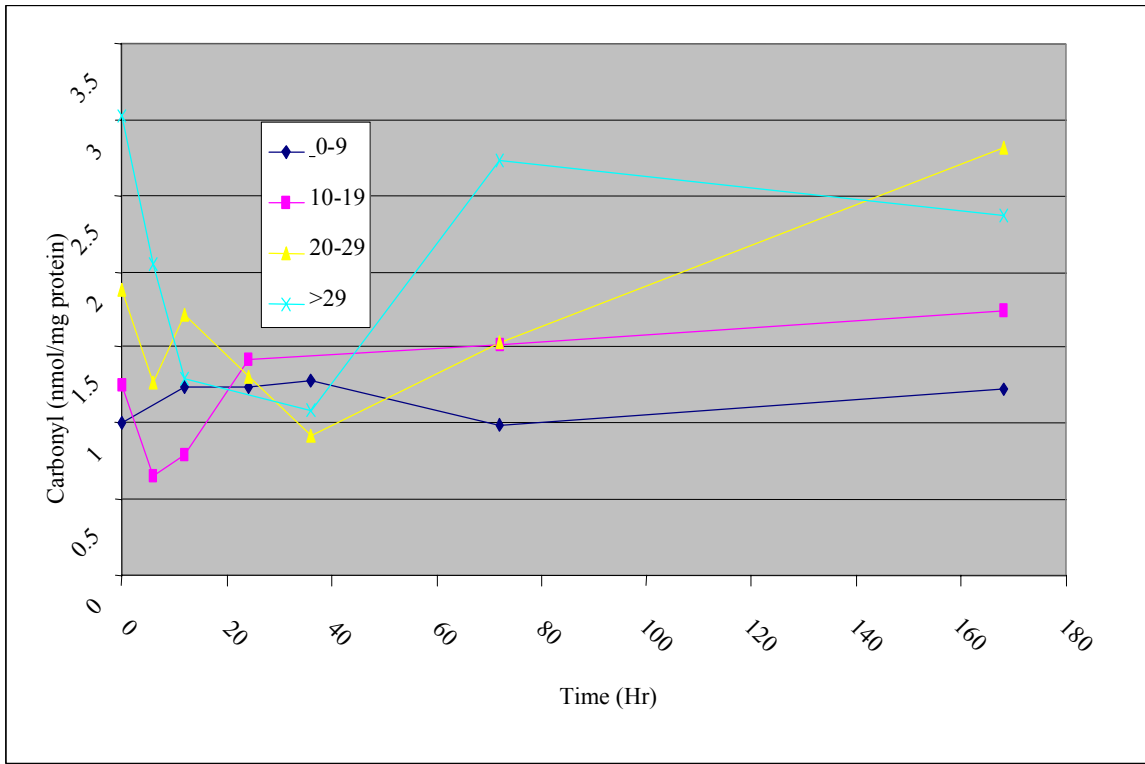


Figure 6.16: Carbonyl content was directly related to trauma severity but the difference between these four sub-groups was not statistically significant (p=NS).

Figure 6.17: Plasma Carbonyl versus Oxidant Levels In Trauma Patients

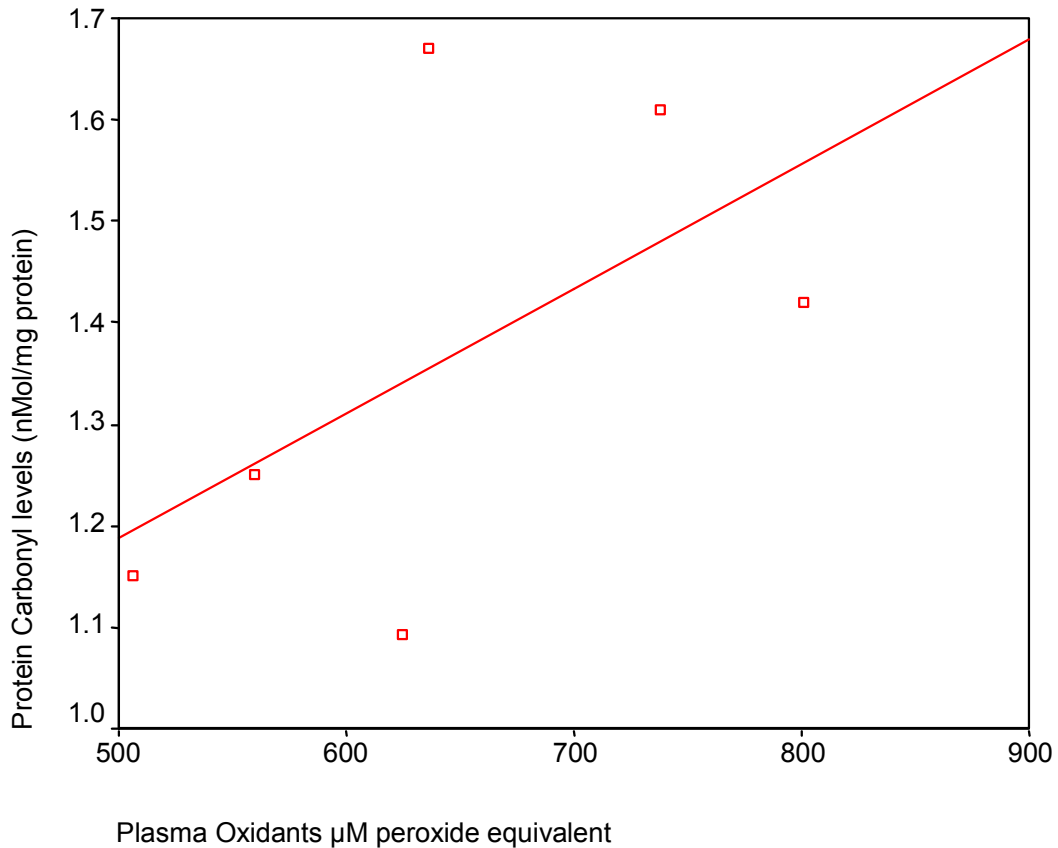


Figure 6.17: Linear relationship is observed between plasma oxidant and carbonyl formation ($p=0.031$).
''

6.6.2. Urine Carbonyl Analysis

Mixing urine with 0.2% 2,4-DNPH or 0.2% 2,4-DNPH + equal volume of 30% TCA in 1 mM EDTA produced no precipitate in the Eppendorf tube irrespective of injury severity. There were large precipitates (0.01 ml in an Eppendorf tube) in all urine samples using the recommended 0.4% 2,4-DNPH irrespective of the trauma score. Using our modification of 0.4% 2,4-DNPH with equal volume of 30% TCA in 1 mM EDTA, it was easy to distinguish small from large precipitates with variation according to trauma score (Figure 6.18). A positive correlation between the presence of precipitate and trauma

severity ($p= 0.000$) was also observed (Figure 6.19), however the presence of precipitate was not necessarily predictive of trauma severity ($p= 0.36$).

Figure 6.18: Grades of Urine Precipitation



Figure 6.19: Appearance of Urine Precipitate with Trauma Severity Over Time

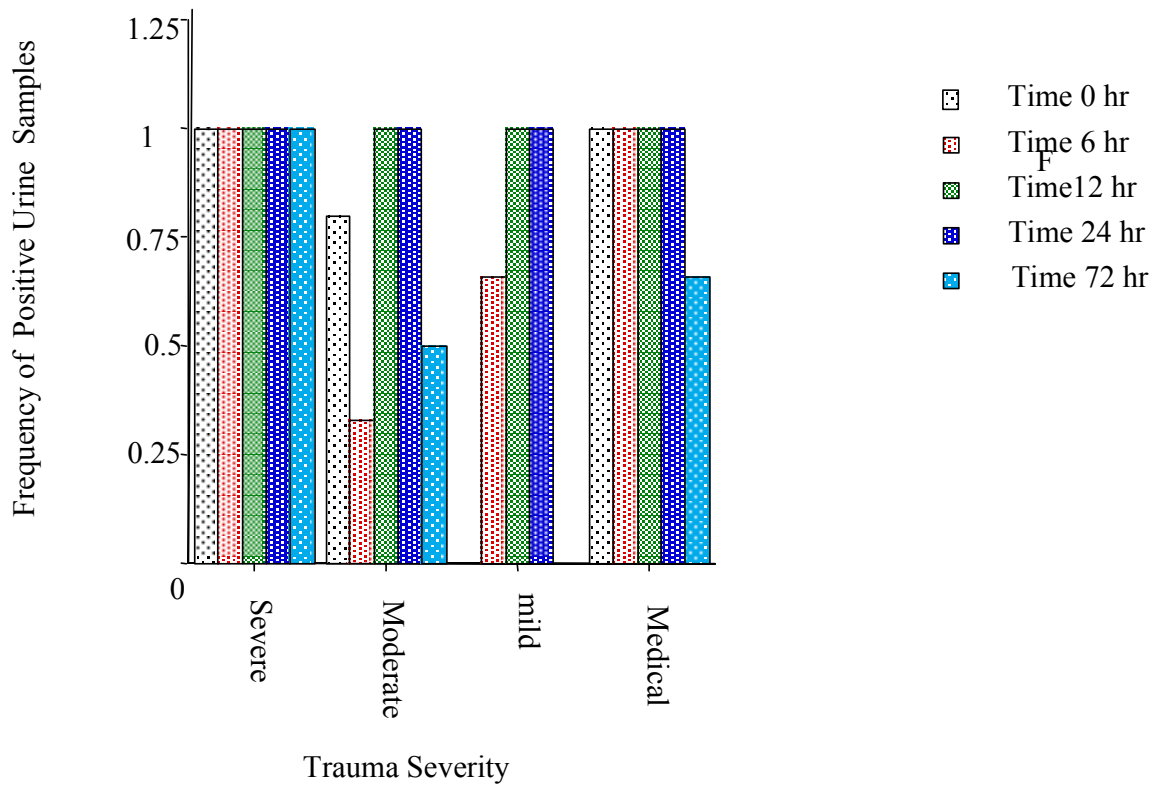


Figure 6.19: Positive correlation between the presence of precipitate and trauma severity ($p= 0.000$) but precipitate not necessarily predictive of trauma severity ($p= 0.36$).

6.7. Discussion: The Effect of Trauma on Plasma Oxidative Homeostasis:

Plasma is the unique site to study oxidative homeostasis in the body not only because it contains antioxidants [103] and the targets of oxidative stress but it is in constant interaction with the red cell which is source of antioxidant regeneration [42]. The choice of trauma patients as the study population is also important in that polytrauma results in generation of reactive oxygen species and could play a role in the pathophysiology of systemic inflammatory response and depletion of antioxidants following trauma.

6.7.1. Plasma Antioxidants:

The highest levels of antioxidants in our patient population was on admission which is similar to the findings of Mayer et al [130] and Kretzschmar et al [18] in multiple trauma patients. These levels are likely to be the patient's baseline before the trauma because patients were recruited within 6 hr of trauma and were previously healthy. The full effect of strong oxidants production particularly from neutrophil activation takes about 6hr as observed in our study and others [221]. Previous studies have also reported no significant change in strong oxidant levels within 6 hr of trauma [371]. This however is the only study using the FRAP method to measure antioxidant capacity in trauma, other studies have used TORC [130] or plasma GSH levels [18]. The FRAP method has however been used in other clinical conditions such as eclampsia, diabetes and following bone marrow transplant [372-374]. It was used in this study because it is an easy, stable, reliable, sensitive, inexpensive, and fully automated method for measuring total antioxidant capacity [375]. While most studies [18, 376, 377] report a depletion in individual plasma antioxidants at 24 hr we observed significant depletion in antioxidant capacity as early as 6 hr and the trend continued over the next 18 to 24 hr after trauma. Mayer et al [130] also observed early depletion in antioxidant capacity in multiple trauma patients but they monitored these changes for only 21 hr after which they reported a return to normal levels. We observed a slight recovery in the antioxidant capacity over the next 7 days but the initial admission levels were not attained. Maderazo (1990) also observed this failure to regain baseline levels by the 8th day when reviewing changes in serum ascorbic acid and alpha-tocopherol in trauma patients [377]. Some

studies have also reported many days of antioxidant decline [376] or a complete lack of recovery at 8 days after trauma [18]. The pattern of change in antioxidant levels was similar in all patients irrespective of trauma severity or premorbid medical condition, a feature observed by other studies [18, 376].

6.7.2. Plasma Oxidants

Plasma oxidants levels present a cyclical graph pattern with two peaks in our study - the first on admission and the second was observed between the 12 and 18 hr. A similar initial peak was noted by Arand et al [378] however in their study the 2nd peak was at 24 hr. The study period also differs, while ours was 7 days theirs was only 24 hr. These results differ from those of Kretzschmar et al [18] in which the plasma oxidants in the study population only began to increase after 7 days, and Winterboun et al [209] in which there were fluctuations and a peak value at 12 days. The reason for the difference might be because these studies measured plasma oxidant levels daily while our study had more frequent evaluation during the first 24 hr of the trauma. It is well known that trauma results in ischemia, hypoxia, necrosis and shock, thus the initial peak observed could be due to the production of reactive oxygen species from the ischemic bed during resuscitation [1, 18, 376] and neutrophil infiltration which is a known source strong oxidant production after trauma [334, 369, 379]. The cyclic pattern observed in plasma may be a result of the neutralization of oxidants by antioxidants in order to maintain oxidative homeostasis. This view is also shared by Lalonde et. al., (1997) who stated that maintaining circulating antioxidants may prevent distant organ lipid peroxidation and may be of clinical use in devising treatment strategies for smoke inhalation injury with the availability of antioxidants [380].

6.7.3. Plasma Protein Carbonyl:

The presence of carbonyl groups has been widely accepted as a measure of oxidative damage under conditions of oxidative stress [329]. The amino acids of normally oxidized and degraded proteins are reutilized for protein synthesis however during oxidative stress the proteolytic capacity of the cells is not sufficient to cope with the number of oxidized protein molecules being generated. Under such circumstances,

oxidized proteins cross link with one another or form extensive hydrophobic bonds which are detrimental to normal cell function and lead to further problems [381]. We observed that trauma causes extensive generation of oxidized protein as seen by protein carbonyl production about 10 times the normal levels. These levels were highest on admission and there was another peak at 168 hr. The second peak of carbonyl content is likely due to complications associated with the severity of the trauma resulting in further oxidative stress. These findings are similar to those of Winterbourn et al (2000) [209] but differs in that we had another peak at 168 hr while their patient population showed continuous decline 24 hr after trauma in a 12 day study. Theirs was however a small study of 8 severe trauma (ISS 25 –50; mean ISS = 35) patients most of whom were recruited 2 days after admission so the oxidative changes occurring in the first 48 hr could not be accounted for. Our study has been able to demonstrate changes from the time of trauma making it more beneficial in understanding the effect of oxidative stress.

This research also aimed to understand the relationship between strong oxidant production and protein damage. The observed linear correlation and symmetry between carbonyl and oxidant levels with peaks occurring at similar intervals ($p=0.031$) in this research suggests increased oxidant levels results in protein damage therefore oxidized proteins could be used as an early marker of disturbances in oxidative homeostasis.

6.8. Discussion: Urine and Oxidative Homeostasis

There has however been no previous study on urine markers of oxidative stress after trauma. This may be because most available methods of urine analysis for oxidative stress require 24 hr urine samples that may be of little benefit in the management critical trauma patients. In addition the methods used are usually technically involved and cumbersome so timely results may not be available for clinical management. Urine an ultra-filtrate of plasma represents a non-invasive, and potentially accurate method of measuring oxidative stress [382-384]. Plasma and urine analysis may yield divergent results thus emphasizing the importance of measuring a biomarker both in the circulation fluid (plasma) and in the elimination fluid (urine) in order to have a general idea of what is occurring in the organism [328]. This research decided to explore the use of urine

markers in evaluating oxidative stress because of its focus on identifying clinically applicable and beneficial diagnostic methods. The Oxistress assay was used to measure urine oxidants thereby evaluating the patients' oxidative status. Urine carbonyl analysis was used to evaluate excreted protein degradation products resulting from strong oxidant damage. Similar markers had been evaluated in plasma and comparisons enabled us to obtain a total picture of oxidative stress in the same trauma patient.

6.8.1. Urine Oxidants

The various mechanisms by which trauma causes increased production of strong oxidants including shock, hypoxia, ischemia, necrosis and bacteria translocation may be responsible for the two-fold increase in urine oxidant levels in our study population as compared with normal values. These levels were highest on admission irrespective of trauma severity. The changes in urine oxidant levels in our study population were similar to the trend in plasma however the peak was observed at 24 hr compared to 18 hr in plasma. The difference in peak oxidant levels between the plasma and urine markers of oxidative stress may be due to urine representing the summation of the reaction between the antioxidants and the oxidants in plasma while the difference in the timing of the peaks may be a time-phased relationship [385]. Buss et al (2001) observed similar results in a spinal ischemia model reporting that trauma results in increased measurable free radical (strong oxidant) production within 60 minutes in plasma and 90 minutes in urine [386]. There was significant decline in strong oxidant levels in both urine and plasma 72 hr after trauma that seems to be associated with a rebound in the red cell GSH seen at the same time in our study. This may be the result of the body's attempt at restoring oxidative homeostasis through increased antioxidant production.

6.8.2. Urine Carbonyl

There are several methods of measuring protein carbonyl in urine all of which use DNPH. Most urine carbonyl analysis has been done in animals using the HPLC, mass spectrometry and gas chromatography methods [387, 388] and very few human studies have been reported [389]. There appears to be much controversy on the benefits of urine carbonyl analysis, some studies suggest it is a useful tool [383, 389] while others disagree

[390]. Our study adopted the clinical method involving mixing equal quantities of filtered urine with DNPH to produce carbonyl precipitates. We modified this method by the addition of equal volume of 30%TCA in 1mM EDTA because TCA reduces solubility of protein and enhances precipitation [391]. We tried lower concentrations of DNPH and failed to get a reaction even when TCA was added. This confirms that the recommended 0.4% 2,4-DNPH is the ideal concentration for the reaction.

We observed large precipitates in all patients using 0.4% 2,4-DNPH alone which was non-diagnostic. The addition of TCA however improved the diagnostic accuracy of this test enabling us to distinguish between small and large precipitates. Grading the precipitate was based on both the composition of the precipitate and the clarity of the solution. We defined small precipitates as particles still in suspension that can be easily displaced, while large precipitates are particles that have formed a hard substrate at the bottom of the tube that cannot be displaced by tilting the tube (Figure 6.19). Large urine Carbonyl precipitates were associated with plasma carbonyl levels greater than 1.5nmol/mg protein and this correlated with the level of plasma protein carbonyl in each trauma group. Trauma in patients with previous medical morbidity appears to be an independent predictor of the presence of urine precipitate. There is a positive correlation between trauma severity, plasma carbonyl and the appearance of large carbonyl precipitates in urine. Based on the evidence-based medicine guidelines provided by Archibald et al., (2001) [392] urine carbonyl test appears to be a simple preliminary test that can be used in patients prone to oxidative stress and a positive result is indicative of high plasma carbonyl in patients.

7. THE PROSPECTIVE ENTERAL ANTIOXIDANT (ALANYL-GLUTAMINE) CLINICAL STUDY

7.1. Introduction

The prospective enteral antioxidant study was designed as a follow up to the natural history of oxidative stress reported in Chapter 6 which identified head injured, combined head and body, and body injured patients as most susceptible to oxidative stress in comparison to those with minor and single system trauma. As well antioxidant depletion has been reported in major elective surgery patients. Lou et al., (1996) demonstrated a 40% depletion in skeletal glutathione as well as 20% depletion in plasma glutathione after selective abdominal surgery compared with pre-operative levels [226]. Following minor elective surgery (mastectomies and lumpectomies) a depletion in total plasma antioxidant 6 hr post-op compared with pre-operative levels and a return to basal levels after 12 hr similar to our findings in minor trauma has been reported by Morris et. al., (2000) [225].

The choice of major elective surgery patients for the enteral antioxidant study was based on the expectation of similar antioxidant response as observed in trauma patients. The justification for this clinical trial is based on anecdotal evidence in clinical and animal studies [17, 135, 342, 343] suggesting that the introduction of antioxidant therapy reduces oxidative stress and its complications. This is an interventional study with the aim of evaluating the effect of glutamine (a precursor to glutamate and a conditional essential amino acid that enhances glutathione synthesis) on antioxidant homeostasis and patient outcome. Glutamine was chosen as the enteral supplement because it is a direct precursor of glutamate and an indirect precursor of glutathione (Smith, 1990) (Lacey and Wilmore 1990). Glutamate has a two-fold way of increasing glutathione by alleviating the GSH inhibition of GCS and by regulating uptake of cystine [223]. Glutathione is a major antioxidant made up of glutamate, cystine and glycine, previously discussed in Chapters 2 and 6.

A double blind prospective randomized controlled trial as well as an outcome study in elective surgery patients was conducted at the Royal University Hospital between the October 2002 and April 2003. Ethics approval was obtained from both the University and the Saskatoon Health Region. The methodology is described in Chapter

4.6 – 4.7. The study patients were recruited from the preoperative assessment clinic and divided into two groups (treatment and non treatment). The graphs and most of the tables are generated using the SPSS program.

7.2. Demographics

The study consisted of eligible patients with craniotomy, significant head and neck, abdomen, thoracic and back surgery. A total of 283 patients were approached for participation, 200 declined, 14 withdrew and 69 completed the study (Table 7.1). The 69 study patients had a Male: Female ratio of 1.1:1 (Table 7.2). The study group is quite similar to the normal surgery patient population with 80% of the patients over 40 yr in age and 43.5% over 60 yr (Table 7.3; Figure 7.1). Cancer patients constituted 55.7 % of the study population (Table 7.5), most patients >60 yr had cancer while most < 40 yr had no cancer, and the 40-60 yr age group was mixed (Fig 7.2). There were comparable cancer and non - cancer patients in the treatment and non- treatment group irrespective of the age group (Figure 7.3). All craniotomy patients in the treatment group were over 40 yr while most of those in the non- treatment group were between 40-60 yr. The thoracic patients were between 40-60 yr. Laparotomy patients in the non-treatment group and 66% of those in the treatment group were over 40 yr. Laparotomy made up 76% of study population followed by craniotomies (14.5%) [Table 7.4]. The non-treatment group included 57% of the laparotomies while 70% of the craniotomies and 100% of the thoracic patients were in the treatment group (Figure 7.5).

Table 7.1: Patient Selection Process For The Alanyl – Glutamine Study

Eligible Patients (All patients approached)	283
Declined Participation (Did not want Nasogastric tubes)	200
Patients enrolled (agreed to participate)	83
Withdrawal from the study (Could not tolerate tube/ inability to insert tube)	14
Final Study Patients	69

Table 7.2: Sex Distribution

	Frequency	Percent	Valid Percent	Cumulative Percent
Valid Female	33	47.8	47.8	47.8
Male	36	52.2	52.2	100.0
Total	69	100.0	100.0	

SPSS derived table.

Table 7.3: Age Distribution of Study Patients

	Frequency	Percent	Valid Percent	Cumulative Percent
Valid Ages 20-40	14	20.3	20.3	20.3
41-60	25	36.2	36.2	56.5
>61	30	43.5	43.5	100.0
Total	69	100.0	100.0	

SPSS derived table.

Figure 7.1: Age Distribution of Study Patients (Bar Chart)

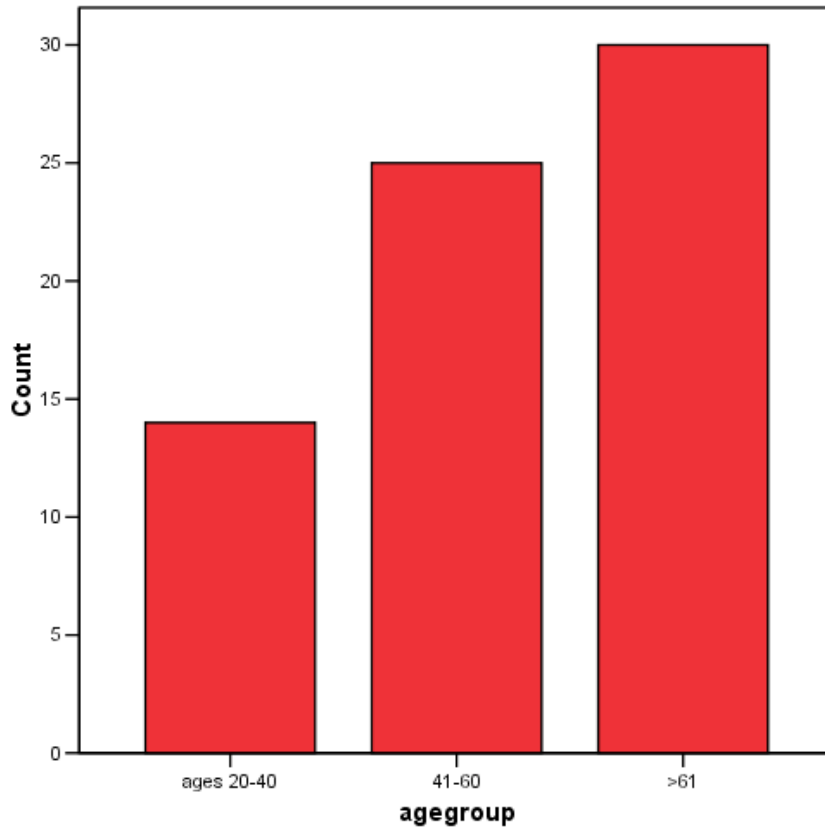


Figure 7.1: y-axis = no. of patients; x-axis = age group with 80% of patients over 40 yr in age and 43.5% over 60 yr.

Table 7.4: Surgery Type:

SURGTYPE					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Back	1	1.4	1.4	1.4
	chest	3	4.3	4.3	5.8
	crainy	10	14.5	14.5	20.3
	H&N	1	1.4	1.4	21.7
	LAP	53	76.8	76.8	98.6
	plastic	1	1.4	1.4	100.0
	Total	69	100.0	100.0	

Table 7.4: SPSS derived table. H&N = head and neck dissection; LAP = laparotomy.

Table 7.5: Cancer Versus Non Cancer Patient Distribution:

	Frequency	Percent	Cumulative Percent
Cancer	39	56.5	56.5
No cancer	30	43.4	100.0
Total	69	100.0	

Table 7.5: SPSS derived table. Cancer patients were slightly higher than non-cancer patients.

Figure 7.2: Age Distribution - Cancer Versus Non Cancer:

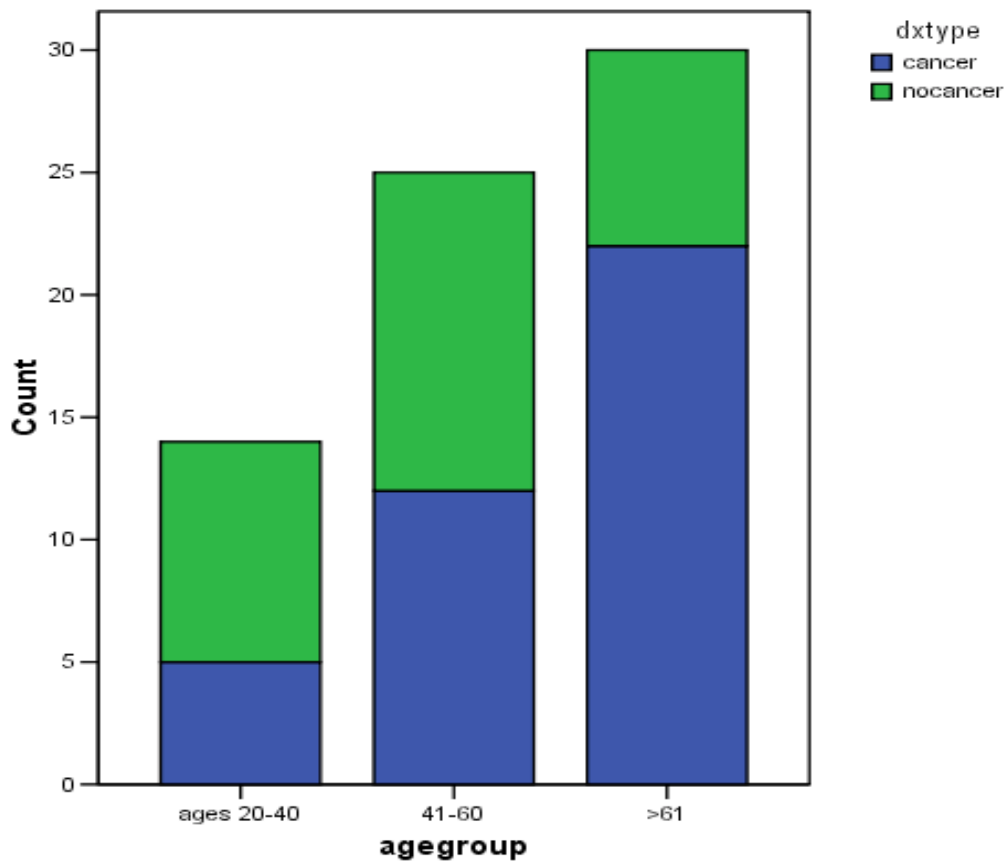


Figure 7.2: Graph showing the distribution between cancer and non-cancer patients in each age group. Most between 20-40 yr were non-cancer; 40-60 yr were almost equally distributed; ≥ 61 were mostly cancer patients; and dx= disease type

Figure 7.3: Treatment Specific Demographics - Cancer versus Non Cancer:

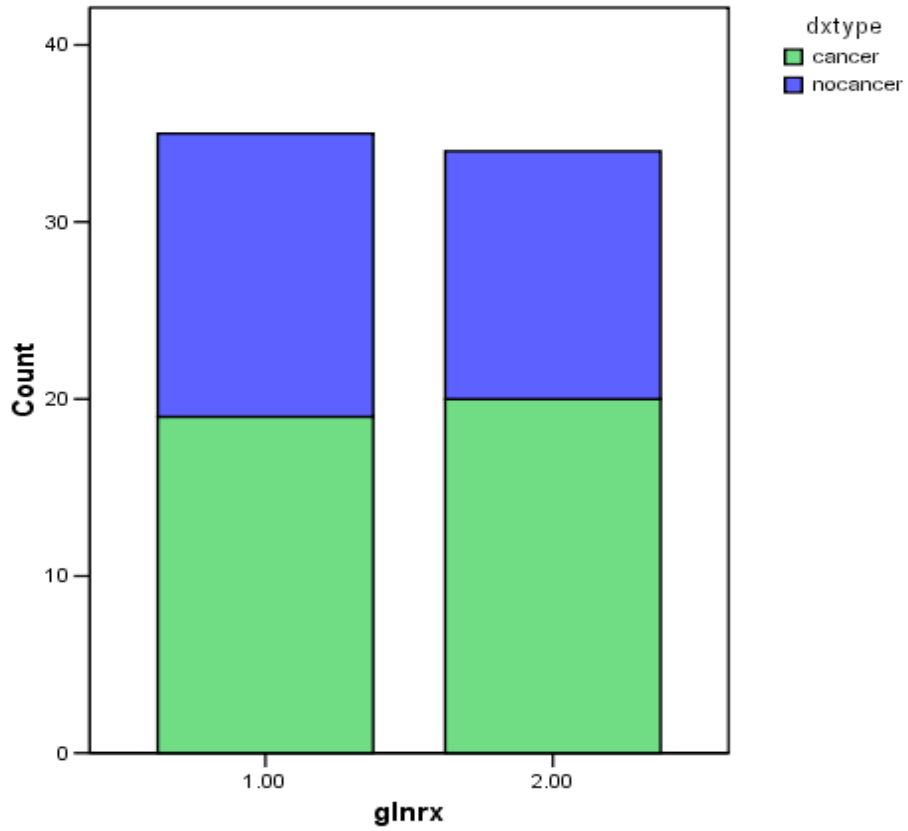


Figure 7.3: dxtype = disease type; glnrx 1.00 = controls; glnrx 2 = treatment group. Fairly equal distribution of patients in both groups.

Figure 7.4: Treatment Specific Demographics – Age Distribution:

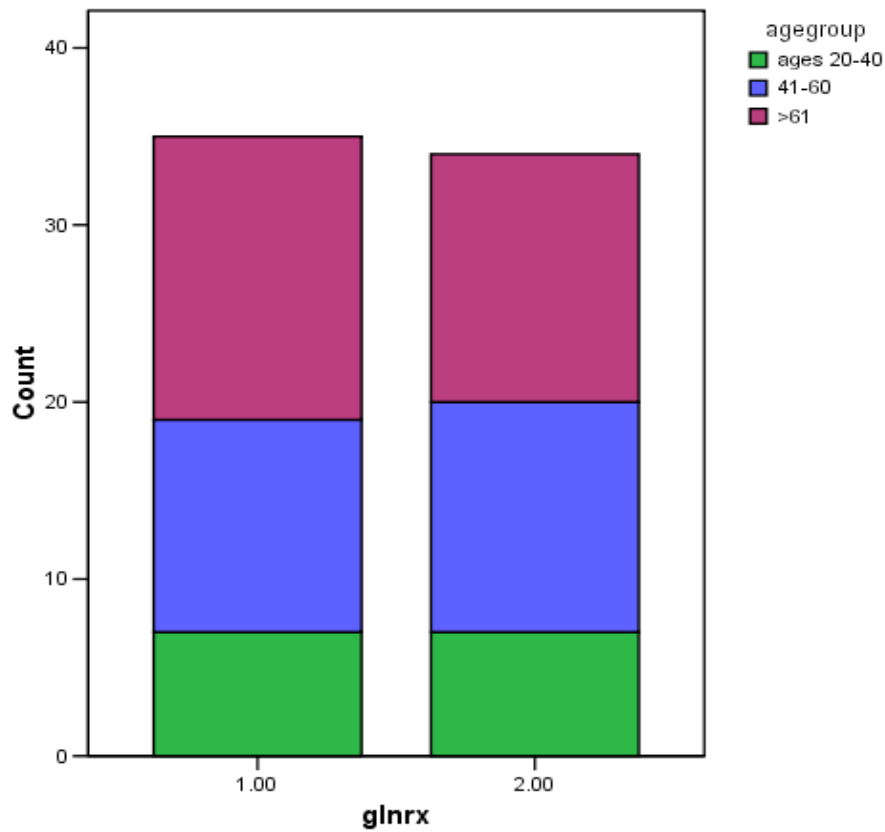


Figure 7.4: Age groups 20-40 yr; 40-60 yr; and ≥ 61 had fairly equal distribution of patients in glnrx group 1.00 (controls) and glnrx 2.00 (treatment group)

Figure 7.5: Surgery Site - Treatment versus Non Treatment

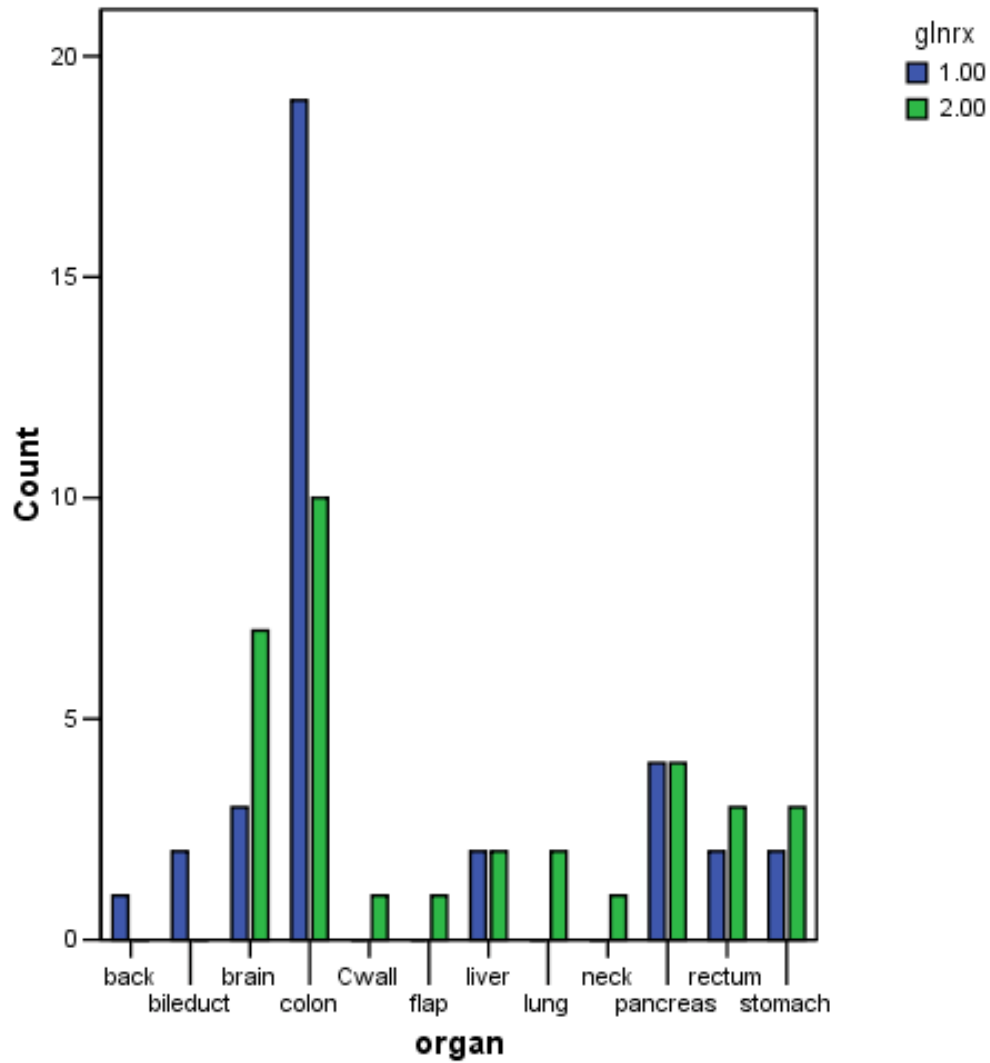


Figure 7.5: glnrx 1.00 = controls and glnrx 2.00 = treatment group. 57% Laparatomies were in non-treatment group; 70% of craniotomies and 100% of thoracotomies were in treatment group.

7.3. Results: The Biochemical Outcome of Glutamine Treatment

7.3.1. Changes in Plasma Glutamine

Surgery appears to reduce plasma glutamine levels in all patients combined with a progressive decline until 72 hr (Figure 7.6). The pre-operative plasma glutamine levels in the treatment group were much lower than in the non-treatment group ($p= 0.002$), however a reduction was observed in the non-treatment group in contrast to an increase observed in the treatment group [Figure 7.7]. With respect to pre-operative glutamine levels the treatment group had an increase of 10 μM while the controls had a 26 μM drop, with trend continuing till 72 hr. However glutamine treatment did not yield a significant increase at post-op ($p= 0.602$; Figure 7.7-7.8; Table 7.6) but the observed increase at 72 hr i.e. 48 hr after completion of treatment had a greater tendency towards significance ($p = 0.196$) [Figures 7.7; 7.9; Table 7.6]. Patients between 41 and 60 yr had lower plasma glutamine levels post-surgery but age does not appear to be an independent predictor (Figure 7.10).

Figure 7.6: Changes In Plasma Glutamine Levels In All Patients

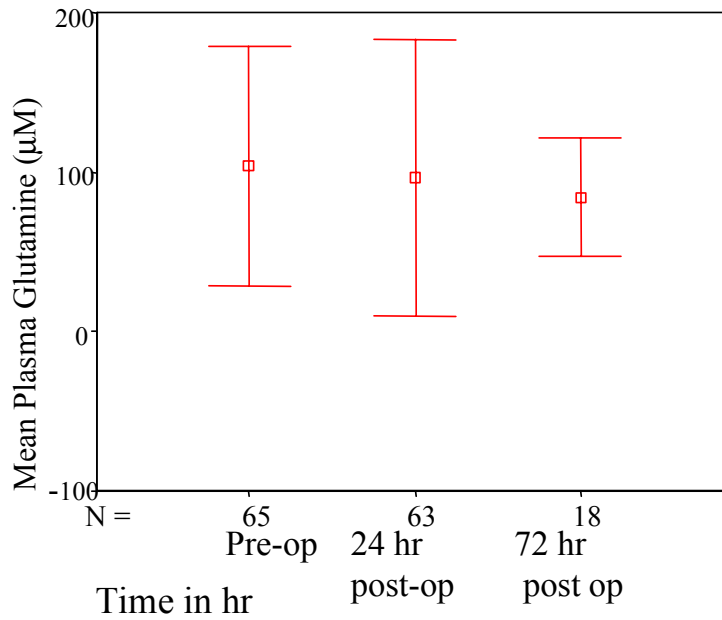


Figure 7.6: Surgery reduces plasma glutamine levels in all patients combined up till 72 hr post-op.

Figure 7.7: Plasma Glutamine Levels In Treatment Versus Non Treatment Groups

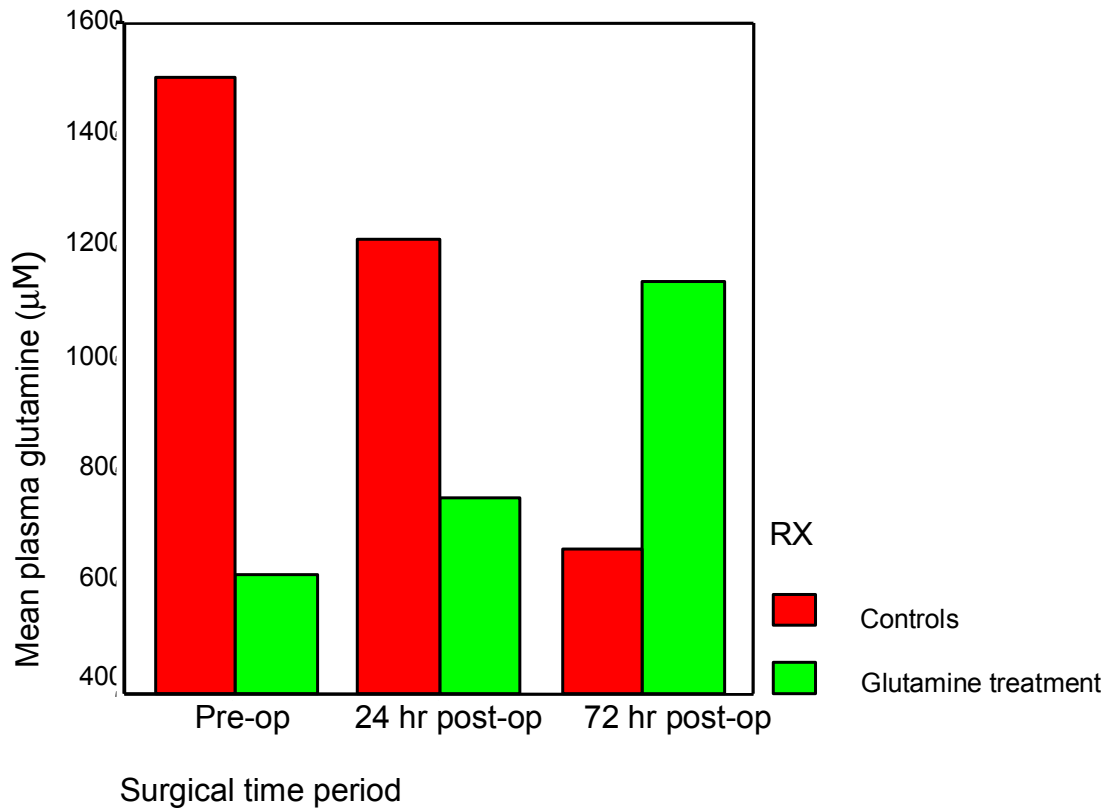


Figure 7.7: Plasma glutamine levels appear to increase in the treatment group compared with controls where a downward trend is observed. Group 1 = controls and group 2 = treatment group. The treatment group had an increase of 10 µM while the controls had a 26 µM drop from pre-op levels.

Figure 7.8: Changes In Plasma Glutamine At 24 Hr Post- Surgery

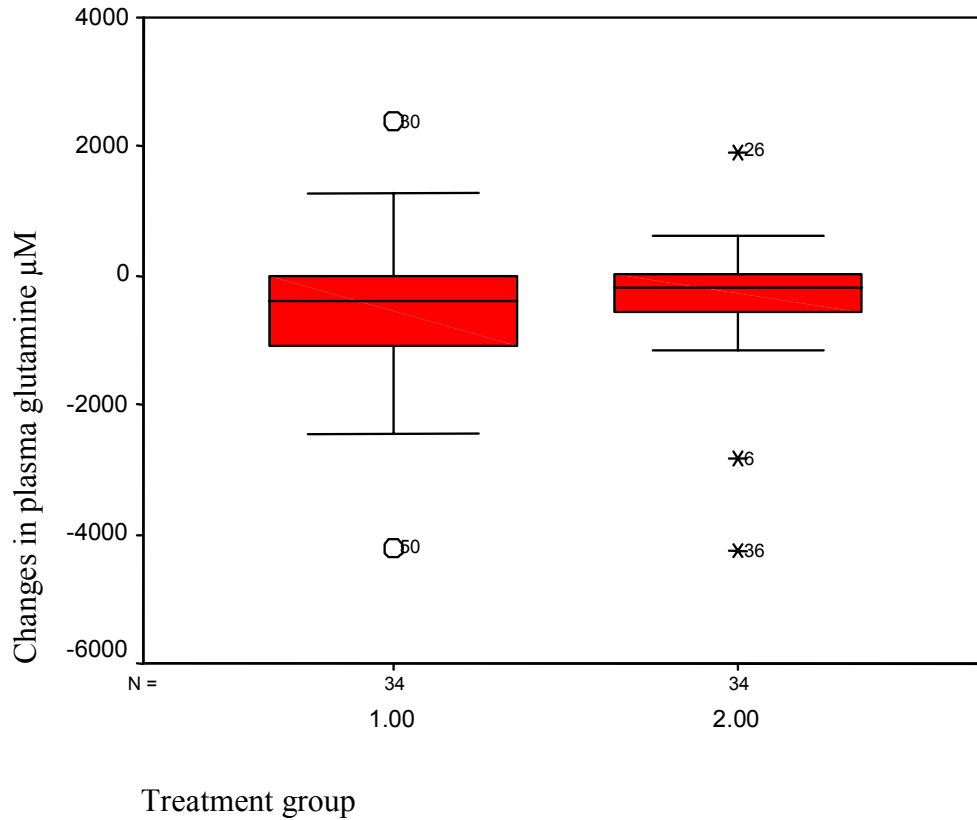


Figure 7.8: The change in plasma glutamine with treatment at 24 hr was not significant ($p= 0.602$). Group 1 = controls and group 2 = treatment group. The symbols * and o identify the outliers.

Figure 7.9: Effect of Treatment and Non Treatment at 72 hr Post-Surgery

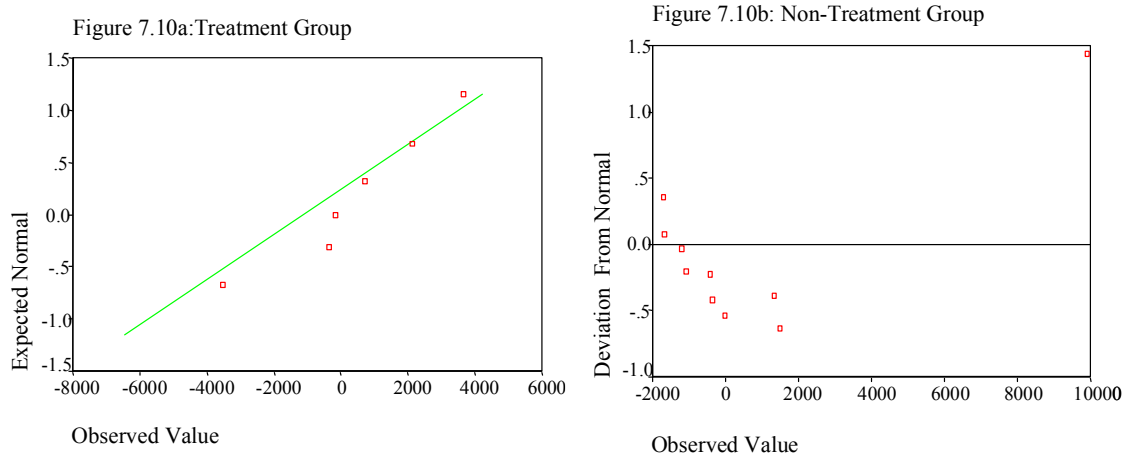


Figure 7.9: is a scatter of the effect treatment on plasma glutamine levels at 72 hr post-op while Figure 7.10b shows plasma glutamine levels in controls at the same time. An increase is observed in the treatment group while a decline is observed in the controls. The change in plasma glutamine with treatment at 72 hr was not significant ($p= 0.196$) but it shows a tendency towards significance. Group 1 = controls and group 2 = treatment group.

Table 7.6: Changes In Plasma Glutamine At 24 Hr And 72 Hr Post-Surgery

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
G24	Between Groups	82691053	1	82691053.39	.275	.602
	Within Groups	1.83E+10	61	300609816.2		
	Total	1.84E+10	62			
G72	Between Groups	24427128	1	24427128.43	1.818	.196
	Within Groups	2.15E+08	16	13434103.08		
	Total	2.39E+08	17			

Table 7.6: SPSS derived ANOVA table showing a summary of the degree change in plasma glutamine at 24 hr (G24) and 72 hr (G72).

Figure 7.10: Age versus Changes in Plasma Glutamine Levels

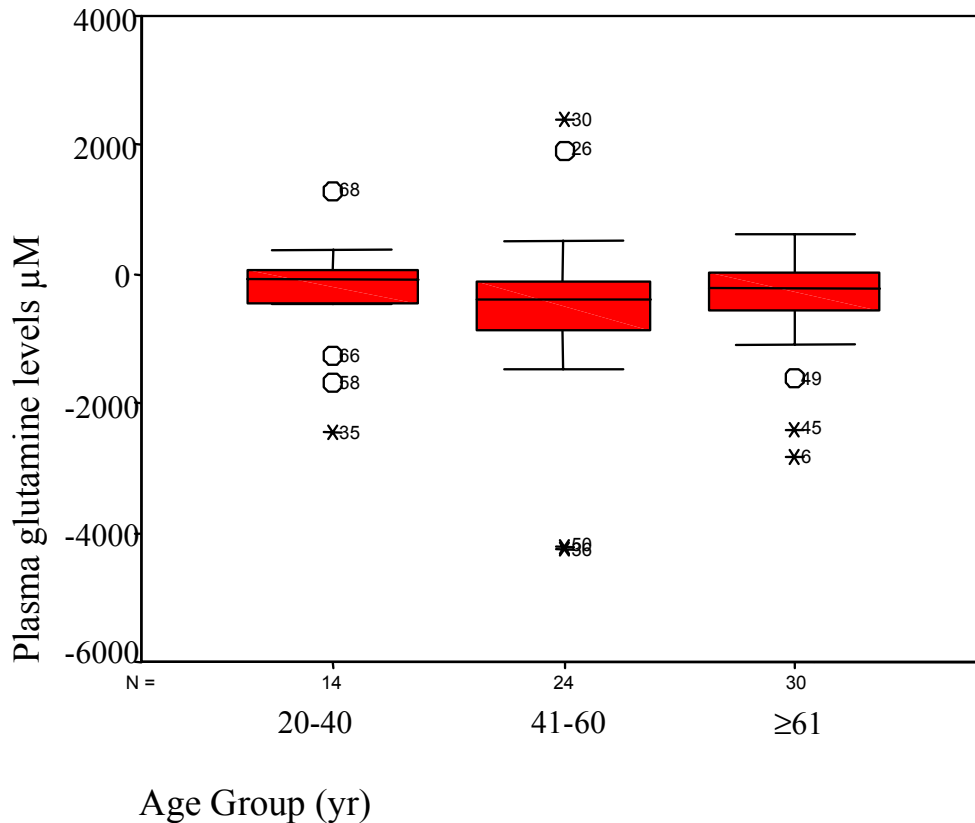


Figure 7.10: There was no significant difference in plasma glutamine levels in relation to age. The symbols * and o identify the outliers.

7.3.2. The Effect of Glutamine Treatment on Plasma Antioxidants

The pre-operative plasma antioxidant capacity in the controls was higher than that of the treatment group. However treatment resulted in an increase while surgery reduced plasma antioxidant capacity (Table 7.7). Post-operative glutamine treatment increased the total plasma antioxidant levels after 24 hr ($p=0.005$) [Table 7.8a]. The increase was also evident but not significant at 72 hr ($p= 0.142$) [Table 7.8b].

Table 7.7: Plasma Antioxidant Capacity in Treatment and Non-Treatment Group At 0 hr And 24 hr (FRAP Method).

Report

GLNRX		FRAP0	FRAP24
1.00	Mean	692.9355	527.5806
	N	31	31
	Std. Deviation	392.21218	259.76794
2.00	Mean	548.6250	627.9063
	N	32	32
	Std. Deviation	289.04891	275.16029
Total	Mean	619.6349	578.5397
	N	63	63
	Std. Deviation	348.56523	270.30382

Table 7.7: SPSS derived. GLNRX 1.00 = controls; GLNRX 2.00 = treatment group; FRAP0 = Plasma antioxidant capacity on admission; FRAP24 = Plasma antioxidant capacity at 24 hr. Glutamine treatment increased plasma antioxidant capacity in the treatment group the controls had reduced antioxidant capacity (p=0.005).

Table 7.8a: Glutamine Rx Versus Plasma Antioxidant Levels At 24hr (FRAP Method).

ANOVA

DFRAP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	942349.9	1	942349.863	8.295	.005
Within Groups	6929968	61	113606.026		
Total	7872317	62			

Table 7.8a: SPSS derived. Treatment resulted in significant difference in plasma antioxidant capacity (p=0.005).

Table 7.8b: Glutamine Rx Versus Plasma Antioxidant Levels At 72 hr (FRAP Method)

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	158487.4	1	158487.384	2.212	.142
Within Groups	4371490	61	71663.775		
Total	4529978	62			

Table 7.8b: SPSS derived. The difference in plasma antioxidant between treatment and control groups was not significant (p=0.142) but had the tendency.

7.3.3. The Effect of Glutamine Treatment on Red Cell Glutathione:

The pre-operative mean red cell GSH level in all patients was 910.27 μ M and the two groups were homogenous (p= 0.619) [Table 7.9]. Glutamine therapy resulted in increased glutathione levels in the red cell at 24 hr (Figure 7.11) but not sustained at 72 hr and no further increase observed (Figure 7.12). In the non-treatment group a decline was noticed at 24 hr followed by a rebound at 72 hr. Non-cancer patients had significant depletion in red cell glutathione levels at 24 and 72 hr compared to cancer patients who had an increase irrespective of treatment category (Figure 7.13; Table 7.10).

Table 7.9: Preoperative Red Cell Glutathione Levels

Treatment Category	Numbers	Mean	T-test for equality of means (Sig. 2-tailed)
No treatment	30	954.52	0.619
Glutamine Rx	30	866.02	
All Patients	60	910.27	

Table 7.9: mean red cell GSH level was homogenous in both groups (p=0.619)

Figure 7.11: Glutamine Treatment Vs Red Cell Glutathione At 24 Hr

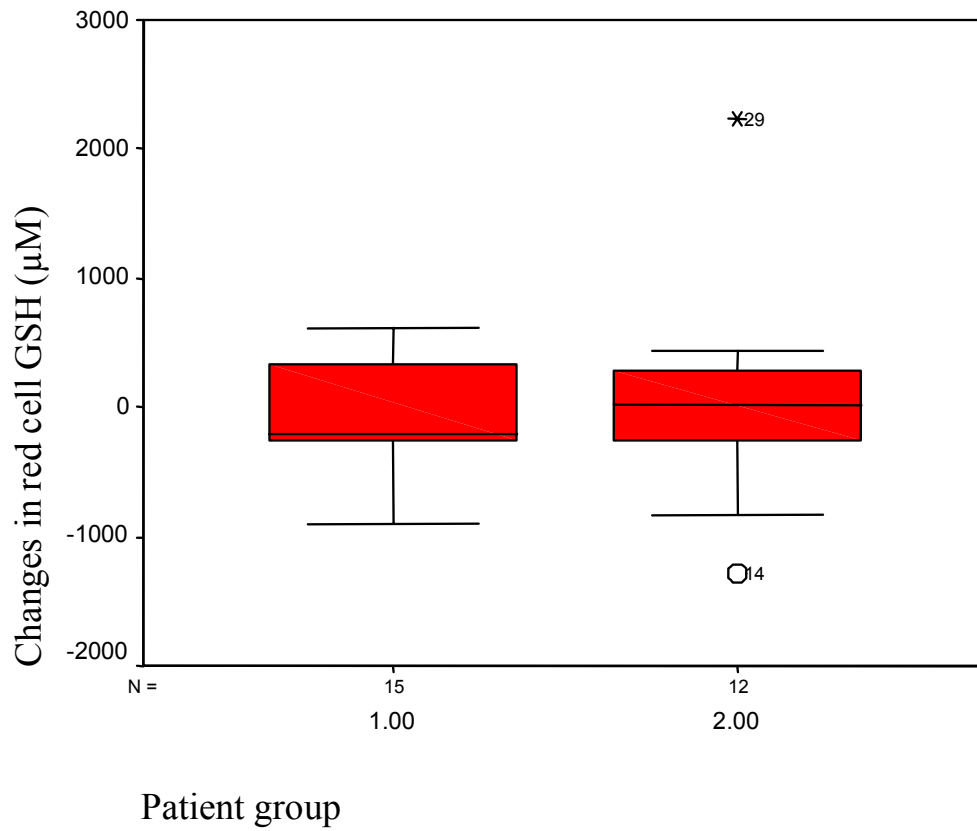


Figure 7.11: Treatment increased red cell GSH at 24 hr ($p= 0.614$). The symbols * and o identify the outliers.

Figure 7.12: Glutamine Treatment Vs Red Cell Glutathione at 72 Hr

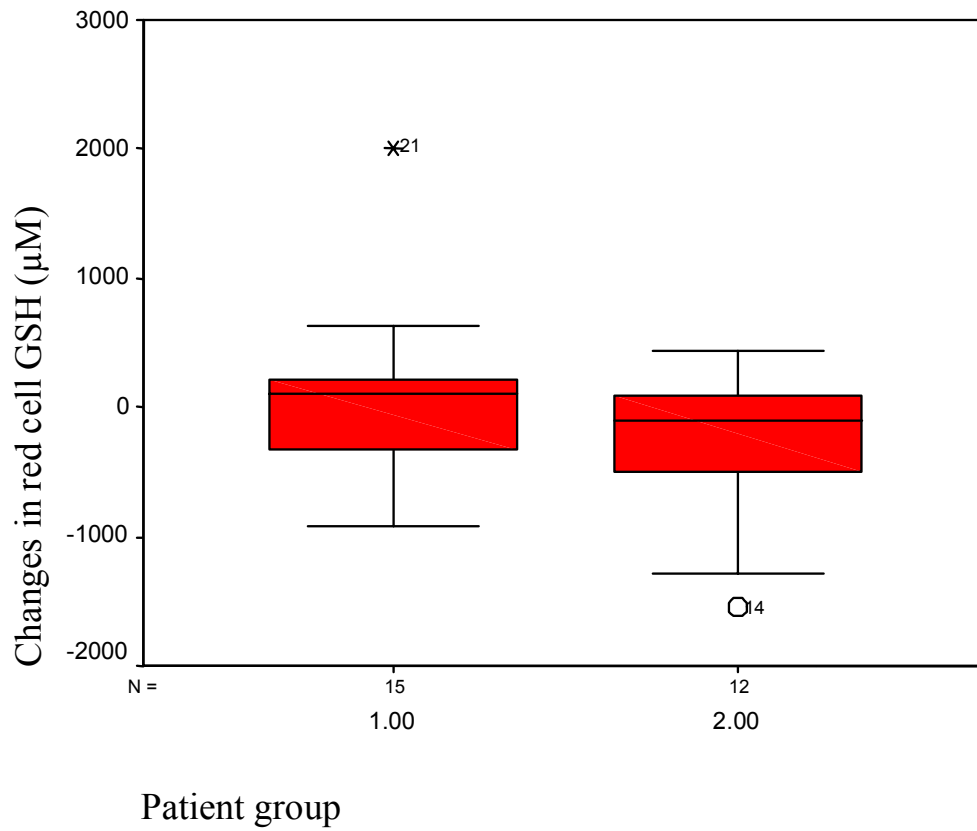


Figure 7.12: Depletion in red cell GSH with treatment at 72 hr ($p=0.27$). The symbols * and o identify the outliers.

Figure 7.13: Glutamine Treatment Vs Red Cell Glutathione (Cancer Versus Non Cancer At 24 Hr)

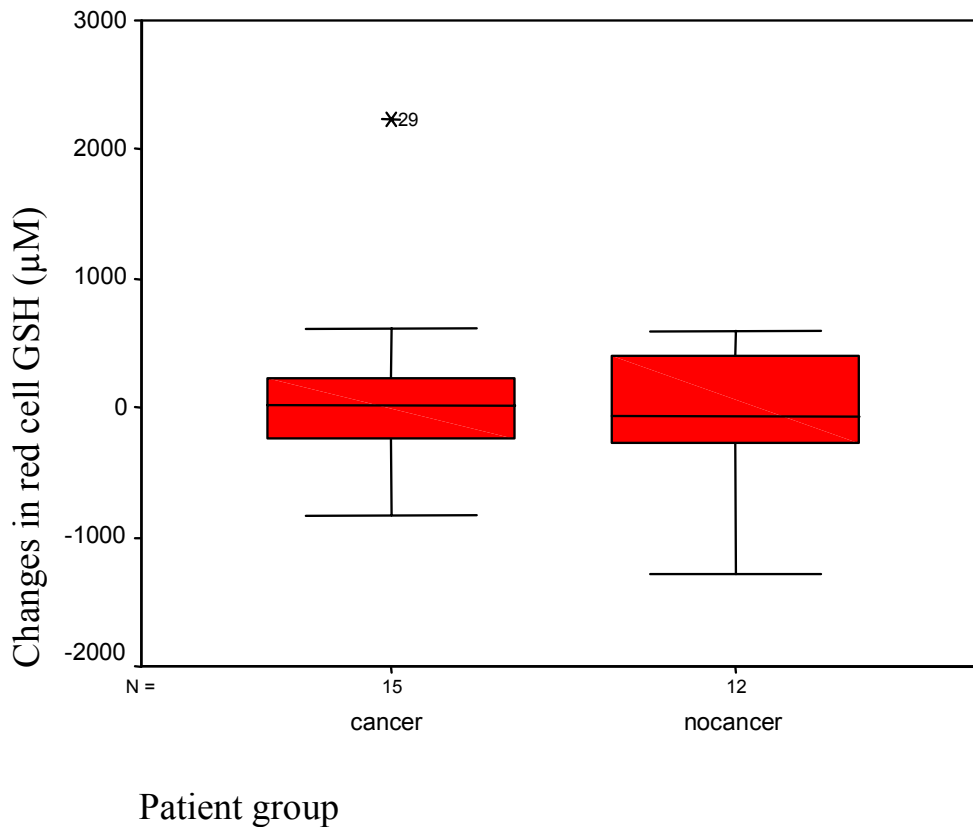


Figure 7.13: Non-cancer patients had greater depletion in red cell glutathione levels at 24 (P=0.134) and 72 hr (P=0.055) compared to cancer patients who had an increase irrespective of treatment category. The symbols * and ○ identify the outliers.

Table 7.10: Glutamine Treatment Vs Red Cell Glutathione (Cancer versus Non Cancer at 24 And 72 Hr)

Time	Statistics	Cancer	Non Cancer
@ 24 hr	Mean Change	72.5467	-90.0000
	SEM	181.19834	162.9104
@ 72 hr	Mean Change	62.7200	-293.0833
	SEM	189.12185	155.21268

Table 7.10: Non-cancer patients had greater depletion in red cell glutathione levels at 24 (P=0.134) and 72 hr (P=0.055) compared to cancer patients who had an increase irrespective of treatment category

7.3.4. The Effect of Glutamine on Strong Oxidants:

The strong oxidants levels in plasma reflected a similar trend in both treatment and control groups. The levels were highest pre-op, there was depletion at 24 hr (Figure 7.14) and a slight increase but not up to pre-op levels at 72 hr (Table 7.11). Patients in the age-group 40-60 yr had the highest level of plasma oxidants pre-op and the greatest depletion post-op. Non-cancer patients had greater reduction in post-op oxidant levels than cancer patients.

Free radical production was less in the treatment group than the non-treatment groups ($p= 0.036$ @ 24 hours) but the absolute difference between these groups was not significant ($p= 0.143$) (Figure 7.15). While the treatment group had a tendency towards reduced strong oxidant levels, the controls tended towards increased levels (Figure 7.16).

Table 7.11: Plasma Oxidant Levels at 0 hr, 24 hr and 72 hr

		Report		
GLNRX		PLASMA0	PLASMA24	PLASMA72
1.00	Mean	1205.74	644.79	710.35
	N	34	34	34
	Std. Deviation	961.947	638.035	568.562
2.00	Mean	987.56	647.82	696.61
	N	34	34	33
	Std. Deviation	1009.049	539.038	602.904
Total	Mean	1096.65	646.31	703.58
	N	68	68	67
	Std. Deviation	984.548	586.192	581.308

Table 7.11: SPSS derived. GLNRX 1.00 = controls; GLNRX 2.00 = treatment group. Highest levels were on admission; At 24 hr there was depletion in both groups; At 72 hr slight increase in both groups but not up to pre-op levels.

Figure 7.14: Effect Of Glutamine On Changes In Plasma Strong Oxidants At 24 Hr.

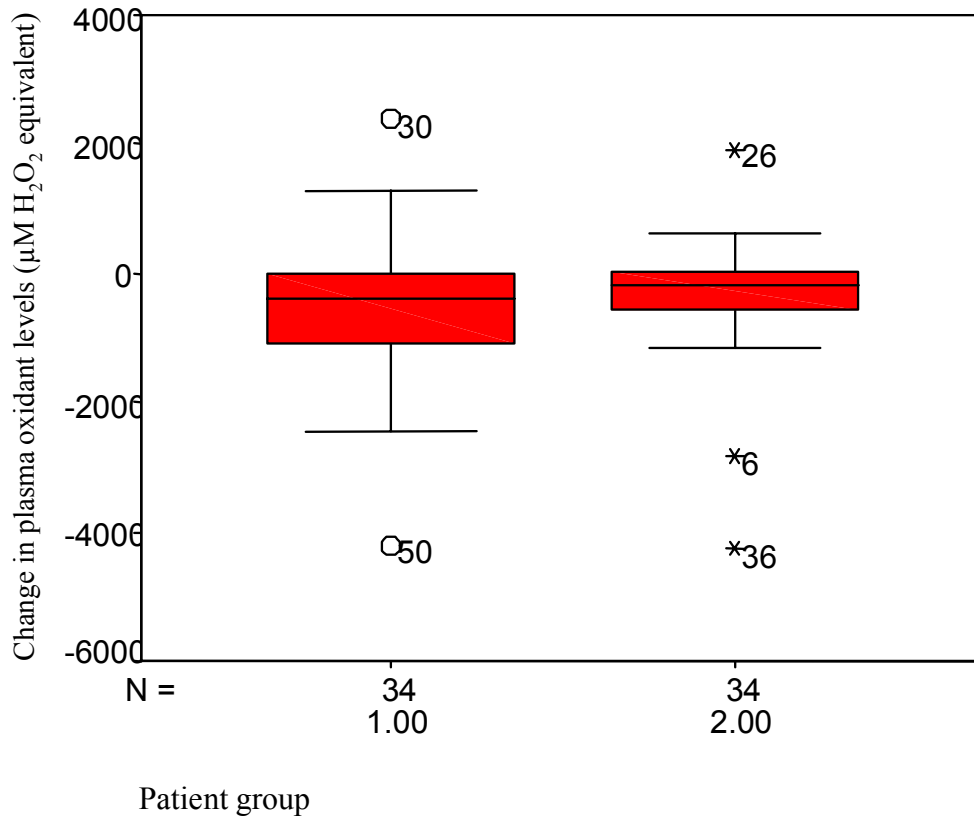


Figure 7.14: Depletion at 24 hr in plasma oxidant levels was similar in both treatment and control groups. Patient group 1.00 = control group; group 2.00 = treatment group. The symbols * and ○ identify the outliers.

Figure 7.15: Effect Of Glutamine On Changes In Urine Strong Oxidants At 24 Hr.

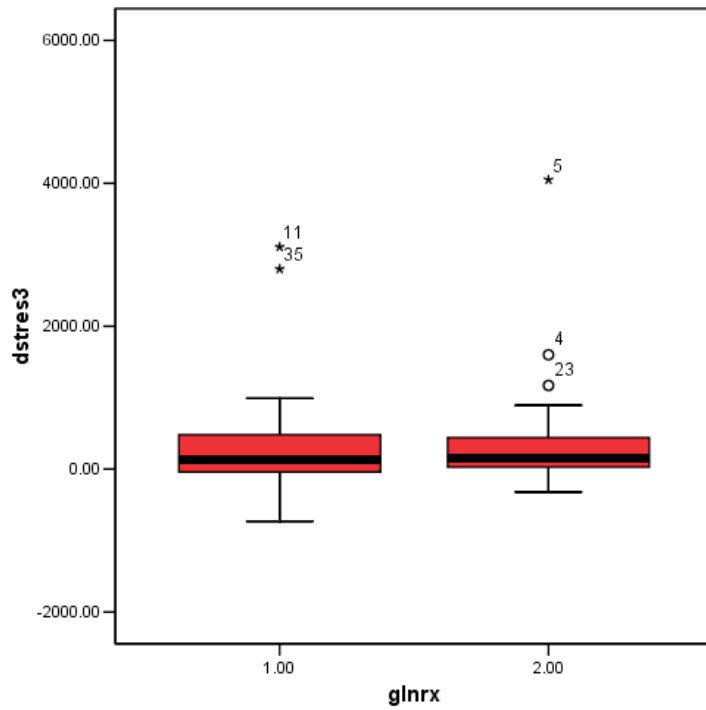


Figure 7.15: No significant difference in the changes in strong oxidants in urine between the treatment (glnrx 1.00) and the controls (glnrx 2.00). dstress3 = change in strong oxidants pre-op and post-op. The symbols * and o identify the outliers.

Figure 7.16: Scatter-gram Of Glutamine Treatment Vs Urine Strong Oxidants at 24 Hr.

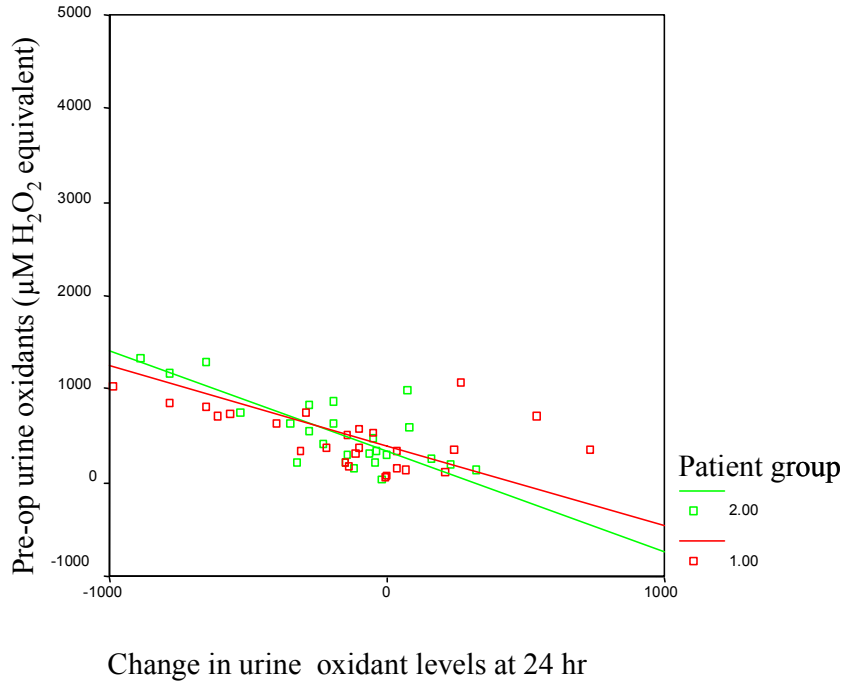


Figure 7.16: While the treatment had a tendency towards reduced strong oxidant levels, the controls tended towards increased levels.

7.4. Result: Clinical Outcomes of Glutamine Therapy

The evaluation of clinical outcome in this study is based on the RIW scoring system previously described in Chapters 2 and 4. The major components of this scoring system include patients' complexity and length of hospital stay. Complexity is based on the following criteria: no complexity; complexity related to chronic conditions; complexity related to serious/ important conditions; life threatening. The effect of glutamine treatment on the length of hospital stay was evaluated based on the type of surgery and the affected organ.

The pre-operative glutamine levels were dependent on disease complexity whereby patients with greater complexity had higher plasma glutamine levels ($p= 0.001$) (Table 7.12). Disease complexity also appeared to be evenly distributed between the

treatment group and controls ($p= 1.000$) therefore the results rule out any selection bias (Table 7.12; Figure 7.17). Disease complexity was also a determinant of plasma glutamine levels in patients post-operatively at 24 hr and 72hr however treatment improved glutamine levels in the presence of disease complexity at 24 hr ($p = 0.044$) and at 72 hr ($p= 0.002$) (Table 7.12).

Disease complexity also provides useful estimate of length of hospital stay ($p= 0.000$) (Table 7.13) wherein patients with greater complexity stayed longer in the hospital than those with less complexity. Glutamine treatment however reduced hospital stay in patients with greater complexity when compared with similar patients in the non-treatment group (Figure 7.17- 7.18).

Glutamine treatment reduced length of hospital stay in all patients by about 1 day (Table 7.14). In cancer patients there is half-day reduction in hospital stay while in non-cancer patients there is 7-day reduction following glutamine treatment (Table 7.15). The treatment also significantly reduced hospital stay after craniotomy, however length of stay was not changed in Chest surgery patients (all in group 2) nor did laparotomy patients appear to achieve any benefit (Figures 7.19).

Glutamine treatment reduced the RIW score in the study patients ($p=0.125$) but this reduction was significant in patients with greater complexity ($p= 0.0005$) (Figure 7.20; Table 7.16).

Table 7.12: Complexity of Condition versus Plasma Glutamine Levels

Paired Samples Test									
		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	GLN - COPLX	98.70	320.055	27.963	43.38	154.02	3.530	130	.001
Pair 2	RX - COPLX	.00	1.384	.100	-.20	.20	.000	191	1.000
Pair 3	G24 - COPLX	4855.4483	17952.68066	2357.302	135.0315	9575.8650	2.060	57	.044
Pair 4	G72 - COPLX	4056.0769	3761.08330	1043.137	1783.2770	6328.8768	3.888	12	.002

Table 7.12: SPSS derived paired sample test. GLN-COPLX = relationship between glutamine levels and disease complexity; RX – COPLX between treatment status and disease complexity; G24 – COPLX = Glutamine level at 24 hr versus disease complexity; G72 – COPLX = Glutamine level at 72 hr versus disease complexity. Pre-op glutamine levels were dependent on disease complexity (p= 0.001). There was no difference in disease complexity between the treatment group and controls (p= 1.000). Treatment improved glutamine levels in the presence of disease complexity at 24 hr (p = 0.044) and at 72 hr (p= 0.002).

Table 7.13: Complexity versus Length Of Hospital Stay

ANOVA					
LOS					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5024.537	4	1256.134	10.130	.000
Within Groups	7191.780	58	123.996		
Total	12216.317	62			

Table 7.13: Patients with greater complexity stayed longer in the hospital than those with less complexity (p= 0.000).

Figure 7.17: Complexity of Condition in Treatment vs. Non-Treatment Groups

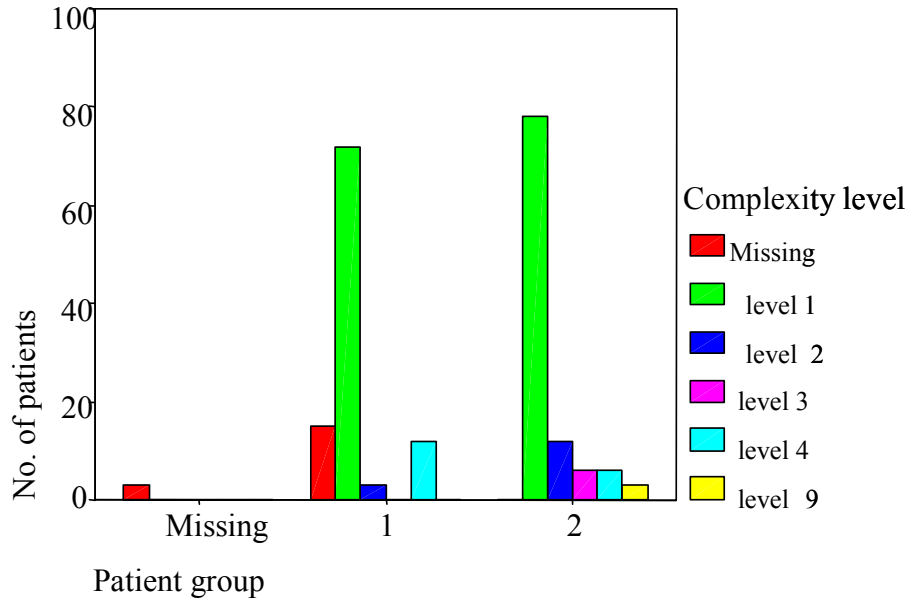


Figure 7.17: No significant difference in disease complexity between treatment group (patient group 2) and controls (patient group 1) ($p=1.000$)

Figure 7.18: Complexity of Condition vs. Length of Hospital Stay

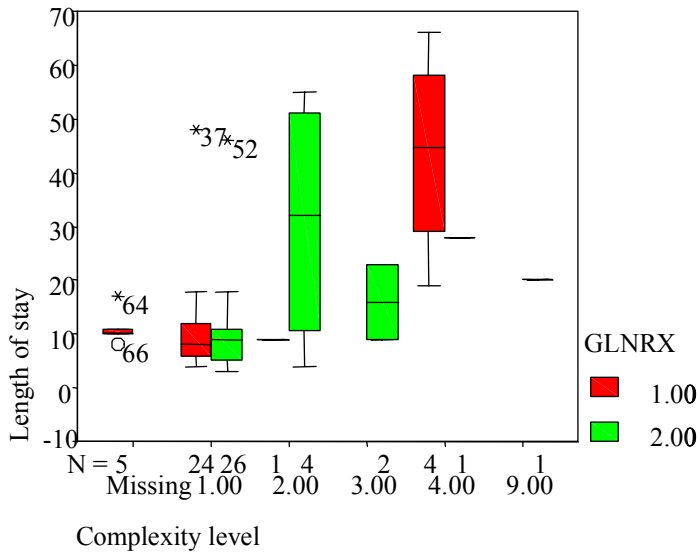


Figure 7.18: Hospital stay was reduced in treated patients with greater complexity when compared with similar controls. The symbols * and o identify the outliers. N= Number of patients in each complexity group and complexity levels range from 1 to 9.

Table 7.14: Effect Of Treatment on Length of Hospital Stay in All Patients

LOS * GLNRX

LOS			
GLNRX	Mean	N	Std. Deviation
1.00	14.32	34	14.480
2.00	13.65	34	12.766
Total	13.99	68	13.552

Table 7.14: SPSS derived. Treatment reduced length of hospital stay in all patients combined.

Table 7.15: Summary Table- Effect of Glutamine Treatment in Cancer versus Non-Cancer Patients

DFRAP DSTRESS2 DLOS * DXTYPE

DXTYPE		DFRAP	DSTRESS2	DLOS
cancer	Mean	-126.4722	-433.3590	.2711
	N	36	39	38
	Std. Deviation	352.03315	983.50328	6.82285
nocancer	Mean	72.7407	-328.2667	7.4667
	N	27	30	30
	Std. Deviation	335.27057	607.14924	14.45469
Total	Mean	-41.0952	-387.6667	3.4456
	N	63	69	68
	Std. Deviation	356.33252	836.96002	11.36217

Table 7.15: SPSS derived. DXTYPE = disease type; DFRAP = change in plasma antioxidant levels; DSTRESS2 = difference in urine strong oxidants at 72 hr; DLOS = difference between the estimated and actual length of stay. Treatment increases the plasma antioxidants in non-cancer patients. There was 7-day difference between LOS in treated non-cancer patients and controls. Treated cancer patients had 0.2 days reduction in hospital stay when compared with controls.

Figure 7.19: Length of Hospital Stay Versus Type of Surgery

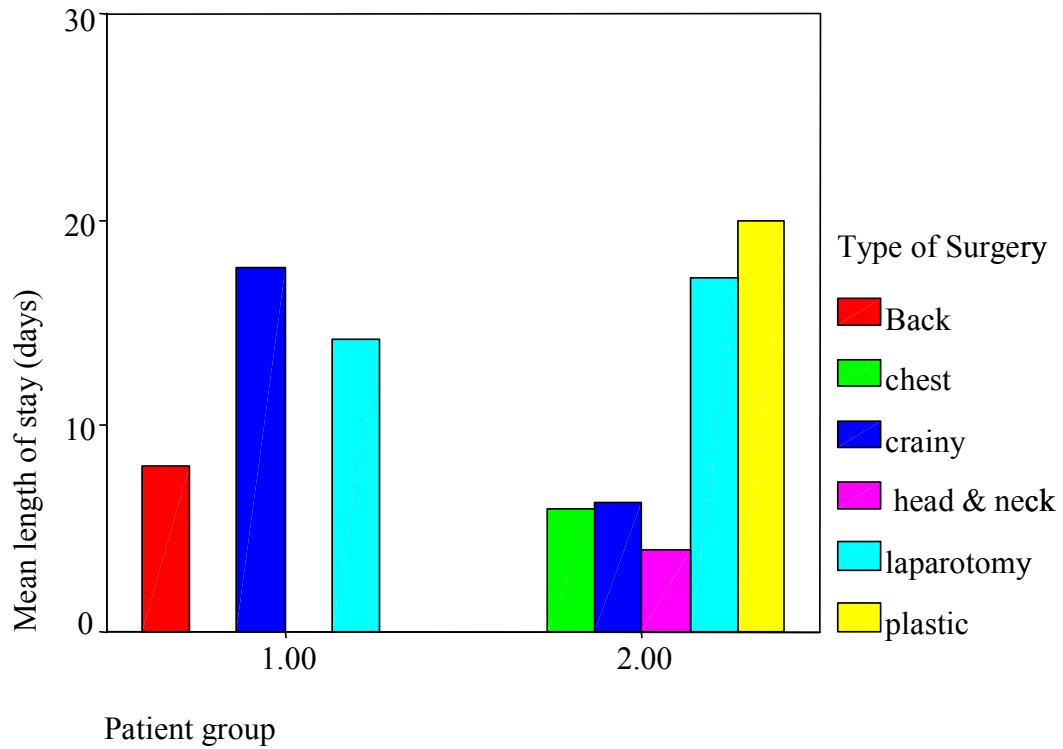


Figure 7.19: Craniotomy patients in treatment group had significant reduction in length of stay when compared with controls. Chest surgery patients (all in group 2) maintained the expected length of stay while treatment did not appear to benefit laparotomy patients.

Figure 7.20: Glutamine Treatment versus RIW Score

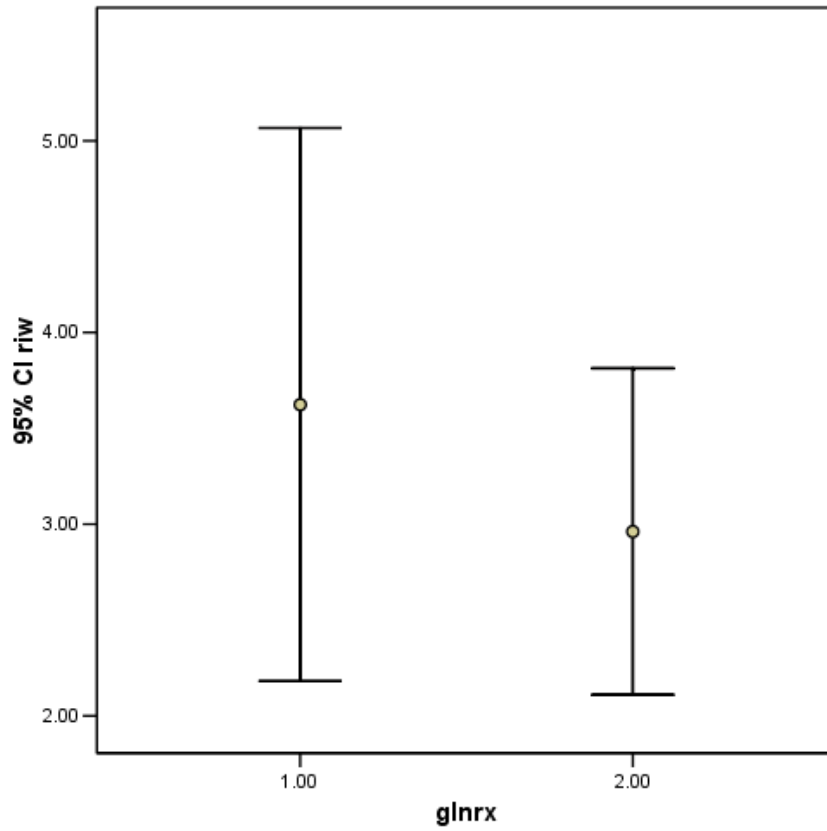


Figure 7.20: Glutamine treatment (glnrx 2.00) reduced RIW scores implying cost reduction and improved clinical outcome in elective surgery patients in comparison with controls.

Table 7.16: Glutamine Treatment or Complexity Versus RIW Score

Coefficients^a

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.810	1.038		1.745	.086
	complex	1.951	.229	.731	8.507	.000
	glnrx	-.945	.608	-.134	-1.555	.125

a. Dependent Variable: riw

Table 7.16: SPSS derived table. Both complexity and glutamine treatment are determinants of RIW scores but complexity is an independent determinant of the score (0.000).

Table 7.17: Glutamine Treatment and Complexity Versus RIW Score

ANOVA^b

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	438.416	2	219.208	37.269	.000 ^a
	Residual	358.786	61	5.882		
	Total	797.202	63			

a. Predictors: (Constant), glnrx, complex

b. Dependent Variable: riw

Table 7.17: SPSS derived table. Glutamine treatment reduces RIW scores in patients with greater disease complexity (p=0.000).

7.5. Discussion:

Evidence based medicine is the method of assessing optimal treatment based on the best current knowledge. This allows the transfer of the results of different clinical studies into numbers that can be used in practice. Evidence-based therapy is essential for the preservation and especially for further development and evolution of high-quality surgery with, at the same time, quality assurance in the new millennium [393-395]. Randomized controlled trials are the best methods to compare the effectiveness of different therapeutic interventions which may directly impact patient care [396-398]. Our study which will be classified as level 1b represents the top of the hierarchy for finding evidence for medical routine which may be used as a reference standard for other clinical studies [398, 399]. Most double blind randomized studies are narrow and focused on – single issues such as trauma, critical care [400], cancer or abdominal surgery [401] which makes generalization difficult. Although our study had rigid entry criteria, it focused on all major elective surgeries making it a useful tool for developing clinical practice guidelines. It also has an outcome component in which the surgical decisions and discharge criteria were surgeon dependent for individual patients [344, 402].

7.5.1. Demographics or Patient Selection or Study Population:

Our study population had 283 intention to treat and 69 candidates who completed the protocol making this larger than most similar double blind enteral studies with 40 or less patients enrolled [403] [404] [401] [405] [406]. There was no need for power analysis since this is the first study looking at the use of oral alanyl-glutamine as a precursor of glutamate and its effects on plasma and red cell antioxidant in surgical patients [407].

7.5.2. Treatment Selection (Oral Alanyl-Glutamine):

Oral alanyl-glutamine has been shown to be safe in patients with dengue fever and has been used a component of elemental diet [408]. Ala-Gln-supplemented PN is clinically safe well tolerated with no side effects as documented by previous researchers [409-411]. Kircher et al [411] also showed that alanyl-glutamine had similar effects as glutamine on the immune system. Oral glutamine supplement with TPN was shown to be

effective in preventing macrophage mesenteric blood mononuclear cells (M-MNC) activation thus avoiding excessive production of cytokines [412] and alanyl-glutamine has the advantage of better stability [413]. Our study used the lowest effective dose of 0.3 g/kg although glutamine doses of up to 0.75 g/kg have been shown to be safe [405, 414]. The lowest dose was used since this is the first study administering enteral alanyl-glutamine in the immediate postoperative period (within 8 hr). The dose was well tolerated, and it appears the observed changes were somewhat limited due to the low dosage. We therefore recommend higher doses within the reference range to obtain optimal effect in patient outcome.

7.5.3. Biochemical Outcomes:

Laboratory outcome test could be used to assess the efficacy of the mode of measurement or treatment. The challenge of biochemical evaluation of glutamine rests on its multiple direct and indirect functions, not just as a conditional essential amino acid, but as a precursor for nucleotide synthesis in fibroblast, lymphocytes and macrophages as well as the role it plays in glutathione synthesis [415, 416]. After surgery the availability of glutamine becomes critical for the activation of immune cell (neutrophils, monocytes and lymphocytes) [235]. Many studies used interleukin 6 and 10 (IL-6 and IL-10) while others used TNF α because it is a major product of macrophage activity which is dependent on glutamine for energy production [417, 418]. Some studies have shown that glutamine therapy helps post-operative patients maintain nitrogen balance in the treatment group. The use of the DEXA scan during the study which would have allowed us to assess the impact of glutamine on lean body mass was not feasible. The attempt to use plasma 3-methyl-histidine to assess the degree of muscle break thereby quantify the nitrogen balance [403] was also not possible due to equipment failure.

This study focused on the role of glutamine as a precursor of glutamate which enhances the uptake of cystine and alleviates the glutathione-induced inhibition of L- γ -glutamyl-L-cysteine synthase (GCS) thereby enhancing glutathione production [223, 419]. We therefore evaluated changes in plasma glutamine levels after oral alanyl-glutamine intake and the impact of this treatment on oxidative parameters. The Oxistress assay as a measure of total oxidative stress was also used in evaluation. There was a

blinding during the evaluation of the biochemical changes resulting from two post-operative doses of alanyl-glutamine given at 8 and 24 hour respectively.

The non-treatment group had a significantly higher mean pre-op plasma glutamine levels than the treatment group even though the basis of randomization was the extent of surgery (major surgery). Surgery resulted in decreased plasma glutamine levels in all patients similar to the study by Exner [405] where glycine-glutamine infusions were given to prevent immunosuppression post-surgery. This may be explained by the increased demand for glutamine as most studies have shown that during stress or catabolic states, glutamine is absorbed in the gut, and liver absorption is increased ten-fold. This glutamine is also used by the immune system and for the production of gut antioxidant.

Therapy resulted in increased plasma glutamine at 24 hr and this continued until 72 hr post-op in comparison with pre-op levels while a decrease was observed in the controls. Although these were not statistically significant findings they appear to be relevant because the oral supplement at the lowest effective dose has resulted in these changes. It is also noteworthy that this is the first report of the sole use of oral alanyl-glutamine in *post-operative patients*. Valencia et al [420] reported similar increases in glutamate and glutamine levels in plasma of *healthy subjects* after oral glutamine. In addition some animal studies also showed increased blood glutamine after oral glutamine therapy [421]. Human studies using parenteral glutamine have made similar observations [403] [406] [401] [409] while others reported no difference [248]. Overall it appears that measuring changes in plasma glutamine is an important mode of evaluating glutamine therapy.

The increase in glutamine levels observed after treatment was translated to changes in plasma antioxidants measured using the FRAP assay. A significant difference ($p=0.01$) between total antioxidants in the treated group at 24 hr compared to the control was observed with the trend present at 72 hr however not significant ($p=0.09$). The transient increase (non-significant) in red cell GSH in the first 24 hr disappeared at 72 hr. This was likely due to the increased use of glutamine by the liver and gut to produce antioxidants thus maintaining normal total plasma antioxidants. Kaufmann [421] had made similar observation in animal studies reporting an increase in gut GSH after oral

glutamine supplementation. On the contrary Valencia et al [420] observed significantly lower concentrations of red cell GSH (37%; $P = 0.00091$) with oral glutamine use in healthy volunteers thus concluding GSH biosynthesis and preservation of GSH stores in red blood cells may involve rate-limiting substrates other than glutamine. This may be explained by the fact that glutamine increases plasma antioxidant levels resulting in a negative feedback on red-cell GSH production.

In both patients groups strong oxidants in plasma were highest pre-operatively because both groups had similar disease complexity. The two groups also had depletion at 24 hr followed by a slight increase but not up to pre-op levels at 72 hr. The reduction in oxidant levels is likely due to patient optimization during surgery and in the immediate post-operative period. Age did not appear to be an independent predictor of plasma oxidant levels in this study even though there is literature evidence that aging predisposes to increased strong oxidant production [381, 422]. Post-op oxidant levels were higher in cancer than in non-cancer patients likely because non-cancer patients are chronically ill patients that have been shown to produce higher levels of strong oxidants [423, 424]. Therefore non-cancer patients are more likely to derive greater benefit from both the surgical care and external antioxidant source. Strong oxidant production in urine was however less in the treatment than the non-treatment group ($p = 0.036$ at 24 hr post-op) similar to findings in plasma following parenteral dipeptide administration. The absolute difference between these groups was however not significant ($p = 0.143$). While the treatment group had a tendency towards reduced strong oxidant levels, the controls tended towards increased levels. However some of the controls also had reduced oxidant levels at 24 hr probably due to the high baseline glutamine and plasma antioxidant levels in these patients. The lack of absolute difference between the treatment and the control might be because elective surgery is a controlled trauma that may be physiologically equivalent to mild or moderate trauma. The low dose of glutamine used in the study and the fact that many patients have access to over the counter antioxidant medication may also explain these findings. Treatment did not affect urine oxidant production at 72 hr post-op likely because the last dose of alanyl-glutamine was given at 24 hr post-op and it has a short half-life. Overall this study suggests that oral glutamine supplementation

prevents oxidant production and may be a valuable agent for prevention of oxidative stress in surgery patients.

7.5.4. Clinical Outcomes

Measuring outcome is not only relevant for research purposes but also in daily surgical practice [344, 425]. The study patients were classified based on their complexity, type of surgery and their disease. The determinants of clinical outcome in the study were the length of hospital stay (used in many surgical studies [272, 426]) and the RIW score. Forty-two percent of the study population were in the 60 yr and above age group making this comparable with a previous study by Desrosiers et al [426] and many others. The reduced length of hospital stay in our treatment group was similar to that in other studies with *parenteral glutamine supplementation*, however our controls had a shorter length of stay than these studies reported. Oral alanyl-glutamine supplementation is therefore as effective as the parenteral in improving clinical outcome.

The complexity of patients' condition appears to be an independent predictor of both the length of stay and plasma glutamine at 24 and 72 hr. Alanyl-glutamine supplementation seemed to reduce length of stay by 7 days in non-cancer patients but by only a half day in cancer patients. This could be due to the replenishment of the pre-op depletion of glutamine observed in non-cancer patients as compared with the cancer patients. Thus supplementation becomes necessary to improve the body's immune and reconstructive ability. There was one mortality (3%) in the treatment group due to complications from surgery remote from antioxidant intervention, this is less than other studies in major surgery patients [426]. It is quite presumptuous to attribute this to the intervention, thus further studies and meta-analysis are needed to answer this question. In the earlier study on the natural history of oxidative stress, head injured patients were most prone hence the significant reduction in hospital stay in treated craniotomy patients is evident of the benefit of oral glutamine supplementation in this group.

We had considered using the E-PASS as a supplementary mode of assessment however it was technically not feasible to compute all the appropriate parameters.

Many studies have tried to evaluate the economic benefit of glutamine. Using the RIW score which is based on resource utilization, the study showed significant benefit

patients with greater complexity, similar to the findings of Mertes [401] who reported that parenteral glutamine supplementation reduced the cost of care in major abdominal surgery patients. The RIW score also reduced with alanyl-glutamine treatment ($p=0.125$) but this did not appear to be an independent predictor. A similar study by Pytlik, et al on transplant patients however did not show any economic benefit with parenteral alanyl-glutamine [427]. This is probably because the major cost of transplantation is the surgery and organ retrieval and changes in supportive care may not affect the cost.

This study has demonstrated improved clinical outcome with post-operative oral alanyl-glutamine supplementation at a relatively low dose therefore greater benefits are likely to be derived from the higher dose of 0.75 g/kg body weight.

8. CONCLUSION

The study of the natural history and modulation of oxidative stress in surgery and trauma patients was undertaken based on an identified knowledge gap in the subject both in the literature and clinical practice. The three research components include studying the natural history of oxidative stress using trauma as a model, developing the novel Oxistress assay, and modulating oxidative stress in surgery patients. The research was based on the hypothesis that an understanding of the natural history of oxidative stress will guide appropriate modulation that will invariably improve patient outcome.

The concept of oxidative homeostasis involves the intrinsic maintenance of a balance between strong oxidant (ROI) and antioxidant production by the body [19]. Antioxidants are responsible for preventing the harmful effects of strong oxidants. The major oxidants in the body include superoxide anions, hydroxyl ions, lipid radicals and hydrogen peroxide [30, 38, 101], while the intrinsic antioxidants include vitamins C & E and GSH [39, 40, 42, 43]. Oxidative stress results from an imbalance in which strong oxidants are produced at a higher rate than antioxidants [1, 19]. Predisposing conditions to oxidative stress include acute and chronic illnesses such as trauma, major surgery, infection, diabetes mellitus, inflammatory bowel disease (IBD), renal diseases, cardiac diseases and neurological diseases [47-49].

Previous research on the natural history of oxidative stress had been on animal models while most human studies have been on selective aspects and groups of patients thus limiting clinical application of oxidative stress. Various laboratory methods were evaluated (Chapter 2) and the most relevant ones were applied in studying the natural history in patient populations. The selected methods needed to be easy, simple, sensitive, and accurate and have potential benefit to the clinical setting. Most methods do not individually measure overall oxidative status hence a combination of methods has been adopted in previous research to obtain an estimate. A notable aspect of this research is the invention of the novel Oxistress assay, a non-invasive single test for overall evaluation of oxidative status. The FRAP method [126] was used to estimate total antioxidant capacity in plasma, the Asensi modification of Brigelius' method [150] was chosen for red cell glutathione measurement, protein carbonyl levels (measure of protein oxidant damage) were evaluated with the colorimetric method [167], and plasma and urine oxidant levels

were measured with the novel Oxistress assay. The role of neutrophils in oxidative stress was also evaluated. Neutrophil activity was evaluated with the myeloperoxidase assay [428] which though not fast provides accurate and reliable results, while the neutrophil count was done by the hospital laboratory.

The invention of the novel Oxistress assay aimed at an easier and rapid clinically applicable method of measuring strong oxidants (H_2O_2 equivalent) in biological fluids such as plasma and urine, occurred at the preliminary phase of the research while evaluating existing laboratory methods. The assay can be used with or without the EDTA component, the latter being a very good laboratory tool for measuring high levels of hydrogen peroxide making it useful in both human and non-human samples. It measures antioxidant status, which is a summation of the reaction between antioxidants and strong oxidants produced in urine. It also allows an evaluation of circulating strong oxidants thereby providing a total picture of the oxidative status of the patient. It is therefore an assay that can be singularly used to diagnose oxidative stress in a clinical setting. In addition it is fast (5 min), inexpensive and produces a visible color change at $37^{\circ}C$. The assay has proven to be a reliable clinical diagnostic tool with high sensitivity and specificity for oxidative stress based on trials in adults and neonates. Reference values were determined in both neonates and adult populations and comparisons with patient populations in both groups yielded highly significant differences ($p=0.0001$). The estimated reference value range for the normal population range is $136\ \mu M$ – $260\ \mu M$, while that of the neonatal population is (140-208 μM).

The study of the natural history of oxidative stress was conducted on trauma patients at the Royal University Hospital, Saskatoon between April and September 2000. There were four patient sub-groups - three based on the injury severity score (ISS) and the last had pre-morbid medical factors. The change in antioxidant homeostasis in our study population was monitored in red cell, plasma and urine over 7 days.

The results showed trauma depletes red cell GSH level and this effect is maximal between 24 and 30 hr. The most important determinants of the depletion in red cell GSH were trauma severity, presence of pre-existing medical problems and the body system affected by the trauma. Similar depletion in plasma GSH have been reported following severe multiple trauma thus validating our findings [18, 429].

A direct relationship between the systemic increase in neutrophil count maximal within 6 hr of trauma, trauma severity and depletion of red cell GSH maximal within 24 hr in the same group of patients was observed. Trauma severity was directly associated with increases in both neutrophil count and plasma myeloperoxidase levels (neutrophil activity), the latter likely a consequence of the former. Patients with pre-morbid medical condition had greater neutrophil activity even with mild trauma. The changes in neutrophils are likely responsible for the observed increase in strong oxidant production and antioxidant depletion. These results are therefore in keeping with those of other authors which suggest neutrophils play a key role in tissue injury and the etiology of oxidative stress [330, 331, 370].

There was significant depletion in plasma antioxidants ($p = 0.017$) within the first 30 hr and slight recovery from 72 hr but never reaching admission levels by day 7 in all patients. These results are in keeping with those of other authors that trauma results in depletion of plasma antioxidants [130, 376]. Trauma severity and pre-morbid medical conditions had similar trend as observed in all patients combined. Plasma oxidant levels had two peaks, 1st on admission, second higher peak between 12-18 hr and a significant decline at 72 hr. A ten-fold increase in plasma protein carbonyl levels over the study period (mean = 1.49 nmol/mg protein; normal is <0.1 nmol/mg protein) was observed following trauma. The peaks were noticed on admission and day 7 with declines within 6-12 hr and 24 hr therefore plasma protein carbonyl appears to be an early index of oxidative damage in trauma. A positive correlation was also observed between raised plasma protein carbonyl levels and trauma severity. There was a similarity in the pattern of changes in the plasma oxidant and plasma protein carbonyl levels. A linear relationship between oxidant and carbonyl levels in the plasma was observed thus implying that the degree of oxidative damage to proteins is dependent on the level of strong oxidants in circulation. The overall effect of trauma on plasma oxidative homeostasis therefore includes increased free radical production leading to protein degradation, antioxidant depletion and subsequent oxidative stress.

Urine analysis proved to be an informative tool in evaluating oxidative stress and promises to be beneficial in clinical diagnosis. Similar to plasma, urine oxidant levels (measured with the oxistress assay) correlated with trauma severity, however the peak for

urine was 24 hr compared with 18 hr for plasma. The difference in time is due to the time required for excretion. Urine oxidant level is the summation of the reaction between plasma antioxidants and antioxidants thus making it an index of antioxidant homeostasis. The presence of protein carbonyl precipitates in urine was directly related to plasma levels and trauma severity as well as the presence of pre-existing medical factors. It is therefore a useful screening method for protein degradation after trauma.

Oxidative stress is associated with increased trauma severity resulting in antioxidant depletion, strong oxidant production and protein degradation. The presence of pre-morbid medical factors also increases oxidative stress in trauma patients. Therefore early introduction of prophylactic antioxidant therapy may be beneficial following trauma and is highly recommended.

Modulation involves the various ways of modifying the body's response to oxidative stress through the use of direct or indirect antioxidant sources. The direct sources include vitamins C & E, N-acetyl cysteine, L-2-oxothiazolelidine-y-carboxylase (OTC), glycine and glutamine, while indirect sources include phase 2 enzyme inducers such in foods such as broccoli, soya milk etc. While there is evidence that *parenteral* glutamine improves clinical and biochemical outcome in patients following major surgery [251, 255, 405, 430], no study has reported any clinical benefit with *enteral* glutamine supplementation. Most studies using enteral glutamine have only reported improvement in biochemical and immunological parameters [412]. The enteral route of nutrition has been shown to be superior to the parenteral route with significantly fewer complications. The most significant side effect of enteral nutrition is diarrhea and vomiting. The use of elemental diet allows absorption when the gut is in a transition phase [270, 284, 285, 431]. There is ample evidence that early enteral feed is cost effective and reduces hospital stay [258, 275]. The recommended post operative dose of parenteral glutamine is 0.1 – 0.3 g/kg body weight, however this dose is quite low and patients tolerance has been documented at doses as high as 0.75 g/kg body weight without any side effects. Outcome evaluation involves laboratory and clinical components, with the latter being both patient and provider focused. The clinical trial of enteral alanyl-glutamine (an indirect precursor of glutathione via glutamate)

supplementation in modulating oxidative stress after major elective surgery was to evaluate these two components of patient outcome.

The prospective double blind trial conducted on surgery patients between November 2002 and April 2003 was designed to treat oxidative stress in previously identified susceptible patient groups. Glutamine was chosen with the aim of increasing GSH levels thereby correcting oxidative stress, and it was clinically acceptable because previous use. Oral alanyl-glutamine was found to be a safe supplement in the immediate post-operative period. The study revealed depletion in plasma glutamine levels following surgery in all patients. Oral alanyl-glutamine was effective even at very low doses (0.3 g/kg body weight) in increasing plasma glutamine ($p= 0.002$) and antioxidant levels as well as in reducing strong oxidant production ($p= 0.005$). The difference in plasma glutamine levels between the treatment and control groups was however limited probably due to the following possible reasons. There is increasing awareness and over the counter use of antioxidant supplements hence the higher preoperative plasma glutamine levels observed in the control group. Elective surgery is classified as mild to moderate trauma, radical production and the need to increase erythrocyte glutathione is therefore limited. The other reason for the transient increase observed could be due to the low dose used in this study. We therefore recommend increasing the dose up to 0.75g/kg in clinical practice in order to maximize the benefit derived from the supplement. Glutamine supplement reduced hospital stay in non-cancer and higher complexity groups but not in the cancer group. This may be because surgery is first line of cancer therapy while it is a consequence of inadequate medical treatment in non-cancer patients who usually have pre-existing medical factors and previous glutamine depletion. Despite its limitation as a measure of economic benefit as shown by Girroiti et al [301], the RIW score improved with glutamine supplementation. The intervention resulted in significant economic benefit in patients with greater disease complexity that will invariably benefit the health care system significantly. Higher doses of oral alanyl-glutamine supplementation are therefore recommended in major surgery patients both in the pre and post-operative periods to enhance improved patient outcome.

The research on oxidative stress in trauma and surgery patients has provided better understanding of the mechanism of oxidative stress, a bedside method of

measurement and the evaluation of the effect of treatment on patient outcome. The limiting factors for the trial included the number of available patients (n=69) within the time frame for the PhD thesis as well as the fact that only one centre was used. It is however important to note that this is the first study of its kind using enteral alanyl-glutamine with a population higher than that in most similar clinical trials making it an ideal reference study.

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Appendix to Chapter 5: Further Evaluation of the Oxistress Assay

Procedure:

The process involved the use of different concentrations of the components of the assay including two different types of EDTA (acid and sodium) as follows:

- 1) Deoxyglucose reagent (100 mM), Thiobarturic acid (TBA) (50 mM), EDTA (1.4 mM) & Ferrous sulfate (1 mM).
- 2) Deoxyglucose reagent (100 mM), Thiobarturic acid (TBA) (50 mM), EDTA with sodium (1.4 mM) & Ferrous sulfate (1 mM).
- 3) Deoxyglucose reagent (100 mM), Thiobarturic acid (TBA) (50 mM) & Ferrous sulfate (1 mM).
- 4) Deoxyglucose reagent (50 mM), Thiobarturic acid (TBA) (50 mM) Glucose (100 mM) & Ferrous sulfate (1 mM).
- 5) Glucose reagent (100 mM), Thiobarturic acid (TBA) (50 mM) & Ferrous sulfate (1 mM)
- 6) Hydrogen peroxide standard was used in all cases in the following concentrations 50, 100, 250, 500, 1000, 2500, 5000, 10,000 & 20,000 μM

The reagents were incubated at either 37⁰C or room temperature overnight or used freshly prepared. The hydrogen peroxide and the reagents were mixed at a ratio of one in ten (1:10) in both microtitre plates and one ml curvettes. The results were read using both the microtitre plate reader and a normal spectrometer. The color changes were also scanned over a period of 5 hr. The end results were standard curves and color changes.

Evaluating EDTA:

The peak reading from the spectrometer occurred between 500- 800 μM of hydrogen peroxide concentrations with both acid and basic EDTA (Figure 5.6). A decline in the spectrometry reading at higher concentrations of hydrogen peroxide produced a U-curve (Figure 5.7). The removal of EDTA however resulted in a linear graph at all hydrogen peroxide concentrations ranging from 0 -2,000 μM working solution (Figure 5.8). At lower hydrogen peroxide concentrations (less than 800 μM), a rapid consistent colour change from pink to red was observed, while at higher concentrations (Figures 5.9

A-D) the color change was not sustained (10 min). The removal of EDTA from the solution resulted in stability of the color and the standard curve at all concentrations over 90 min and up to 5 hr (Figures 5.10-5.11).

Figure 5.6: Oxistress Assay (with EDTA) With Hydrogen Peroxide Standards

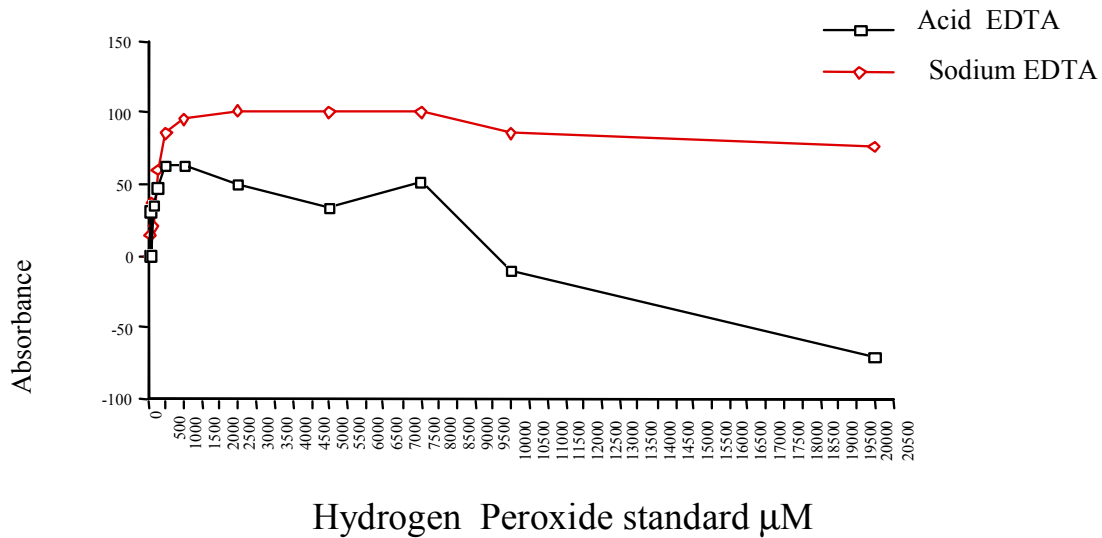


Figure 5.6: This graph indicates that the assay is linear up to 800 μM hydrogen peroxide concentration after which the absorbance reduces. Sodium EDTA appears to produce slightly better graph than acid EDTA.

Figure 5.7: U-Curve of Oxistress Assay (with EDTA) With Various Dilutions of Hydrogen Peroxide Standards

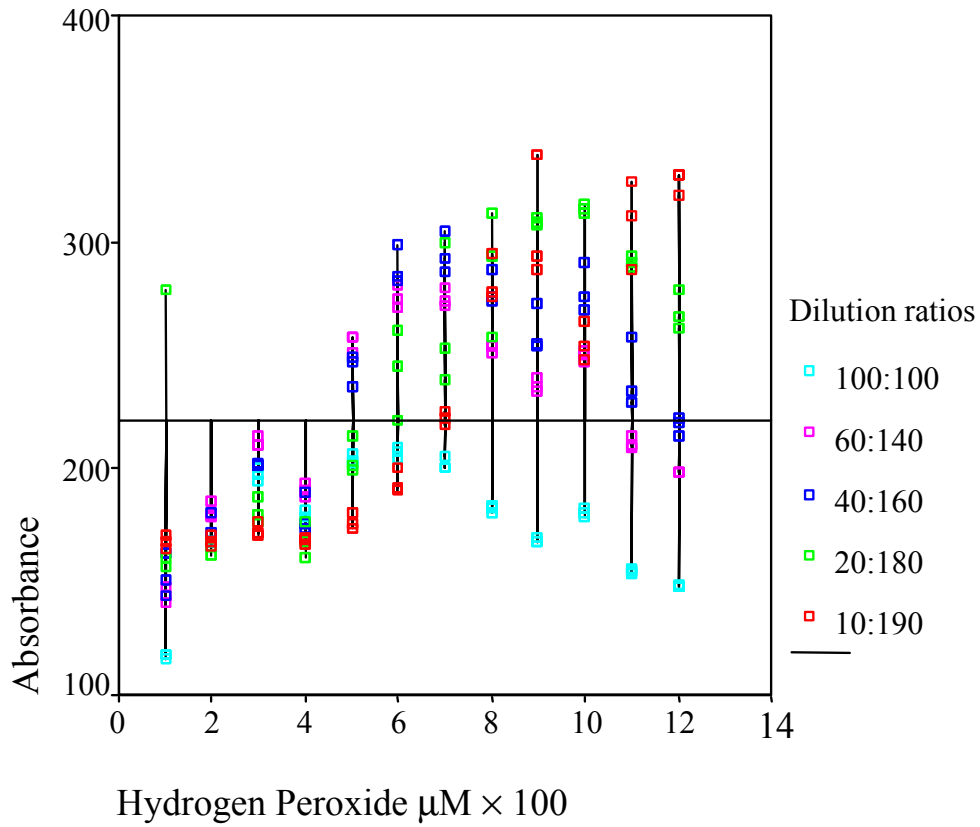


Figure 5.7: This curve indicates that the assay is linear up to 800 μM hydrogen peroxide concentration after which the absorbance reduces.

Figure 5.8: Oxistress Assay (With And Without EDTA) With Hydrogen Peroxide Standards

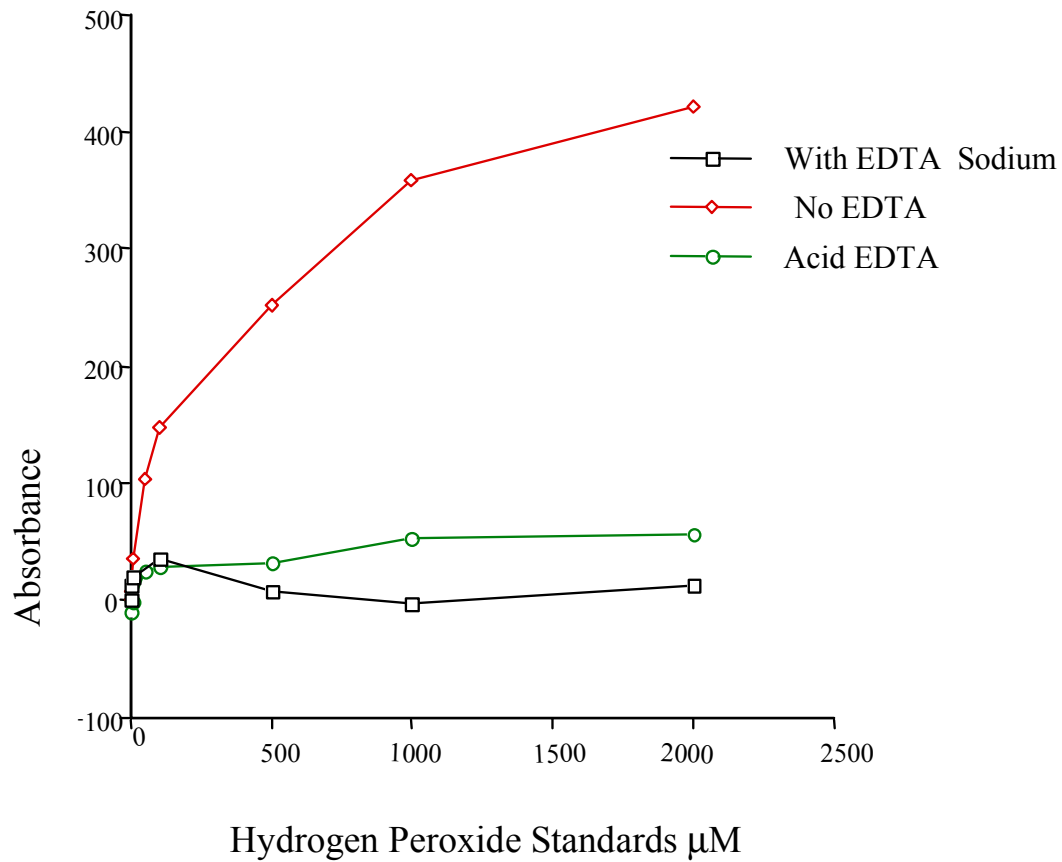


Figure 5.8: Removal of EDTA resulted in a linear graph at higher peroxide concentrations (up to 2500 μM).

Figures 5.9 A-D: Color Changes - Oxistress Assay (with EDTA) and 0.88 M Hydrogen Peroxide Standard

Figure 5.9A: At 5 min After The Reaction:



Figure 5.9B: Between 10 –15 min After The Reaction



Figure 5.9C: At 25 min After The Reaction



Figure 5.9D: At 30 min After The Reaction



Figure 5.10: Colour Change In The Oxistress Assay (Without EDTA) Versus Hydrogen Peroxide Standards (Up To 2500 μ M)

Figure 5.10A: Colour reaction @ 10 min without EDTA



Figure 5.10B: Colour reaction @ 90 min without EDTA



Figure 5.11: Oxistress Assay (without EDTA) Reaction Over Time

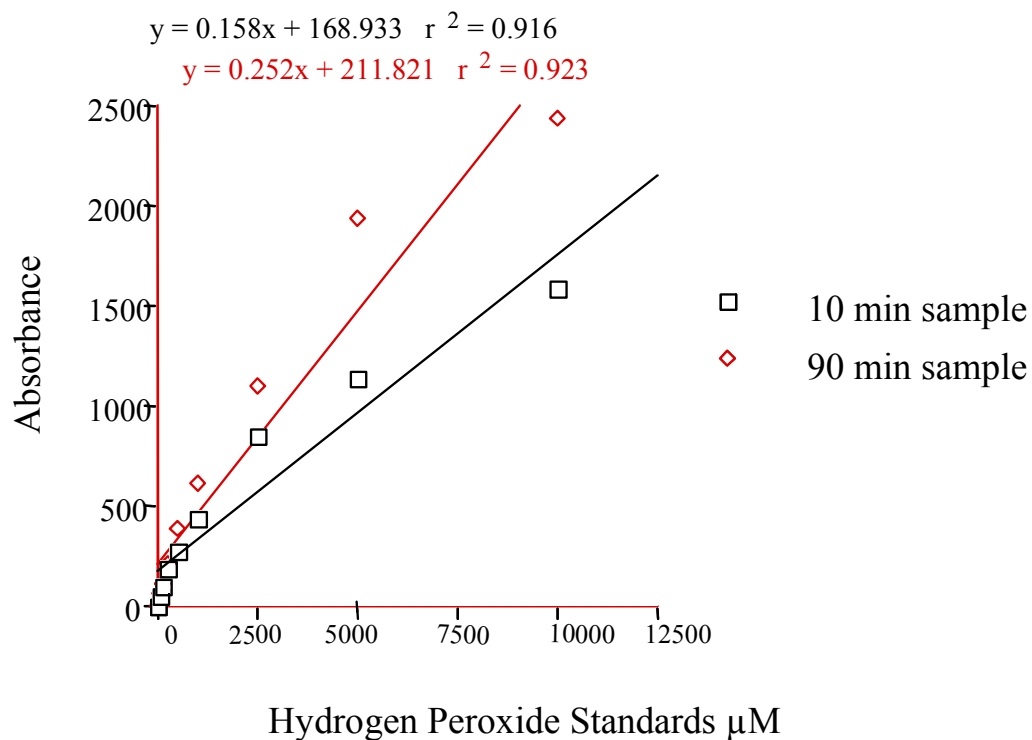


Figure 5.11: Oxistress assay is an end-point assay that is not affected by the time of reading.

Evaluating the Role of Deoxyglucose (Or Glucose)

This evaluation was done without the EDTA component of the Oxistress Assay in order to clearly identify the role of deoxyglucose (or glucose). There was visible colour

change within 10 min of the reaction using the Eppendorf tubes and microtitre plates at different peroxide concentrations (Figure 5.12). The change was seen at 250 μM of hydrogen peroxide for 50 mM deoxyglucose concentration compared with 100 μM of hydrogen peroxide standard for 100 mM deoxyglucose concentration. The colour persisted for 4 days at room temperature in both cases but it was more pronounced in the 100 mM deoxyglucose concentration than 50 mM deoxyglucose concentration.

With 100 mM glucose as the assay sugar a visible color change was not observed until 500 μM of hydrogen peroxide was used. This peroxide level is five times the amount required for similar colour change using equivalent deoxyglucose concentration. Deoxyglucose in the assay produced a better linear curve with a correlation coefficient of 95% compared to 82% for glucose (Figure 5.13).

Figure 5.12: The Effect Of Variable Deoxyglucose Concentrations In The Oxistress Assay

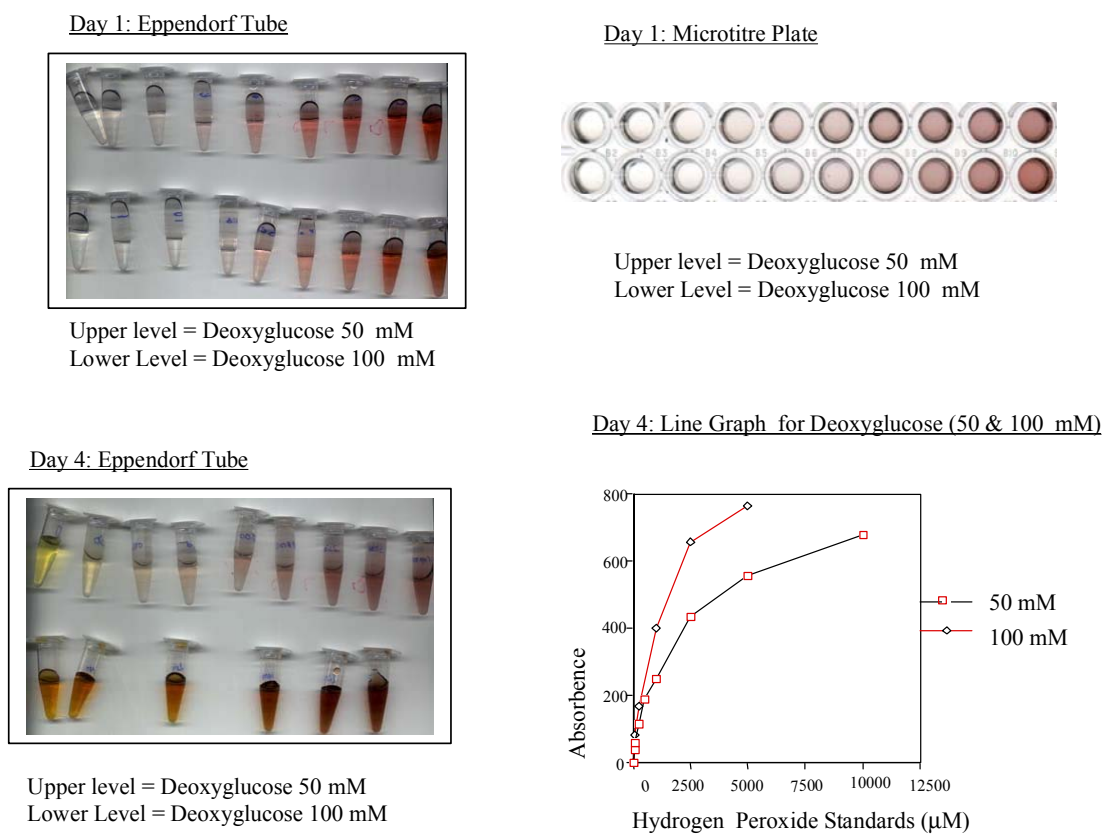


Figure 5.13: Glucose Versus Deoxyglucose In Oxistress Assay

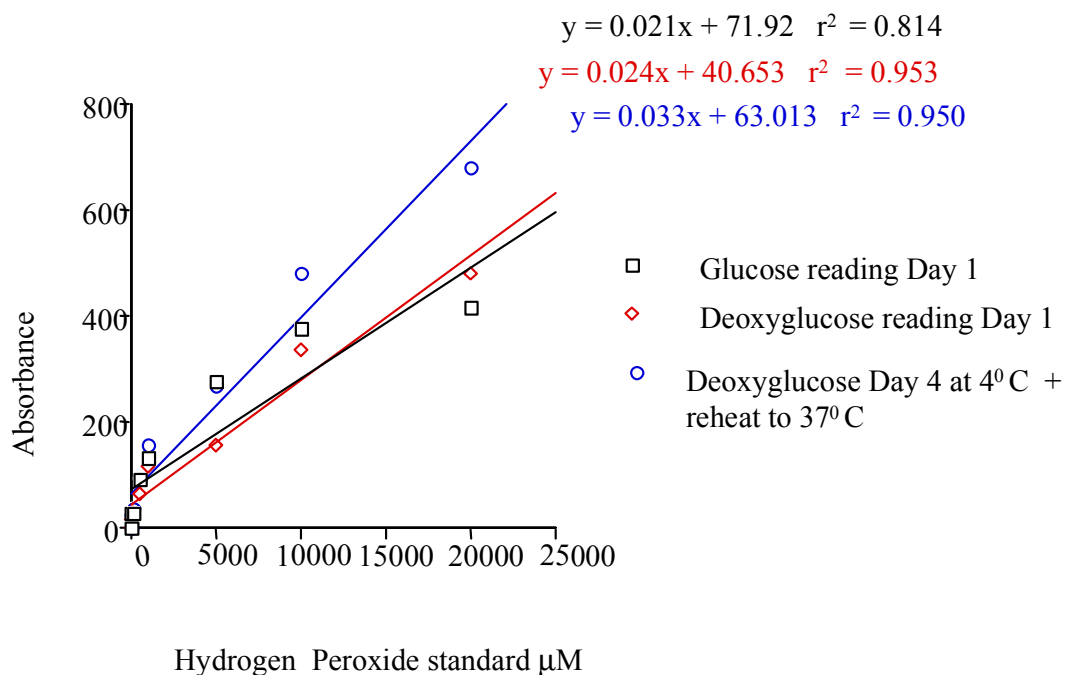


Figure 5.13: Stored Deoxyglucose produced better correlation coefficient with hydrogen peroxide standards at day 4 than freshly made glucose.

EDTA-free Oxistress Assay:

As a result of the observed limitations with EDTA and glucose, the assay was subsequently used without EDTA and deoxyglucose was the preferred sugar. The EDTA-free Oxistress Assay was thus evaluated using three different methods of preparation to determine stability and colour changes with storage and hydrogen peroxide reactions.

Stability and Colour of EDTA-free Oxistress Assay with Storage

The colour changes in the assay reagent with these storage methods are shown in Figure 5.14.

Method 1: The assay reagent was stored in the fridge (4°C) for 5 days where it maintained a clear colour. Some precipitates were noticed but they dissolved once the solution was re-warmed to 37°C leaving a slight yellowish colour tinge.

Method 2: The assay reagent was deoxygenated with nitrogen and stored at room temperature for 4 days. Nitrogen was chosen because of its' ability to reduce oxygen

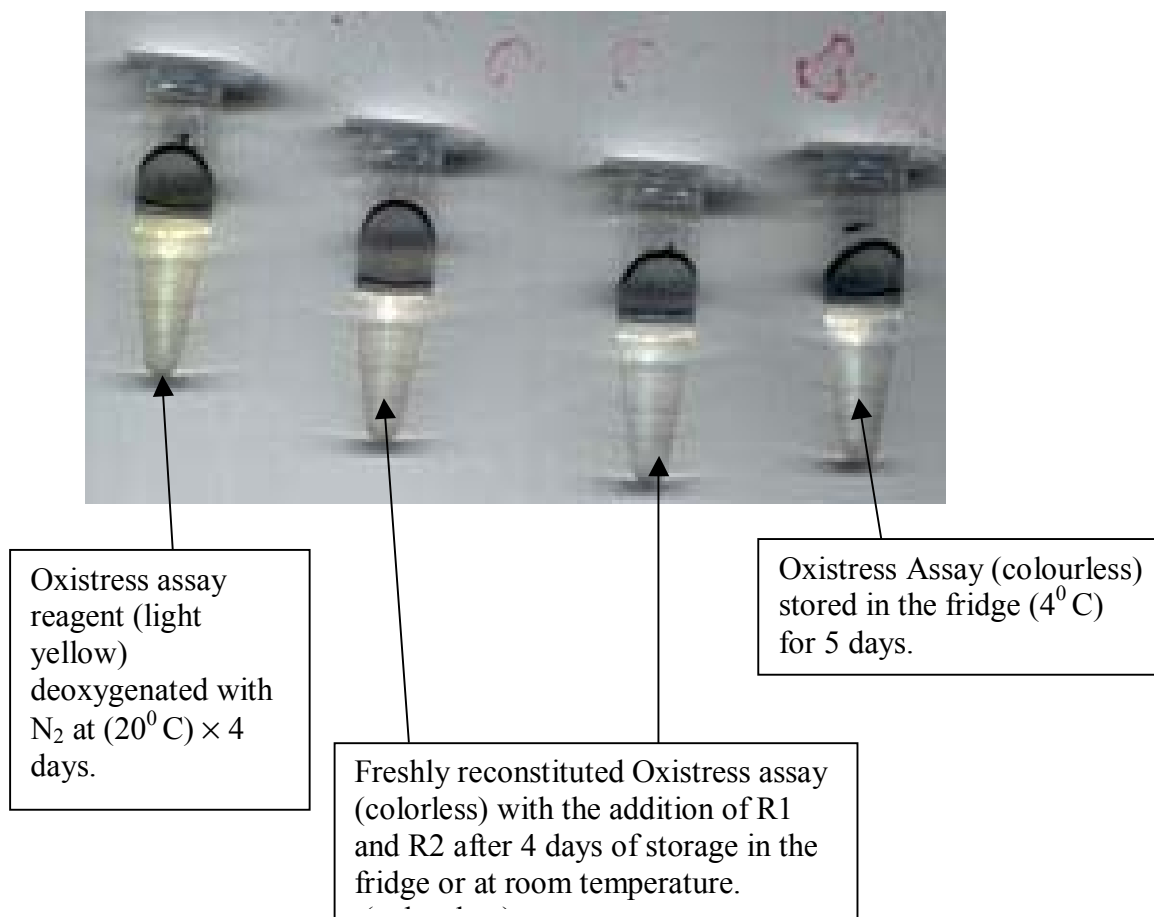
concentration in solutions thus preventing oxidation and possible colour change. The result was a light yellow colour change contrary to expectation.

Method 3: The assay reagent was divided into two components:

- ◆ Reagent 1 (R1) is a mixture of thiobarturic acid (TBA) (50 mM) & Ferrous sulfate (1 mM).
- ◆ Reagent 2 (R2) is the deoxyglucose reagent (100 mM).

Reagents 1 and 2 were each sub-divided into two parts stored separately at room temperature or in the fridge. Equal volumes of R1 and R2 were mixed prior to use and a clear solution was obtained. This result was reproducible after 4 wk of storage of these components at room temperature or in the fridge.

Figure 5.14: Colour Changes With The Different Assay Storage Methods



Storage Method Vs. EDTA-Free Oxistress Assay Reaction With Hydrogen Peroxide Standards:

Using the same hydrogen peroxide standards with all the assay methods, a correlation coefficient ranging from 82% to 91% was observed (Figure 5.15). The best long-term correlation was noticed with the reconstituted R1 and R2 (Method 3). Based on these observations the EDTA-free Oxistress assay was either prepared fresh or stored in its R1 and R2 components for the rest of the study. The assay was also able to maintain its clear colour with reconstitution after storage as well produce colour change with hydrogen peroxide standards (Figure 5.16).

Figure 5.15: Scattergram Of Various Storage Methods For EDTA-Free Oxistress Assay

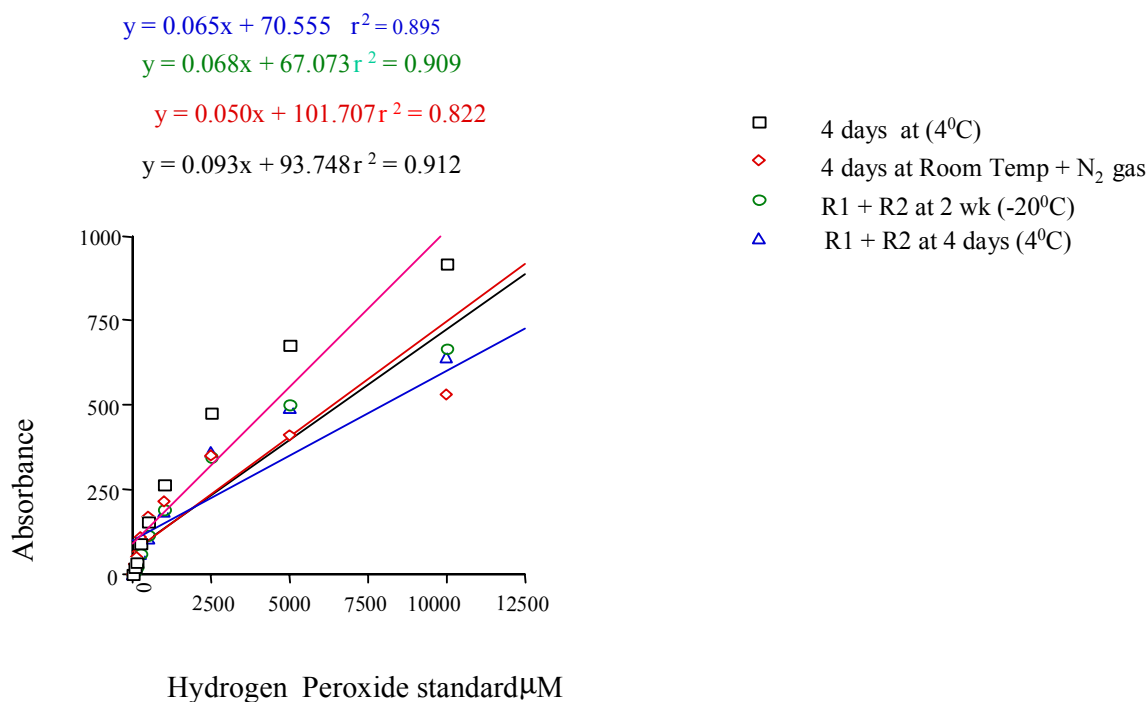


Figure 5.15: Reagents are stable up to 2 wk with the best correlation at 4 days at 4⁰C but storage at room temp appears to reduce assay performance.

Figure 5.16: Sustaining Color Change Of After The Reaction:

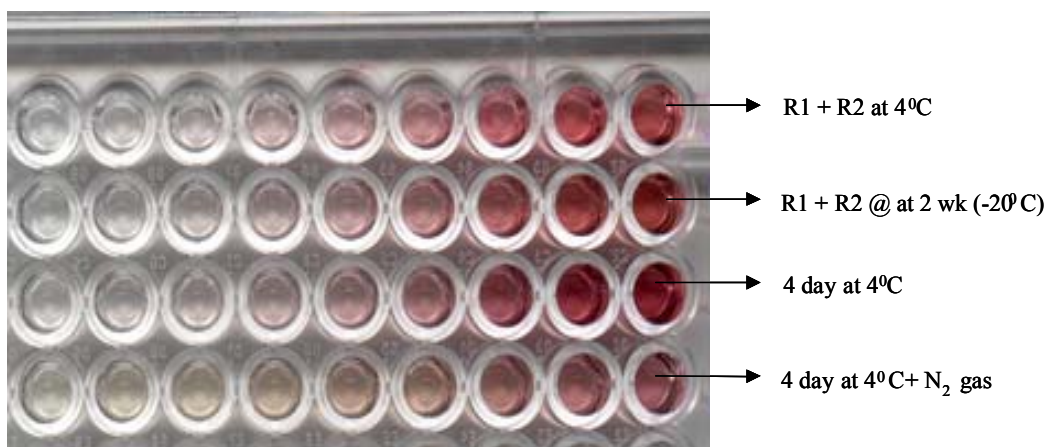


Figure 5.16: Storage of the assay components for up to 2 wk did not appear to affect colour change.