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3. MATERIAL AND METHODS

3.1 PLACE OF STUDY:
The study entitled "Evaluation of the effects of N-acetylcysteine and vitamin C on the oxidative stress following abdominal sepsis", was conducted in the Intensive Care Unit of the Dept of Anaesthesiology, J.N. Medical College, AMU, Aligarh and the biochemical analyses was performed in the Dept of Biochemistry, F/O Life Sciences, AMU, Aligarh.

3.2 MATERIALS:

3.2.1 SAMPLE SIZE:
After obtaining approval from the Board of studies, 60 patients suffering from sepsis, admitted in the ICU and 15 normal people (as a normal control) (a total of 75 patients) of either sex, age ranging between 18 – 60 years, were enrolled for the study. Written informed consent was obtained from the patients or patient’s relatives.

3.2.2 SELECTION CRITERIA:
- Age not less than 18 years and not more than 60 years of either sex
- Patients diagnosed as peritonitis (abdominal sepsis)
- Presence of two or more of criteria for systemic inflammatory response syndrome (SIRS) and
- Presence of definite site of infection (abdominal)
- Post surgical septic peritonitis

Note: The criteria for the diagnosis of SIRS is as follows
1. Temperature greater than or equal to 38°C or less than 36°C,
2. Heart rate greater than 90 bpm,
3. Respiratory rate greater than 20 breaths/min or PaCO₂ more than 32 mm Hg, and
4. WBC count greater than 12,000 per mm³ or less than 4000 per mm³ or the presence of more than 10 percent immature bands.

3.2.3 EXCLUSION CRITERIA:
The following patients were not included in the study:
- Age less than 18 years,
- Glasgow coma scale (GCS) less than 9,
- patients with un-recordable pulse and blood pressure,
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- with Acute coronary syndrome,
- with Active gastrointestinal hemorrhage,
- patients with hepatic or renal failure,
- with history of seizure,
- with drug overdose,
- with history of poisoning,
- with burn injury,
- history of malignancy,
- with do not attempt resuscitation (DNAR) status.

3.2.4 GROUP DIVISION:

**Group A** | (normal control) | comprised of 15 normal subjects.

60 patients fulfilling the selection criteria were enrolled for the study. The patients were randomly divided into four groups. Block randomization was done with an intention to have equal number of patients in each group. Randomization was done after the patients underwent abdominal surgery (laparotomy). Each group comprised of 15 patients with abdominal sepsis.

**Group B** | post surgical septic peritonitis patients received 100 ml of 5% dextrose 8 houry for 3 days (9 doses).

**Group C** | post surgical septic peritonitis patients receiving N-acetylcysteine @ 70 mg/Kg dissolved in 100 ml of 5% dextrose 8 hourly for 3 days (9 doses).

**Group D** | post surgical septic peritonitis patients received vitamin C @ 25 mg/kg dissolved in 100 ml of 5% dextrose 8 hourly for 3 days (9 doses).

**Group E** | post surgical septic peritonitis patients received a combination of N-acetylcysteine @ 70 mg/kg and vitamin C @ 25 mg/kg dissolved in 100 ml of 5% dextrose 8 hourly for 3 days (9 doses).

3.2.5 COLLECTION OF SAMPLE:

10 ml of blood was withdrawn with 10 ml disposable syringe from peripheral veins of all those enrolled in the study. Sample was collected only once from the normal control group (group A) to estimate the control values of the variables in the general population. From the sepsis patients (groups B, C, D, and E) blood was collected on the following days:

- after admission: prior to any surgical intervention (preoperative sample)
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- post operative day 1 in ICU
- postoperative day 4 in ICU (after completion of total 9 doses).

The samples were sent to the Main Biochemistry department, F/O Life Sciences, AMU and the Dept of Pathology, J. N. Medical College, AMU, Aligarh.

For the biochemical analysis the sera were separated from blood as soon as possible by centrifugation at 2000 x g at 4°C for 10 min in Beckman J2-M1 (Beckman instruments. Inc Palo Alto, C.A. USA) refrigerated centrifuge. The serum obtained was stored in aliquots at -20°C until further analysis.

The study ended on the fourth day after collection of the third (last) sample that was one day after the administration of the ninth dose. The duration and dose of drug therapy was within the range of the previous studies (Galley et al., 1997; Molnar et al., 2003; Fraga et al., 2011). However, in our study we intentionally limited the therapy for 3 days (9 doses). We wanted the postoperative supportive therapy to be protocol based, standardised and uniform in all the patients. This was possible only if the study was conducted in the ICU. In our institution because of patient load and crisis of beds postoperative peritonitis are kept for a maximum period of 3-4 days. However, patients who needed prolonged ICU treatment were kept in the ICU though our study ended on the fourth day.

In situations when the patient expired or required ventilator support postoperatively, after collection of the first sample, the patient was excluded from the study and a fresh case was included in the same group.

All the patients in various study groups were evaluated clinically and all routine investigation was done. All patients received same standard therapy as follows:

- antibiotics as per the ICU protocol for abdominal infections.
- inj. multivitamin I ampoule IV daily
- fluids to maintain the MAP > 65 mmHg. and a CVP > 8 cm H2O
- vasopressors / inotropes as per requirement depending upon the CVP and MAP.
- blood transfusion if the Hb is < 7 gm%
- hydrocortisone @ 10 mg/kg IV bolus if not responsive to vasopressor or inotropes.
3.3. METHODS:

3.3.1 ESTIMATION METHODS OF ANTI-OXIDANT ENZYMES

- All enzymes were assayed at zero order kinetics unless otherwise specified.
- The activities of each enzyme from various comparing groups were determined simultaneously under similar conditions by using same solutions to avoid day to day experimental variations.
- One unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 μmole of product per min or hour under the specified experimental conditions.

3.3.2 MATERIALS FOR ENZYME ASSAYS:

Substrates for enzymes:
Sodium pyruvate for lactate dehydrogenase, D-glucose-6-phosphate for glucose-6-phosphate dehydrogenase, pyrogallol for superoxide dismutase, hydrogen peroxide for catalase, CDNB for glutathione-S-transferase, and oxidised glutathione for glutathione reductase were purchased from SRL (Mumbai, India). Glutathione reductase required in assay of glutathione peroxidase was purchased from Sigma Chemical Co. (St Louis, MO, USA).

Miscellaneous: All chemicals used were of the finest quality commercially available and their sources are indicated against them. Glass double distilled water was used in all experiments.

SRL, India: Acetic acid, Bovine serum albumin, Copper sulphate (CuSO₄), 5, 5’-Dithio-bis 2-nitrobenzoic acid (DTNB), Folin’s phenol reagent, Glutathione reduced (GSH), Glycine, Glycylglycine, Hydrochloric acid (HCl), Magnesium chloride (MgCl₂), Nicotinamide adenine dinucleotide phosphate, Nicotinamide adenine dinucleotide phosphate reduced, Nicotinamide adenine dinucleotide reduced, Sodium azide, Sodium carbonate (Na₂CO₃), Sodium potassium tartarate.

Sigma Chemical Co., USA: Glutathione reductase, Cysteine hydrochloride, Sodium dodecyl sulphate, Thiobarbituric acid (TBA).
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**Material and Methods**

**Oualigens, India:** Di-potassium hydrogen orthophosphate (K$_2$HPO$_4$), Di-sodium hydrogen orthophosphate (Na$_2$HPO$_4$), Ethylene diamine tetra-acetic acid (EDTA), Hydrogen peroxide, Potassium dihydrogen orthophosphate (KH$_2$PO$_4$), Sodium dihydrogen orthophosphate (NaH$_2$PO$_4$), Succinic acid, Trichloroacetic acid (TCA), Tris-(Hydroxymethyl) aminomethane (Tris-base).

### 3.3.3 LACTATE DEHYDROGENASE (L-Lactate: NAD oxidoreductase; LDH; E.C. 1.1.1.27):
It was assayed by the method of Komberg (1955). The reaction mixture, in a total volume of 3 ml, contained 150 μmoles Tris-HCl buffer pH 7.4; 10 μmoles MgCl$_2$; 5 μmoles sodium pyruvate; 0.24 μmoles NADH and 25 – 50 μL serum sample. The activity was measured as decrease in absorbance, by pyruvate dependent NADH oxidation to NAD$^+$ for 5 min at 340 nm.

### 3.3.4 Assay of enzymatic antioxidant parameters:

**SUPEROXIDE DISMUTASE (SOD, E.C. 1.15.1.1):** It was assayed by the method of Marklund and Marklund (1974). To 0.1 ml of supernatant 2.72 ml of 0.05 mM Tris-succinate buffer, pH 8.2 was added, mixed well and incubated at 25 °C for 20 min. The reaction was started by adding 0.1 ml of 8 mM pyrogallol solution. Change in absorbance per minute was immediately recorded for the initial 3 minutes at 420 nm. A reference set, containing 0.1 ml distilled water instead of serum sample, was run simultaneously.

**CATALASE (E.C. 1.11.1.6):** This enzyme was assayed by the method of Giri et al., (1996). The mixture consisted of 1.95 ml of 0.05M potassium phosphate buffer pH 7.0, 1 ml of 0.019 M H$_2$O$_2$ and 0.01 ml of serum sample in a final volume of 3 ml. The decrease in absorbance at 240 nm was immediately noted after every 30 seconds for 3 min. Enzyme activity was calculated using molar extinction coefficient of H$_2$O$_2$ (436M$^{-1}$cm$^{-1}$ at 240 nm).

**GLUTATHIONE PEROXIDASE (GPX, E.C. 1.11.1.9):** The activity of GPX was determined by the method of Flohe and Gunzler (1984). The assay mixture contained 500 μl of 0.1 M potassium phosphate-1 mM EDTA buffer (pH 7.0), 50 μl of 1 mM of sodium azide, 50 μl of serum, 100 μl glutathione reductase (0.24 U), and exactly 100 μl of 10 mM GSH. The mixture was preincubated for 10 minutes at 37°C. Then 100 μl of 1.5 mM NADPH solution was added and the hydrogen peroxide independent
consumption of NADPH was monitored for 3 minutes. The overall reaction was started by adding 100 μl of prewarmed 1.5 mM hydrogen peroxide solution and the decrease in absorbance at 340 nm was monitored for 5 minutes (ε = 6.22 x 10^3 M^-1 cm^-1).

GLUTATHIONE-S-TRANSFERASE (GST, E. C. 2. 5. 1. 18): It was assayed by the method of Habig et al., (1974), with some modification by Raisuddin et al., (1994). The reaction mixture contained 1.65 ml sodium phosphate buffer (0.1 M, pH 6.5). 0.1 ml of 1mM reduced glutathione, 0.05 ml of 1mM CDNB and 0.05 ml serum in a total volume of 2 ml. The change in absorbance was recorded at 340 nm and the enzyme activity calculated using a molar extinction coefficient of 9.6 x 10^3 M^-1 cm^-1.

GLUTATHIONE REDUCTASE (GR, E. C. 1. 8. 1. 7): The activity of GR in the serum was assayed by the method of Carlberg and Mannervik., (1985). The reaction mixture of 1 ml contained 100 μl of 10 mM GSSG, 50 μl of 2 mM NADPH, 800 μl of 0.5 mM EDTA-0.1 M sodium phosphate buffer (pH 7.6). Then 50 μl serum sample was added to give a change in absorbance of 0.05 to 0.30 per min.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (D-Glucose-6-phosphate NADP oxidoreductase; G6PDH; E.C. 1.1.1.49): This enzyme was assayed by the method of Shonk and Boxer (1964). The reaction mixture, in a total volume of 3 ml, contained Tris-HCl buffer, pH 7.4, 150 μmoles; 10 μmoles MgCl2; 5 μmoles glucose-6-phosphate; 0.24 μmoles NADP+; and 25 – 50 μL serum sample. The activity was measured by monitoring increase in absorbance, by glucose-6-phosphate dependent reduction of NADP+ to NADPH for 5 min at 340 nm (Shonk and Boxer, 1964).

3.3.5 Assay of non-enzymatic antioxidant parameters:

TOTAL -SH GROUPS: They were assayed by the method of Sedlak and Lindsay (1968). To 0.050 ml of serum sample, 2.35 ml of 0.1 M Tris-HCl (pH 8.2), 0.5 ml of 10% SDS and 0.3 ml of 0.1 M EDTA were added. The reaction was incubated in a boiling water bath for 5 min. Then, 0.1 ml DTNB (40 mg/100 ml methanol) was added. After 30 min at room temperature, the absorbance was read at 412 nm. A calibration curve with different amounts of cysteine (20-160 nmoles) was constructed by the same procedure as described above and used to calculate the total SH groups in the samples.
**LIPID PEROXIDATION:** It was determined spectrophotometrically by the method of Ohkawa et al., (1979). To 0.2 ml of serum sample 1.5 ml of 20% trichloroacetic acid, 0.2 ml of 8% SDS, 0.6 ml of distilled water and 1.5 ml of 0.8% thiobarbituric acid were added and after mixing incubated at 95°C for 20 min. After cooling to room temperature and centrifugation at 10,000 rpm for 10 min the absorbance of the supernatant was read at 532 nm against reagent blank (ε = 1.56 x 10^5 M⁻¹ cm⁻¹).

**Parameters / Investigations recorded by the Researcher**

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Material and Methods

Record sheet for the Data Analyser

Record sheet to be maintained by the data analyser

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<td>SOD (Units/mL)</td>
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<tr>
<td>GR (Units/L)</td>
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Record sheet maintained by the observer not a part of the study, preparing and administering the drugs

Name

Groups

Code no.

3.4 BLINDING THE STUDY

Blinding was assured by making appropriate dilutions such that equal volumes of study drug based on patient weight were administered. Study drug was administered over 30 min, every 8 hourly, for a total of 9 doses during a 3-day treatment period. Neither the researcher who collected the sample nor the patient was aware of the drug administered. The researcher who collected the sample gave a code number to the sample and maintained the record of the samples sent (name, date & code no.). The observer who was not the part of the study prepared and administered the drug. He also simultaneously maintained a record of the name of the patient, code no. and
the group. The sample from each patient was sent thrice, first, before operation (preoperative sample), second on day 1 of ICU and the third on day 4 of ICU stay. The samples were sent for the investigations with a separate code number for each sample so that the data analyser was blind to the name of the patient and the day of the collection. The results of all the samples were sent back by the analyser after the completion of the study.

3.5 STATISTICAL ANALYSIS

There are different statistical methods for testing a hypothesis. The appropriate method is usually governed by the design of the study, the type of data collected and the type of relationship being evaluated.

Statistical analysis was performed using Microsoft Excel and Statistical package for the social sciences (SPSS) version 17 software. The results were presented in number, percentage, mean and standard deviation as appropriate.

In chapter – I, the demographic profile of the patients were compared between the normal control and the sepsis patients applying chi square test for proportions. The demographic profile of the patients between the groups was compared applying oneway ANOVA.

In chapter – II, the variables between normal subjects (normal control) and all the sepsis patients together, on admission were compared with each other applying unpaired ‘t’ test.

In chapter – III, the analysis of relationship between two quantitative variables APACHE II score and the dependent variables (sepsis markers) was done applying the Pearson’s Correlation analysis and the correlation coefficient (r) was calculated. Subsequently linear regression analysis was applied to understand the statistical dependence of one variable on other. It showed what proportion of variance between variables was due to the dependent variable, and what proportion was due to the independent variables. The relation between the variables was illustrated graphically, or more usually using an equation $Y = a + bX$ (where a, b are constants).

In chapter – IV, the results were compared between preoperative and postoperative day 1 level of sepsis markers to evaluate the surgical effect if any. The level of postoperative day 1 was then compared with postoperative day 4 levels to evaluate the
effect of drugs (antioxidants) if any. The results were statistically analysed applying paired t-test.

Finally, the difference in the level of sepsis markers between day 4 and day 1 within each group were computed and then compared between the groups using ANOVA followed by Post Hoc Tukey test. In addition the difference in the mean values of all the sepsis markers was computed and the percentage change was calculated between day 4 and day 1 and between day 4 and normal level. This was done with an intention to identify the drug (group) that had the most significant effect on the sepsis markers.

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<tr>
<td>Less than 0.3</td>
<td>Poor</td>
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</table>

A p value of < 0.05 was taken as statistically significant. A p value of < 0.001 was considered highly statistically significant.