MATERIAL AND METHODS

Endogenous opioid system has been implicated in the pathophysiology of hemorrhagic shock and opioid antagonist naloxone has been demonstrated to improve cardiovascular variables offset by the opioid peptides in shock. A therapeutic role of opiate receptor antagonist naloxone in shock has been proposed in hemorrhagic shock and a number of subsequent studies in experimental animals, including primates have established this effect on cardiovascular function. The effect of naloxone on renal function in hemorrhagic shock is not yet investigated and hence in the present study, an attempt has been made to investigate various parameters of renal function in hemorrhaged dogs and to compare its effect with clinically used drugs like noradrenaline and dopamine.

The study was mainly divided into five parts:

1) Effect of hemorrhage on renal function, cardiovascular function and metabolic status of the animals.
2) **Effect of naloxone on various parameters measured in hemorrhagic shock.**

3) **Effect of catecholamines (noradrenaline and dopamine) and saline in hemorrhage shock.**

4) **Effect of above drugs on angiohistopathological changes in hemorrhaged dog's kidney.**

5) **Effect of drugs on survival rate.**

Renal function was assessed by using (1) inulin clearance, (2) PAH clearance, (3) urine flow and (4) excretory rate of sodium and (5) angiohistopathological studies.

Cardiovascular function was monitored by recording arterial blood pressure and heart rate.

Metabolic status of the animal was investigated by estimating (1) arterial lactic acid concentration (2) partial pressure of oxygen ($\text{PO}_2$) in the arterial blood, (3) Arterial PH (4) Arterial bicarbonate ($\text{HCO}_3^-$) concentration, (5) partial pressure of carbon dioxide ($\text{PCO}_2$) in the arterial blood, (6) serum sodium ($\text{Na}^+$) and serum potassium ($\text{K}^+$).
Survival rate was studied by observing the animals for one week after the experiment.

**Selection of animals:**

Dogs were used in the current study as there are plenty of references in the literature using dog as an animal model in experimental hemorrhagic shock. Healthy mongrel dogs of either sex, weighing 10-18 kg from the central animal house of Medical College, Rohtak, were used for the present study. These animals were kept under observation at least for one week prior to the experiment in separate cages, in the animal house having controlled room temperature. They were given food from the hospital kitchen and water ad libitum.

**Dissection of the animals:**

After twelve hours of fasting each animal was anaesthetised using nembutal (Loba Chemicals, Bombay) 30 mg/kg intravenously. The dogs were intubated with soft endotracheal tubes and allowed to breath room air spontaneously. Each animal was kept
euthermic by controlling room temperature. Bilateral groin incisions were made to expose the femoral vessels. A polyethylene cannula was inserted into the left femoral artery and connected to a polygraph for recording blood pressure. The cannula put in the left femoral vein was connected to a slow injector (INCO) for infusing inulin and para amino hippuric acid (BDH) in saline at a constant rate. The femoral artery on the right side was cannulated for collecting blood samples and to produce hemorrhage and the vein on the same side was used for administering the drugs. Both ureters were exposed by giving a flank incision, and catheterised as near the kidney as possible to reduce the dead space in the catheters, for collecting urine samples. Heart rate was monitored continuously by feeding lead II of ECG connected to the polygraph.

Procedure:

After cannulation and catheterisation, a priming dose of PAH and inulin (8 mg/kg of PAH and 50 mg/kg of inulin) dissolved in 10 ml of 0.9% saline was given. This was followed by continuous intravenous infusion of PAH and inulin in saline at
a rate of 0.5 ml per minute (0.5 ml saline containing 2 mg of PAH and 10 mg of inulin calculated on the basis of the clearance values of these substances) throughout the experiment (Smith et al, 1956). 30 minutes were allowed for the equilibration and after that collection of urine samples started for basal clearance studies. Two samples of urine of 15 minutes durations were collected in graduated tubes. Blood samples were collected in heparinized centrifuging tubes at mid point of any urine collection period.

The animals were given heparin intravenously (250 units/kg body weight) and at time \( t_{15} \), bled over a period of 15 minutes into a sterile heparinized bottle until a mean arterial pressure (MAP) of 45 mmHg was achieved (time zero \( t_0 \)). MAP was maintained at this level for 60 minutes \( (t_0 - t_{60} \) minutes) by adjusting the level of the bottle. During this period also urine and blood samples were collected for clearance studies. At \( t_{60} \) minutes the reservoir was clamped and the drug therapy started. The effects of different drugs like naloxone hydrochloride (Endo Laboratories, U.S.A.), noradrenaline (Nor-Drin-Unchem Laboratories, Bombay) dopamine-
hydrochloride, (Pharma Research and Analytical Laboratories, Madras) and sodium chloride on various parameters were studied for one hour \(t_{60}-t_{120}\) min by collecting urine samples and blood samples.

The details regarding the various groups of dogs, their number, dose and mode of administration of various drugs are given in table 3.

For the estimation of lactic acid, blood samples were collected freshly from femoral artery in perchloric acid (5 ml of 70% perchloric acid diluted with 100 ml of double distilled water) in a ratio 1:1 \(t_{15}, t_{60}\) and \(t_{120}\) minutes centrifuged and supernatent fluid was separated and estimation was done enzymatically (Varley, 1976).

For the blood gas analysis, blood samples were collected at \(t_{-15}, t_{60}\) and \(t_{120}\) minutes anaerobically from the femoral artery in air tight glass syringes and kept in ice. The estimation was done within one hour of sample collection by using radiometer.

For serum sodium and potassium estimations, blood samples were collected in small vials, sera separated and estimations were done by using flame photometer.
Table 3

<table>
<thead>
<tr>
<th>Group(n)</th>
<th>Drug</th>
<th>Dose</th>
<th>Mode of administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (15)</td>
<td>Naloxone</td>
<td>1 mg/kg</td>
<td>Intravenous-bolus</td>
</tr>
<tr>
<td>II (15)</td>
<td>Noradrenaline</td>
<td>2 µg/kg/min</td>
<td>Intravenous infusion</td>
</tr>
<tr>
<td>III (15)</td>
<td>Dopamine</td>
<td>10 µg/kg/min</td>
<td>Intravenous infusion</td>
</tr>
<tr>
<td>IV (10)</td>
<td>Sodium chloride (.9%)</td>
<td>0.5 ml/min</td>
<td>Intravenous infusion</td>
</tr>
<tr>
<td>V (10)</td>
<td>Naloxone</td>
<td>2 mg/kg</td>
<td>Intravenous bolus</td>
</tr>
</tbody>
</table>
Recording of blood pressure and heart rate:

The instrument used was Polyrite (Inco). The procedure adopted for recording the blood pressure was as follows:

It is a Statham type pressure transducer which convert mechanical energy into electrical energy. A silicon diapharm is connected to a movable inner frame which has a pair of resistances. A fixed inner frame has the other pair of resistances forming a bridge. The resistances of bridge are matched during balancing. The deflections of the diaphram due to pressure changes are converted into resistance changes of the bridge. The bridge output is of the order of microvolts, so that a high gain DC amplifier is required to follow the bridge.

The pressure transducer was balanced and calibrated manometrically every time. For calibrating the pressure transducer, the bridge was included in the circuit. Two air-tight 3-way stop cocks were placed on its outlets and the transducer dome was
filled with heparinized saline without entrapping any air bubbles. The outlets of the transducer were closed and the input selector switch of the amplifier was turned to 'bridge' and its polarity switch in 'use' position. The bridge was balanced to the atmospheric pressure first by turning the coarse adjustment and then by the fine adjustment knobs, so that the stylus was at the centre of the chart paper. One outlet of the transducer was connected to a sphygmomanometer, through a water seal bottle containing heparinized saline. The sensitivity of the amplifier was kept according to the range of blood pressure recording. The manometric pressure was increased in steps by 40 mmHg. The switch was kept on at every step to record the amount of deflection of the stylus. After reaching the expected range of blood pressure, the outlet of the transducer was closed and disconnected from the manometer and connected to the already cannulated femoral artery from which the blood pressure was recorded. The upper level was considered as systolic pressure and the lower level as diastolic pressure. Mean arterial pressure was calculated as diastolic pressure + ½ of the pulse pressure.
A time marker was put up at the bottom and the heart rate was calculated from E.C.G. recordings.

Renal Clearance Studies:

1) Determination of Inulin in Plasma and Urine
   Resorcinol method (Varley, 1976)

   In this method, the red colour which inulin gives on hydrolysis with resorcinol reagent in HCl have been used.

Reagents used:

1. Acid cadmium sulphate solution (17.84 gms of cadmium sulphate dissolved in 84.55 ml of N. sulphuric acid and made upto 500 ml.
2. Sodium hydroxide 1.1N.
3. Resorcinol 0.15% in absolute alcohol.
4. Conc. Hcl containing 7.5 mg of ferric chloride per litre.
5. Standard solution of inulin containing 2 mg/100 ml.
Procedure:

Proteins in the plasma are precipitated by adding 5 ml of distilled water, 3 ml of acid cadmium sulphate and 1 ml of 1.0N sodium hydroxide to 1 ml of plasma. 2 ml of the filtrate is pipetted out into a stoppered test tube and added 3 ml of conc. HCl containing ferric chloride and 3 ml of resorcinol reagent and placed in the water bath at 80°C for 40 minutes. At the same time a standard using 2 ml of the standard and a blank using 2 ml of distilled water were treated in the same way as the test. At the end of 40 minutes, the tubes were removed from the water bath, cooled and read in the colorimeter with a transmission at 480 millimicrons.

To find out the inulin concentration in urine, urine was diluted 100-200 times and treated in the same way as plasma. Each test was done in duplicate.

Calculations:

Since 2 ml of standard contain 0.04 mg of inulin and 2 ml of filtrate are equivalent to 0.2 ml of plasma, mg of inulin in 100 ml of plasma=
Inulin clearance was calculated by using the formula:

\[
\text{Inulin clearance} = \frac{\text{mg of inulin per 100 ml urine}}{\text{mg inulin per 100 ml plasma}} \times \frac{100}{\text{ml of urine per minute}}.
\]

II) PAH Clearance:

This method depends on diazotising the P-amino group of PAH with HNO\(_2\), destruction of excess HNO\(_2\) with sulphamate and coupling with N-(1-napthy)ethylene diamine.

Reagents:

1. Acid cadmium sulphate (as for inulin)
2. Sodium hydroxide 1.1N
3. Hydrochloric acid 1.2N
4. Sodium nitrate 0.1% solution prepared freshly every few days by dissolving 100 mg/100 ml.
5. N-(1-naphthyl)ethylene diamine dihydrochloride 0.1% solution kept in dark.

6. Ammonium sulphamate 0.5% solution prepared freshly every fortnight.

7. Standard solution of PAH containing 0.2 mg/100 ml.

**Procedure:**

To 1 ml of plasma or 1 ml of urine (diluted 200 times) added 10 ml of distilled water, 3 ml of acid cadmium sulphate and 1 ml of 1.1N sodium hydroxide, to precipitate protein. Centrifuged and to 10 ml of the filtrate added 2 ml of 1.2N HCl and 1 ml of 0.1% sodium nitrite. After mixing and waiting for 5 minutes added 1 ml of ammonium sulphamate. After 3 minutes 1 ml of N-(1-naphthyl) ethylene diamine dihydrochloride was added and mixed waited for 10 minutes and read in the calorimeter by using a transmission at 520 millimicrons. As standard 4 ml of weak standard solution plus 6 ml of distilled water and for blank 10 ml of distilled water were used and treated in the same way as that of the test. Every test was conducted in duplicate.

**Calculations:**

Since the standard contains 0.008 mg of
PAH, and 10 ml of filtrate is equal to 10/15 ml of plasma.

\[
\text{mg of PAH per 100 ml of plasma} = \frac{\text{Reading of the test}}{\text{Reading of the standard}} \times 0.008 \times \frac{15}{10} \times 100
\]

\[
= \frac{\text{Reading of the test}}{\text{Reading of standard}} \times 1.2
\]

PAH clearance was calculated in the same way as that of inulin clearance.

**Lactate estimation:**

**Enzymatic method** (Varley, 1976):

Pyruvic acid is completely converted into lactic acid by NADH₂ in the presence of lactate dehydrogenase. The reverse reaction to the left, the conversion of lactate to pyruvate can be brought if an alkaline solution of excess NAD is used and the pyruvic acid could be removed as it is formed by hydrazine. The NADH⁺ H⁺ formed can be read at 340 millimicrons and a quantitative measure of lactic acid could be made.
Reagents used:

1. Glycine buffer 0.5M, PH 9.0, solution contains 0.4M hydrazine, prepared a solution containing 3.753g glycine and 2.925g sodium chloride in 100 ml. 89 ml of this added to 11 ml of 0.5N sodium hydroxide. Dissolved 1.28 gm of hydrazine per 100 ml of this. Checked the PH and adjusted with NaOH.

2. NAD (Nicotinamide adenine dinucleotide) 0.027H: 20 mg/ml.

3. LDH (Lactate dehydrogeanse) containing 2 mg enzyme protein per ml.

4. Per chloric acid-5 ml of the 70% acid was diluted with twice distilled water to 100 ml.

5. Lactic acid standard 20 mg/100 ml.

Technique:

1 ml of freshly taken arterial blood was mixed with 1 ml of the perchloric acid and centrifuged. 0.1 ml of the supernatent fluid was pipetted out into a test tube containing 2 ml of the glycine buffer and 0.03 ml of LDH. Finally 0.2 ml of NAD was added and mixed well by shaking and kept in a water bath at 25°C for one hour. Then read against a reagent
blank at 340 millimicrons.

**Calculations:**

Since a change of 0.100 extinction corresponds to 0.0161 μmol per ml of NADH⁺ and hence to 0.0161 μmol per ml lactic acid, a volume of 2.33 thus corresponds to 3.375 μg/m of lactic acid.

\[
\text{mg of lactate per 100 ml of blood} = \frac{\text{Change in extinction} \times 3.376}{0.1} \\
\times 2 \times \frac{1}{0.1} \times \frac{100}{1000} \\
= \text{Change in extinction} \times 67.5
\]

**Determination of sodium and potassium in plasma and in urine:**

**By using flame photometer:**

**Principle:**

The sample solution is introduced in the form of a fine spray into a non luminous gas flame. The various ions present produce their characteristic emission spectra. By the use of proper colour filters or other means, the emitted light characteristic of
the ions, being determined is isolated, allowed to excite photoelectric cell. The response of the photoelectric cell is measured on a suitable meter. Under controlled conditions, the meter reading is a measure of the concentration of ion being determined. A calibration curve is established by analysing a series of standard solutions of the ions being determined. From the meter reading of the sample being analysed, the concentration is established by reference to the calibration curve.

**Standards for sodium:**

Standard solutions covering the range of 100 to 150 milliequivalents of sodium per litre at a dilution of 1:250 were made for calibration purposes. A stock standard was prepared containing 100 milliequivalents of sodium per litre by dissolving 5.88 g of NaCl in water and diluting to one litre. Working standards were made from this stock standard by measuring out 4.0, 5.0 and 6.0 ml aliquots in to one litre volumetric flasks and diluting to the mark with water. These standards represent 100, 125, and 150 milliequivalents of sodium per litre at a 1:250 dilution.
Standards were always run both before and after analysing a series of unknown solutions to be sure that the calibration was constant.

Procedure for Sodium:

1 ml of plasma diluted to 250 ml in distilled water was used for sodium analysis. In order to get the sodium content of the sample the reading obtained was referred to the calibration curve previously obtained.

For Potassium: Since the potassium content of plasma is much lower than the sodium content, the sample was diluted 1:100. Standard solutions covering a range from 3 to 6 milliequivalents of potassium per litre, at a dilution of 1:100 were made for calibration purposes. A stock standard solution was made by dissolving 0.746 gm of KCl in water and diluting to 1 litre. Working standards equivalent to 3.0, 4.0, 5.0 and 6.0 milliequivalents of potassium per litre at a dilution of 1:100 are prepared by diluting respectively 3.0, 4.0, 5.0 and 6.0 ml of stock solution to 1 litre with water and a calibration curve was made.
Blood Gas Analysis:

Heparinised arterial blood samples were collected under anaerobic condition in glass syringes and was securely capped and transported in ice to the acid base laboratory for immediate analysis.

The acid-base analysis was done on a completely automated acid base analyser (Radiometer, ABL3) which measures PH, PCO₂ and PO₂ using specific electrodes and automatically computes (HCO₃⁻) from the Henderson Hasselbach equation (HCO₃⁻) = 0.03 x PCO₂ x Antilog (PH-6.1).

Following are the principles of electrodes used for measuring various parameters.

PH measurements:

PH is measured by the glass electrode which consists of a PH sensitive thin glass membrane that separates the solution of unknown PH from a buffer solution of known PH.

A potential difference which develops across the membrane according to the H⁺ activity on the respective sides, is measured by a potentiometer which is connected to the sample by means of a calomel
electrode and salt bridge (20%) KCl solution). The electrode is calibrated first with two buffer solutions of different PH prior to measurement.

PCO₂ measurement: PCO₂ electrode is essentially a PH electrode which is immersed in a bicarbonate solution whose PH depends on the PCO₂ of this solution. The bicarbonate solution is separated from unknown sample by a polypropylene membrane which permits diffusion of neutral gases like CO₂ but is impermeable to H⁺ or other ions. Therefore the PH of the bicarbonate solution which is measured is a function of PCO₂ of the unknown sample. The electrode is calibrated with two gases of different PCO₂ prior to the measurement.

PO₂ measurement: At specific voltage (polarizing voltage) only specific molecules get reduced. The PO₂ electrode consists of a slender platinium cathode which is sealed in glass with only its tip exposed. The cathode is immersed in phosphate buffer with KCl which is also in contact with the Ag/AgCl₂ anode. A potential difference of 650 mv is applied to the electrodes and current (in pico amperes) flows through the solution which depends on O₂ present in the solution. The solution is separated from the test
sample by a polypropylene membrane which is permeable to $O_2$ gas. The current so produced is measured by an amperometer and is a function of $PO_2$ of the test sample. The electrode is calibrated with solution of known $PO_2$ prior to measurement.

**Angiohistopathology:**

In dogs used for angiohistopathological studies of the kidney, after one hour of treatment with various drugs at $t_{120}$ minutes, India ink was infused through the renal artery till it started coming out through the renal vein. Immediately the renal vessels were ligated, the kidneys were removed before sacrificing the animals, with over dosage of nembutal. Since the saline treated animals died before $t_{120}$ minutes, the kidneys were taken in this group at $t_{90}$ minutes.

For gross section, each kidney was cut longitudinally and was fixed in 10% formaline solution. Blocks were made from the cortical region of the kidney and sections were cut and stained with hemotoxylin and eosin after routine processing. Kidneys of three normal dogs without hemorrhage were also perfused with India ink and processed in the same way for control
Survival study of the animals:

For survival studies, the experiment was conducted under aseptic conditions and ureters were not exposed and clearance study was not made. Five animals randomly selected from each group were reinfused with the shed blood at $t_{120}$ minutes over a period of 30 minutes. Cannulae were removed, wound closed and the animals were sent to their cages to note the survival rate. Survival rate was noted for seven days.

Statistical Analysis:

The various data obtained were statistically analysed using students paired 't' test and analysis of variance test.

For each animal various estimations and measurements were made before hemorrhage ($t_{-15}$) during hemorrhagic shock ($t_0-t_{60}$) and during drug treatment ($t_{60}-t_{120}$). The values were expressed as mean±SE in each case.
A comparison of various parameters were made between values of $t_{-15}$ and $t_{60}$, as well as $t_{60}$ and $t_{120}$ to find out the statistical significance by using paired 't' test.

**Analysis of variance test (ANOVA Test):**

This was used to find out whether there was any significant difference between groups in any of the parameters measured during the base line period of observation and during the period of hypotension.