Immobilization-induced increases of systolic blood pressure and dysregulation of electrolyte balance in ethanol-treated rats

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Abstract: Clinical and experimental studies revealed that alcohol drinking and life event stresses are predisposing factors to hypertension. Intra and extra cellular levels of electrolytes may play important role in the pathogenesis and treatment of hypertension. Dietary intake of sodium, potassium, calcium and magnesium is suggested to have a role in the regulation of blood pressure. The present study was designed to monitor the effects of acute exposure to 2h immobilization stress and ethanol administration at a dose of 2.5g/kg body weight (i.p.) and combined effect of acute administration of ethanol and immobilization stress on systolic blood pressure (SBP), intraerythrocyte, serum and tissue electrolytes in rats. Results showed that acute exposure to 2h immobilization increased SBP, intraerythrocyte sodium and decreased intraerythrocyte potassium in water as well as in ethanol injected rats. The concentration of Na^+ and Ca^{2+} increased while that of K^+ and Mg^{2+} decreased in the heart and kidney tissue. Ethanol administration also increased Na^+ and Ca^{2+}levelsand decreased K^+ and Mg^{2+} levels in the heart and kidney tissue. Restraint stress decreased serum levels of Na^+, K^+, Ca^{2+}, P, and Cl^- and increased serum Mg^{2+}, glucose and haematocrit. Ethanol administration also decreased serum levels of Na^+, K^+, Ca^{2+}, P, and Cl^- and increased serum Mg^{2+}, glucose and haematocrit. The effects of ethanol and stress on the changes of blood and tissues electrolytes were additive and may be involved in the greater occurrence of hypertension in alcoholics. Our results suggested an important role of intra and extra cellular electrolytes in both stress and ethanol-induced hypertension. The findings may help to develop strategies for the treatment of hypertension in alcoholics.

Keywords: Ethanol, stress, RBC sodium, rbc potassium, electrolytes, SBP, ATPase, glucose.

INTRODUCTION

Worldwide, ethanol is a compound of enduring research interest because its consumption involves both social as well as medical implications significantly. Chronic alcohol consumption is widely reported to be associated with an increased prevalence of hypertension, stroke, cardiomyopathy and arrhythmias (Klatsky, 2010; Kodavali *et al.*, 2006). A large number of epidemiological studies have suggested a positive association between high ethanol consumption and blood pressure (Miller *et al.*, 2005). It is well known that both acute and chronic alcohol drinking is associated with a number of abnormalities in electrolyte balance, among which magnesium deficiency is especially common and significant (Romani, 2008; Hermans *et al.*, 1996). The role of intracellular Na^+ content in the development of hypertension is well documented (Orlov *et al.*, 2001). Studies on the effects of ethanol on serum electrolytes are not very consistent. Ethanol administration has been shown to induces hypocalcaemia and hypophosphatemia in dogs, rats and human subjects (Hermans *et al.*, 1996, Peng *et al.*, 1972; Elisaf *et al.*, 1995; Territo and Tanaka., 1974). Although moderate doses of alcohol did not decrease serum Ca^{2+} concentrations (Ljunghall *et al.*, 1985).

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Studies on the effects of various types of stress on electrolyte levels in humans and animals also had shown that exposure to stress produces alteration in electrolytes levels. Others have reported that elevation of arterial blood pressure could result from audiogenic stress (AS) (Altura *et al.*, 1992). Alterations in Mg^{2+} metabolism may also be involved in noise stress-induced hearing loss (Scheibe *et al.*, 2000). Two hours of noise stress produced a mean reduction in erythrocyte Mg^{2+} and a simultaneous increase serum Mg^{2+} in guinea pigs. Chronic noise stress increased in serum Mg^{2+} and decreased it in erythrocyte and myocardia (Joachims *et al.*, 1983; Ising *et al.*, 1986). Exposure to noise stress also decreases erythrocyte Mg^{2+}. Life events stresses increase blood pressure in human (Markovitz *et al.*, 2004). Parallel studies on animals show that exposure to an uncontrollable stressor such as 2h restraint stress increases systolic blood pressure (SBP) in rats (Mahboob *et al.*, 1996). In view of greater prevalence of hypertension and stroke in alcoholics than in normal population (Zeppa *et al.*, 1988). The present study investigates effects of 2h restraint on serum, erythrocytes and tissue electrolytes balance in rats injected with ethanol.

MATERIALS AND METHODS

Animals

Twenty-four locally bred male Albino - Wistar rats (BW: 200-220g) purchased from The Aga Khan University,

Pakistan, were housed individually under a 12-h Light/Dark cycle (lights on at 06:00) with access to cubes of standard rodent diet and tap water for at least 1 week before experimentation. All experiments were performed according to International European Ethical Standard and a protocol was approved by the local Animal Care Committee.

Experimental protocol

Twenty-four animals were randomly divided into two equal experimental groups. Each group was assigned as water - treated and ethanol treated rats. Ethanol was injected intraperitoneal (i.p.) at a dose of 2.5g/kg body weight in ethanol-treated rats whereas water- treated groups received equal volume of deionized water through the same route. Both water- and ethanol- treated groups were further divided into water and ethanol - treated unrestrained rats and water or ethanol - treated restrained rats. A group of water injected animals and another group of ethanol- injected animals were immobilized for 2hr commencing between 9: 00 to 11:00h. The animals of unrestrained groups were left to their home cages during this period in a separate room. All the treatments were carried out in a balanced design to avoid order effects.

Restraining procedure

The animals were immobilized on wire grids of 10×9 inches fitted with a Perspex plate of 9×6.5 inches. Restraining procedure was the same as describe earlier (Haleem *et al*., 1988) for 2h (between 9:00 to 11:00). Immobilization was produced by pressing the fore legs of the rats through the gaps in the metal girds and taping them together with zinc Oxide plaster tape. Hind limbs were also taped and the head of animal rested on the Perspex plate.

Blood pressure measurements

Rats were placed in plastic restraining tube for approximately 10 minutes before the onset of BP determinations. Systolic blood pressures of both restrained and unrestrained groups were monitored in terms of mmHg by Rat Tail Cuff Blood Pressure Systems (IITC Life Science Inc.). In restrained animals blood pressure was monitored immediately before termination of stress. The mean of three-artifact free determination (not differ by 10%) served as an index of SBP.

Animals were sacrificed immediately after the termination of restraint period to collect blood samples. Serum was separated and analyzed for serum electrolytes.

Analytical methods

Serum Na^+, K^+, and Ca^{2+} were estimated by Corning- 410 flame photometer. Concentration of Mg^{2+} was estimated by method described by Hallry & Skypeck (1964). For this purpose a protein free filtrate was prepared by mixing TCA (5%, w/v) and centrifuged for 10 minutes. Supernatant was mixed with titan yellow (0.05%) and of NaOH (4N). Blank was prepared by taking deionized water and treated similarly as test and standards. After through mixing, all the tubes were allowed to stand at room temperature for 15 minutes and emission intensity was measured against blank at 540nm in Shimadzu spectrophotometer UV-120-01. P was determined by Goldenberg method (1966) and serum Cl^- by chemistry analyzer titration method of Schales (Schales & Schales, 1941). Glucose was determined by "GOD- PAP" method (Barham and Trinder, 1972) and haematocrit was determined by centrifuging blood in a Hawksley microhematocrit centrifuge.

Erythrocytes preparation

Heparnized blood was centrifuged, plasma was separated, buffy coat was aspirated and discarded. Red cells were washed three times at room temperature by suspension in the $MgCl_2$ solution (112mmol/L) and centrifugation. Washed red cells were then used for the estimation of intraerythrocyte Na^+ and K^+ by the method of Fortes Meyer & Starkey (Meyer & Starkey, 1977).

Tissue preparation

Frozen tissues (heart & kidney) were digested in 20 ml deionzed water followed by 10ml of concentrated nitric acid and percholoric acid (equal volume) for three hours at room temperature and then at 70^{o}C for another three hours. The samples were initially heated very gently. After foaming subsided the temperature was increased to produce steady state boiling. The excess acids were boiled off to near (not complete) dryness. The digest then cooled to room temperature and analyzed for Na^+, K^+ and Ca^{2+} content by flame photometer and Mg^{2+} by the method of Hallry and Sky Peck (Hallry and Skypeck, 1964).

STATISTICAL ANALYSIS

Results were presented as means ± SD. Significant differences between control and test values were evaluated by 2-way ANOVA. Post-hoc comparisons were done by Newman-Keuls statistics. P-values<0.05 were considered significant

RESULTS

Fig. 1 (A, B, C) show effects of single 2h restraint on SBP, intraerythrocyte Na^+ and intraerythrocyte K^+ concentrations in water- and ethanol- injected rats. Two-way ANOVA showed significant effect of ethanol on SBP ($F=23.14$, df 1, 20, $p<0.01$), intraerythrocyte Na^+ ($F=9.46$, df 1, 20, $p<0.01$) and K^+ ($F=11.44$, df 1, 20, $p<0.01$). Effects of single 2h restraint on SBP ($F=31.14$, df 1, 20, $p<0.01$), Na^+ ($F=18.69$, df 1, 20, $p<0.01$) and K^+ ($F=18.70$, df 1, 20, $p<0.01$) concentration in red cell were significant. Interaction between ethanol and stress was not significant for SBP, intraerythrocyte Na^+ and K^+.

Post- hoc comparisons by Newman-Keuls test showed that single 2h restraint significantly increased ($p<0.01$) SBP in water- and ethanol- injected rats. Single restraint significantly ($p<0.01$) increased intraerythrocyte Na^+ and decreased K^+ in water- and ethanol-injected rats. Intraerythrocyte levels of Na^+ increased and K^+ decreased in ethanol- injected unrestrained than water- treated unrestrained rats. Levels of Na^+ were significantly greater and K^+ were significantly smaller in ethanol- injected restrained than water- injected restrained rats.

Fig. 1 (D, E, F) show effects of single 2h restraint on serum concentrations of Na^+, K^+ and chloride in water - and ethanol-injected rats. Two-way ANOVA revealed significant effect of ethanol on serum Na^+ ($F=19.05$, df 1, 20, $p<0.01$), K^+ ($F=13.86$, df 1, 20, $p<0.01$) and chloride ($F=85.52$, df 1, 20, $p<0.01$). Effects of single restraint stress on serum Na^+ ($F=26.87$, df 1, 20, $p<0.01$), K^+ ($F= 20.92$, df 1, 20, $p<0.01$) and chloride ($F=149.50$, df 1, 20, $p<0.01$) were also significant. Interaction between two factors was not significant for Na^+, K^+ and chloride.

Post-hoc comparison by Newman-Keuls test showed that serum concentration of Na^+, K^+ and chloride were significantly ($p<0.05$) decreased in water- and ethanol-injected restrained compare to unrestrained rats. Serum levels of Na^+, K^+ ($p<0.05$) and chloride ($p<0.01$) were smaller in ethanol- injected unrestrained than water-injected unrestrained rats. Levels of Na^+ and chloride ($p<0.01$) and K^+ ($p<0.05$) were significantly lower in ethanol-injected restrained than water-treated rats.

Fig. 2 (A, B, C) show effects of single restraint 2h on serum concentrations of Ca^{2+}, Mg^{2+} and phosphorus in water-and ethanol- injected rats. Data analyzed by Two-way ANOVA revealed significant effect of ethanol on serum Ca^{2+} ($F=49.23$, df 1, 20, $p<0.01$), Mg^{2+} ($F=17.80$, df 1, 20, $p<0.01$) and phosphorus ($F=13.79$, df 1, 20, $p< 0.01$) concentrations. Effect of stress on serum Ca^{2+} ($F= 93.07$, df 1, 20, $p<0.01$), Mg^{2+} ($F=14.84$, df 1, 20, $p<0.01$) and phosphorus ($F=30.07$, df 1, 20, $p<0.01$) were also significant. Interaction between ethanol and stress were not significant for Ca^{2+}, Mg^{2+} and phosphorus.

Follow-up comparison by Newman-Keuls test indicated that single restraint stress significantly ($p<0.01$) decreased serum concentration of Ca^{2+} and phosphorus and increased Mg^{2+} ($p<0.05$) in water- and ethanol-injected rats. Serum levels of Ca^{2+} ($p<0.01$) and phosphorus ($p<0.05$) were lower and Mg^{2+} higher in ethanol-injected unrestrained than water- injected unrestrained rats. Similar decreases in Ca^{2+} and phosphorus ($p<0.05$) and increases in Mg^{2+} ($p<0.01$) concentration were observed in ethanol- treated restrained than water-injected restrained rats.

Fig. 2 (D, E, F) show effects of single restraint stress 2h on serum concentrations of glucose, haematocrit and effective plasma osmolality in water- and ethanol-injected rats. Data analyzed by Two-way ANOVA showed significant effect of ethanol on glucose ($F= 29.80$, df 1, 20, $p<0.01$) and effective plasma osmolality ($F= 21.33$, df 1, 20, $p<0.01$). Effects of ethanol for haematocrit were not significant. Stress effects were significant for glucose ($F=24.42$, df 1, 20, $p<0.01$), and effective plasma osmolality ($F=29.17$, df 1, 20, $p<0.01$) and not significant for haematocrit. Interaction between ethanol and stress was significant for haematocrit ($F=19.44$, df 1, 20, $p<0.01$) and not significant for glucose and effective plasma osmolality.

Post-hoc comparison by Newman-Keuls test showed that 2h restraint stress significantly decreased levels of glucose and effective plasma osmolality and increased haematocrit in water- and ethanol- injected rats. Levels of glucose and effective plasma osmolality were lower and haematocrit were higher in ethanol- injected unrestrained than water- injected unrestrained rats. Similar decreases ($p<0.01$) in glucose and effective plasma osmolality and increases ($p<0.05$) in haematocrit were observed in ethanol- injected restrained than water- injected restrained rats.

Fig. 3 (A, B, C, D) show effects of 2h restraint stress on concentration of Na^+, K^+, Ca^{2+} and Mg^{2+} in heart tissue in water- and ethanol-treated rats. Data analyzed by Two-way ANOVA revealed significant effect of ethanol on Na^+ ($F=160.15$, df 1, 20, $p<0.01$), K^+ ($F=18.63$, df 1, 20, $p<0.01$), Ca^{2+} ($F=19.94$, df 1, 20, $p<0.01$), and Mg^{2+} ($F=36.21$, df 1, 20, $p<0.01$). Effect of single restraint on Na^+ ($F=17.84$, df 1, 20, $p<0.01$), K^+ ($F=34.42$, df 1, 20, $p <0.01$), Ca^{2+} ($F=28.21$, df 1, 20, $p<0.01$) and Mg^{2+} ($F=69.71$, df 1, 20, $p<0.01$) were also significant. Interaction between ethanol and stress was not significant for Na^+, K^+, Ca^{2+}, and Mg^{2+}.

Post- hoc comparison by Newman-Keuls test showed that 2h restraint stress significantly increased Na^+, Ca^{2+} and decreased K^+ and Mg^{2+} content in water- and ethanol-injected rats. Ethanol- injected unrestrained rats exhibited significantly higher Na^+, Ca^{2+} and lower levels of K^+ and Mg^{2+} concentration than water- injected unrestrained rats.

Fig. 4 (A, B, C, D) show effects of single 2h restraint stress on concentration of Na^+, K^+, Ca^{2+} and Mg^{2+} in kidney tissue in water- and ethanol-injected rats. Data analyzed by Two-way ANOVA revealed significant effect of ethanol on Na^+ ($F=13.17$, df 1, 20, $p<0.01$), K^+ ($F=71.11$, df 1, 20, $p<0.01$), Ca^{2+}($F=16.05$, df 1, 20, $p<0.01$), and Mg^{2+} ($F=35.43$, df 1, 20, $p<0.01$). Effect of single 2h restraint on Na^+ ($F=21.04$, df 1, 20, $p<0.01$), K^+ ($F=49.55$, df 1, 20, $p<0.01$), Ca^{2+} ($F=13.10$, df 1, 20, $p<0.01$) and Mg^{2+} ($F=83.15$, df 1, 20, $p<0.01$) were also significant. Interaction between ethanol and stress was significant for Mg^{2+} ($F=8.01$, df 1, 20, $p<0.01$) and not significant for Na^+, K^+ and Ca^{2+}.

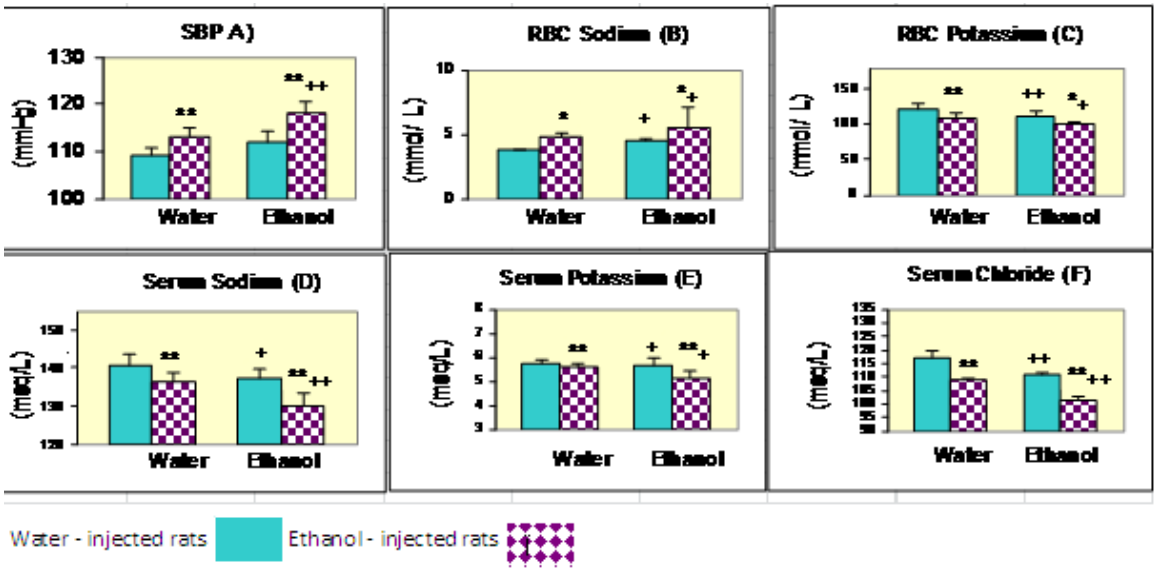

Fig. 1: Effects of single restraint (2h) on SBP (A). RBC Na^+ (B) and RBC K^+ (C), Serum Na^+(A), K^+ (B) and Chloride (C) levels in water- and ethanol- injected rats. Values are + SD (n=6). Significant differences by Newman- Kuels Test; *P<0.05, **P<0.01 from respective unrestrained rats;$^+$P<0.05, $^{++}$P<0.01 from respective water treated rats; Following Two- way ANOVA.

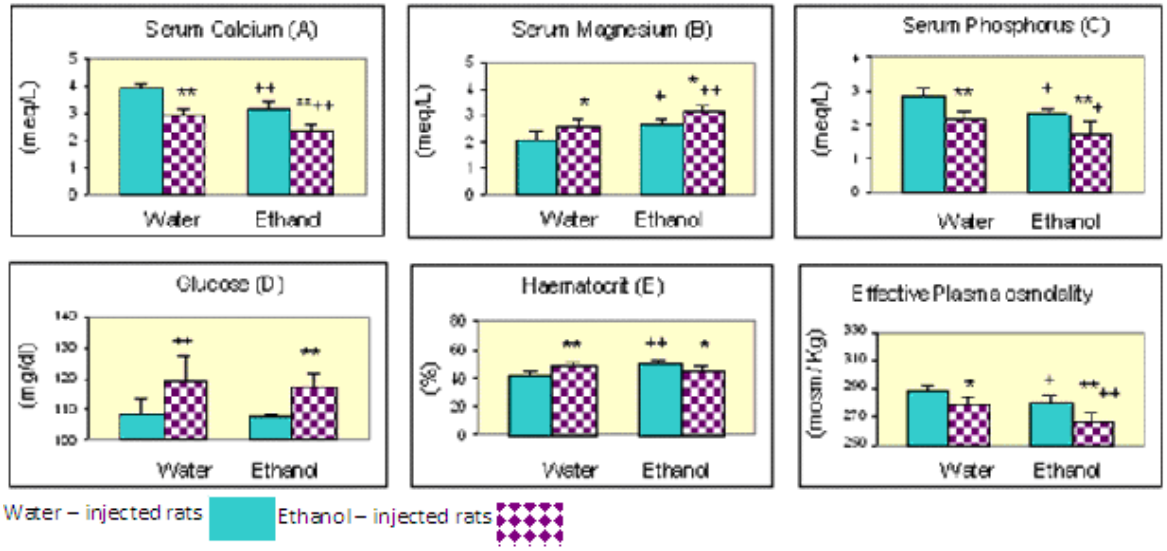

Fig. 2: Effects of single restraint (2h) on Serum Ca^{2+} (A), Mg^{2+} (B) and Phosphorus (C), Glucose (D), Haematocrit (E) and Effective plasma osmolality (F) levels in water- and ethanol- injected rats.
Values are + SD (n = 6). Significant differences by Newman-Kuels Test; *P<0.05, **P<0.01 from respective unrestrained rats; $^+$P <0.05, $^{++}$P<0.01; from respective water treated rats; Following Two-Way ANOVA.

Post- hoc comparison by Newman-Keuls test showed that 2h restraint stress significantly increased Na^+ and Ca^{2+} and decreased K^+ and Mg^{2+} content in water- and ethanol-injected rats. Ethanol-injected unrestrained rats exhibited significantly higher levels of Na^+, Ca^{2+} and lower levels of K^+ and Mg^{2+} than water- injected unrestrained rats. Ethanol- injected unrestrained rats exhibited significantly higher levels of Na^+ and Ca^{2+} and lower levels of K^+ and Mg^{2+} than water- injected restrained rats.

DISCUSSION

The present study revealed that exposure to forced immobilization stress elevates SBP in water as well as ethanol (2.5g/kg; i.p) injected rats (fig. 1A). Previous studies have also shown an increase in systolic blood pressure following 1h immobilization (Mahboob *et al.*, 1996) and acute administration of absolute ethanol administration at a dose of 0.4ml (Fink *et al.*, 1985).

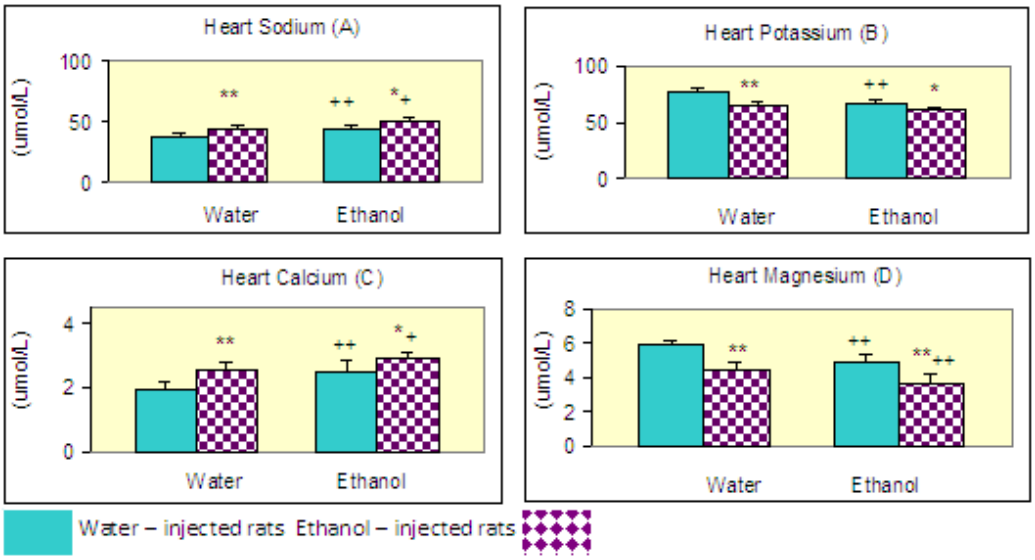

Fig. 3: Effects of single restraint (2h) heart Na^+ (A), K^+ (B) Ca^{2+} (C) and Mg^{2+} (D) levels in water- and ethanol-injected rats. Values are + SD (n=6). Significant differences by Newman-Kuels Test; *P<0.05, **P<0.01 from respective unrestrained rats; $^+$P< 0.05, $^{++}$P<0.01 from respective water- treated rats; Following Two- way ANOVA

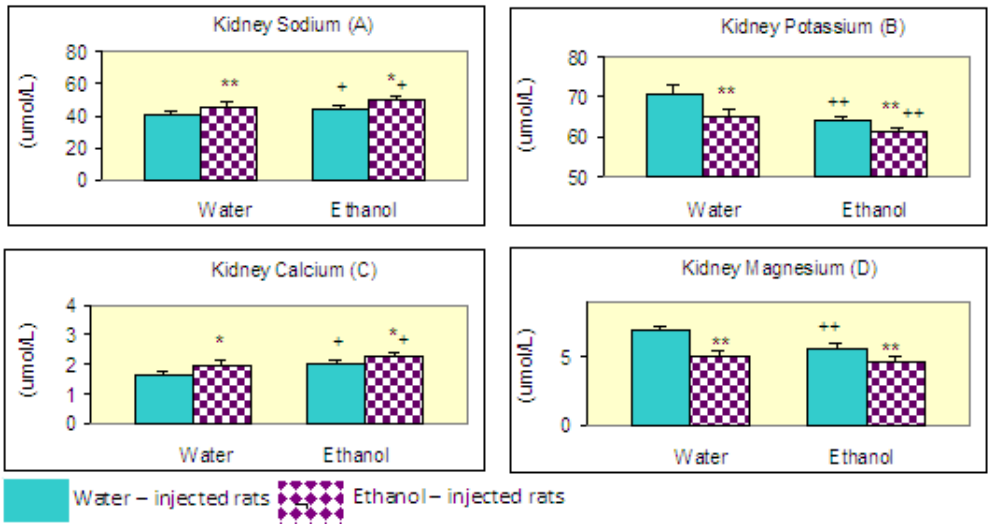

Fig. 4: Effects of single restraint (2h) kidney Na^+ (A), K^+ (B) Ca^{2+} (C) andMg^{2+} (D) levels in water- and ethanol-injected rats.

Values are + SD (n=6). Significant differences by Newman-Kuels Test; *P<0.05, **P<0.01 from respective unrestrained rats; $^+$P< 0.05, $^{++}$P<0.01 from respective water- treated rats; Following Two-way ANOVA

Literature survey revealed that acute administration of ethanol leads towards different effects on blood pressure (Varga and Kunos. 1997; Malinowska *et al*., 1989; McDougle *et al*., 1995; Rahman *et al*., 1987). These differences may be attributed to the variation in the route and dose of the day. Differences in the length of time after which blood pressure was monitored could also be responsible for generating these differences in results.

Restraint- induced increases of systolic blood pressure as observed in the present study may be due to the stress-induced release of catecholamine (Pshennikova *et al*., 1990; Carlsson and Carlsson, 1989). Stress stimulates sympathetic nervous system to increase the synthesis and release of catecholamines (Pshennikova *et al*., 1990) that increases cardiac output and cause vasoconstriction. Both increases may lead to rise in blood pressure. Ethanol administration at a dose of (3.2g/kg (i.g.) is reported to increase circulating catecholamines in normotensive rats (Netter, 1983). Acute alcohol administration of (2.0g/kg; i.g) has been also shown to increase plasma corticosterone (Thiagarajan *et al*., 1989).

Possible explanation for the observed decrease in Na^+ concentration following ethanol administration could be an inhibition of antidiuretic hormone release resulting in a

decrease of the osmo- Na^+ receptors and enhanced urine formation (Eisenhoffer and Johnson, 1983). It is often suggested that atrial natriuretic peptide (ANP) could also play an important role in alcohol- induced diuresis (Colantonio *et al*., 1991).Acute ethanol intakes cause an increase in plasma ANP (Guillaume *et al*., 1994). Stress has been also reported to increase the release of ANP (Horky *et al*., 1985). Higher levels of ANP suppress arginine vasopressin (AVP) release from the supraoptic nucleus (Clark *et al*., 1991). In fact, at physiological concentration, ANP causes a large inhibition of vasopressin- stimulated osmotic water permeability in the rat terminal inner medullary-collecting duct (Nonoguchi *et al*., 1988) causing diuresis and natriuresis.

Mahboob *et al* reported an increase in the intracellular levels of Ca^{2+} in most tissues (1996). A decrease of serum Ca^{2+} levels as observed in the present study (Figure 2C) suggests an increase in the intracellular concentration of Na^+ (fig. 1B, fig. 3A, and fig. 3B). Therefore, Ca^{2+} was inversely related to Na^+-K^+ ATPase activity. A decrease of the latter might increase through increased intracellular Na^+ (Mahboob *et al*., l996). A decrease in Na^+-K^+ ATPase activity seems to be a common feature in vascular smooth muscle cells with accomplished arise in intracellular Ca^{2+}, it could be an important pathogenic factor in the development of hypertension.

Hypokalemic effects of immobilization stress and also of ethanol are explainable in terms of an increase in plasma concentration of catecholamines (Zgombick *et al*., 1986) as epinephrine's action via β- 2 receptor mechanisms could promote the movement of K^+ from extra cellular fluid to intracellular compartment to decrease serum levels of K^+ (Clausen and Flatman, 1980).

Moreover, increases of intracellular Na^+ and decreases of K^+ levels in RBCs, heart and kidney tissue following exposure to immobilization stress as observed in present study (fig. 1B, 1C, 3A, 3B, 4A, 4B) may be a consequence of inhibition of the activity of the Na^+ –K^+ ATPase (Eaton *et al*., 1984).

A decrease in serum level of Ca^{2+} and corresponding increase of Mg^{2+} following 2h acute immobilization stress observed in the present study (fig. 2A and 2B) suggests an association between these two electrolytes in serum that is the decrease of Ca^{2+} in serum may be a consequence of increased intracellular Ca^{2+} concentration required for excessive firing during stress (Bickler and Hansen, 1998; Meerson, 1983).In addition, stress increases levels of Mg^{2+} in serum. The increases of Mg^{2+} might act as physiological damping of stress that includes vasoconstriction and norepinephrine secretion (Ising *et al*., 1986). The present data also showed a decrease of serum phosphorus level following repeated 2h immobilization stress in both water and ethanol treated rats (fig. 2C). The mechanism underlying the hypophosphatemia has not been extensively studied. The observed decreases may be due to any of these three reasons. 1) The intracellular shifting of phosphate due to the presence of excess catecholamines, glucagon and androgens (Body *et al*, 1983; Guthrie *et al*., 1978). 2) The inhibition of Na^+-K^+ ATPase activity may decrease ATP hydrolysis to enhance inorganic phosphate. This observation has also been proved by the result shown in (fig. 2C). 3) - The possible cause of hypophosphatemia is loss of phosphorus from cells and excretion in urine (Knochel, 1982).

Stress-induced increases of glucocorticoids facilitate glycogenolytic actions of glycogen and epinephrine. So that cortisol's prime role is to stimulate gluconeogenesis (Rudolph *et al*., 1989). It induces the enzyme phosphoenolpyruvic carboxykinase and fructose-1, 6-diphosphatase, which is the key in the hepatic process of gluconeogenesis (Baxter and Forsham, 1972).

In conclusion the present study showed that the increases in the intracellular Na^+ and Ca^{2+} and associated decreases of Mg^{2+} may be involved in stress and alcohol induced hypertension and also in the prevalence of hypertension in alcoholics. Because effects of stress on both systolic blood pressure and electrolyte changes were greater in ethanol treated than water treated rats. The study tends to provide a reason for the greater occurrence of hypertension in alcoholics. The finding may help to develop strategies for the treatment of hypertension in alcoholics.

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