Possible Mechanisms of Cardiac Contractile Dysfunction and Electrical Changes in Ammonium Chloride Induced Chronic Metabolic Acidosis in Wistar Rats

Noha N. Lasheen[1] & Ghada F. Mohamed[2]

[1] Lecturer of Physiology, Physiology department, Faculty of Medicine, Ain Shams University

[2] Professor of Histology, Histology department, Faculty of Medicine, Ain Shams University

Beside El-Nour Mosque, Abbasia, Cairo, Egypt. (Egypt)

A short title: **Cardiac Contractile Dysfunction in Chronic Metabolic Acidosis in Wistar Rats**

Corresponding Author: Noha Nooh Lasheen
Lecturer of Physiology, Physiology department, Faculty of Medicine, Ain Shams University
Email: nohalasheen@med.asu.edu.eg
    & nlashen@yahoo.com

Summary:

Metabolic acidosis could occur due to either endogenous acids accumulation or bicarbonate loss from the gastrointestinal tract or commonly from the kidney. This study aimed to investigate the possible underlying mechanism(s) of chronic acidosis-induced cardiac contractile and electrical changes in rats. Twenty four adult Wistar rats, of both sexes, were randomly divided into control group and chronic metabolic acidosis group, which received orally 0.28 M NH_4Cl in the drinking water for 2 weeks. At the end of experimental period, systolic and diastolic blood pressure values were measured. On the day of sacrifice, rats were anaesthetized by i.p. Pentobarbitone (40 mg/kg B.W.), transthoracic echocardiography and ECG were performed. Blood samples were obtained from abdominal aorta for complete blood count and determination of pH, bicarbonate, chloride, sodium, potassium, troponin I, CK-MB, IL-6, renin

and aldosterone levels. Hearts from both groups were studied for cardiac tissue IL-6 and aldosterone in addition to histopathological examination.

Compared to control group, chronic metabolic acidosis group showed anemia, significant systolic and diastolic hypotension accompanied by significant reduction of ejection fraction and fraction of shortening, significant bradycardia, prolonged QTc interval and higher widened T wave, as well as, significantly elevated plasma levels of renin, aldosterone, troponin I, CK-MB and IL-6, and cardiac tissue aldosterone and IL-6. The left ventricular wall of the acidosis group showed degenerated myocytes with fibrosis and apoptosis. Thus, chronic metabolic acidosis induced negative inotropic and chronotropic effects and cardiomyopathy, possibly by elevated aldosterone and IL-6 levels released from the cardiac tissue.

Key words:
Aldosterone, Cardiac, Chronic Metabolic Acidosis, ECG, IL-6

List of Abbreviation:
CK-MB: Creatine Kinase MB subform, ENaC: Epithelial Sodium Channel, IL: Interleukin, LV: Left Ventricle, NHE1: Na^+/H^+ exchanger, RAAS: Renin–Angiotensin–Aldosterone System, TNF α: Tumor Necrosis Factorα

Introduction:

Metabolic acidosis, a common clinical condition, is caused by a decrease in blood pH and bicarbonate concentration (**Wagner 2007**). It could occur acutely, lasting for a few hours to a day, or as a chronic condition when normal pH could not be fully restored. Metabolic acidosis, also, could occur frequently as a secondary complication, and have detrimental effect on patients' outcome with various life-threatening conditions (**Weiner and Hamm 2007**). It was observed that metabolic acidosis could result from many conditions such as chronic kidney diseases (**Kraut and Kurtz 2005**), lactic acidosis (**Lorenz 2009**), diabetic ketoacidosis (**Kraut and Madias 2012**) and diet induced acidosis (**Yancy** *et al.* **2007**)**.**

Acidosis, as a dominant type of acid base disturbance, has been reported to cause complex dysregulation of cardiovascular functions (**Curley** *et al.* **2010**). **Allen and Orchard (1983)** reported that acidosis could induce a negative inotropic effect in the heart**,** through alteration of electrical activity, pumps and channels, and modifications of myofilament sensitivity to calcium (**Crampin and Smith 2006**)**.** Also, **Biais** *et al.* **(2012)** reported that metabolic acidosis

could impair the positive inotropic effect of β-adrenergic stimulation. However, **Schotola** *et al.* **(2002)** found insignificant inotropic effect of mild metabolic acidosis on isometrically contracting muscle strips from non-failing sheep hearts.

Also, **Perez** *et al.* **(1979)** found that metabolic acidosis induced by HCl or NH_4Cl was associated with increased aldosterone production without concomitant changes in plasma renin activity. In a later study, **Henger** *et al.* **(2000)** suggested that inhibition of aldosterone action by spironolactone in chronic metabolic acidosis resulted in exacerbated acidosis by a non-renal mechanism possibly mediated by glucocorticoids rise. **Mihailidou (2012)** reported that sustained aldosterone rise could induce cardiovascular damage independently from renal sodium regulation and blood pressure effects.

Therefore, it was observed that studies on the cardiac effects of acidosis have been performed mostly in isolated tissues or myocytes, and the pH values used were rather low (~6.0 to 6.5), corresponding to local acidosis in cardiac tissue during ischemia. The alterations of cardiac function *in vivo,* also, depend on other factors such as changes in venous return, afterload, autonomic nervous system, and compensatory mechanisms **(Biais** *et al.* **2012)**. However, few researches dealing with the effect of chronic metabolic acidosis on contractile function of the heart *in vivo* are available. Thus, it is of great interest to study the *in vivo* cardiac contractile and electrical changes in response to chronic metabolic acidosis.

Aim of the Work:

The current study was designed to demonstrate the effects of chronic acidosis induced by ammonium chloride administration on the contractile and electrical activity of the heart, and to elucidate the possible underlying mechanism(s) of such effects.

Site of Research:

Physiology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

Materials and Methods:

Experimental Protocol:
Animals:

Animals used were twenty four adult Wistar rats, of both sexes, initially weighing 150-180 grams, purchased from Vacsera animal house (Helwan),

Cairo, Egypt, and housed in animal cages (4 rats / cage) with suitable ventilation, temperature of 22-25 $^{\circ}$C, 12 hours light dark cycle and free access to food and water- *ad libitum-* in the Animal House, Physiology Department, Faculty Of Medicine, Ain-Shams University.

Animals were randomly divided into:

GROUP I: Control group(C) (n=12): Rats of this group received rodent chow and tap water *ad libitum.*

GROUP II: Chronic Metabolic Acidosis group (initial n=14, two rats died during the experiment): They received orally 0.28 M NH_4Cl in the drinking tap water for 2 weeks **(Kwon** *et al.* **2002)** *ad libitum* with rodent chow similar to that of control group. The daily acid load administered was 11 ±1 mmol/day/rat.

Throughout the experimental period, the daily water intake for each cage was measured by specific water bottle for 3 rats in the cage in both groups. No differences in either water consumption or food intake were found between the groups all through the two weeks.

At the end of experimental period, all rats were subjected to arterial blood pressure measurement using non-invasive small animal tail blood pressure system (NIBP200A, Biopac systems Inc; USA).

On the day of sacrifice, overnight fasted rats were anaesthetized with intraperitoneal injection of Pentobarbital (40 mg/kg B.W.). Transthoracic echocardiography was performed; then, rats were subjected to ECG recording, using the ECG recorder Cardimax FX-2111(Fukuda Denshi Co., Ltd., Japan). All leads were established by subcutaneous needle electrodes. From lead II–ECG tracing, the heart rate, the voltages of R and T waves, as well as, the QRS and T wave duration and the durations of P-R and Q-T intervals were calculated. Corrected QT interval (QT-c) was calculated according to **Goldschlager and Goldman (1984)**:

$$QT_{\text{-}C} \text{ interval} = \frac{Q\text{-}T \text{ interval (in seconds)}}{\sqrt{R\text{-} R \text{ interval (in seconds)}}}$$

Transthoracic echocardiographic evaluation was performed using Vivid 7 Dimension, GE (Vingmed ultrasound AS N-3190 Horten, Norway), left ventricular (LV) systolic function was determined by estimation of ejection fraction and shortening fraction **(Cloez** *et al.* **1988)** *(Courtesy of the Pediatric Department, Faculty of Medicine, Ain Shams University).* The left hemi-thorax

was carefully shaved, and a prewarmed ultrasound transmission gel (Parker Laboratory, Orange, NJ) was applied to the precordium.

Ejection fraction was calculated from the left ventricle (LV) cross-sectional area (2-D short-axis view) using the equation:

Ejection Fraction (%) = [(LVDA−LVSA) /LVDA] ×100

Fraction of shortening, a measure of LV systolic function, was calculated from the M-mode LV dimensions using the following equation:

Fraction of shortening (%) = [(LVEDD−LVESD) /LVEDD] ×100

Then, an abdominal midline incision was performed, blood samples from the abdominal aorta were collected into two tubes, EDTA containing tube for complete blood count, the other tube was heparinized, and thereafter, centrifuged at 4000 rpm for 15 min., then the separated plasma was used for subsequent determination of pH and plasma levels of sodium bicarbonate, chloride, sodium, potassium, aldosterone, troponin I, creatine kinase (CK-MB), interleukin 6 (IL-6).

Also, cardiac tissue samples from both groups were dissected and preserved at -80 ° C for subsequent determination of cardiac tissue aldosterone and IL-6. Also, hearts from both groups were subjected to histopathological examination.

Red Blood Cells Parameters: were performed by the use of coulter T- 660, according to the method described by **Coulter (1956)**, depending upon electronic counting to study Erythrocyte count (millions /µL), Hemoglobin level (gm/dL), and Haematocrite value (%).

Blood pH and sodium bicarbonate: were measured using gas analyzer BEARS supplied by CIBA corning Diagnostics Corp. (medfield, MA, USA).

Determination of plasma levels of chloride, sodium, potassium, renin, CK-MB, troponin I, IL-6 and aldosterone, and cardiac tissue levels of IL-6 and aldosterone were performed using commercially available kits.

Histological Examination of Heart: Samples were fixed in 10% formalin for light microscopy. Paraffin embedded sections of 5-µm thickness were stained with Hematoxylin and Eosin (H & E) and Mallory stain for subsequent microscopic examination under high power **(Bancroft and Gamble 2002).**

Also, the hearts of both groups were studied by immunohistochemistry by using antibodies against Caspase III.

Statistical Analysis

All results in the present study were expressed as mean ± SE of the mean. Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL, USA) program, version 20.0 was used to compare significance between the two groups. Comparisons were made using unpaired ''t'' test. Differences were considered significant when $P \leq 0.05$.

Ethics Committee

The current study was approved by the Ethics Committee of Faculty of Medicine, Ain Shams University.

Results:

As regards ECG changes, as shown in table 1, chronic metabolic acidosis (MA) group showed significant bradycardia compared to control (C) group (297.27 ± 14.39 versus 369.3 ±17.07, $P < 0.005$), also, T wave duration was significantly prolonged (85.46 ± 2.82 versus 58 ±4.67, $P < 0.001$), and of significantly higher voltage (300 ±30.15 versus 215 ±15, $P < 0.05$), in addition to significant prolongation of QTc interval (255.27 ±7.43 versus 221.5 ± 9.26, $P < 0.02$). However, insignificant changes in QRS duration, R voltage and PR interval between the two groups were observed. Also, elevated ST segment was prominent in some rats of chronic MA group.

Regarding echocardiographic parameters of left ventricular systolic function, as shown in table 1, chronic MA group showed significant reduction in ejection fraction and in fraction of shortening compared to C group (55.14% ±2.14 versus 70% ±3.8, $P < 0.01$ and 29% ±3 versus 40% ±3.16, $P < 0.05$, respectively), systolic and diastolic blood pressure values were significantly decreased in chronic MA group compared to C group (94.1 ± 1.23 versus 126.8 ±1.32, 68 ±0.76 versus 85.8 ±0.8, $P < 0.001$ for each).

Plasma levels of troponin I, CK-MB and IL-6 were significantly elevated in the chronic MA group compared to C group (8.77 ± 0.28 versus 0.99 ±0.08, 30.85 ±6.65 versus 7.05 ±0.81, 127.67 ±20.57 versus   61.38 ±1.26, $P < 0.001$, < 0.005, <0.01 respectively), as shown in table 2. Similarly, cardiac tissue IL-6 was significantly increased in chronic MA group compared to C group (62.29 ±1.45 versus 45.41 ±1.15, $P < 0.001$). As shown in table 2, rats received NH_4Cl for 2 weeks showed significant decrease in both plasma pH and plasma bicarbonate level compared to the C rats (7.19 ±0.004 versus 7.4 ±0.003, $P <$

0.001; 16.36 ±0.25 versus 25.81 ±0.3, P < 0.001 respectively), and significantly elevated plasma chloride level (106.22 ±3.26 versus 94.47 ±2.94, P <0.02). Thus, this group showed chronic hyperchloremic MA (non-anion gap MA). Chronic MA group was found to consume almost equal daily water intake compared to C rats, as the daily water intake for each cage was measured by specific water bottle for 3 rats in the cage in both groups. No differences in water consumption were found between the groups all through the two weeks.

Chronic MA group showed significant increase in plasma sodium level (213.41 ±17.6 versus 138.42 ±19.3, P <0.05), significant decline in potassium level (3.73 ±0.36 versus 5.28 ±0.25, P <0.01), and significant rise in plasma renin and aldosterone (37.8±1.07 versus 17.65 ±1.01, P <0.001; 62.6 ±7.14 versus 25.14 ±3.47, P <0.001 respectively). Also, there was significant rise in cardiac tissue aldosterone level in chronic MA group compared to C group (40.04 ±1.88 versus 23.03 ±1.49, P < 0.001).

As regards red blood cell parameters, erythrocyte count (RBC) (5.62 ±0.7 versus 6.73 ±0.2, P < 0.005), hemoglobin level (Hb) (11.74 ±0.22 versus 13 ±0.36, P < 0.01) and hematocrit value (Hct) 30.64 ±0.49 versus 32.07 ±1.16, P < 0.005) were all significantly lowered in chronic MA group compared to C group, as shown in table 3.

Histological Results:

As shown in fig.1-a, b & c, the wall of the apical region of the left ventricle of the C group revealed regularly arranged cardiac muscle fibers, appearing branching, anastomosing and running in various directions. The myocardial cells were attached end to end. The nuclei appeared central and vesicular, and the sarcoplasm appeared acidophilic and striated. In transverse section, the cardiac myocytes appeared more or less comparable in size with noticeable myofibrillar content.

The cardiac muscle specimens of chronic MA rats exhibited congestion of blood vessels with apparent affection of cardiac myocytes (Fig.2-a), in the form of mononuclear cellular infiltration of the cardiac muscle. Also, distortion of some cardiac myocytes, together with some areas of complete degeneration and fragmentation of the cells was observed (Fig.2-b). Loss of the regular arrangement of the cardiac myocytes was observed in (Fig.2-c &2-d), together with marked cell vacuolation (Fig.2-d) in addition to leukocytic infiltration (Fig.2-e).

As shown in fig.3-a, there was less or minimal fibrous tissue around the blood vessels in the C group as stained by Mallory, while the heart of chronic MA group showed dense fibrous tissue distorting the normal arrangement of cardiac myocytes (as shown in fig.3-b).

Immunohistochemistry study, by using antibodies against caspases, revealed that the heart of chronic MA group showed large areas of positive reaction compared to the heart of C group which caused minimal areas with positive reaction (as shown in fig.3-c&d).

Discussion:

Rats received NH_4Cl in drinking water developed hyperchloremic (non-anion gap) metabolic acidosis (MA), confirmed by a blood pH of 7.19, less bicarbonate and higher chloride levels in plasma. The death rate was 14% in rats received NH_4Cl. During the two weeks period, the renal compensation to restore acid base balance were present but ineffective proved by decrease in alkali reserve found in chronic MA group at the end of experimental period. Chronic metabolic acidosis could raise urinary net acid excretion, together with adaptive renal tubular changes to increase urinary acidification such as rise in both Na^+/H^+ exchanger activity (**Alpern 1990**), and increased bicarbonate reabsorption capacity in proximal tubules and the medullary collecting ducts, and enhanced $NH4^-$ secretion in the proximal tubule (**Good and Knepper 1985**). The elevated plasma chloride level could be, also, caused by chloride load (present in food plus the added NH_4Cl to drinking water).

Chronic MA group showed significant bradycardia compared to C group. Similar results were obtained by **Satoh and Hashimoto (1983)** and **Satoh and Seyama (1986)**, who found a negative chronotropic effect of acidosis in canine and rabbit SA nodal tissues respectively. The acidosis induced bradycardia could be explained by a direct inhibitory effect of acidosis on the SA node (**Maruna 1958**).

The electrocardiographic changes observed in the present study, could be attributed to ventricular action potential duration changes. In acidosis, varied action potential duration and configurational changes were noticed such as small depolarization of the resting potential caused by regional differences in channel expression (**Orchard and Cingolani 1994**). Acidosis, present in ischaemia, caused a marked depression of the sub-epicardial action potential, and altered repolarization pattern resulting in arrhythmias (**Antzelevitch** *et al.* **1991; Crampin** *et al.* **2006),** manifested in the present study as elevated ST

segment in chronic MA group. Also, the lowered plasma potassium level, observed in the current study, could explain widened T wave.

However, prolonged QTc interval and widened T wave observed in the current study disagree with the study of **Stengl** *et al.* **(2013),** who found no effect of acidosis on the action potential. This could be due to presence of a complex humoral and nervous regulation present *in vivo* and/or to inherent limitations in the QT-interval correction.

The insignificant change in QRS duration in chronic MA group, despite significant bradycardia, is similar to **Aberra** *et al.* **(2001)**, who reported that acidosis slowed cardiac pacemaker activity without affecting time course of the action potential spread through the ventricles.

The different models, *in vivo* and *in vitro*, used in studying cardiac functions in acidosis should be considered because the isolated heart was not working against a load, and was not stretched in addition to plasma potassium changes and chemoreceptor mediated reflexes could alter the ECG *in vivo* (**Aberra** *et al.* **2001**).

Chronic MA caused left ventricular systolic dysfunction detected by transthoracic echocardiographic heart examination, which is an excellent non-invasive tool to assess left ventricular structure and function (**Picard** *et al.* **1990**), however, it is limited by the small sized rat animal and rapid heart rate (**Litwin** *et al.* **1994)**. The negative inotropic effect of acidosis, in the present study, agrees with many previous studies (**Paulsen** *et al.* **2006; Stengl** *et al.* **2013**). **Paulsen** *et al.* **(2006)** reported that diabetic ketoacidosis caused impaired left ventricular function and developed myocardial necrosis that could be due to increased caspase concentration in the heart, which was evident in the current study by immunostaining with caspases antibodies. **Stengl** *et al.* **(2013)** found reduced cardiac contractile function in MA despite maintained cardiac output caused by adaptive sympathetic changes. **Biais** *et al.* **(2012)** reported that acidosis could impair the positive inotropic effect of β-adrenergic stimulation, and could affect cardiac muscle lusitropy, which could be due to cardiac tissue fibrosis observed histologically, also, in the current study. In addition, acidosis could decrease calcium sensitivity of troponin I (**Orchard 1987**), a target protein of protein kinase A-dependent phosphorylation during β-receptor stimulation (**Biais** *et al.* **2012).**

In addition, Na–H exchange (NHE), the primary process by which the cardiac cell extrudes protons especially in intracellular acidosis, is expressed in all tissues. Cardiac cells have primarily the ubiquitous NHE-1 subtype which

could contribute to chronic maladaptive myocardial responses to injury with development of heart failure (**Karmazyn** *et al.* **2008).** NHE could be activated by autocrine, paracrine and hormonal factors such as endothelin-1, angiotensin II, and $\alpha 1$ adrenoceptor agonists via phosphorylation-dependent processes (**Fliegel and Karmazyn 2004**). Thus, the elevated hydrogen protons in extracellular fluid could cause systolic dysfunction (**Biais** *et al.* **2012),** by reducing Na^+-K^+-ATPase activity in myocardial cells (**Brown** *et al.* **1978**), producing heart failure (**Mitchell** *et al.* **1972**).

In the present study, the significantly elevated aldosterone in both plasma and cardiac tissue in chronic MA group could be one of the underlying mechanisms of depressed cardiac contractile function in acidosis. Aldosterone induced cardiac fibrosis evidenced histologically, is in agreement with **Brilla** *et al.* **(1990)**, who found that aldosterone rise could affect regulation of collagen synthesis within the cardiac interstitium and in the adventitia of intramyocardial coronary arteries.

In addition, significant hypotension in chronic MA group is similar to **Kopple** *et al.* **(2005)** who found hypotension in chronic MA, in chronic kidney diseases patients. The observed hypotension could be attributed to chronic MA-induced depressed myocardial contractility, noticed by echocardiography and ECG, as well as, histological cardiac changes in the form of degeneration and fibrosis. **Kraut and Madias (2012)** reported that acidosis caused depressed cardiac contractility, increased susceptibility to cardiac arrhythmias and hypotension.

The acidosis-induced hypotension, in the present study, is the stimulating factor for the significant rise of plasma aldosterone level and plasma renin activity observed in this study according to **Vendeloo** *et al.* **(2011)**. They reported that less sodium reabsorption, in the proximal tubules, could occur early in metabolic acidosis due to lowered glomerular filtrated bicarbonate amount, thus, the distally increased sodium could cause a hyperreninaemic hyperaldosteronism state. Similarly, **Györke** *et al.* **(1991)** suggested that aldosterone rise in MA could be mediated by the combined effect of sodium and water diuresis-related increased plasma renin activity, early hyperkalaemia and direct stimulation of adrenal steroidogenesis. Also, **Schambelan** *et al.* **(1987)** suggested that chronic NH_4Cl-induced acidosis could cause a sustained stimulation of aldosterone secretion without ACTH secretion change. They mentioned that other factors than increased renin secretion and raised plasma potassium concentration might be involved in, at least the early phase of

aldosterone stimulation, suggesting that plasma hydrogen ion concentration might be a separate regulator of aldosterone secretion.

The acidosis-induced myocardial damage, manifested by elevated plasma levels of CK-MB and troponin I in chronic MA group, agrees with **Wu** *et al.* **(2013)**.

However, the elevated plasma levels of cardiac enzymes, the hypotension and the bradycardia observed in chronic MA group disagree with **George** *et al.* **(1996)**, who observed unaltered heart rate, cardiac enzymes and arterial pressure in diabetic ketoacidosis. They suggested that short-term ketoacidosis did not contribute to the abnormalities of ventricular function in diabetes mellitus. Thus, the deteriorated cardiac functions, observed in the current study, could be due to longer duration of MA.

Chronic MA caused disruption of myocytes arrangement, fibrosis together with apoptosis, which might be caused by high plasma and cardiac aldosterone levels, thereby affecting left ventricular systolic function and electrical activity with subsequent elevation of plasma troponin I and CK-MB.

The renal handling of sodium could be dependent on the time course of acidosis. Within the first 24 hours after acidosis induction, decreased salt and water reabsorption in the proximal tubule with subsequent natriuresis **(Wiederkehr and Krapf 2001; Faroqui** *et al.* **2006),** could be correlated with a reduced bicarbonate level in the peritubular capillaries **(Hebert** *et al.* **1972)**. Therefore, natriuresis caused extracellular volume decrease, resulting in a secondary hyperaldosteronism **(Wiederkehr and Krapf 2001)**. However, in prolonged chronic MA, there was progressive rise of sodium reabsorption, together with a progressive increase in Na^+/H^+ Exchanger 3 isoform protein abundance in the apical membranes along the proximal tubule and thick ascending limb **(Ambühl** *et al.* **1996)**. **Faroqui** *et al.* **(2006)** found that after 5 days of chronic MA, there were aldosterone-induced upregulation of serum- and glucocorticoid-regulated kinase, epithelial Na^+ channel, Na^+/Cl^- cotransporter leading to lowered urinary sodium excretion rate. Therefore, **Amlal** *et al.* **(2004)** reported that enhanced sodium reabsorption in the distal tubule could be a compensatory response to impaired proximal tubular function.

Therefore, the observed hyperaldosteronism in chronic MA group could have deleterious cardiovascular effects. **Stowasser** *et al.* **(2005)** reported that increased aldosterone level could induce cardiac and vascular damage in young humans, before the onset of hypertension. Moreover, mineralocorticoids receptor signalling could be directly involved in macrophage-dependent cardiac

remodelling and fibrosis **(Usher** *et al.* **2010),** which could explain the fibrosis observed by Mallory stain which could be caused by increased cardiac tissue IL-6.

Further, the elevated plasma aldosterone level caused lowered plasma K^+ level in chronic MA group, which disagrees with **Afzal** *et al.* **(2013),** who found moderate to severe hyperkalemia in high anion gap MA. Acute hyperkalemia could be present in MA, and could be caused by extracellular potassium shift **(Schales and Schales 1941).** However, **Menegon** *et al.* **(1998)** reported that chronic MA in rats might increase urinary K^+ excretion. In acidosis, there could be other factors indirectly affecting potassium level such as aldosterone, impaired renal function, volume depletion, and diarrhea **(Hamm** *et al.* **2013)**. Thus, the significantly lowered plasma K^+ level present in this study could be due to overriding of elevated plasma aldosterone on the potassium shift, as the hyperreninaemic hyperaldosteronism could stimulate distal sodium reabsorption, causing potassium wasting. The persistent higher plasma aldosterone level, despite lowered plasma potassium level in the present study, is in accordance to **Magner** *et al.* **(1988)**. They observed a prompt plasma potassium rise in acute MA, and after 3 to 5 days of acidosis, hypokalemia was developed due to increased urinary K^+ excretion mediated by renal aldosterone action.

Local synthesis of Ang I and II could be present in the heart muscle **(Danser and Schalekamp 1996).** Aldosterone, produced in the heart like Ang II, could be attributed not only to ACE but also to a serin protease named heart chymase (CMA) **(Gumprecht** *et al.* **2002)**.Thus, the elevated plasma and cardiac tissue aldosterone levels in the current study could be suggested as underlying mechanism(s) of cardiac fibrosis and left ventricular systolic dysfunction. **Funder (1997)** reported that mineralocorticoid receptor and 11b-hydroxysteroid dehydrogenase, which increases mineralocorticoid selectivity to aldosterone target tissues, could be present in heart. Also, aldosterone could have stimulatory effect on cardiac collagen synthesis, thereby producing cardiac fibrosis **(Brilla** *et al.* **1990; Robert** *et al.* **1994)**, however, its mechanism was unclear **(Robert** *et al.* **1995)**. In addition, aldosterone was reported to cause baroreceptor dysfunction **(Wang 1994)** and preventing myocardial uptake of norepinephrine **(Struthers 1996).** Also, the potassium changes in MA could affect cardiovascular function **(Terkildsen** *et al.* **2007)**.

The significantly elevated IL-6 observed in chronic MA group is in accordance to **Zampieri** *et al.* **(2014)**, who reported that MA was positively

associated with tumor necrosis factor (TNFα), IL-6, IL-8, and IL-10. Also, lower bicarbonate level was associated with higher inflammatory biomarkers **(Farwell and Taylor 2010)**. **Kalantar-Zadeh** *et al.* **(2004)** and **Kellum** *et al.* **(2004)** mentioned that severe MA could stimulate an acidosis inflammatory state and macrophage interleukins production. Plasma IL-6 level only gives a systemic IL-6 rise and it could be a response to acidosis by liver, adipose tissue and macrophages. Therefore, cardiac tissue IL-6 was evaluated in this study, and it was found to be significantly increased in chronic MA group, suggesting its role in cardiac tissue fibrosis induced by MA. This could be supported by the study of **Mann (2002),** who reported that inflammatory cytokines might modulate myocardial functions via different mechanisms including stimulation of hypertrophy and fibrosis through direct effects on cardiomyocytes and fibroblasts, manifested in the current study by dense fibrous tissues. Also, IL-6 could impair myocardial contractile function through direct effects on intracellular calcium transport and signal transduction through β-adrenergic receptors, induction of apoptosis, which was evident in the current study by positive reaction to immunostaining with anti-caspases antibodies. Thus, the left ventricular contractile dysfunction could be attributed partially to the elevated IL-6 mainly in cardiac tissue in the present study **(Aukrust** *et al.* **1999; Damås** *et al.* **2000)**.

It is worth noting that the direct effect of acidosis on the previously described changes could be present, however, the rat survival for 2 weeks period with acidosis could ensure that the daily slight H^+ ions addition when being uncompensated, development of metabolic acidosis could ensue. This could be supported by the study of **Biais** *et al.* **(2012)**, who studied acute metabolic acidosis effect on papillary muscles in Wistar rats at pH=7.10. They reported that myocardial consequences occurred below a pH of 7.20 which could be clinically relevant.

The acidosis-induced anemia in the present study could be due to increased RBCs hemolysis, iron deficiency or bone marrow depression. The increased hemolysis might be due to reduced Na^+-K^+-ATPase activity in RBCs **(Levin** *et al.* **1972),** or by chronic MA induced inflammatory mediators **(Andrews 2004),** present in this study by plasma IL-6 rise. In addition, **Khositseth** *et al.* **(2008)** reported that acidosis could aggravate the degree of hemolytic anemia. **Andrews (2004)** reported that the inflammatory cytokines, such as IL-6, could induce production of hepcidin, an iron-regulatory hormone inhibiting macrophage iron release and intestinal iron absorption, leading to hypoferremia.

Thus, the observed increased IL-6 in chronic MA group could further explain the associated anemia in this group. In addition, **Mann (2002)** suggested that the indirect effect of inflammatory mediators in progression of heart failure through induction of systemic inflammation and impairment of bone marrow function with secondary anemia. In addition, **Silverberg** *et al.* **(2004)** reported that the RBCs contain many antioxidants, thereby, anemia might be associated with increased oxidative stress **(Siems** *et al.* **2000),** causing myocardial cell function impairment. Therefore, the observed anemia could cause cardiac dysfunction in chronic MA.

Moreover, the chronic MA- induced anemia might cause aldosterone level rise. This might be explained by peripheral ischemia, peripheral arteriolar vasodilatation, with subsequent hypotension (detected in the present study) as a consequence of anemia, with the resultant activation of the renin-angiotensin-aldosterone system **(Anand** *et al.* **1993)**, which was also evident in the present study by significant rise of both plasma renin and aldosterone.

Conclusion:

Chronic metabolic acidosis induced by NH_4Cl had detrimental effects on cardiac function in the form of negative chronotropic and inotropic cardiac effects, hypotension, arrhythmia which could be due to fibrosis and apoptosis in addition to anemia. The acidosis-related alterations in cardiac functions could be due to elevated plasma and cardiac tissue of both aldosterone and IL-6 levels.

Conflict of Interest:

The authors declared no conflict of interest in the present work.

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Table (1): ECG parameters, echocardiographic parameters of left ventricle systolic function and systolic and diastolic blood pressure (mmHg) in the different studied groups.

	Control group (n = 12)	Chronic Metabolic Acidosis group (n = 12)
Heart Rate (bpm)	369.3 ±17.07	297.27 ±14.39
P		<0.005
PR interval (msec.)	44 ±4	50.91 ±4.95
P		NS
QRS duration (msec.)	30 ±3.33	27.28 ±3.04
P		NS
R voltage (µV)	600 ±53.75	572.73 ±44.91
P		NS
T duration (msec.)	58 ±4.67	85.46 ±2.82
P		<0.001
T wave voltage (µV)	215 ±15	300 ±30.15
P		<0.05
QT-c interval (msec.)	221.5 ±9.26	255.27 ±7.43
P		<0.02
Ejection Fraction (%)	70 ±3.8	55 ±2.14
P		<0.01
Fraction of Shortening (%)	40 ± 3.16	29 ±3
P		<0.05
Systolic Blood Pressure (mmHg)	126.8 ±1.32	94.1 ±1.23
P		<0.001
Diastolic Blood Pressure (mmHg)	85.8 ±0.8	68 ±0.76
P		<0.001

P: Significance by LSD at P <0.05 from control group.

Table (2): Biochemical changes in plasma and cardiac tissue in the different studied groups.

	Control group (n = 12)	Chronic Metabolic Acidosis group (n = 12)
Troponin I	0.99	8.77
(ng/ml)	±0.08	±0.28
P		<0.001
Creatine Kinase	7.05	30.85
(CK-MB) (U/L)	±0.81	±6.65
P		<0.005
Plasma IL-6	61.38	127.67
(pg /ml)	±1.26	±20.57
P		<0.01
Cardiac Tissue IL-6	45.41	62.29
(pg /ml)	±1.15	±1.45
P		<0.001
Blood pH	7.4	7.19
	±0.003	±0.004
P		<0.001
Plasma bicarbonate	25.81	16.36
(m M)	±0.3	±0.25
P		<0.001
Plasma chloride	94.47	106.22
(mEq/L)	±2.94	±3.26
P		<0.02
Plasma Na$^+$	138.42	213.41
(mEq/L)	±19.3	±17.6
P		<0.05
Plasma K$^+$	5.28	3.73
(mEq/L)	±0.25	±0.36
P		<0.01
Plasma renin	17.65	37.8
(pg/ml)	±1.01	±1.07
P		<0.001
Plasma Aldosterone	25.14	62.6
(ng/ml)	±3.47	±7.14
P		<0.001
Cardiac Tissue Aldosterone	23.03	40.04
(ng/ml)	±1.49	±1.88
P		<0.001

P: Significance by LSD at P <0.05 from control group.

Table (3): Changes in the red blood count (RBC), hemoglobin content (Hb) and hematocrit value (Hct) in the different studied groups.

	Control group (n = 12)	Chronic Metabolic Acidosis group (n = 12)
RBC	6.73	5.62
(10^6/μL)	±0.2	±0.7
P		<0.005
Hb	13	11.74
(g/dL)	±0.36	±0.22
P		<0.01
Hct	32.07	30.64
(%)	±1.16	±0.49
P		<0.005

P: Significance by LSD at P <0.05 from control group.
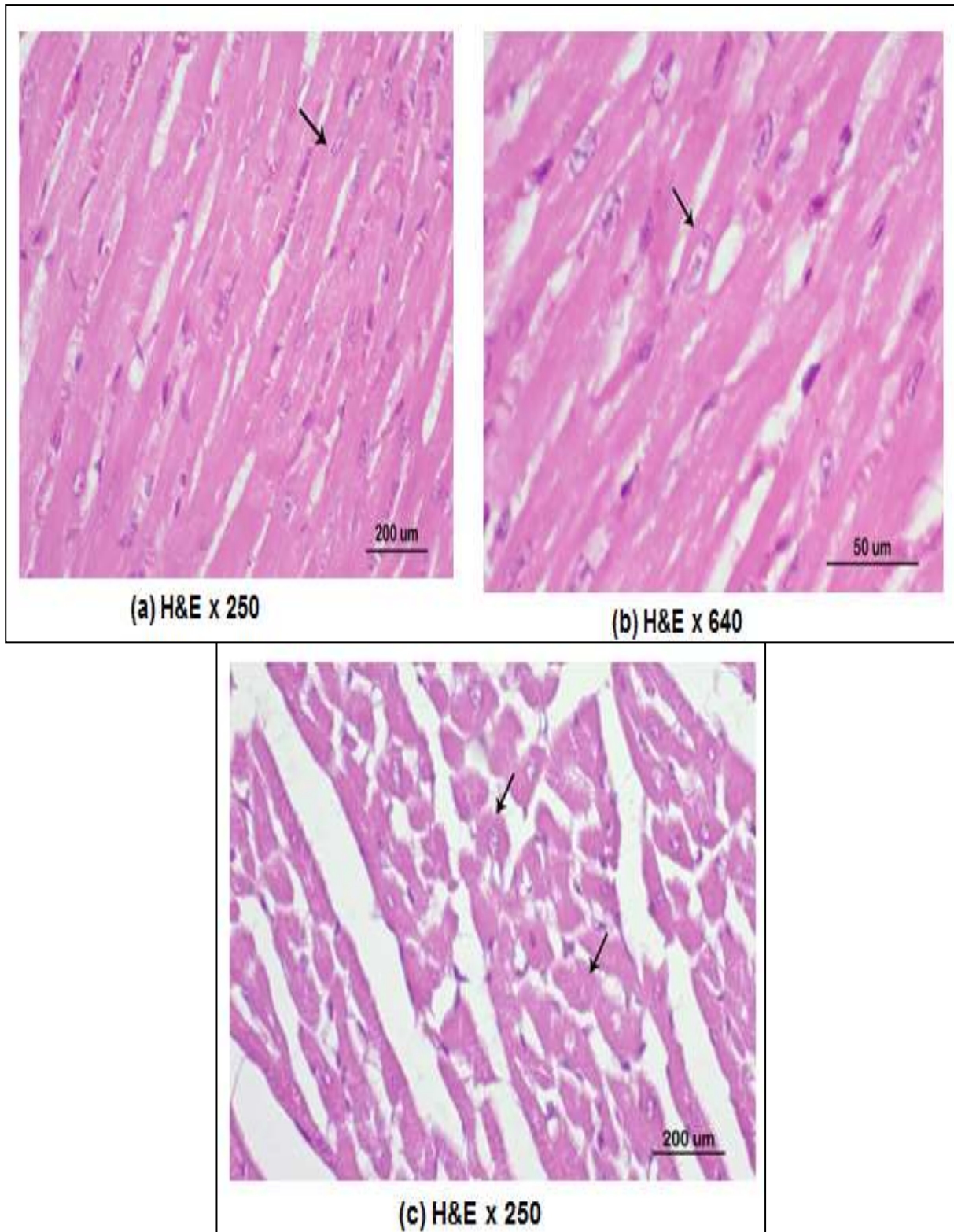
Fig. (1): Photomicrographs of control heart (a & b) longitudinal section showing regularly arranged cardiac muscle fibres branching, anastomosing and attached end to end (black arrow in a), with central and vesicular nuclei (black arrow in b), and acidophilic sarcoplasm. (c) Transverse section in control heart showing normal sized cardiac myocytes (black arrow).
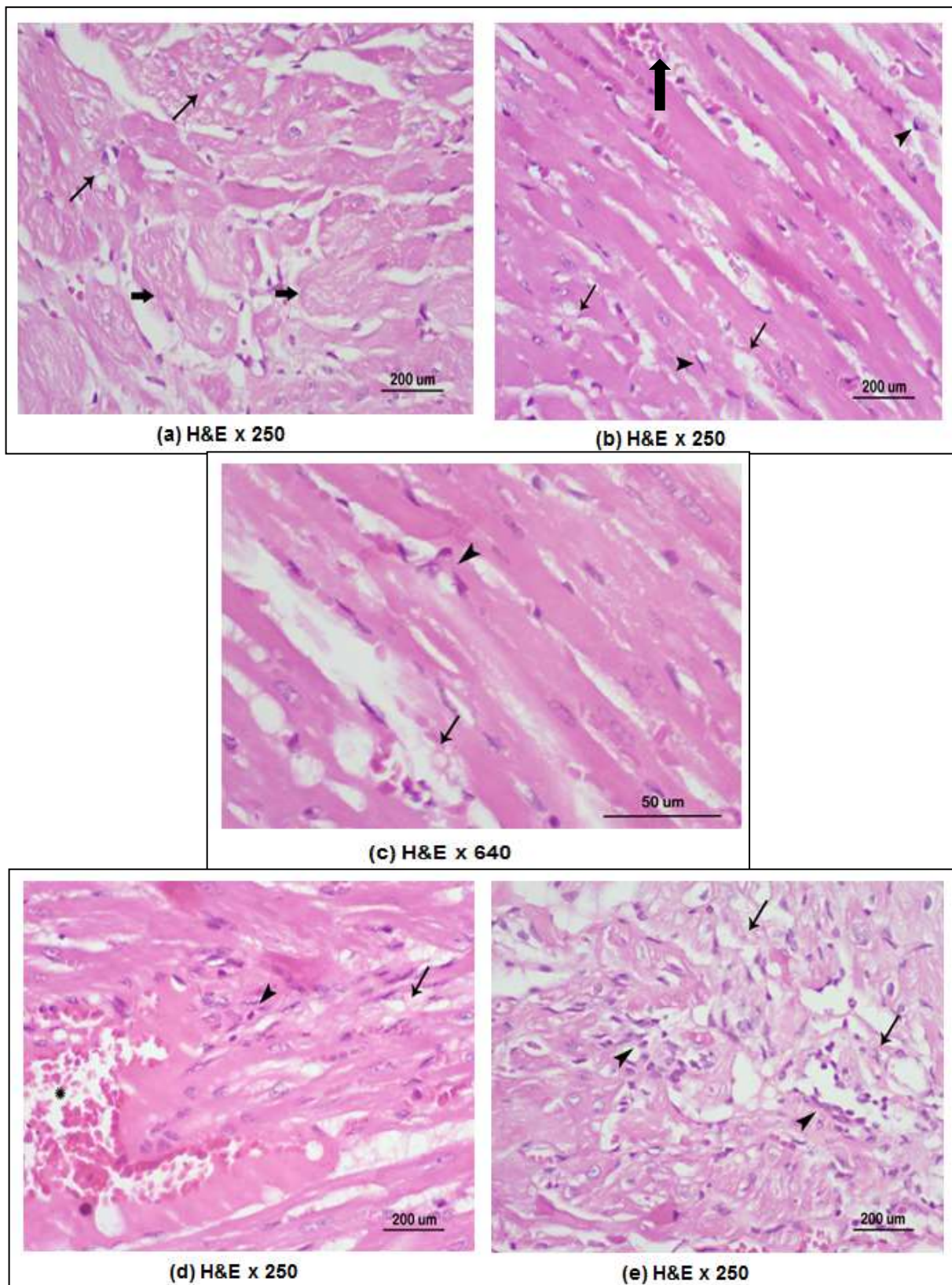
Fig. (2): Photomicrographs of heart of chronic metabolic acidosis group showing acidophilic sarcoplasm and vesicular nuclei (black heads {^}). Areas of complete degeneration and fragmentation of cardiac myocytes (↑ in c), with observed loss of the regular arrangement of the cardiac myocytes together with marked cell vacuolation (↑ in d), congested blood vessels (* in d), and mononuclear cellular infiltration of the cardiac muscle (↑ in e).
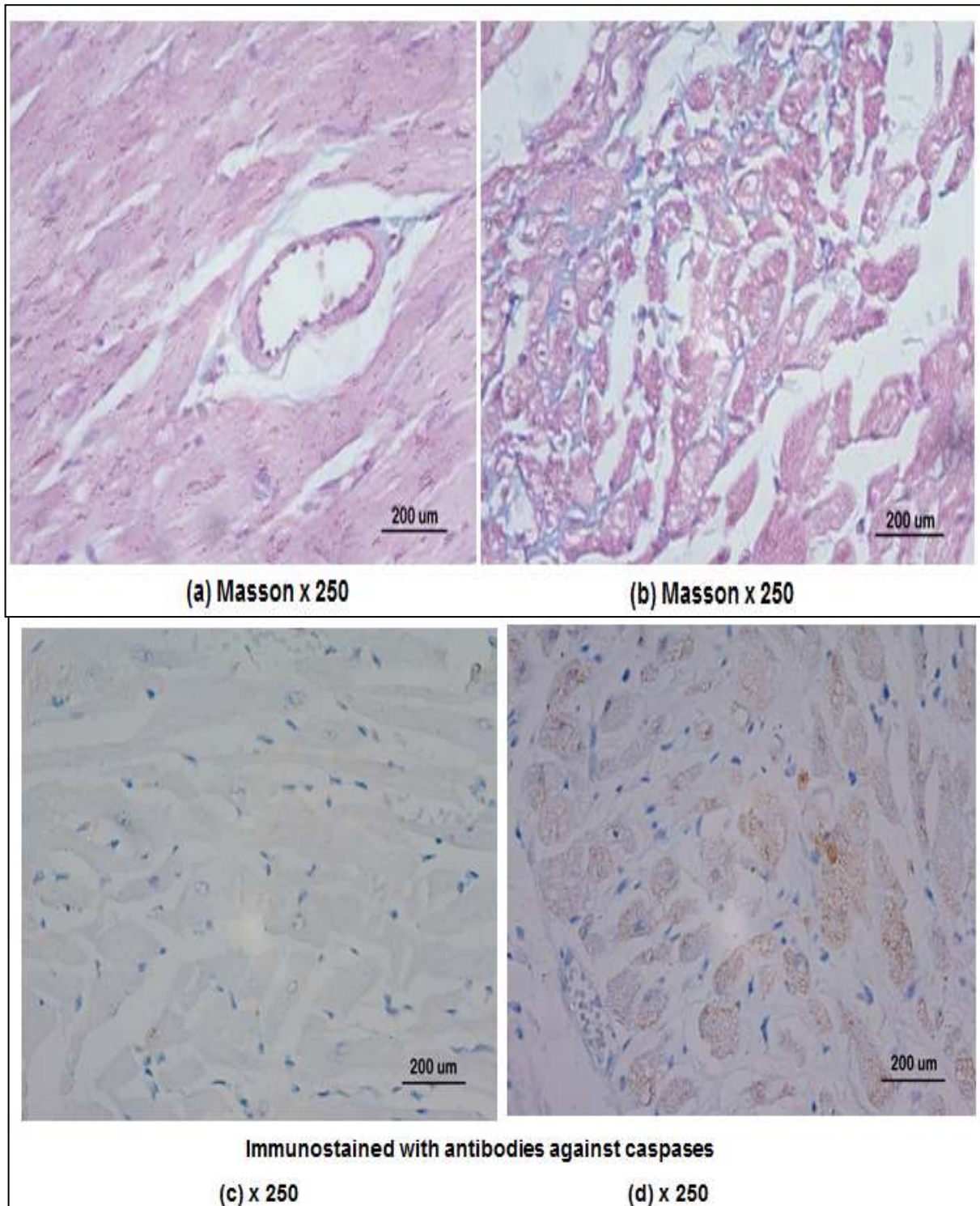
Fig. (3): (a) Photomicrograph of control group heart stained with Masson showing minimal fibrous tissues. (b) Photomicrograph of chronic metabolic acidosis group heart stained with Masson showing dense fibrous tissues distorting cardiac myocytes appeared in blue colour. (c) Photomicrograph of control heart immunostained with antibodies against caspase showing no areas with positive reaction. (d) Photomicrograph of chronic metabolic acidosis group immunostained with antibodies against caspase showing large areas with positive reaction.