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Cardiac adaptation in prolonged inverted bats *(Eidolon helvum)*

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SUMMARY

Inversion (head-down positioning) is often the regular resting position for some species of bats. The objectives of the study was are to determine the differences in body weight, relative heart weight; serum cardiac troponin I and creatine kinase MB; and cardiac left ventricular general histology, deoxyribonucleic acid (DNA) synthesis, elastic fibre and collagen fibres, glycogen accumulation, fat accumulation in bats exposed to prolonged inversion. Bats obtained from Bowen University roosting colony, Osun State, Nigeria were grouped into A, B, C and D with each group containing ten bats. The bats were sacrificed at first, eight, fifteenth, twenty-second day of experiment respectively. Blood samples collected for the estimation of cardiac troponin I and creatine kinase MB. Histological examination of the left ventricle was done using Haematoxylin and Eosin and other stains. The results showed elevation of cardiac function markers (cTnI and CK-MB) indicated cardiac myocyte damage and increased energetics was associated with prolonged inversion in bats. Histological findings revealed mechanically-stressed heart muscles which later adapted followed by myocyte regeneration. The study concludes that bats cardiac tissue possess positive adaptation to prolonged inversion and could become a suitable ground-based model for simulating microgravity while for bat laboratory acclimatization protocols need to be revisited.

Key words: Bats – Inversion – Cardiac hypertrophy – Heart

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INTRODUCTION

Bats are considered as unique and enigmatic group of mammals with characteristics, most importantly for being considered the only true flying mammals (Fenton, 1995; Teel et al., 2005). They are also known to rest head-down positioned/ inverted and which is a reverse of the system that most animals possess (Riskin, 2009; NASA, 2012). The cardiovascular structural adaptations involved are poorly understood (Flanagan, 2001; Flanagan, 2002). The cardiovascular system (CVS) plays a critical role amongst other body systems on exposure to altered gravitational environment (Blomqvist and Stone, 1983).Since the cardiovascular system is heavily dependent on gravity and cephalad fluid shift was said to be its potent stimulus (Blomqvist and Stone, 1983). The CVS is constantly exposed to mechanical perturbation from shear and tensile stresses (Bishop and Lindahl, 1999), meanwhile, changes in the normal level of mechanical perturbation from shear and tensile stresses have profound effects on their cell of the heart and results in abnormal changes in cellular microstructure and function (Bishop and Lindahl, 1999).

Differential adaptation exists along the aortic column of prolonged inverted bats in which the ascending aorta was hypertrophied and descending aorta became atrophied (Ashaolu et al., unpublished). The results of these studies demonstrate that cranial fluid shift is associated with inversion in bats. There is scarcity of information regarding cardiac adaptational responses in either long or short term inverted bats. The present study was designed to histoarchitectural changes, deoxyribonucleic acid (DNA) synthesis, elastic fibre and collagen fibres, glycogen accumulation, fat accumulation in left ventricles of bats exposed to varying duration of prolonged inversion. Also, to deter-

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mine differences in cardiac troponin I and creatine kinase MB in serum of bats exposed to varying duration of prolonged inversion.

MATERIALS AND METHODS

Animal grouping

The experiment was approved by the Bowen University Bat Conservation Committee and the University Ethical Clearance Committee on the use of animals for experimental studies. Forty (40) presumably healthy bats were obtained by netting from the Bowen University Campus bat roosting colony, Iwo, Osun state, Nigeria. The bats were obtained in the periods between November and December 2011, when bats had maintained their routine daily flight and resting activities (i.e. they were not hibernating).

Experimental procedures

After capture and conveyance to the laboratory, the bats were weighed to obtain their initial body weights and were then transferred to wooden cages. Immediately after the closure of the cage openings, all the bats were hanging inverted on the wire gauze roof of the cages. The hook-like hind limbs of the bats were used in griping the wire gauze on the roof of the cage. Bats are known to rest continuously in inverted positions, and this is an inherited trait in bats. Foods and water were positioned on a platform close to the roof of the cages to which the bats were hanging to prevent downward creeping of the bats. Movement of the bats hanging (to the roof of the cages) was also unrestricted.

The bats were divided randomly into four groups, A, B, C and D. Group A bats were taken directly into the laboratory for immediate sacrifice. Group B, C, and D underwent inversion for 8 days, 15 days, and 22 days respectively. Throughout the period of the experiment, the roofs of the cages to which all the bats hung their hind limbs remained unopened.

The bats were intermittently observed to ensure they remained in the inverted position throughout the period of the experiment. The opening at the lower front part of the cage was the access point used in maintaining regular cleaning of the cages while the another upper and frontward opening was the access point for the feeding of the animals.

The bats were weighed using a top loader sensitive balance (Adams Equipment) and recordings were taken as final body weight. The bats were anesthetized with sodium pentobarbital 40mg/kg. A longitudinal incision was made through the mid-thoracic and mid-abdominal walls of the bats to obtain the hearts of the bats. The heart of the animals were excised and weighed to obtain their absolute cardiac weight.

Collection of blood samples

Four ml of blood was collected by cardiac puncture from each animal and dispensed into heparinized tubes, care was taken to prevent hemolysis. The blood sample collected was incubated at 37°C for 10 minutes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was dispensed into plain bottles and refrigerated at 4^{o}C.The obtained serum was used for determination of cardiac creatine kinase MB and troponin I.

Serum cardiac marker estimation

The Creatine kinase MB (CK-MB) Enzymatic Assay Kit and Cardiac Troponin-I ELISA (Xpress bio product, USA) and their appended manual instructions were used for the demonstration of se-

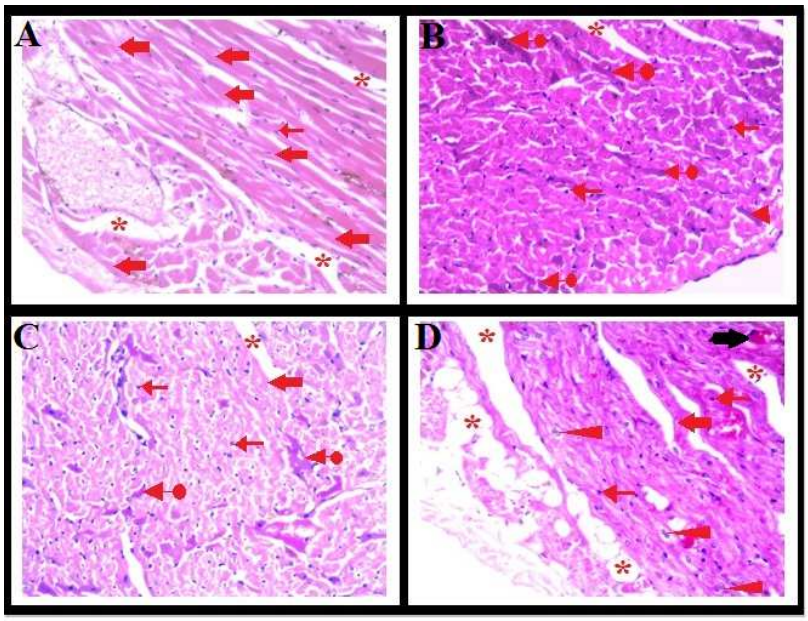


Fig. 1. Representative micrographs showing epicardial aspect of left ventricle in the experimental groups with Hematoxylin and Eosin staining to demonstrate cardiac general histology. **(A)** (no laboratory inversion group) displays regularly arranged cardiac myocytes and interstitial spaces. **(B)** (8 days laboratory inversion group) shows irregular arrangement of cardiac myocytes and interstitial spaces with numerous and diffuse nuclear appearance. **(C)** (15 days laboratory inversion group) displays haphazard myocyte and interstitial spaces orientation with numerous and diffuse nuclear appearance. **(D)** (22 days laboratory inversion groups) displays syncytial-like myocardium with cardiac multinucleation and vacuolar spaces. *Coloured block arrow*, myocyte, black blocked arrow indicates apoptotic cardiac myocyte, line arrow indicates cardiac myocyte nucleus, star- interstitial spaces. Bullet arrow - diffused nuclei, arrow head- dividing nuclei (diploid nuclei). Magnification, x400.

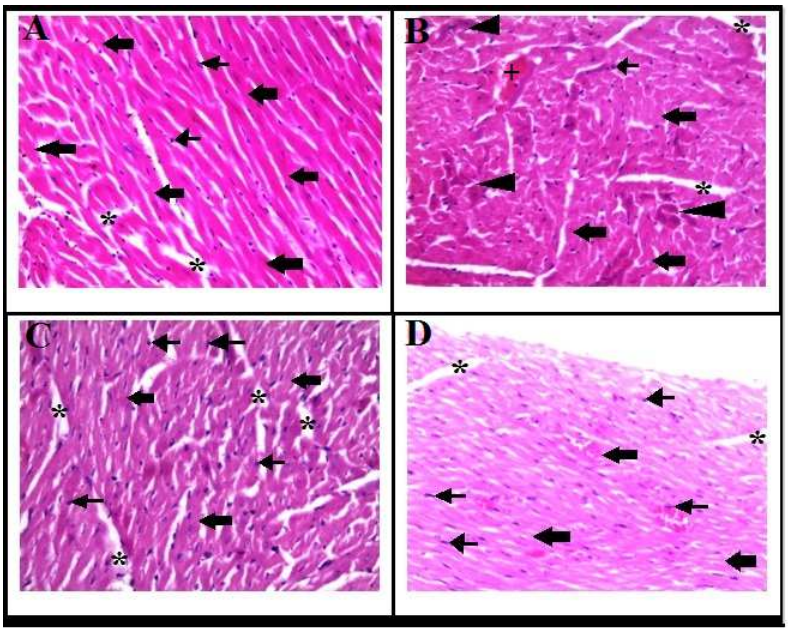


Fig. 2. Representative photomicrographs showing left ventricular myocardium for bat experimental groups demonstrated with Hematoxylin and Eosin staining (H&E). **(A)** (No laboratory inversion group) displays regularly intercalating cardiac myocytes. **(B)** (8 days laboratory inversion group) shows apoptotic and irregular arrangement of cardiac myocytes and diffuse nuclear appearance. **(C)** (15 days laboratory inversion group) displays cardiac myocyte with irregular dimensions and interstitial spaces. **(D)** (22 days laboratory inversion groups) displays denser tinnier cardiac myocte architecture. Block Arrow=myocyte, blocked arrow, line blocked arrow-n=cardiac myocyte nucleus, star- interstitial spaces. Bullet arrow - diffused nuclei, arrow head- diffused nuclei. Plus indicate apoptotic myocytes. Magnification, x400.

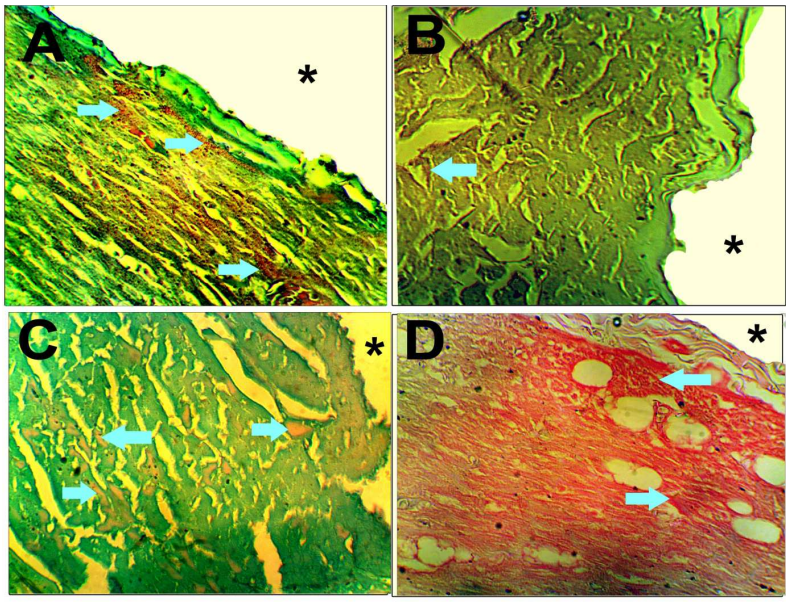


Fig. 3. Representative photomicrographs showing bat cardiac DNA for the experimental groups demonstrated by Feulgen DNA reaction. **(A)** (no laboratory inversion group) displays considerable DNA synthesis activities. **(B)** (8 days laboratory inversion group) shows sparse DNA. **(C)** (15 days laboratory inversion group) also displays sparse DNA staining. **(D)** (22 days laboratory inversion groups) displays profound DNA synthesizing activities. Blue arrows indicate DNA material, star: pericardiac space. Magnification = x400.

rum creatine kinase MB concentration and serum cardiac Troponin-I concentration respectively. The creatine kinase MB kit measures the concentration of creatine kinase MB using a coupled, plate-based, colorimetric reaction. The cTnI ELISA test is based on the principle of a solid phase enzyme-linked immunosorbent assay. Absorbance is measured spectrophotometrically for Creatine kinase MB and Cardiac Troponin-I at 340 nm and 450 nm respectively with a Plate Reader (Biotek, China).

Histological tissue processing for histo-architectural evaluation

Histological tissue processing was performed using methods described by Culling (1974) as described below. The apical part left ventricle was carefully dissected and excised for histological processing. The tissues were fixed in 10% formo-saline. The fixed tissues were transferred to graded series of ethanol and then cleared in xylene. The tissues were then infiltrated in molten paraffin wax in the oven at 58^{o} C and mounted on plastic cassette. Serial sections were obtained from a solid block of tissue at 5um, Sections were fixed on clean slides. The cardiac left ventricle were stained for Hematoxylin and eosin, Orcein , Van Gieson, Periodic Acid Schiff, Sudan Black B and Feulgen staining for the demonstration of general histology, elastic fibres, collagen fibres, glycogen accumulation, fat accumulation and DNA synthesis respectively. Slides were examined using light microscope. Photomicrographs were taken with a high definition digital camera Leica ICC50 (Leica Microsystems, England) mounted on a microscope Leica DM 750 (Leica Microsystems, England).

Statistical Analysis of Results

SPSS (Version 20) statistical analysis software was used to analyse the results and test for testing differential hypothesis between parameters obtained from experimental groups. Specifically, mean and standard error of mean was compared between groups. One way ANOVA was utilized with LSD and Scheffe assortment after Post-hoc variance treatment. $p<0.05$ was taken for statistical significance.

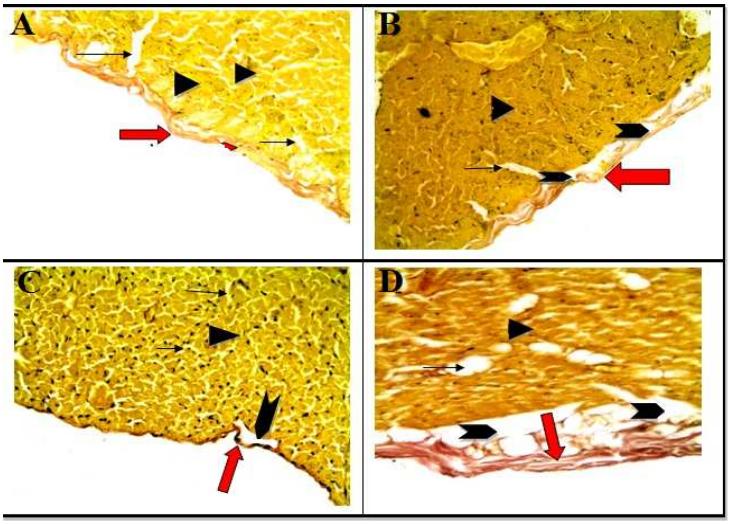
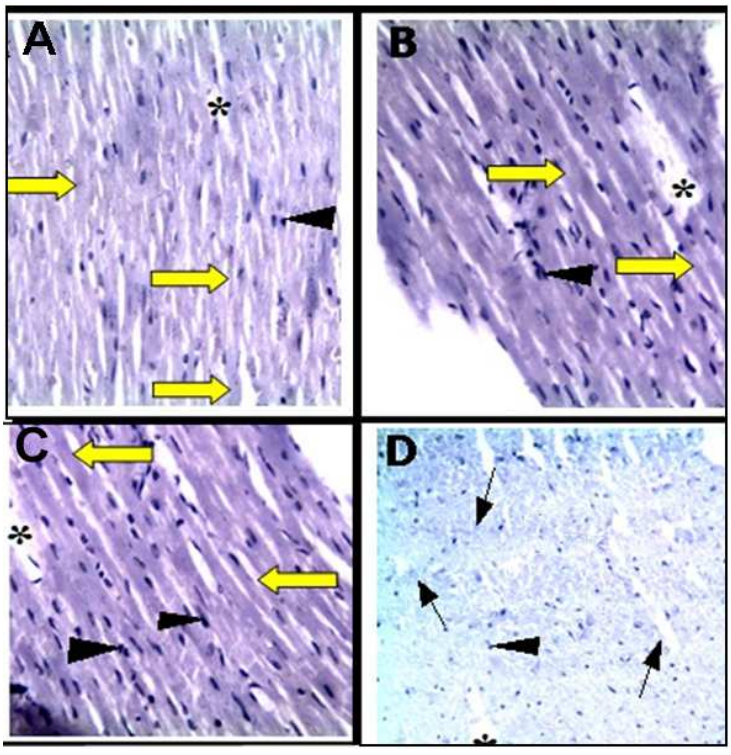


Fig. 4. Representative photomicrographs showing collagen demonstration in cardiac left ventricular epicardium for the experimental groups with Van Gieson staining. **(A)** (No laboratory inversion group) displays consistent collagen fibres firmly apposed to cardiac epicardium. **(B)** (8 days inversion group). Collagen fibres are inconsistently conformed and loosely attached to the cardiac epicardium. **(C)** (15 days laboratory inversion group) displays diminished collagen showing signs of loose apposition to the cardiac epicardium. **(D)** (22 days laboratory inversion groups) present with thickest collagen fibre assemblage and also possess loose attachment to epicardium. Red block Arrow- indicate collagen fibres, Flow arrow indicate collagen detachment from epicardium, Arrow head indicate cytoplasm. Line arrow indicate interstitial spaces. Magnification, x400.



RESULTS

Quantitative analysis

Morphometric changes

Initial body weight, final body weight, absolute cardiac weight and relative cardiac to final body weight was compared between groups A, B, C and group D. There was significantly higher absolute cardiac weight in group D (2.66±0.03g) compared to A (2.0 1±0.03g) (P< 0.05). We also found significantly higher relative cardiac weight only in group D (1.10±0.04g) compared to A (0.81±0.05g) (P< 0.05). Comparison of final body weight between the groups showed there are no significant animal weight changes with varying durations of inversion (P< 0.05) (see Table 1).

Cardiac function markers

Creatine kinase MB level increased with statistically significant differences in groups B (94.7 ± 20.01µg/L), C (116.34 ± 18.92 µg/L) and D (332.9 ± 23.55 µg/L) compared to values in group A (58.4 ± 13.55 µg/L) (P< 0.05). It was found that cardiac troponin I level increases significantly and progressively from A (585.76±41.12), B (602.3±49.15), C (1030.74±58.22) through D (558.19±42.64) at P< 0.05 as shown in Table 2.

Qualitative analysis

In early and mid-phases of the inversion (groups B and C), cardiac myocytes and interstitial spaces have haphazard orientation with numerous and diffuse nuclear appearance. The prolonged stage of inversion (group D) present the heart with syncytial-like myocardium with cardiac multinucleation and vacuolar spaces. Cardiac myocytes appear smallest in D. There are evidences of dividing nuclei in group D (Figs. 1 and 2). DNA material was demonstrated in all the groups but group D shows DNA abundantly compared to group A (Fig. 3).

Inversion (groups B, C and D) resulted in collagen fibers being partly detached from the outermost epicardium. The early and

Fig. 5. Representative micrographs showing bat cardiac left ventricular elastic fiber demonstration in experimental groups (A, B, C, and D) with Orcein staining. **(A)** (No laboratory inversion group) displays regularly and moderately stained elastic fibres. **(B)** (8 days laboratory inversion group) Elastic fibres with intensive staining relative to A. **(C)** (15 days laboratory inversion group) Elastic fibres with intensive staining relative to (A) also. **(D)** (22 days laboratory inversion group) Only faint amorphous elastin deposition was observed. Block yellow arrow- indicate elastic fibre, Arrow head indicates nuclei. Line arrow –elastin, star indicate interfibrillar spaces. Magnification, x400.

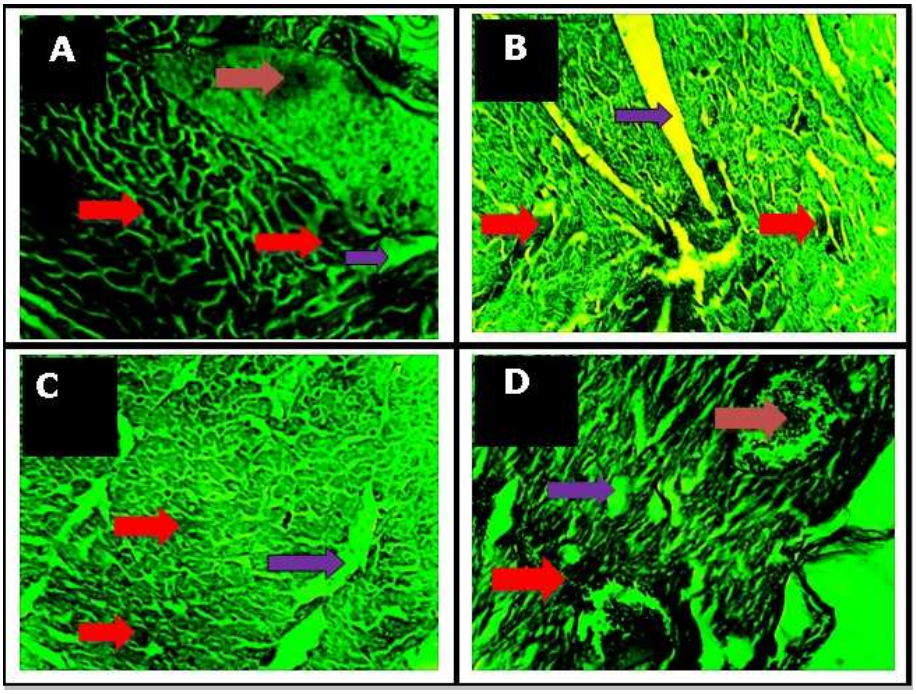


Fig. 6. Representative micrographs showing cardiac left ventricular lipid deposition in experimental groups with Sudan Black B staining. **(A)** (no laboratory inversion group) displays intensively stained lipid accumulation. **(B)** (8 days laboratory inversion group) shows sparse demonstration of lipid bodies. **(C)** (15 days laboratory inversion group) shows sparse demonstration of lipid bodies. **(D)** (22 days laboratory inversion groups) shows abundantly restored lipid bodies. Red arrows indicate lipid demonstration, brown arrows indicate extra-myocyte lipid vacuole, Purple arrows indicate interstitial spaces. Magnification, x400.

mid-phases of the inversion (groups B and C) showed collagen fibers were faintly demonstrated in the epicardium. The late phase of inversion (group D) presents the heart with the thickest layer of collagen fibers in the epicardium when compared with those present in the other groups (Fig. 4).

Elastic fibers staining intensity increased in the early and mid-phases of the inversion (groups B and C) while elastic fibres were not demonstrated in the prolonged phase of inversion (group D) which only had faint appearances of elastin molecules (Fig. 5).

The early and mid-phases of the inversion (groups B and C) demonstrate notably diminutive and dispersed lipid deposition. The prolonged phase of inversion (group D) showed intense lipid demonstration and also showed extra-myocardial lipid vacuoles (Fig. 6).

In group A, glycogen deposition was intensive. Groups B and C have reduced glycogen deposition which was restored in group D (Fig. 7).

DISCUSSION

The increment in cardiac mass in response to inversion in bats found in the present study could have been potentiated by aortic regurgitation leading to ventricular distension and cardiac hypertrophy in the inverted state. It has been reported that hindlimb unloaded rodents developed reduced left ventricular mass (Bao et al., 1999) but Ray and collaborators (2001) reported that cardiac mass and function were not affected by exposure to simulated microgravity. Although, dearth of information exists as regards bat cardiac mass adaptation in the inverted state, the increasing mass of the bat heart would help to-

Table 1. Changes in body weight, absolute and relative cardiac weights

Experimental groups	Final Body weight (g)	Absolute cardiac weight (g)	Relative cardiac weight (g/100g) to final body weight
A	249.65 ± 4.35	2.0 1±0.03	0.81 ± 0.05
B	247.43±6.13	2.09 ± 0.04	0.83 ± 0.04
C	246.03±7.02	2.13± 0.12	0.89 ± 0.02
D	241.01±3.23	2.66± 0.03 [a]	1.10±0.04[a]

Values are mean ± SEM n=5 in each group. [a] statistically different from Group A values at P< 0.05 Weight changes were not comparable between the groups

Table 2. Creatine kinase MB and cardiac troponin I levels in the experimental groups

	Group A	Group B	Group C	Group D
Creatine Kinase (µg/L)	**58.4 ± 13.55**	**94.7 ± 20.01**	**116.34 ± 18.92**	**332.9 ± 23.55**
Cardiac Troponin I (ng/L)	**585.76± 41.12**	**602.3± 49.15**	**1030.74±58.22**	**1558.19± 42.64**

Values are mean ± SEM n=5 in each group. * statistically different from Group A (p<0.05)

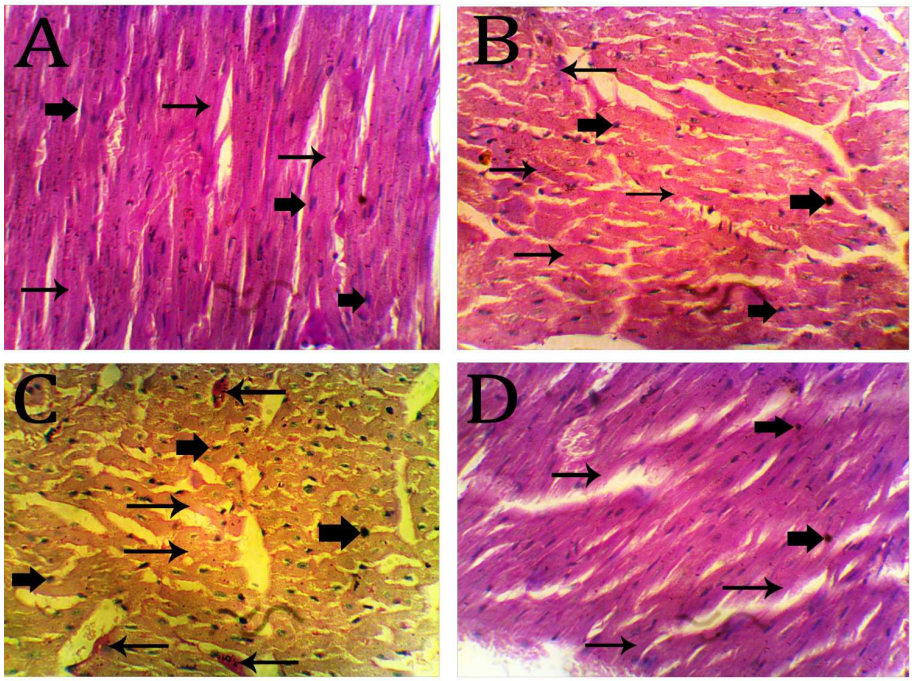


Fig. 7. Representative photomicrographs of left ventricle of the experimental groups (A, B, C and D) with Periodic Acid Schiff staining for glycogen demonstration. Glycogen deposition is substantially demonstrated in all the groups. **(A)** (No laboratory inversion group) Intensive glycogen accumulation. **(B)** (8 days laboratory inversion group) Less intensive glycogen staining relative to (A). **(C)** (15 days laboratory inversion group) Less intensive glycogen staining relative to (A). **(D)** (22 days laboratory inversion groups) Glycogen staining intensity similar to (A). Yellow block arrow indicates glycogen deposition, which are stained bluish. Black arrow indicates interstitial spaces. Magnification, x400.

wards propulsion of rapidly returning blood from the caudal region in the inverted state. The contradiction of the present study with previous studies might be due to the physiological impact of the inversion degree which is higher in bats compared to that in hindlimb unloaded rodents.

The prolonged inversion posed stress on bat cardiac myocyte and induced their disarrayed organization but compact tensegrit myocytes were observed in the latter phase of the present study. The influence of the gravitational stress was such that both the transversely and longitudinally orientated cardiac myocytes were found to be disorganized by the early and mid-phase of the inversion and could affect cardiac syncytial functionality (Williams et al., 1995; Shaw and Rudy, 2010). There were evidences of apoptotic cardiac myocytes which were thought to be due to volume overload and elevated end-diastolic left ventricular pressure that initiated the events of myocyte apoptosis (Sarkar et al., 2004). The interplay of cell damage and cell regeneration was imminent in this study. However, apoptosis and cell regeneration can result from combined effect of neurohumoral changes and mechanical factors in addition to increased cardiac mass, which triggered the heart to go to failure which has severely compromised bat cardiac function (Sarkar et al., 2004). Cell division and cardiac myocyte remodeling in the prolonged inverted groups buttresses the adaptive nature of bat heart in the face of profound gravitational stress.

The finding of cardiac regeneration following prolonged inversion in bat is in contrast to the believe that mammalian cardiac myocytes are terminally differentiated, that their life span corresponded to that of an animal and that hypertrophy is their only means of growth postnatally (Kajstura et al., 1998; Poss et al., 2002 Dimmeler et al., 2005; Rubert & Field, 2005). The dividing myocytes found in this study might have originated from cardiac stem cells or from migratory stem cells (Sarkar et al., 2004).

Pertaining to cardiac function markers demonstrated in the present study, it was found that prolonged inversion is associated with high serum level of creatine kinase MB and cardiac troponin I. Creatine kinase (CK) is an intracellular enzyme which catalyzes the phosphorylation of creatine using ATP and it is found primarily in the brain and muscle tissues (Welsh, 2002). Equally, its MB isozyme is particularly located in the heart (Welsh, 2002) while Troponin I is the inhibitory and contractile regulating protein complex of striated muscles, located periodically along the thin filament of the muscle (Welsh, 2002). The serum levels of these enzymes are often increased in situations such as myocardiac infarction and traumatic injuries to the heart muscles. The elevated serum creatine kinase MB and cardiac troponin I as reported

in this present study may be due to damages of the cardiac muscles occasioned by the interchange between the active state and the resting position of the bats. The pathophysiology of the mechanisms of the ischemic reperfusion injury is poorly understood and efforts are ongoing at determining the anatomical basis of the adaptational mechanisms as it affects the cardiac activities of the animals. The histological findings from the present study demonstrated regions of ischemic reperfusion injuries within the section areas which corroborate the findings of the elevated serum creatine kinase MB and cardiac troponin I activities analyzed. The reasons for the evidences of hypertrophied muscular changes seen on the histology may be due to the effect of the gravitational stress on the prolonged inversion in which the bats were subjected to.

The bi-phasic deposition of collagen and elastic fibres observed in the bat cardiac tissue in the course of prolonged inversion is considered as an adaptive capability. The reduced collagen deposition and increased elastic fibres deposition at the initial and mid-inversion phases, would increase the elastic modulus of the cardiac tissue and permit ventricular distension potentiated by gravitational stress. The role of cardiac fibrillar collagen network in cardiomyocyte alignment, stiffness and prevention of excess myocyte and sarcomere stretch would be reduced (Reiser, 1992; McCormick, 1998; Cleutjens, 1995) while elastic fibre role in elasticity, resilient recoil and structural architectures maintenance would increase in the cardiac tissue (Reiser, 1992; Cleutjens et al., 1995; Cleutjens et al., 1999). However, elastic modulus of the cardiac tissue at the latter phase of the inversion would decrease because elastic fibres were worn out and collagen deposition had increased. It is therefore inferred that the extracellular re-moderations observed was meant to rescue the heart from rupturing, since collagen increment in ventricular dysfunction or overdistension is often aimed at protecting cardiac myocyte integrity (Zile, 2002; Jugdut, 2003; Liu et al., 2003).

It is evident in our study that the bat heart is well adapted to continuously changing energy demands and that myocardial fuel utilization is highly influenced by the inversion conditioning. The reduced cardiac glycogen and lipid demonstrated in the early and mid-phase of inversion indicated that such phases demanded high cardiac energetics. Bat cardiac fatty acid oxidation would play an important role in cardiac energetics and would usually supply about 60% to 90% of myocardial ATP whereas the balance, 10% to 40% would usually come from glucose and lactate metabolism (Shipp 1961; Wisnecki, 1987), also cardiac tissues possess high turnover of fatty acids (Saks et al., 2006; Schaffer, 2003) while their changing composition may help clarify the extent of tissue energy demand or utilization (Christoffersen et al., 2003).The ameliorated lipid and glycogen component in the latter phase of inversion indicated that efficient energy utilization was acquired and this corroborates the histological finding of cardiac tensegrit remodeling also found in the latter phase of prolonged inversion (Huss and Kelly; 2004, Schneider et al., 2006). Previous studies (Ayettey et al., 1986: Navaratnam et al., 1994) have provided evidences that the bat heart is exceptional, in that it possesses greater numerical densities of lipids when compared to some typical terrestrial mammals and that such structural design would allow for more efficient metabolic activities and higher exercise tolerance. Our finding agrees with such findings, as lipid bodies were clearly demonstrated in the cardiac tissue of prolonged inverted bats.

The comparison between the inverted and non-inverted bats following capture was based on the presumption that the experimental bats were not subjected to prolonged inversion prior to capture. We consequently employed graded-time of laboratory inversion, to understand the trend of responses of bat during prolonged inversion since true control of non-inverted bats were not obtainable, meanwhile attempts to create non-inverted model in our laboratory proved abortive due to 100% mortality about two days in such position. We also ensured the bats were not hibernating prior to capture.

CONCLUSION

To the best of our knowledge, this work is the first to demonstrate that cardiac remodelling is associated with long-time inversion in bats. The remodelling involved both cardiac cellular and non-cellular elements. All examined parameters on the heart showed it was mechanically-stressed, partially damaged and latter recuperated by exhibiting surprising myocyte regeneration and remodelling characteristics, being mammalian. The varying structural make-up of tissues in long-time inversion may create confusion if investigating scientists do not take note of the duration of bat inversion in laboratory-based studies. We recommend bats as suitable models for understudying cardiac damage and regeneration.

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