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## ABSTRACT

Cardiac troponins (cTnT and I) are reliable biomarkers for detecting myocardial injury. New ultrasensitive assays have been able to detect baseline serum cTn levels even in healthy patients. Recently, an ultrasensitive cTnI immunoassay (Erenna IA, Singulex, CA) has detected increases from baseline cTnI levels due to drug-induced myocardial injury in rats, dogs and monkeys and baseline cTnI ranges in Sprague-Dawley (SD) rats. The present study was initiated to use the Erenna cTnI assay to further document baseline cTnI levels in normal control animals from multiple strains, including spontaneously hypertensive (SHR), SD, Wistar, Wistar-Kyoto, Fisher, SHR ovariectomized and SHR castrated rats. Baseline cTnI concentrations were detected in all 115 rats tested. Male rats had higher mean cTnI concentrations than females of the same strain. (SHR 26 vs 10 pg/ml) SHR male rats had the highest baseline levels (mean=26 pg/ml and the largest cTnI variability(3.9-70.2 pg/ml)). Increased cTnI levels were observed for castrated SHR compared to unaltered male SHR, (51 vs 16 pg/ml) while ovariectomized SHR had lower cTnI concentrations than normal female SHR (mean=4 vs 9 pg/ml).. These results show quantifiable differences in cTnI concentrations between strains, sexes and non-cardiac surgical alterations in control animals. Thus, there is a continuing need to develop an expanded knowledge base of control information in order to realize maximal potential from monitoring small changes in cardiac troponin by new ultrasensitive assays.

## INTRODUCTION

A major advance in the detection of myocardial injury occurred with the development of immunoassays that specifically measure cardiac troponin T (cTnT) and cardiac troponin I (cTnI) isoforms in plasma or serum. Cardiac troponins have become the gold standard biochemical means for diagnosis of acute coronary syndromes and further evidence has suggested that these biomarkers can also be used to detect non-ischemic myocardial alterations (Gaze and Collinson 2005).

Increased specificity and sensitivity of troponin immunoassays have expanded the utility of troponins from diagnoses of acute myocardial infarction to detecting subtle cardiac injury resulting from non-ischemic alterations (Mair 1997). Commercial cardiac troponin T and I assays have been used in nonclinical situations, and both cTnT and cTnI have been used as biomarkers to detect drug-induced cardiac injury in humans (Herman and Ferrans 2001) and animals (O'Brien 2008). There has been increasing interest in quantifying baseline concentrations of cTnI in healthy populations to develop reference ranges from which to measure these subtle increases. Though cardiotoxin-induced increases in cardiac troponins have been well documented in rats, data related to baseline concentrations of these proteins in rats has been limited or unavailable.

Recently, an ultrasensitive cTnI assay (Erenna immunoassay system) has been described (Todd et al. 2007) with an approximate 50-fold improvement in sensitivity over many currently utilized troponin immunoassays. This assay uses a single-molecule counting system that significantly reduces background noise and improves sensitivity of detection (Todd et al. 2007) and is one of only a few available assays that have the sensitivity and precision to detect baseline cardiac troponin concentrations in a healthy population (Wu et al. 2009a). The Erenna ultra sensitive cTnI assay has been utilized in studies as a means to monitor isoproterenol-induced increases in cTnI above baseline in healthy rats, dogs and monkeys (Schultze et al. 2008) and to establish the preliminary reference range of cTnI in healthy rats under standard laboratory handling conditions (Schultze et al. 2009).

In the present study, we utilized this ultrasensitive assay to further document baseline concentrations of cTnI in healthy normal control animals from multiple strains commonly used in cardiotoxicity studies, including SHR, SD, Wistar, WKY and Fisher strains. These studies investigated the degree of variability for ultra-sensitive cTnI baseline measurements of control animals between strains, sexes and surgical alterations (i.e. castration or ovariectomization) that are common to control animals in an effort to identify potential contributing factors to cTnI baseline variability.

By identifying these critical factors and taking into account expected cTnI variability in control animals being utilized in each study, researchers will be able to improve upon pre-clinical study design when using ultrasensitive cTnI as a biomarker to assess cardiotoxicity.

## MATERIALS/METHODS

**Animals:**  
Serum from 125 rats (32 male and 19 female spontaneously hypertensive (SHR), 36 male and 15 female Sprague-Dawley (SD), 5 male and 6 female Wistar, 5 male Wistar-Kyoto (WKY) and 7 male Fisher).

Rats were obtained from Taconic (Hudson, NY) or Harlan Industries, Inc (Indianapolis, IN).

Samples selected for analysis came from non-treated control animals that were part of several in-house studies. In a separate study, the baseline cTnI concentrations from groups of castrated male SHR and ovariectomized female SHR were compared to groups of normal male SHR and female SHR.

**Collection of Hearts:**  
At necropsy the entire heart was removed and fixed in 10% neutral buffered formalin. After fixation the heart was embedded either in glycol methacrylate resin (sectioned at a thickness of 1 µm and stained with toluidine blue) or paraffin (sectioned at 5µm and stained with hematoxylin and eosin). Heart sections were examined by light microscopy.

**Serum cardiac troponin- I measurement:**  
Cardiac troponin I was measured with the ultrasensitive Erenna immunoassay system (Singulex Inc., Alameda, CA) which uses a microparticle immunoassay and single-molecule counting in a capillary flow system. The assay is described in detail elsewhere (9). All samples were analyzed in duplicate.

**Statistical methods:**  
Outliers were identified as being greater than 4 standard deviations from the mean for each cohort included in the study (> 4 SD) and were excluded from further statistical analysis. For each cohort, the mean, SD, and range of values was determined.

Biological variation (%BV) was calculated for each cohort independently as the percentage of the mean for each cohort divided by the standard deviation of measurements of all individuals within the corresponding cohort.

Analytical variation (CVA) of the Erenna cTnI test (Singulex) was determined by calculating the percentage of the mean of three replicates divided by the standard deviation between replicates for each sample tested. Because data was not always normally distributed and because sample sizes in some groups were small, non-parametric Kruskal-Wallis (with Dunn's multiple comparison) and Mann-Whitney U-tests were used to evaluate significance of differences amongst various groups in rat serum concentrations of cTnI and cTnT. Alpha was set at 0.05 and all tests were two-tailed. The InStat and Prism (GraphPad Software, Inc., San Diego, CA) statistical software packages were used for all analyses.

Table 1. Cardiac TnI concentration (Mean ± sdev) and significance (p > 0.05) of various rat strains and sexes.

Clinical Parameters	Males					Females		
	SHR	SD	Wistar	WKY	Fisher	SHR	SD	Wistar
Mean [cTnI], pg/ml	26.0	4.8	3.9	5.6	17.5	10.0	2.4	1.0
± sdev	18.9	4.2	2.3	3.2	5.3	9.4	1.0	0.3
Range [cTnI], pg/ml	3.9-70.2	0.7-20.1	1.7-7.6	1.7-9.9	11.8-27.3	2.8-22.2	1.2-5.5	0.7-1.5
N	31	23	5	6	7	21	15	5
Biological Variation (%BV)	73	87	58	58	30	54	43	34
Analytical Variation (Mean, Range %CV)	5.3	6.0	7.1	3.6	2.1	3.9*	8.3	9.8
Statistical Matrix** (P < 0.0001)	SHR	SD	Wistar	WKY	Fisher	SHR	SD	Wistar
Males	SHR	---	0.0001	0.0009	ns	< 0.0001	< 0.0001	0.0004
Female	SHR	---	0.0001	0.0002	0.0002	---	---	---
	SD	---	ns	ns	0.0004	0.0162	0.0018	---
	Wistar	---	---	ns	0.0112	ns	0.0079	---
	WKY	---	---	---	ns	ns	0.0043	---
	Fisher	---	---	---	---	---	---	---
	SHR	---	---	---	---	---	< 0.0001	0.0007
	SD	---	---	---	---	---	---	0.0017
	Wistar	---	---	---	---	---	---	---

\* One poor quality sample was removed with CV of 69.3%, with this sample included mean analytical variation = 7.0% CV.  
\*\* Kruskal-Wallis with Dunn's multiple comparison's post-test (two-tailed, ns if P > 0.05)  
\* All pair-wise calculations are Mann-Whitney U-tests (two-tailed, ns if P > 0.05)

## RESULTS

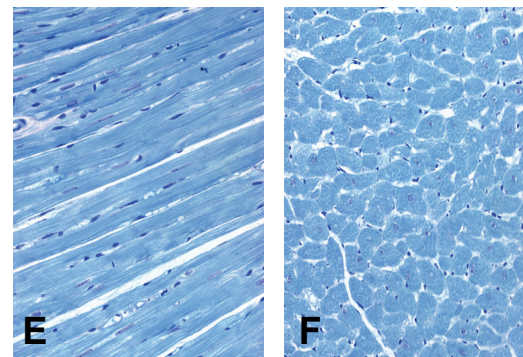
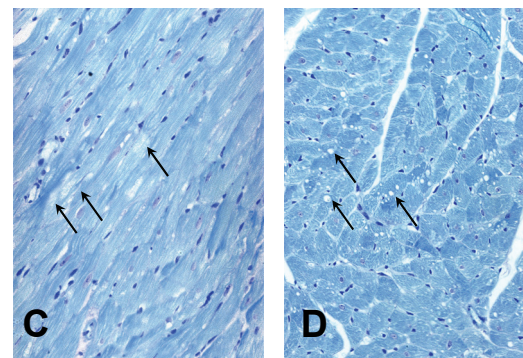
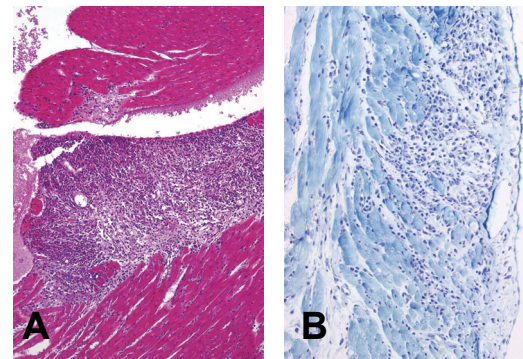


Figure 1. Light micrographs showing myocardial alterations in the hearts from SD male rats given distilled water orally for 14 days.

(A) Focal inflammation is observed in the left ventricular endocardium and a papillary muscle from a rat with an elevated level of serum cTnI (202pg/ml). H&E stain, X100.

(B) Focal inflammation is noted in the left ventricular epicardium from a rat with an increased serum level of cTnI (202pg/ml).

(C) Minimal myofibrillar loss in the left ventricular myocardium (arrows) from a rat with an elevated concentration of cTnI (142 pg/ml).

(D) Minimal cytoplasmic vacuolization in the left ventricular myocardium (arrows) from a rat with an increased serum concentration of cTnI (93 pg/ml).

(E & F) Normal left ventricular myocardial morphology from a rat with a baseline (non elevated) cTnI level.

Figures B-F, one-micron-thick sections of plastic-embedded tissue, alkaline toluidine blue stain, X 400.

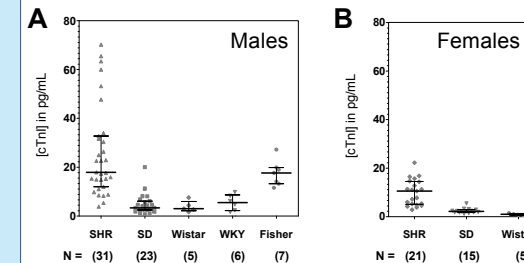


Figure 2. Distribution of cardiac troponin-I concentrations detected by the ultrasensitive Erenna cTnI Immunoassay in various strains of (A) male, and (B) female control rats. Lines indicate the median and interquartile range, with cTnI concentration in pg/ml. Each data point represents the mean of triplicate measurements from an individual rat. The number of rats per cohort is indicated.

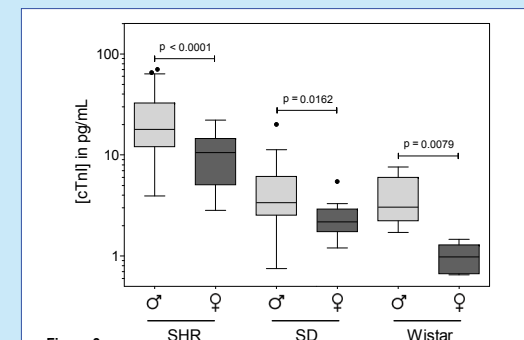


Figure 3. Comparison of serum cTnI concentrations between male and female SHR, SD and Wistar rats. Concentrations of cTnI are shown for male (light gray) compared to female (dark gray) SHR, SD and Wistar rat strains. Significance was determined using a Mann-Whitney t-test, with alpha set at p<0.05. Results are shown as median and interquartile range, with cTnI concentration in pg/ml.

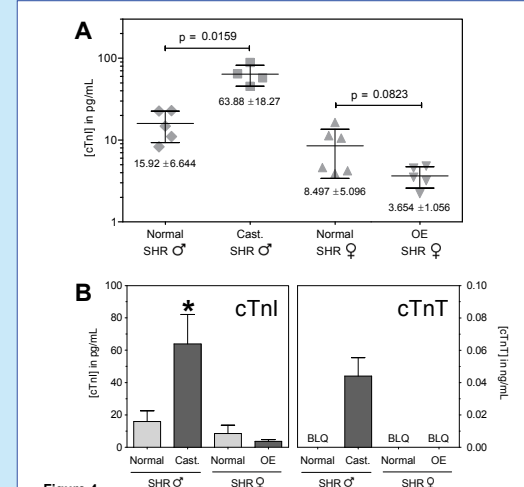


Figure 4. Effect of castration (Cast.) or ovariectomization (OE) on cTnI concentration in male and female SHR. (A) Distribution of cTnI concentrations between normal and castrated male SHR and normal and ovariectomized female SHR. (B) Comparison of baseline results utilizing cTnI (Erenna immunoassay) and cTnT (Roche Elecsys Stat immunoassay) in normal and castrated male SHR and normal and ovariectomized female SHR. Lines in (A) and bars in (B) indicate mean +/- SD. BQL=Below Quantifiable Limit. \* Mean value significantly than that of normal male SHR, normal and ovariectomized female SHR (P<0.05).

## CONCLUSIONS

• Cardiac troponins (T and I) have proved to be reliable blood biomarkers for identifying a variety of myocardial alterations in humans and animals.

• Recently, an ultrasensitive cTnI assay (Erenna IA) has been used to demonstrate increases in baseline cTnI resulting from drug-induced myocardial injury in rats, dogs and monkeys.

• The present study was initiated to use the Erenna cTnI assay to document baseline cTnI concentrations in normal control animals from multiple strains, including hypertensive, ovariectomized and castrated rats. Baseline cTnI concentrations were detected in all rats tested.

• Male rats exhibited higher mean baseline cTnI concentrations than females of the same strain.

• Spontaneous hypertensive male rats exhibited the highest baseline concentrations and the largest cTnI variability.

• Increased cTnI concentrations were observed for castrated SHR compared to unaltered male SHR, whereas ovariectomized SHR had lower cTnI concentrations than normal female SHR.

• These results show quantifiable differences in cTnI concentrations between strains, sexes and non-cardiac surgical alterations in untreated control rats. Thus, there is a continuing need to develop an expanded knowledge base of control information in normal rats, order to realize maximal potential from monitoring small changes in cardiac troponin by new ultrasensitive assays.

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