Reviewer's report

Title: Cardiac-Specific Catalase Overexpression Rescues Anthrax Lethal Toxin-Induced Cardiac Contractile Dysfunction: Role of Oxidative Stress and Autophagy

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Reviewer: Serguei Popov

Reviewer's report:

The article by Machender R. Kandadi, Xuejun Yu, Arthur E. Frankel, and Jun Ren describes cardiac contractile dysfunction induced by anthrax lethal toxin in mice and cultured cells. They demonstrate that cardiac-specific catalase overexpression rescues the toxin-induced oxidative stress associated with the induction of autophagy. The topic of the study is important for the anthrax field. The authors made original observations regarding the mechanical, proteosome and autophagic effects of LeTx in the WT and catalase overexpressing myocytes. However, some of the results represent nearly an exact duplication of the data they previously reported (for example, observation of autophagy in H9C2 cells). Overall the article is suitable for publication in BMC Medicine. The following corrections need to be made before it can be accepted.

General comments
(The manuscript is presented without line numbers, so I refer to pages only)

The article’s general problems are typical for this type of studies. The major question is a biological relevance of the obtained data. LeTx compromises the essential cellular functions, so the list of potential effects can be almost endless. Which of the observed effects are important for the outcome of disease? The answers to these questions are difficult and I don’t expect the authors to address all of them in a single study. Nevertheless, in order to avoid an over-interpretation of results, it has to be clearly stated that the intoxication murine model may not reflect many features of the infectious process. Next, many conclusion of the authors are limited to the in vitro experiments with cultured cells exposed to a single dose of LeTx, NAC and inhibitors for a single period of time.

The article does not report a susceptibility of the used mouse strain FVB to anthrax and LeTx. Did mice die upon administration of the toxin, and what was the toxic dose? How does the toxic dose relate to the doses used in the in vivo experiments? What was a rational of choosing a 2 mg/g challenge dose? If the mice are resistant to the toxin, what could be the relevance of the reported myocardial abnormalities to the death of infected mice?

The methods used in the study are appropriate, but not always well described. Many sufficient details are missing. Many of the statements are difficult to interpret exactly. For example, a sentence on p. 7 says: “... isolated myocytes from WT and catalase mice with or without lethal toxin treatment were loaded
with 10 µM of the nonfluorescent dye H2DCFDA”. It is completely obscure whether the myocytes were treated in cell culture or isolated from LeTx-challenged mice.

The majority of the data are sound and well controlled. However, in many instances the authors tend to over-interpret their results, even in contradiction with their previous results. For example, in the previous publication they referred to the LeTx-induced autophagy as “subtle although significant”, while in the current study they call it “acute”. They also advocate for the mitochondrial origin of ROS in contrast to the previous data implying the NADPH. Overall the manuscript adheres to the relevant standards for reporting and data deposition, but some of the references need to be added to give credit to previous studies.

The manuscript is plagued with typos and awkward expressions. Some of them are commented below.

Compulsory changes

P2. Infection by inhalation of B. anthracis spores can result in a mortality rate up to 80%. -- This is incorrect. The mortality rate is up to 100%. The historical inhalation rate mortality is 92% (Holty JE, Bravata DM, Liu H, et al. Systematic review: a century of inhalational anthrax cases from 1900 to 2005. Ann Intern Med. 2006;144:270-280).

P4. At least brief descriptions of toxin production and CAT mice generation need to be included. The manuscript requires at least a minimal introduction regarding their properties (what is the level of overexpression, etc.?). The FVB strain of mice needs to be characterized regarding its sensitivity to the toxins and infection. The effect of LeTx on cardiomyocyte viability in the described experiments needs to be presented.

P12. According to the data presented in Fig. 4, the effect of O2- production was “significantly attenuated by catalase overexpression”. In addition, “the effect of ROS generation [in] WT cardiomyocytes was mitigated by catalase overexpression”. --- It indicates that hydrogen peroxide was the primary cause of fluorescence in both cases. This is at odds with the mechanism in which fluorescence of oxyethidium due to formation of superoxide should appear before the superoxide dismutation to hydrogen peroxide. This fluorescence should be resistant to catalase. Literature data confirm that the formation of oxyethidium is inhibited by superoxide dismutase but not catalase and does not occur upon the addition of H2O2, peroxynitrite, or hypochlorous acid (Fink et al. Am J Physiol Cell Physiol 287: C895–C902, 2004). However, transient exposure of myocytes to H2O2 (30 µmol/L H2O2 for 5 min followed by 10 U/mL catalase for 5 min to degrade the H2O2) was reported to cause 66% increase in dihydroethidium signal compared with controls exposed to only catalase (Violla et al. Circ Res. 2007 Apr 13;100:1036-44). Therefore, the appearance of superoxide in myocytes could be induced by the external hydrogen peroxide. Whether or not it is the case upon LeTx treatment needs to be investigated. The authors need to explain why the catalase influences the process which is supposed to be catalase resistant.
Is the effect of catalase overexpression directly relevant to the degradation of intracellular hydrogen peroxide formed in mitochondria? The arguments in favor of mitochondria are indirect and other sources of hydrogen peroxide formation in the cell (NOX enzymes, for example) need to be considered. The NOX system can generate an extracellular hydrogen peroxide. Alternatively, the intracellularly released superoxide can be rapidly dismutated into hydrogen peroxide. Then the nonpolar hydrogen peroxide can diffuse through membranes and reach the extracellular space (Bedard, Krause, Physiol Rev. 2007;87(1):245-313). To my surprise, the authors indeed presented data in favor of the NADPH-induced ROS generation in the previous publication. They wrote: These data favor the notion that NADPH oxidase may play a crucial role in lethal toxin-induced cardiomyocyte contractile dysfunction. Why did they abandon this evidence? Please explain.

Figs 1, 2. Although the authors demonstrate statistically reliable difference in cardiomyocyte mechanics and Ca signaling, the biological significance of these changes is not clear. -- Please comment.

Fig. 3. What are the 3 gel lanes for each protein? The figure can be moved to the supplementary material because it does not reveal any positive result.

Fig. 4. There are dark corners in every image. What is the explanation of it? Were the whole images or their parts evaluated? In B and D the Y axes are labeled differently. Is it a relative fluorescence in D as well as in B?

In Fig. 4 C, what does the acronym DCF mean? Material and Methods refer to H2DCFDA or CM- H2DCFDA. Why did the authors limit the demonstration of ROS generation to one time point? -- please comment.

Quantitation of images needs to be described for this and other figures.

Fig. 6. The ubiquitination, chymotrypsin-like and caspase-like proteosome assays used in Fig. 6 need to be described. It is not clear what is presented in Fig. 6 A. Is it a western blot of what and using what antibody? What do the triplicate lanes mean? There is high variability within WT and CAT triplicates of a 60 kD band intensity. What does it reflect?

Data in page 13 need to be appropriately referred to the corresponding parts of Fig. 6 and 7. “Total” references to whole figures are insufficient.

Fig. 7. Panel A remains completely unexplained. What do the arrows point to? There needs to be higher magnification to see the punctuate expression of LC3. Images in rows 1 and 2 can be removed, because the merged image is sufficient and does not demonstrate any co-localization. Overall these data seem to be a duplication of the results in the previous paper.

The LC3 scoring system needs to be described. It is preferred to count the number of puncta per cell, not the number of cells with puncta.

The results of Fig. 7 C western blot are marginal and inconclusive. The figure
legend is useless to determine whether the experiments were performed with the cells isolated from LeTx-treated mice or the cells treated in culture after isolation. The legend does not say what cells were tested. The text on p.13 says about H9C2 cells but the figure legend statistics description refers to 6 mice per group. Does it mean that the western blot LC3 data were obtained independently from 6 toxin-challenged mice, not from H9C2 cells or isolated cardiomyocytes? It seems that LC3 WB quantitation is based on 3 repeats in the same gel in one experiment. The quantitation needs to be described.

The legend is further confusing with a description of the panels. It says, “D: Representative gel blots depicting expression of Beclin-1, Atg-7, LC-3 and GAPDH (used as loading control); E: Beclin-1; F: Atg-7; and G: LC3II”. Panels E, F, and G remain unexplained. Are those in E, F, and G also representative western blots? What is the origin of antibodies? It is not clear which lanes belong to LeTx and control in panel C.

P12. For some reason the authors consider O2- separately from ROS. It makes wrong impression that myocardium in vivo generates O2- while cardiomyocytes in culture generate other ROS but not O2-.

P13. Antioxidant treatment with NAC needs to be described in Materials and Methods. What was the purpose of using external hydrogen peroxide treatment? Do the authors believe that this situation is relevant to LeTx-treated myocytes? It is not clear if NAC was removed before addition of hydrogen peroxide? If not, NAC would obviously react with hydrogen peroxide and inactivate it. The NAC capacity to reduce externally added hydrogen peroxide is obvious and merely serves as a control.

P13. However, catalase significantly attenuated lethal toxin-induced increase in LC3-II levels without altering lethal toxin-induced responses in Beclin-1 and Atg-7 (Fig. 7). -- There are no detectable LeTx-induced responses in Beclin-1 and Atg-7 to be altered.

P14. ...autophagosome formation in ~26% of cells compared with < 2% in non-treated group. -- These data need statistical evaluation. The observations are limited one time point and LeTx concentration. It remains unknown whether the effect is concentration dependent.

P14. To further consolidate a role ROS in lethal toxin-induced autophagy, levels of Beclin-1, Atg-7 and LC3 were determined. Lethal toxin exposure elicited significant upregulation of LC3-II expression without affecting Beclin-1 and Atg-7. -- Why did the authors decided to duplicate (“consolidate”) the Fig. 7 data in Fig. 8? By the way, the LC-3 western blots are presented 3 times in Fig. 7C, D, and fig. 8A. In all cases they look substantially different. For example, in Fig. 7D the LC3-I band in predominant compared to LC3-II. The opposite is true in Fig. 8A.

Fig. 8. The NAC pretreatment needs to be described. Fig. 8 legend gives an impression that NAC was not removed after the pre-treatment.

In Fig. 8 legend, there have to be spaces between values and units.
Duplicate lanes in panel A are not explained.
The effect of NAC on LeTx is not confirmed by detection of ROS.
Fig. 8 E-J is too big and difficult to comprehend. I suggest moving negative results such as panels E and I to supplementary data.
Conditions of rapamycin pre-treatment need to be clarified. P. 14 refers to the pre-treatment, but Fig. 8 legend indicates co-incubation.
The conclusion on permissive role of autophagy in lethal toxin-induced cardiomyocyte contractile anomalies at the end of results section needs to be explained.

Discussion
The first paragraph of Discussion is very convoluted and needs to be simplified. The last sentence of it is especially confusing. It says about catalase- and lethal toxin-induced contractile responses. What catalase-induced responses do the authors refer to? It is also strange grammatically. Why is the dash used in “catalase enzyme – offered”?
The statement of devastating hemodynamic and cardiac anomalies following acute anthrax exposure [1, 12-14, 16, 30, 31] needs to be rephrased. First, what is “acute anthrax exposure”? If the authors mean anthrax as a disease, is there a non-acute, chronic anthrax? Secondly, what exactly does the “devastating” mean? Although the referenced studies revealed cardiac effects of the toxins likely contributing to the lethal outcome of infection, it needs to be clearly said that they did not prove a direct cardiac cause of patients’ or animals’ death in anthrax (although the heart ultimately stops in every death case). In Ref. 1 the patients died from gastrointestinal and lung complications. In Ref. 12, mild cardiac pathology was detected in the intoxicated rabbits without lethal outcome. Ref. 13 deals with the in vitro effects of the toxin. In Ref. 14 the uniquely LeTx-sensitive rats were used. All these details need to be taken into account instead of making a general, indiscriminating summary statement.
The authors are not the first to discover the autophagy-related effect of LeTx, and need to reference previous studies.
The statement about the overt autophagy displayed by the LeTx treatment does not seem to be in agreement with the presented results showing substantial variation of the subtle LeTx effects without detectable changes in other markers.
The limitations of the study need to be clearly stated. First, the presented data LC-3 deal only with the autophagosomal punctuation and the increased level of LC3-II. Next, the autophagy observations are limited to the in vitro-treated H9C2 cells or isolated cardiomyocytes. A common negative autophagy control with wortmannin was not used. Also the starved cells more sensitive to autophagy have not been tested. The same is true regarding the effects of catalase overexpression and NAC. The proteasome effects revealed by the authors also refer to the in vitro system. The intoxicated or infected animals have not been studied and it remains unknown if the full-blown autophagic process takes place in vivo. It also needs to be taken into consideration that LeTx was reported to
inhibit the PI3K pathway controlling the autophagy. Therefore the effect of LeTx on the autophagy can be quite complex, from initial stimulation at low concentration to inhibition at higher concentrations.

Minor Essential Revisions

Abstract.

Anthrax infection is a fatal pathological condition that often results in cardiovascular complications, depicting detrimental cardiovascular effects of toxins secreted by Bacillus anthracis namely lethal and edema toxins. -- Please rephrase.

P2. “toxin induced” requires a hyphen, “Zinc-metalloprotease” does not require a hyphen and a capitalization.

P2. PA binds the cellular receptors tumor endothelial marker 8 and gene. -- PA does not bind to the capillary morphogenesis gene, but capillary morphogenesis protein 2.

P2. The combination of LF and the receptor binding PA yields the highly cytotoxic lethal toxin [9]. -- The toxin is not highly lethal. It is not even a controlled substance. In 1954, Smith and Keppie wrote: The toxin is not particularly toxic.

P2. “Anthrax exposure” is a slang.

P2. However, the underlying mechanisms behind lethal toxin-induced unfavorable cardiac effects remain elusive. As a result, effective therapeutic remedy against lethal toxin-induced cardiac dysfunction is somewhat dismal at this time. -- This is a misstatement. An effective toxin inhibitor would be sufficient without knowledge of the exact mechanism.

P3 and other pages. The toxins’ acronyms are defined but not used in the rest of the manuscript. The LC3, Beclin-1, Atg-7 and GFP-tagged LC3 puncta are not explained when they first mentioned and are not on the Abbreviations list. The latter refers to LeTx as Anthrax lethal Toxin (Anthrax and Toxin are capitalized but lethal is not [?]).

P4. Recent evidence suggested that anthrax lethal toxin initiates ROS accumulation in particular generation of superoxide and other ROS in a variety of cells including macrophages and neutrophils [13, 19, 20]. -- These references include three cell types: macrophages, neutrophils and myocytes. This is not a variety.

Ref. 20 (Hanna PC, Kruskal BA, Ezekowitz RA, Bloom BR, Collier RJ: Role of macrophage oxidative burst in the action of anthrax lethal toxin. Mol Med 1994, 1(1):7-18) is not recent. Also, it is controversial. It claims the cytokine storm and oxidative burst of macrophages never confirmed by other studies. In the myocyte case, viability is only slightly influenced by LeTx-induced ROS.

P5. well-controlled does not require a hyphen.

P5 and throughout the manuscript. The abbreviations for hour are used
inconsistently as hr or hrs. Metric system recommends h.

P5. PBS saline is a tautology.

P5 and throughout the manuscript. Subscript symbols shall be used appropriately in chemical formulas.

P5. Studies was. -- Please correct

P6. Oil objective is slang.

P6. Metric symbols in 75-W lamp or 380-nm filter should be used without hyphens.

P7. OCT compound should be explained.

P7. Frozen sections incubated with DHE for 45 min at room temperature is nonsence. The authors probable mean thawed sections of frozen tissues.

P7 and further in the manuscript. “Catalase mice” is slang.

P7. Lethal toxin treatment requires a hyphen.

P7. KRH buffer needs to be explained.

P7. Measurement of ROS production is confusing. Which compound was used with cardiomyocytes, H2DCFDA or CM-H2DCFDA?

P 8 and 9. The volume in the proteasome assay reaction needs to be indicated.

P9. Full stop is required after “soluble fraction”.

P9. Cell supernatant (50 µg protein) was incubated in 50 mM Tris-HCl buffer… Please rephrase.

P9. Twenty four hours later, cells were visualized for autophagy using confocal microscopy [13]. -- Please explain the visualization procedure. Were they just observed?

P9. The rpm should be replaced with the corresponding g force.

P10. For analysis of autophagy, cells were visualized at 40x magnification. -- Please see above.

P10. 1 hrs. -- Please correct.

P10. … before gel band was developed and gel intensity was determined. -- There is no gel on the membrane after transfer and the intensity of the protein band, not a gel, was determined. Please rephrase.

P11. Acute lethal toxin challenge. -- What does “acute” means in this context?

P11. “the effect of which was either significantly attenuated” -- I guess the authors wanted to say “The effects of challenge were significantly attenuated.”

P11. … none of the other intracellular Ca2+ indices was altered by catalase
overexpression. -- What “other intracellular indices” are meant?

P12. Please correct “ROS generation WT cardiomyocytes” LC3-I and LC3-II should be written consistently with a hyphen or not.

P17. “…anthrax exposure [43]” needs to be replaced with “…anthrax toxin exposure [43]”

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I've no competing interests in relation to this paper.