Reviewer's report

**Title:** Effects of Ischemic Preconditioning On Ischemia/Reperfusion-Induced Arrhythmias by Upregulatation of Connexin 43 Expression

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**Reviewer:** G Salama

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Chen et al., investigate the changes in Cx43 expression in hypertrophied rabbit hearts after an ischemic episode and ischemic preconditioning. Although there are several studies on I/R and Cx43 and preconditioning and Cx43, and on hypertrophy and Cx43, the current study is new because it attempts to link preconditioning in hypertrophied hearts to changes in Cx43. There are numerous major concerns that reduce enthusiasm for the study: a) technical experimental concerns, b) serious grammatical errors and lack of clarity, c) unjustified interpretation of the data.

Major points:

1. The introduction focuses on clinical aspects of the problem but does not refer to previous studies on changes in gap junctions and Cx43 in IP, ischemia-reperfusion, and hypertrophy. The role of possible changes in gap junctions during these pathologies is not mentioned and it does not link changes in gap junction expression to arrhythmia susceptibility.

2. A 20% increase in heart to body weight in hypertrophy is used as criteria for cardiac hypertrophy. There is no justification for this rather mild hypertrophy and whether their findings apply to more severe hypertrophy. Would IP still be protective?

3. What is the link between decreased Cx43 expression and increased arrhythmia susceptibility (or Curtis-Ravingerova score)?

4. On page 11, the authors claim that IP plays a direct anti-arrhythmic role in hypertrophied myocardium during ischemia-reperfusion by maintaining the integrity of Cx43 formed gap junction channels (permeability and conductance) or maintain normal Cx43 coupling is not justified and could be false. There are no measurements of gap junction conductance or altered electrophysiological properties like conduction velocity or reentry caused by conduction blocks etc… Hence the significance of altered levels of antibody detection of Cx43 is not followed up by physiological measurements. The detection of greater levels of protein does not guarantee these channel proteins are functional and have any impact on electrical properties of the myocardium.

5. HE staining: is it H&E? The abbreviation should be defined; data with H&E staining should be shown or at least mentioned. Was H&E data used to verify
hypertrophy

6. Surgery: they should describe more clearly the surgical procedures, and detail whether body temperature, arterial blood gases, concentrations of serum electrolytes, basic hemodynamic parameters were checked and controlled during the in vivo studies.

7. What is the effect of a sham operation on Cx43 expression?

8. IP was achieved by a 5 min LAD occlusion followed by a 5 min reperfusion before the 30 min ischemia. The protocol for IP is rather surprising; most studied repeat the period of ischemia and reperfusion 3 times and a final period of 30 min ischemia is rather long and would typically be irreversible under physiological rates and temperatures. A reference should be provided to justify this protocol.

9. Curtis-Ravingerova should be referred to in the ‘methods section. However, the original C-R scoring is a 5-step scale, instead of the 10-step scale described in the Methods section. So this is a C-R scoring scheme? It is not clear if the arrhythmias were scored for the whole I/R period of 120 min or for a 30 or 90 min period. The 120 min should be dissected to smaller periods of time, and arrhythmia scores should be assessed for each period. At the very least, ischemia and reperfusion should be scored separately.

10. The methods include measurements of QRS duration, but the data are not presented.

11. The description of histological procedures is unclear. It should be more detailed and more proper – excision, fixation and preparation of the samples, both for electron microscopy and for immunofluorescence.

12. How was “connexin expression” analyzed? Was it merely a measurement of sarcolemmal or intercalated disc-Cx43 density? The Cx43 density should be measured at the ends of myocytes and in the lateral orientation along the sarcolemma. Lateralization of gap junctions is often a feature of ischemic myocardium. How were “Cx43 expression area” and “Fluorescence intensity” measured? Representative images and a clear description about where (whole tissue, whole cell, sarcolemma, intercalated disc, etc.) and how did they quantify their images is necessary.

13. The authors did not show how ST elevation and QRS duration changed as a function of time. One ST elevation value and no QRS measurements were shown; it would be important to show their changes during I/R, and reveal differences between these groups.

14. Despite a discussion of an arrhythmia scoring scale, in the Methods section, the table describing the arrhythmias does not report the scoring, just the incidence of arrhythmias. The scoring should be equally used to evaluate arrhythmias for ischemia and reperfusion separately, and should be compared to healthy and hypertrophic control groups.
15. Is it not clear from the Statistical analysis, where T test and where the ANOVA analysis were applied.

16. A correlation analysis between the arrhythmia score and the amount of Cx 43 is mentioned but not shown or discussed.

17. Cx43 expression area and conclusions about Cx43 expression should be re-interpreted because there is a lack of evidence: no Northern or Western blots or RT-PCR. Cx43 is known to be quickly translocated between several organelles under pathologic conditions such as ischemia. The changes reported in membrane connexin or gap junction plaque density may be a result of translocation of Cx43 molecules, rather than changes in expression levels.

18. In the Discussion, the authors claim that IP maintains effective spatial distribution of Cx43-based gap junction channels. This is not true, the amount of Cx43 in gap junction plaques, compared to the controls, remains significantly reduced in the IP group. The slight increase in Cx43 density in the IP group may be of no significance to any real functional change.

19. Afterdepolarization may be part of the arrhythmia mechanisms but it is entirely speculative.

20. The PKC discussion is unclear and is perhaps not needed; which PKC isoforms affect gap junction function?

21. The last paragraph about KATP channels should be justified.

22. How are hemichannels relevant to this study?

Minor comments

1. Abstract: 33 rabbit models of myocardial hypertrophy -> 33 rabbits with myocardial hypertrophy.
2. Methods: ECG recorded and the mean value was used… -> mean value of what?
4. Figure legends: instead of SEM, the abbreviation TEM should be used (as those images are not from scanning electron microscope)
5. Discussion: arrhythmias occurrence -> arrhythmia occurrence
6. PKC – badly defined, it is not phosphate kinase but protein kinase.
7. Fix: Coupling of Cx43 molecules – cells can be coupled, not Cx molecules.
8. “…shift from gap junctions to myocardial cell surface” – Do they mean ‘translocation from gap junction plaques, or intercalated discs to non-plaque membrane areas, or a translocation from the cell ends to the cell sides. Lateralization has been shown to occur in ischemia.

X I have concerns about the statistics
Declaration of competing interests:

I declare that I have no competing interests.