PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Glucocorticoid receptor expression in patients with cardiac arrest in the early period after the return of spontaneous circulation: A prospective observational single-center study
AUTHORS	Yu, Yanan; Tang, Ziren; Xie, Miaorong; Li, Jiabao; Hang, Chen-Chen; An, Le; Li, Chunsheng

VERSION 1 – REVIEW

REVIEWER	Granfeldt, Asger Aarhus Univ, Intensive Care
REVIEW RETURNED	09-Mar-2022

GENERAL COMMENTS	The study by Yanan al investigates glucocorticoid receptor expression after the return of spontaneous circulation. First of all the reviewer would like to acknowledge the work by the authors in completing a complicated study and trying to answer an important question; however some concerns exist. The title says after the return of spontaneous circulation in patients who experienced cardiac arrest: It is not possible to obtain return of spontaneous circulation without a cardiac arrest, hence do the authors need to include both return of spontaneous circulation and cardiac arrest in the title? Its interesting that only 544,000 people die from sudden CA in China. This must be heavily underreported when compared to the population size. Its unclear from the methods section whether the patients had an in-hospital or out-of-hospital cardiac arrest? The inclusion criteria ROSC 6 h after CA is a bit unclear. Please elaborate within 6 hours or after 6 hours? Page 7 line 112 the authors write both adrenaline and epinephrine Since ACTH and cortisol was analysed based on residual samples of heparin blood it is unclear how the blood sample for flowcytometry was collected? Was the samples taken at the same timepoint? Then further down it is stated that “Venous blood samples were collected in ethylenediaminetetraacetic acid tubes” This needs to be clarified. Was the study truly prospective? collecting samples from the laboratory seems more of a retrospective character? The statistical analysis section is insufficient. ACTH and cortical was skewed why the data was logarithmic transformed yet they were presented with medians and compared using mann-whitney? Why did the authors then transform the data? Page 7 line 171 The authors write “Patients who experienced CA” this makes no sense as all patients had an cardiac arrest? The following sentence makes no sense “Patient consent to

	participate was obtained prior to enrolment in this study." Patients with cardiac arrest could not give consent prior to the cardiac arrest. Be more specific. How was the sample size determined? And why only 40 controls? Please refer to both efigure 1 and the STROBE checklist in the supplemental material in the main manuscript. Normally only 3% of all cardiac arrests are caused by neurological disease. Please describe what it included in the category cerebral cause? MAP is included in table 1. At what timepoint is MAP collected? Please delete p-values from table 1. It would have strengthened the study if the authors had included a more relevant control group. This could have been patients admitted for other reasons at the emergency department. Of course patients who just had a cardiac arrest differ from healthy controls. The authors should include the timepoint for blood sampling/inclusion in the study. Do the authors have data on witnessed status and bystander CPR rates? In the abstract the authors define primary and secondary outcomes. Please also include this in the manuscript. It would have been more interesting if the authors have collected data over time instead of just one timepoint. In the discussion (line 251) the authors first write that the evidence for GCs is controversial. But then in the next sentence write that it improves survival. This is confusing. Also lines 256-258 is not backed by data. Line 281. The authors write that low GR expression is not matched by higher cortisol levels. Yet cortisol levels were higher? Do the authors mean ACTH levels?

REVIEWER	Yang, Wei Duke University Medical Center
REVIEW RETURNED	10-Mar-2022

GENERAL COMMENTS	This clinical study by Yu et al. examined glucocorticoid (GC) receptor (GR) expression in circulatory lymphocytes of patients who were successfully resuscitated after cardiac arrest (CA). They also measured cortisol and ACTH in the plasma. This study provides useful information for further understanding of the role of GC in CA. Below are specific comments. 1. Page 3, line 85. Please specify cell types in which GR expression is decreased. 2. Some information for the blood samples is unclear. When were blood samples collected for flow cytometry analysis and ELISA? At admission or 6 hours after ROSC? Was the same blood sample of each patient split for different analysis? On page 6, line 116, it stated heparin as anticoagulant, while on page 7, line 150, EDTA tubes were used. 3. Fig 1. and Table S3. The data related to T cells appeared difficult to understand. For healthy controls, the total T cell count is 1586. But, the sum of CD4+ and CD8+ T cell counts is only 662. The ratios of CD4+ T cells and CD8+ T cells also seemed too low. However, in the blood, most T cells are either CD4+ T cells or CD8+ T cells. Please explain. 4. Fig 2. The MFI values are in the range of 1-4, which appear to be very low. How were these values generated? Is it because of the specific flow cytometer used? Please explain. 5. Please add a flow cytometry gating strategy scheme with

	representative dot plots to help readers understand each cell population. 6. Discussion. In most studies that showed positive results, GC was used during CPR. Thus, it is unlikely that these beneficial effects of GC are primarily due to its anti-inflammatory properties. A more plausible mechanism is through GC's ability to improve hemodynamic stability during and early after CPR. Based on this, the data in the current study may have limited implications for GC use during resuscitation, but are more related to post-CA immune dysfunction. Therefore, it may be appropriate to have less discussion on GC use, but focus more on potential immune consequences of decreased GR in lymphocytes.

VERSION 1 – AUTHOR RESPONSE

Reviewer #1:
1. The title says after the return of spontaneous circulation in patients who experienced cardiac arrest: It is not possible to obtain return of spontaneous circulation without a cardiac arrest, hence do the authors need to include both return of spontaneous circulation and cardiac arrest in the title?
Thank you for the careful review and valuable suggestion. This study focused on patients in the early stages of post-resuscitation. We modified the title to reduce ambiguity, and modifications are shown in the revised manuscript according to your suggestion.

2.Its interesting that only 544,000 people die from sudden CA in China. This must be heavily underreported when compared to the population size.
Thank you for the careful review and valuable suggestion. Although the reference directly states that the number of sudden cardiac death (SCD) in China is approximately 544,000 annually. We considered cardiac arrest (CA) due to other causes but did not find precise data, so we used "over 544,000" in the manuscript.

3. Its unclear from the methods section whether the patients had an in-hospital or out-of-hospital cardiac arrest?
Thank you for the valuable suggestion. We have modified this question, and modifications are shown in the revised manuscript.

4. The inclusion criteria ROSC 6 h after CA is a bit unclear. Please elaborate within 6 hours or after 6 hours?
Thank you for the valuable suggestion. We have modified this question, and modifications are shown in the revised manuscript.

5.Page 7 line 112 the authors write both adrenaline and epinephrine
Thank you for the careful review. We have modified this question, and modifications are shown in the revised manuscript.

6.Since ACTH and cortisol was analysed based on residual samples of heparin blood it is unclear how the blood sample for flowcytometry was collected? Was the samples taken at the same timepoint?
7.Then further down it is stated that "Venous blood samples were collected in ethylenediaminetetraacetic acid tubes" This needs to be clarified.
Thank you for the careful review and valuable suggestion. We have modified these two questions, and modifications are shown in the revised manuscript.

8.Was the study truly prospective? collecting samples from the laboratory seems more of a retrospective character?
Thank you for the careful review. We looked up statistics books and literature on a similar study[1] for this question, and we think prospective studies are more appropriate. Because the retrospective study

is the outcome of the study that occurred at the beginning of the current study. In this study, subjects did not reach the study outcome (survival on day 28) at the beginning of the study. Glucocorticoid receptor expression was detected during this period. Different outcomes were compared after a certain period of follow-up.

Reference
[1] Vassiliou AG, Floros G, Jahaj E, et al. Decreased glucocorticoid receptor expression during critical illness. Eur J Clin Invest 2019;49:e13073.

9.The statistical analysis section is insufficient. ACTH and cortical was skewed why the data was logarithmic transformed yet they were presented with medians and compared using mann-whitney? Why did the authors then transform the data?
Thank you for the valuable suggestion. The primary purpose of log-transformation conversion is to reduce the absolute value of the ACTH and cortical data for easy calculation. After conversion, the distribution is still non-normal, so the non-parametric test method is selected. For this log-transformation problem, we also consulted a teacher specializing in statistics. The feedback is that this method is used for non-normal distribution data conversion and to convert non-normally distributed data into non-normally distributed or nearly customarily distributed data. The conversion is followed by routine statistical processing.

10.Page 7 line 171 The authors write "Patients who experienced CA" this makes no sense as all patients had an cardiac arrest?
Thank you for the careful review. We have modified this question, and modifications are shown in the revised manuscript.

11.The following sentence makes no sense "Patient consent to participate was obtained prior to enrolment in this study." Patients with cardiac arrest could not give consent prior to the cardiac arrest. Be more specific.
Thank you for the careful review and valuable suggestion. We have modified this question, and modifications are shown in the revised manuscript.

12.How was the sample size determined? And why only 40 controls?
Please refer to both efigure 1 and the STROBE checklist in the supplemental material in the main manuscript.
Thank you for the careful review and valuable suggestion.
The sample size was calculated by PASS15.0 software and a non-parametric test method. The median GR expression was 0.93 and 0.80 in the healthy group, and CA group, respectively, and the interquartile spacing was 0.1 and 0.3. According to the ratio of 1:2 between the two groups, when the test level was 0.05, and the confidence was 0.90, 105 samples were required, including 35 in the healthy group and 70 in the CA group. The number of people included in the two groups in this study was 40 and 85, respectively, which could meet the research needs.
We have added the method description, and modifications are shown in the revised manuscript.

13.Normally only 3% of all cardiac arrests are caused by neurological disease. Please describe what it included in the category cerebral cause?
Thank you for the careful review and valuable suggestion. We have explained this question in "Patient Characteristics," and modifications are shown in the revised manuscript.

14.MAP is included in table 1. At what timepoint is MAP collected?
Thank you for the careful review. The timepoint of MAP in the control group was in the morning. Although we tried our best to ensure the consistency of time, we could not avoid the situation of < 6h after ROSC. The systemic ischemia-reperfusion response in patients who have experienced CA, and the systemic system dysfunction. The body response is different from ordinary people. According to the early stage of ROSC in this study, MAP collection time of CA group was set as follows: the timepoint of MAP in the CA group was >6 hours and < 24 hours after ROSC.

15.Please delete p-values from table 1.
Thank you for the careful review and valuable suggestion. We have modified this question, and

modifications are shown in the revised manuscript.
16.It would have strengthened the study if the authors had included a more relevant control group. This could have been patients admitted for other reasons at the emergency department. Of course patients who just had a cardiac arrest differ from healthy controls.
20.It would have been more interesting if the authors have collected data over time instead of just one timepoint.
Thank you for the careful review and valuable suggestion. No researchers have done similar studies since this is a preliminary observational study. Therefore, we only measured the early changes of glucocorticoid receptors in peripheral blood mononuclear cells of patients with CA. A more relevant control group and a more extended period of dynamic observation will be more helpful in understanding the significance of GR expression in the clinical course of CA after ROSC. Your suggestion provides an essential idea for our future research. Your suggestion provides a fundamental idea for our future research. We have added your suggestion in "Limitations" modifications are shown in the revised manuscript.

17.The authors should include the timepoint for blood sampling/inclusion in the study.
Thank you for the careful review and valuable suggestion. We have modified this question, and modifications are shown in the revised manuscript.

18.Do the authors have data on witnessed status and bystander CPR rates?
Based on our collected information, 83.5% of the patients enrolled in this study received timely and effective CPR after CA.

19.In the abstract the authors define primary and secondary outcomes. Please also include this in the manuscript.
Thank you for the careful review and valuable suggestion. We have modified this question, and modifications are shown in the revised manuscript.

21. In the discussion (line 251) the authors first write that the evidence for GCs is controversial. But then in the next sentence write that it improves survival. This is confusing. Also lines 256-258 is not backed by data.
Thank you for the careful review and valuable suggestion. We have modified this question, and modifications are shown in the revised manuscript.

22. Line 281. The authors write that low GR expression is not matched by higher cortisol levels. Yet cortisol levels were higher? Do the authors mean ACTH levels?
Thank you for the careful review. In this study, plasma total cortisol levels were significantly higher in patients who experienced CA than in healthy controls ($P<0.001$), but ACTH levels were not. We have explained the question, and modifications are shown in the revised manuscript.

Reviewer #2:
1.Page 3, line 85. Please specify cell types in which GR expression is decreased.
Thank you for the careful review and valuable suggestion. We have modified this question, and modifications are shown in the revised manuscript.

2.Some information for the blood samples is unclear. When were blood samples collected for flow cytometry analysis and ELISA? At admission or 6 hours after ROSC? Was the same blood sample of each patient split for different analysis? On page 6, line 116, it stated heparin as anticoagulant, while on page 7, line 150, EDTA tubes were used.
Thank you for the careful review and valuable suggestion. We have modified this question, and modifications are shown in the revised manuscript.

3.Fig 1. and Table S3. The data related to T cells appeared difficult to understand. For healthy controls, the total T cell count is 1586. But, the sum of CD4+ and CD8+ T cell counts is only 662. The ratios of CD4+ T cells and CD8+ T cells also seemed too low. However, in the blood, most T cells are either CD4+ T cells or CD8+ T cells. Please explain.

Thank you for the careful review. In the study, the CD3 marker was used to label population lymphocytes. Although CD3+CD4+ and CD3+CD8+ T cells are the two most common T cell subsets, other lymphocytes of CD3+ are also present, such as CD3+CD4-CD8-, CD3+CD16+, CD3+CD56+CD16+ and CD3+CD56- T cells.[1-4] In addition, the dispersal points of related cells outside the scope of the gating strategy cannot be excluded.

References:
[1] Brandt D, Hedrich CM. TCRαβ+CD3+CD4-CD8- (double negative) T cells in autoimmunity. Autoimmun Rev. 2018;17(4):422-430.
[2] Wu Z, Zheng Y, Sheng J, Han Y, Yang Y, Pan H, Yao J. CD3+CD4-CD8- (Double-Negative) T Cells in Inflammation, Immune Disorders and Cancer. Front Immunol. 2022 Feb 10;13:816005.
[3] Zhou JG, Donaubauer AJ, Frey B, et al. Prospective development and validation of a liquid immune profile-based signature (LIPS) to predict response of patients with recurrent/metastatic cancer to immune checkpoint inhibitors. J Immunother Cancer. 2021;9(2):e001845.
[4] Liu S, Meng Y, Liu L, Lv Y, Yu W, Liu T, Wang L, Mu D, Zhou Q, Liu M, Ren Y, Zhang D, Li B, Sun Q, Ren X. CD4+ T cells are required to improve the efficacy of CIK therapy in non-small cell lung cancer. Cell Death Dis. 2022 May 6;13(5):441.

4.Fig 2. The MFI values are in the range of 1-4, which appear to be very low. How were these values generated? Is it because of the specific flow cytometer used? Please explain.
Thank you for the careful review. The instrument automatically generated the MFI values after the flow cytometry test was completed.

5. Please add a flow cytometry gating strategy scheme with representative dot plots to help readers understand each cell population.
Thank you for the careful review and valuable suggestion. We have modified this question, and modifications are shown in the revised electronic supplemental material (Supplemental Figure 2).

6. Discussion. In most studies that showed positive results, GC was used during CPR. Thus, it is unlikely that these beneficial effects of GC are primarily due to its anti-inflammatory properties. A more plausible mechanism is through GC's ability to improve hemodynamic stability during and early after CPR. Based on this, the data in the current study may have limited implications for GC use during resuscitation, but are more related to post-CA immune dysfunction. Therefore, it may be appropriate to have less discussion on GC use, but focus more on potential immune consequences of decreased GR in lymphocytes.
Thank you for the careful review and precious suggestion. We have modified this question, and modifications are shown in the revised manuscript.

VERSION 2 – REVIEW

REVIEWER	Granfeldt, Asger Aarhus Univ, Intensive Care
REVIEW RETURNED	25-May-2022

GENERAL COMMENTS	Thank you for addressing my concerns. Please include the sample size calculation in the manuscript.

REVIEWER	Yang, Wei Duke University Medical Center
REVIEW RETURNED	08-Jun-2022

GENERAL COMMENTS	I appreciate that the authors have made efforts to address the concerns. However, there are still some points that need clarification. 1. The response to CD4 and CD8 T cell populations in the human

	blood is not convincing. CD3+CD4+ and CD3+CD8+ are the major populations of T cells. They together would account for > 80% of all T cells (vs ~42% in the current study). The cited papers in the response just indicate that there are other small T cell populations. Are they any relevant papers showing CD4 and CD8 T cell populations in healthy human subjects comparable to the current study? 2. In the section of “strengths and limitation of this study”, the future tense is not appropriate. Also, the point 3 is unclear. 3. Page 7, line 138. GR antibody was from Bio-Rad not BD. 4. Supplemental Figure 2B, the Y axis of the second plot should be CD19. 5. Page 15, Line 291-298. Again, in most studies that showed positive results, GC was used during CPR. It is unlikely that the positive effects observed are through GC-GR interaction and its downstream gene expression. Thus, this part of discussion needs to be re-written. 6. As GC therapy for post-resuscitation CA patients has not been well supported by solid clinical evidence, the sentence “The assessment of GR expression in CA patients may help screening for those who are more sensitive to glucocorticoid therapy” should be deleted.

VERSION 2 – AUTHOR RESPONSE

Reviewer #1:
1.Please include the sample size calculation in the manuscript.
Reply: Thank you for the careful review and valuable suggestion. We have added information on the sample size calculation to the revised manuscript.

Reviewer #2:
1.The response to CD4 and CD8 T cell populations in the human blood is not convincing. CD3+CD4+ and CD3+CD8+ are the major populations of T cells. They together would account for > 80% of all T cells (vs ~42% in the current study). The cited papers in the response just indicate that there are other small T cell populations. Are they any relevant papers showing CD4 and CD8 T cell populations in healthy human subjects comparable to the current study?
Reply: Thank you for the careful review and valuable suggestion. We searched the literature again, but found very few studies on CD4 and CD8 T cell populations in healthy human subjects comparable to our cardiac arrest study. We covered the relevant literature as well as possible:
[1]Compared with healthy individuals, CD4+/CD3+ lymphocyte ratio on day 1 or day 3 after ROSC were 57.9 [49.4, 63.0] vs. 55.4 [46.5, 66.5] vs. 55.4 [50.2, 67.0])%.
[2]The median lymphocyte count was 2,042/mm3 [708–3,606], the CD4 T cell count was 858 [366–1,731], and that for CD8 T cells was 448 [166–1,181] for healthy subjects (n=30). For ICU controls (n=15, including six cardiac arrests), lymphocyte counts were 856 [298–2,246], 405 [100–1,265], and 160 [23–687], respectively.
Therefore, the above ratio is likely to occur in absolute lymphocyte counting with fluorescent microspheres. This difference may be due to the different flow cytometry counting methods. The fluorescent microsphere counting method is more accurate [3], so the percentage derived from absolute counting is lower than the relative ratio detected by the conventional flow method.

References:

[1]Qi ZJ, Zhang Q, Liu B, Shao H, Li CS. Early Changes in Circulatory T Helper Type 1, 2, and 17 Cells of Patients with Out-of-Hospital Cardiac Arrest after Successful Cardiopulmonary Resuscitation. Chin Med J (Engl). 2018 Sep 5. doi: 10.4103/0366-6999.239300.
[2]Carvelli J, Piperoglou C, Bourenne J, Farnarier C, Banzet N, Demerlé C, Gainnier M, Vély F. Imbalance of Circulating Innate Lymphoid Cell Subpopulations in Patients With Septic Shock. Front Immunol. 2019 Sep 20. doi: 10.3389/fimmu.2019.02179.
[3]Schlenke P, Frohn C, Klüter H, Saballus M, Hammers HJ, Zajac SR, Kirchner H. Evaluation of a flow cytometric method for simultaneous leukocyte phenotyping and quantification by fluorescent microspheres. Cytometry. 1998 Nov 1. doi: 10.1002/(sici)1097-0320(19981101)33:3<310::aid-cyto4>3.0.co;2-k.

2.In the section of “strengths and limitation of this study”, the future tense is not appropriate. Also, the point 3 is unclear.
Reply: Thank you for your careful review. We have modified the tense and point 3 in the manuscript.

3.Page 7, line 138. GR antibody was from Bio-Rad not BD.
Reply: Thank you for your careful review. We have indicated the GR antibody brand separately in the manuscript.

4.Supplemental Figure 2B, the Y axis of the second plot should be CD19.
Reply: Thank you for your careful review. We have revised the title of the Y axis in Supplementary Figure 2B.

5.Page 15, Line 291-298. Again, in most studies that showed positive results, GC was used during CPR. It is unlikely that the positive effects observed are through GC-GR interaction and its downstream gene expression. Thus, this part of discussion needs to be re-written.
Reply: Thank you for your insightful and very valuable suggestion. We have re-written the repsective part of the discussion.

6.As GC therapy for post-resuscitation CA patients has not been well supported by solid clinical evidence, the sentence “The assessment of GR expression in CA patients may help screening for those who are more sensitive to glucocorticoid therapy” should be deleted.
Reply: Thank you for your insightful and valuable suggestion. We have removed the respective sentence from the manuscript.

VERSION 3 – REVIEW

REVIEWER	Yang, Wei Duke University Medical Center
REVIEW RETURNED	12-Aug-2022

GENERAL COMMENTS	No more concern.