Response to reviewers' comments

ESPS Manuscript NO: 25012

Reviewer's Code: 02446204

1) In page 12, lines11-12, the phrase " …, who also demonstrated increased ROS production in DM (Houstis et al., 2006)" should be replaced by "…, who demonstrated the involvement of increased ROS production in insulin resistance in type 2 DM using a cell culture model and murine models (Houstis et al., 2006)". The work by Houstis et al. was not a clinical study and the work presented in the current manuscript may be the first report to show clinical relevance of the involvement of ROS in pathological progression of type 2 DM.
   The statement in the manuscript is changed as per reviewer's suggestion

2) In page 3, line 3, the word "follow" should be corrected as "follows".
   Corrected as per indicated in the manuscript

Reviewer's Code: 02445758

1) The authors should address the possible mechanism responsible for higher ROS levels in diabetic patients' neutrophiles. The authors might analyze the expression of NADPH oxidase in diabetic and non-diabetic neutrophiles.
   The following paragraph was added to elaborate the possible mechanisms which could potentially responsible for the higher ROS level in diabetic patients' neutrophils.

   '*The elevated oxidative stress which results from superoxide release by neutrophils in diabetic condition is well documented (Hand et al., 2007; Omori et al., 2008; Daoud et at., 2009; Wong et al., 2002). The assessment of neutrophil-mediated respiratory burst activity from the Hispanic diabetic individuals had demonstrated a significant rise in ROS outburst as compared to the normal group. Interestingly, upon treatment with PKC inhibitors and azithromycin the magnitude of the respiratory burst response found substantially reduced (Hand et al. 2007). Similarly, the high levels of glucose and AGE as well induced neutrophil activation, and subsequently, escalate the oxidative stress via RAGE-ERK1/2 pathway (Omori et at., 2008). It becomes clear that the harmful effect of ROS is very much linked with the augmented production of the advanced glycated end-products (AGE) and their cognate receptors (RAGE). The ligation between AGE and RAGE potentially increases the cytosolic ROS; facilitates mitochondrial superoxide production in the hyperglycaemic condition (Stefeno et al., 2016). Although the actual mechanism that governs the production and release of ROS in diabetic patients' neutrophils is still elusive, yet it does not negate the possible role of damaged mitochondria to generate an excess amount of superoxide which is fuelled by a sustained supply of NADH (Coughlan et al., 2009).'*

   It will be good supportive data if the expression of NADPH is explored. However, we are unable to conduct any additional lab works at this period of time due to financial constraint and lack of manpower.

2) The authors could conduct the experiments by measuring ROS level in cell lines such as HL-60 cells after incubation with high glucose concentration.
HL-60 cell line would be an excellent model to explore the relationship between neutrophils and ROS as HL-60 could be differentiated into neutrophils as well. However, we are unable to conduct any additional lab works at this period of time due to financial constraint and lack of manpower.

3) Part of data in Fig 2 is repeatedly presented in Fig.3.
Figure 2 reflects the ROS production over the time period which includes the maximal secretion of ROS and also a pattern of ROS release. However, figure 3 represented the maximal ROS production. Although, it could be partially seen as repeated, yet will provide a clear vision on overall ROS production among normal and diabetic individuals.

4) The authors should carefully discuss that ROS causes diabetes or diabetes leads to increase ROS generation.
The reviewer's concern is noted; possible caution is taken, and discussed based on scientific evidence.

Reviewer's Code: 00225280

1) Page 4, beginning of the second paragraph: Repetition. See end of the first paragraph of the paper.
The first sentence of the second paragraph has been reworded as below
'The prime effector function of neutrophil relies on its ability to generate ROS within the phagolysosome for the degradation of engulfed pathogens.'

2) Page 5: Regarding the study. Why did you not choose patients with the same basic characteristics? i.s. same medical treatment, duration of DM and family history? Did someone of the healthy volunteers receive any medical treatment, e.g., statins? Please refer in detail.
For your kind information, this was a preliminary laboratory study which was conducted by the postgraduate student for the period of three months. Patients and volunteers were recruited based on the fastest availability. Hence, they were recruited among the local lab staffs and relatives. Due to this reason, the stipulated standards were not adhered. The purpose of this study is to obtain preliminary data which will aid the conception of strategically planned research project.

3) Regarding the table, some medications are mentioned with the brand name. Please correct. The title of the table should change to demographics and characteristics of the patients, instead of basic details.
The names of medications were standardised. The title of the table was amended as per reviewer's suggestion.

4) Page 6, first paragraph last line: immediately OR within 2 hours?
The statement has been changed as below
'Peripheral blood samples were processed immediately after the collection.'

5) Page 7: Regarding statistical analysis. How was the study statistically designed? Why patients were 6 and controls only 3? Why did you use such a limited sample of patients and controls?
   This is a preliminary study where the samples were collected from the readily available sources within a short period of time.

6) Page 8: figure 1. This is an image not a figure. Last paragraph refers to "resting condition". The last nine lines however do not belong to the resting condition.
   The title of figure 2 is changed as below
   'Neutrophils from diabetic patients constitutively produced higher amount of ROS in resting and activated conditions.'

7) Page 12: You refer to a study by Alba-Loureiro et al which needs more discussion. Regarding the outcomes of the study: What about statins received by your patients? Could statins influence the results of your study? Please discuss in detail. What about herbals received by one patient? Please discuss in detail
   The scholarly work Alba-Loureiro is elaborated in the discussion as below
   'This observation was based on the assessment of neutrophil activities such as chemotaxis, phagocytosis, ROS production and microbial killing where these activities consume a substantial amount of ATP. Since diabetes affects the energy metabolism, thus it could also result in down-regulation or decrease in neutrophil activities.'

   We are unable to trace the type of statins received by patients and its effect on neutrophils. However, it has been reported that Fluvastatin significantly improved the lipid composition and peroxidation in plasma and neutrophils in hypertensive patients, and caused positive dynamics in their aggregation due to optimization of glycoprotein receptors.
   (Medvedev IN, Skoryatina IA. [The aggregation capacity of neutrophils in patients with arterial hypertension and dyslipidemia treated with fluvastatin]. Klinicheskaia meditsina. 2015;93(1):66-70.)

8) Lack references, report presence or absence of relevant references. Please, update your paper with recent references. Needs minor grammar polishing.
   New references have been added, and manuscript had been subjected to the extensive language editing.

Reviewer's Code: 00742314

1) The main concerns regarding the paper relies on the small sample size (6 diabetics and 3 controls), which limits their capability to generalize the findings, and lack of novelty in the results. Thus, my recommendation is against the publication.
   For your kind information, this was a preliminary laboratory study which was conducted by the postgraduate student for the period of three months. Patients and volunteers were recruited based on the fastest availability. Hence, they were recruited among the local lab

staffs and relatives. Due to this reason, the stipulated standards were not adhered. The purpose of this study is to obtain preliminary data which will aid the conception of strategically planned research project.

2) Regarding the abstract section, it could be better written. The methods is poorly described, since it lacks important information to understand how the study was performed. Also, the results could be improved;
The abstract has been rewritten with improved methods and results sections.

3) The introduction section did not approach properly the neutrophil respiratory burst in diabetes patients. It is not possible to know what the evidence is in the field with the introduction. Several studies could be explored throughout the introduction (PMID: 16959366; PMID: 18390927; PMID: 19519161; PMID: 12196480).
The suggested research works had been included and discussed in the manuscript as below

'The elevated oxidative stress which results from superoxide release by neutrophils in diabetic condition is well documented (Hand et al., 2007; Omori et al., 2008; Daoud et at., 2009; Wong et al., 2002). The assessment of neutrophil-mediated respiratory burst activity from the Hispanic diabetic individuals had demonstrated a significant rise in ROS outburst as compared to the normal group. Interestingly, upon treatment with PKC inhibitors and azithromycin the magnitude of the respiratory burst response found substantially reduced (Hand et al. 2007). Similarly, the high levels of glucose and AGE as well induced neutrophil activation, and subsequently, escalate the oxidative stress via RAGE-ERK1/2 pathway (Omori et at., 2008). It becomes clear that the harmful effect of ROS is very much linked with the augmented production of the advanced glycated end-products (AGE) and their cognate receptors (RAGE). The ligation between AGE and RAGE potentially increases the cytosolic ROS; facilitates mitochondrial superoxide production in the hyperglycaemic condition (Stefeno et al., 2016). Although the actual mechanism that governs the production and release of ROS in diabetic patients' neutrophils is still elusive, yet it does not negate the possible role of damaged mitochondria to generate an excess amount of superoxide which is fuelled by a sustained supply of NADH (Coughlan et al., 2009).'

4) Most references are too old. An update is necessary
Reference has been updated and included with recommended references

5) In the methods section: According to the authors, one of the inclusion criteria was age range of 60-80 years. However, one patient aged 82 years old (patient 3, table 1) was included; Age from the control group was quite different (30-50 years old) from that of the diabetes group. I guess it raises concern about the validity of the comparison, since neutrophil functions are altered with aging;
The inclusion criteria have been changed as 60-82 years. Thank you, we do agree that there will be slight changes in neutrophils activation among various age groups. This is already noted for the future study.

6) What about the Ethics Committee approval? Was there written consent? In the paper, only verbal consent was described
This study was conducted as a part of the Immunobiology postgraduate course that lasted for a period of three months. Thus, the sample size of diabetic patients and non-diabetic subjects was very limited. Since this was a preliminary / pilot study, ethical clearance was not obtained from the FPSK, UPM Ethical, and Research Committee. The diabetic patients (n=6) were recruited from the family members of Immunobiology postgraduate students whilst the non-diabetic subjects (n=3) were enrolled from FPSK, UPM. However, all participants of this study (patients and normal subjects) were briefed on the purpose of the study; agreed to donate blood samples and share the recently measured glucose level, family history & medications. They also signed a standard written consent form prior to the sample collection.

7) The assessment of neutrophil oxidative burst needs a better description. The experiments were performed in duplicate or triplicate? For how long neutrophils were stimulated with PMA?
Experiments were repeated at least twice, and the measurement of ROS was conducted instantly after adding PMA

8) Leishman staining needs to be described in the methods
The Leishman staining procedure has been added in the methodology

9) Why that time scale was chosen, with measurements until 50 seconds? Also, a 'radical escalation index' was cited in last sub-section of the results. Please, describe it better
ROS measurement was conducted until 50 seconds as the maximal ROS secretion of from normal and diabetic patients had fallen within 10 seconds.

10) In the figure 3, mean and SEM are presented, whereas mean and SD are presented in figures 1 and 2. Please, standardize that.
The error bars were standardised using SD.

11) Most of the discussion is not discussion; it is rather a literature review. Please, focus on discussing the study findings;
Discussion has been improved.

12) Please, keep the conclusion answering only the study aim. It is not a place to discuss methodology or give direction to future research.
Conclusion has been improved accordingly.