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# Activation of the MyD88 Pathway of Innate Immune Response to Recombinant BCG Malaria Vaccine Candidate: The Role of TLR-4

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**Abstract**

*Malaria, a highly devastating disease caused by Plasmodium spp. puts half the world's population at risk and has defied the ever-enhanced treatment, control and elimination strategies, necessitating the search for vaccine alternatives. A recombinant BCG (rBCG) expressing the merozoite surface protein 1C (MSP-1C) of Plasmodium falciparum was developed in our laboratory, which exhibited some immunomodulatory effects through undefined mechanisms likely activated by Toll-like receptor-4 (TLR-4). This study tested the hypothesis that TLR-4 mediates the attachment between rBCG and macrophages eliciting an immune response through the myeloid differentiation primary response 88 (MyD88) pathway. In this study, mice (n = 6 per group) were injected with PBS-T80, parent BCG or rBCG in the presence or absence of a TLR-4 inhibitor; TAK-242 and western blot analysis carried out on the macrophages obtained to determine the role of TLR-4 in the activation of the MyD88. The results obtained showed a significant increase in the expression of the proteins in favour of the rBCG construct compared to the parent BCG and PBS-T80. These increase was significantly inhibited in the presence of TAK-242 signifying the role of TLR-4 in the activation MyD88 pathway of innate immune responses against recombinant BCG malaria vaccine candidate, presenting for the first time an empirical evidence of the importance of TLR-4/macrophage attachment mechanism and its effects as a fore-runner in the MyD88 pathway of immune response to our rBCG expressing the MSP-1C of P. falciparum.*

***Key words:*** *Malaria, vaccine, TLR-4, Immune response, MyD88, immunisation, macrophages, MSP*

## INTRODUCTION

Malaria is a highly devastating, life-threatening disease caused by one of the species of *Plasmodium genus*, namely: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* transmitted to humans through the bites of infected female *Anopheles* mosquitoes (Bridget *et al.*, 2017; CDC, 2009). Among the causative agents of malaria, P. *falciparum* is mostly implicated as the cause of malaria in all regions of the world, responsible for about 90% of cases in the Sub Saharan Africa, about 50% of cases in most East Asian countries and 23.3% of malaria in the Americas (WHO, 2012). Malaria leads to high morbidity and mortality in these regions (WHO, 2013) with *P. falciparum* malaria being most devastating among children and pregnant women leading to many complications such as spontaneous abortion, premature birth, stillbirth and severe maternal anaemia (Batool, 2015). Malaria-related ailments account for 50% of in-patient admissions and up to 60% of outpatient visits in Africa and the global death of about half a million people annually (CDC, 2017a).

The vector of malaria parasite, the *Anopheles* mosquito is becoming resistant to all recommended classes of insecticides (CDC, 2017b) and *P. falciparum* is also resistant to all available antimalarials presently in use (Aung *et al.*, 2017) including a fast failing of the artemesinin combination therapies (ACTs) (Fairhurst and Dondorp, 2016). In the quest for alternatives to the antimalarial drugs and vector control, researchers have shifted the emphasis to eradication (Feachem and Sabot, 2008) since the answer to the question of control, elimination and possible eradication of malaria lies on an effective antimalarial vaccine (Peter *et al.*, 2010). Many researchers have beamed their search light towards the search for an efficient and effective malaria vaccine including the use of BCG as a vector of the merozoite surface protein (MSP). Due to the tremendous success achieved with its vaccination against the bacterium, *Mycobacterium tuberculosis* (Rodrigues *et al.*, 2011) and the proven safety, with minimal incidences of complications in the billions of people who received it worldwide

(Hanson *et al.,* 1995) and the fact that it is easily recognised and rapidly phagocytosed by phagocytic cells (Mandavi and Rajiv, 2011), leading to increased specific adaptive responses (Djuardi *et al.*, 2010) and antibody formation (Yu *et al.*, 2012), BCG is being manipulated in vaccine development against other diseases other pathogenic organisms (Stover *et al.*, 1991) such as malaria (Rapeah and Norazmi, 2006). Agonists such as 19 kDa C-terminal region of MSP-1 on the surface of lysed merozoites are used in generating protective immunity against malaria (O'Donnell *et al.,* 2001). These MSP-1 based vaccine candidates have given courage as possible candidates of malaria vaccine development (Bisseye *et al.,* 2011) and are the subjects of this study. Our laboratory used the 19kDa MSP in attempting the recombinant BCG vaccine (rBCG) construct expressing the MSP-1C of *P. falciparum*. This construct was able to generate higher inflammatory response in macrophages (Dhaniah *et al.*, 2014) and elicited higher stimulatory effects of an enhanced phagocytic activity with higher production of and Nitric oxide by macrophages compared to the parent BCG (Rapeah *et al.*, 2010; Dhaniah *et al.*, 2014). The mechanism of the induction immunomodulatory effects of our recombinant BCG vaccine candidate had not been analysed (Dhaniah *et al.*, 2014) but is thought to be due to the effects on toll-like receptor-4 (TLR-4), among other things and this formed the backbone of this study (Zakaria et al., 2018). Toll-like receptor-4 is one of the 13 members of TLRs which are a group of pattern recognition receptor (PRR) components that recognise conserved pathogens structures, termed Pathogen-associated molecular pattern molecules (PAMPs) located on or in cells of the innate immune system. TLR-4 recognises exogenous molecules from PAMPs, especially molecules from gram-negative bacterial lipopolysaccharides (LPS) (Akira and Takeda, 2004), other ligands, such as viral proteins (Tal *et al.*, 2004) and glycosyl phosphatidylinositol (GPI), a glycogen moiety from *P. falciparum* (Gowda, 2007; Gun *et al.*, 2014). The activation of TLR-4 depends on a cascade involving LPS binding and transfer to MD-2/TLR-4 forming the of LPS/MD-2/TLR-4 complex through the use of cluster of differentiation 14 (CD14) protein (Ryu *et al.*, 2017) followed by channeling the reaction into either the MyD88-dependent or MyD88-independent pathways (Lee and Kim, 2007; Gill *et al.*, 2010) leading to cytokine release (Rosadini and Kagan, 2017).

Interactions between TLR-4 and its ligands lead to the induction of innate immune response such as the production of IFN-γ, IL-4, TNF-α and IL-12 and adaptive immune response such as the production of antigen-specific antibodies: IgG, IgG1 and IgG2a (Ailian *et al.,* 2017). In the MyD88-independent pathways MyD88 interacts with Interleukin-1 receptor-associated kinase-1 (IRAK1) (Dunne *et al.*, 2010), IRAK2 (Lin *et al.*, 2010) and other members of the IRAK family forming Myddosome complex and subsequent IRAK kinase activation. The sequence also activates the adaptor molecules TNF receptor associated factor 6 (TRAF6) which in turn activates transforming growth factor-βactivated kinase 1 (TAK1) through an interaction with TAB2/TAB3 adaptor proteins and IKKγ subunit of IKK-complex. TAK1 then leads to the activation of MAPK cascades (Landstrom, 2010). Through the use of a selective Toll-like receptor 4 inhibitor; TAK-242 which blocks the intracellular domain TIR Toll/IL-1 receptor of TLR-4 (Takashima *et al.,*
2009) and thus preventing the interactions of TLR-4 with its adaptor molecules, TIRAP TIR domain containing adaptor protein, this study evaluated the expression of MyD88 protein involved in one of the two signalling pathways of inflammatory responses against PBS-T80, BCG and rBCG.

## MATERIALS AND METHODS

### Ethics

All animal work in this study was carried out in accordance with USM Animal Ethics Committee guidelines and its approval.

### BALB/c Mice

BALB/c mice were purchased from the Animal Research and Service Centre (ARASC) Universiti Sains Malaysia and housed at the facility. The mice were provided with standard laboratory chow and water ad libitum.

### Preparation of BCG and rBCG cultures

The parent BCG (Japan) and the recombinant BCG expressing the MSP-1C of *P. falciparum* (rBCG) earlier cloned in our laboratory (Nurul and Norazmi, 2011) were cultured on a 7H11 agar (Becton Dickinson, USA) supplemented with oleic acid, albumin, dextrose and catalase (OADC) (Becton Dickinson, USA) incubated at 37$^{o}$C with 15 µg/mL of kanamycin (Sigma, USA) added to the rBCG culture. The two cultures were observed for contamination and growth monitoring for 2-3 weeks. When sufficiently grown, a single colony was aseptically picked from each culture and transferred to flasks containing 10 mL of 7H9 broth (Becton Dickinson, USA) supplemented with OADC with 15 µg/mL of kanamycin added to the rBCG culture.

The culture in liquid media was observed for any signs of contamination for another 2-3 weeks until the optical density (OD) of approximately 0.8 (A600 ≈0.8) was obtained. **Determination of Colony Forming Unit (CFU)** Prior to mice immunisation, the colony forming unit of both parent BCG and rBCG were determined. Ten millilitres each of BCG and rBCG was taken and centrifuged in separate tubes at 1500 x g for 10 minutes at room temperature, washed twice with PBS and the pellet resuspended in 1 mL DMEM. The colony forming unit of each sample was then determined from the absorbance reading of a spectrophotometer at 600 nm using the formula described by Norazmi and Dale (1997).

*O.1 OD = 4X 10⁶ CFU/mL*

**Mice Immunisation**

A total of 36 male BALB/c mice aged 4-6 weeks were used for the study. These mice were grouped into six groups of six mice each. Each group of mice was immunised intraperitoneally three times (3 weeks apart) with 200 µL of PBST80, 2 X10⁶ CFU of BCG in 200 µL of PBS-T80 or 2 X10⁶ CFU of rBCG in 200 µL of PBS-T80 intraperitoneally respectively, in the presence or absence of 0.5 mg/kg of TAK-242 administered intraperitoneally, based on (Yao *et al.,* 2013; Yonglin *et al.,* 2016), one hour before each immunisation. The mice were closely observed for any signs of adverse effects from the immunisations, the immunogens or the TLR-4 inhibitor. **Peritoneal Macrophage harvest**

The mice were sacrificed via rapid cervical dislocation three weeks after the last booster. The peritoneal macrophages were then harvested from the mouse as described by Ray and Dittel (2010). Briefly, immediately after sacrificing the mouse, each mouse was sprayed with 70% ethanol and mounted on the Styrofoam block on its back and pinned with pins. A pair of forceps was used lift the skin and a pair of scissors used to make an off-center skin incision over the caudal half of the abdomen and gently pulled back to expose the inner skin lining the peritoneal cavity and the abdominal wall was lifted with forceps. A 10 mL syringe was filled with ice-cold sterile 3% FBS and gently injected into the caudal half of the peritoneal cavity using a 27 G needle. The pins were gently removed and the entire body of the mouse was gently shaken and the peritoneum massaged for 10 seconds to dislodge any attached cells into the PBS solution. A 25 G needle attached to another 10 mL syringe in collecting the fluid which was deposited in tubes kept on ice, after removing the needle from the syringe. This process was repeated and an incision was further made in the inner skin of the peritoneum and a plastic Pasteur pipette was then used to collect the remaining fluid from the cavity. Care was taken not to contaminate the suspension with blood. The suspension thus collected was spun at 125 *x g* at 4°C for 8 minutes, the supernatant discarded and the cells resuspended in DMEM and cultured at 1 × 10⁶ total peritoneal cells/mL for 2 hours at 37°C in a $CO_2$ incubator after which, nonadherent cells were removed by gentle washing three times with warm PBS. Fresh DMEM added and the cells allowed another 2 hours at 37°C in a $CO_2$ incubator. The cells were then gently scraped using a sterile plastic scraper and transferred into a 15 mL tube and harvested by centrifuging at 125 *x g* for 5 minutes at 4°C using ice-cold PBS.

**Preparation of peritoneal macrophage cell lysate for protein analysis**

The peritoneal macrophage cells were placed on ice, the PBS aspirated and the supernatant discarded. Then 100 µL of ice-cold RIPA (0.4 M NaCl, 50mM Tris/HEPES pH 7.5, 1% NP-40, 0.1% SDS, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride and 0.05% protease inhibitor) lysis buffer was added to the pellet of 2 X 10⁶ cells overnight at 4°C. The next day, the lysate was centrifuged at 1650 *x g* for 20 minutes, the supernatant aspirated and placed in a fresh tube kept on ice and the pellet discarded. A bicinchoninic acid (BCA) assay was carried out to estimate the total protein concentration in the supernatant before being stored at -80°C until analysis.

**Western blot analysis**

The expression of MyD88 was determined via western blot analysis. The supernatant from the macrophages lysed with RIPA buffer were recovered from the -80°C freezer. The total cell lysates were then denatured with Laemmli buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.003% bromophenol blue and 5% 2mercaptoethanol). Electrophoresis of the lysate was then carried out on 10% polyacrylamide and then transferred to polyvinylidene difluoride membranes (GE Healthcare). The membranes were blocked for 1 h with 5% skimmed milk PBST blocking solution followed by incubation with rabbit anti-mouse antibody for MyD88 (Abcam, USA) overnight at 4 °C. This was followed by incubation with goat anti-mouse antibody conjugated to HRP for 1 h at 37 °C (Dako). Chemiluminescence was then generated by an ECL western blot detection reagent, as recommended by the manufacturer (GE Healthcare).

An anti-ß-actin antibody (Sigma) was used as the control. The intensity of each protein expression

was measured using the Image J 1.47 (National Institute of Health, USA). Briefly, the film image was inverted before the integrated density of each band was measured. The integrated density value (IDV) of each protein was then normalized with the integrated density of ß-actin to determine the mean relative intensity (MRI).

**Statistical analysis**

Statistical analyses were performed using the statistical package of social sciences (SPSS) software version 22. All Data were representative of 3 experiments; performed in triplicate and presented as mean ± standard error of the mean (SEM). All the data were analysed by one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. The *p*-value of < 0.05 was considered statistically significant.

## RESULTS AND DISCUSSIONS

**MyD88 expression in the peritoneal macrophages of mice immunised with recombinant BCG expressing the MSP-1C of *Plasmodium falciparum***

For its importance in the activation of reactions downstream of the TLR-4 activation in innate immune response and the modulation of the adaptive immune response, MyD88 was investigated in this study. A band of approximately 33 kDa (the expected size of MyD88) was observed in the macrophages harvested from the six groups of mice on western blot analysis (Figure 1A). MyD88 expression was found to be significantly higher in peritoneal macrophages of mice in the group immunised with PBS-T80, BCG or rBCG in the absence of TLR-4 inhibitor, TAK-242, $p$ <0.001. Furthermore, MyD88 expression in peritoneal macrophages from mice immunised with the construct, rBCG was the highest (0.996 ± 0.003 MRI), then those immunised with BCG (0.923 ± 0.012 MRI) followed and those immunised with PBS-T80 was lowest (0.862 ± 0.012 MRI) in the absence of TAK-242. MyD88 expression was significantly inhibited in the presence of TAK242 in the group of mice immunised with PBST80, $p$ < 0.001 and mean difference -0.567 BCG, $p$ < 0.001 and mean difference -0.633 and rBCG p < 0.001 and mean difference -0.617 in the presence of TAK-242 (Figure 1B).

When PRRs recognise and interact with a pathogen an immune response is generated via the MAPKinase pathways (Medzhitov and Janeway, 2002). TLR-4 signal uses both MyD88 and TRIF pathways for a poorly understood reason (Bryant *et al.*, 2015). In malaria infection, the production of pro-inflammatory cytokines was shown to be dependent on the TLR-4/MyD88 dependent signaling pathway of the macrophage which also modulates several symptoms of malarial (Suprabhat *et al.*, 2016). Due to the significance of MyD88 down the ladder of the TLR4-macrophage activation pathways, this study investigated its role in the exhibition of the downstream effects of the rBCG bondage with macrophage. The expression of the MyD88 protein in the presence or absence of TAK-242 was analysed. This study showed that MyD88 adapter protein expression was significantly higher in the mice group immunised with rBCG than in the control groups. These findings signify higher activation and better bonding between TLR-4 and the antigen presenting cells and thus increased downstream product synthesis and expression. These results were similar to the one on LPSinduced MyD88 adapter protein expression and MyD88 mRNA, which found a significant increase in the MyD88 protein and its mRNA when synovial fibroblasts from temporomandibular joint were exposed to lipopolysaccharide (Xuefen *et al.*, 2015). MyD88 expression was also induced by stimulation by pro-inflammatory cytokines such as IL-6 (Lord *et al.*, 1990), IL-12 (Timo *et al.*, 2000), IFN-α. (Wei *et al.*, 2004) and IFN-γ (Zarember and Godowski, 2002). Chronic depression and the consequent chronic inflammation on the background inflammatory cytokine release also led to overexpression of MyD88 gene seen in depressed patients (Hajebrahimi *et al.*, 2014). *In vitro* studies of TLR-4/MyD88 signaling also showed a profound induction of inflammation with the release of TNF-α and IL-1ß when RAW macrophages were infected with the lymphatic filariasis, Setaria cervi (Mukherjee *et al.*, 2017). The production of LPS-induced IL-4 was also found to be TLR-4-dependent generated via both MyD88-dependent and MyD88independent pathways (Sumanta *et al.*, 2009) was reduced in TLR-4 deficiency (Komai-Koma *et al.*, 2014).

This study on the proteins involved in the TLR-4 downstream pathways revealed that mice immunised with PBS-T80, BCG and rBCG led to MyD88 expression which was significantly higher in the group of mice immunised with rBCG than in the other groups. These results were similar to what was obtained in a study of MyD88 cytokine released which found that stimulation of bone marrow-derived macrophages by *Schistosoma mansoni* cercariae elicited TLR-4/ MyD88 activation via MEK/ERK/RSK and p38 led to rapid production of IL-10 (David *et al.*, 2015). Similarly application of saturated free fatty acid, palmitate on monocytes induced MyD88 expression in a TLR-4/MyD88 pattern (Rasheed *et al.*, 2017). The results obtained in this study

showed a significant inhibition in MyD88 expression in the presence of TAK-242. Similarly, an intraperitoneal administration of TAK-242 one and a half hours before exposure to some migraine inducers inhibited MyD88 expression in rat migraine models (Min *et al.*, 2018). TAK-242 inhibition of TLR-4 also inhibited LPS-induced MyD88 expression in lung ischemia-reperfusion injury (Zhou *et al.*, 2014) just as it inhibited MyD88 expression induced by high glucose condition (Peng *et al.*, 2017). Further analysis of the MyD88 expression was carried out via the inhibition of TLR-4 with TAK-242. The results obtained showed a significant inhibition of the expression of MyD88 in the presence of TAK-242. This finding was similar to the inhibition observed in the lungs exposed to cigarette smoke where TAK-242 suppressed the cigarette smoke-induced synthesis of MyD88 (Dan *et al.*, 2016). The result was also similar to the one obtained on pre-treatment with TAK-242 where a significant inhibition of the Myd88 expression was observed in coronary microembolisation induced injury in mice (Wang *et al.*, 2017). Inhibiting TLR4 expression by TAK-242 also led to the inhibition of methamphetamine-induced MyD88 expression (Si-Hao *et al.*, 2017). Essentially, these results show that TLR-4 is crucial to the activation of Myd88 signaling pathway and its subsequent effects which include release of cytokines by immune cells such as macrophages. For example, TNF-α and IFN-γ production in mice infected with *P. chabaudi* was significantly reduced in MyD88 deficiency (Franklin *et al.*, 2007) while TNF-α and IL-12 production were significantly inhibited by MyD88 down-regulation (Kolanowski *et al.*, 2014). MyD88 silencing also led to decreased TLR-4 signal transduction and reduced inflammation responses (Chaohui *et al.*, 2016).

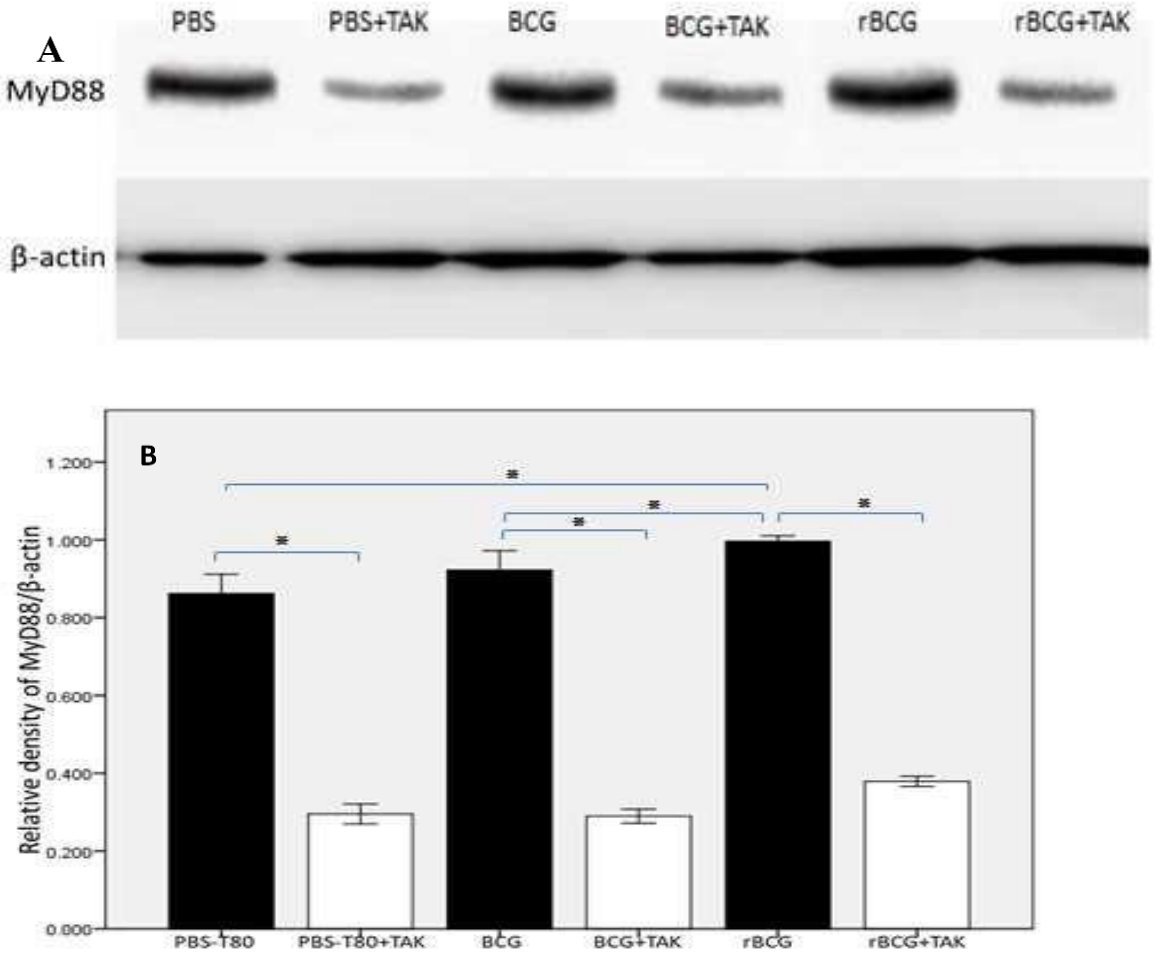


**Figure 1** (A) Representative result of MyD88 and ß-actin protein expression and (B) The relative density of MyD88/ß-actin protein in peritoneal macrophages of mice immunised with PBS-T80, BCG, or rBCG in the presence or absence of TLR-4 inhibitor, TAK-242. Data are presented as mean relative density of MyD88/ß-actin ± SEM for three independent experiments. *$p < 0.001$.

## CONCLUSION

This study has highlighted the role of TLR-4 on the activation of the MyD88 pathways of innate immune responses to recombinant BCG expressing the MSP-1C of P. falciparum. The results showed that the earlier observed enhanced immunomodulatory effects of our recombinant BCG malaria vaccine construct were due to the increased bonding between the macrophage and its ligand, the MSP-1C of plasmodium falciparum which led to activation of MyD88 which in turn led to the activation of the MAPKinases and other protein cascades with the resultant increased cytokine release.

## RECOMMENDATION

In order to have a better understanding of the mechanisms of initiation of the immune response against our vaccine candidate, there is need for further evaluation of other proteins downstream of the TLR-4-macrophage bonding. These studies could include the MAPKinases ERK1/2 and JNK1/2 as well as the proteins involved in the MyD88-independent pathway.

**Disclosure of Potential Conflicts of Interest** No potential conflicts of interest were disclosed.

**Author Contributions**

M.A.A. performed the research and analysed the data. R.S. designed the experiments and analysed the data. Both authors drafted and approved the final manuscript.

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