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Changes in the expression level of the genes involved in the innate and adaptive immunity of divers

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From time immemorial, humans had engaged in breath-hold diving. Developing the scuba (self-contained underwater breathing apparatus) in the last century has made humans increase the capabilities and efficiency of diving. Shallow diving is usually without side effects, but there may be a series of side effects called Decompression Sickness (DCS), which can even lead to severe neurological damages and death in deep and long dives. Scuba diving and its complications alter the pattern of many genes expression involved in innate and adaptive immunity. Researchers have reported various types of these changes in both the genomes of healthy and sick divers. This study surveyed the ten gene expression levels imported into immune responses like apoptosis and inflammation by real-time PCR in Iranian professional fit divers in steady-state. These genes were: Interleukins (*IL-6*, *IL-8*, *IL-10*), Tumor Necrosis Factor (*TNFα*), complement C3 (*C3α*), Tumor Necrosis Factor Receptor Type 1 - Associated Death Domain (*TRADD*), bradykinin receptor B2 (*BDKRB2*), rennin (*REN*), arachidonate 5-lipoxygenase (*ALOX5*), and prostaglandin-endoperoxide synthase 2 (*PTGS2*). The results showed that the expression levels of *TNFα*, *ALOX5*, *TRADD*, and interleukin genes increased, but *PTGS2*, *REN*, and *C3α* genes' expression levels did not change much. *BDKRB2* gene expression level also decreased.

[**Keywords**: Apoptosis, Decompression Sickness (DCS), Inflammation, Innate immunity, Real-time PCR, SCUBA diving]

Introduction

People worldwide are diving for recreational or professional purposes, which was made possible by developing the SCUBA (Self-Contained Underwater Breathing Apparatus) in the 1940s. Scuba gives divers the courage to dive for more extended periods and in deeper areas than breath-hold diving by providing breathing gas equal to the ambient air pressure[1]. Scuba diving is a traditional way to conduct underwater (submarine) operations with human intermediation. During diving, the divers must gradually adapt to high-pressure environments[2].

Technology and equipment improvement have led to increased diving efficiency and safety. However, scuba diving is always associated with risks. Decompression Sickness (DCS) is a severe threat to divers who use scuba. Most divers do not develop the disease. But in cases of the disease, the slightest physiological disorders such as skin rashes to hazardous clinical symptoms such as neurological harm, cardiac failure, and death are on both sides of the spectrum of symptoms in this complex systemic disease[2].

Comprehending DCS development from responses to the normal physiological states at high-pressure underwater environments is complementary knowledge. However, vascular endothelial acute reduction in function in scuba diving has been shown even in DCS's absence in the previous studies[3].

Increased expression of adhesive molecules, coagulation, and increased amounts of circulating microparticles promote pro-inflammatory development in scuba diving. Oxidative stress triggers such reactions that play essential roles in physiological homeostasis upholding. Several factors intensify oxidative stress during DCS, leading to redox homeostasis disruption, which ultimately leads to abnormal responses[4-7].

The increased partial pressure of oxygen (PO_2) stressed the circulatory system during diving and gas bubbles induced by pressure relief on the ascent to the surface during scuba diving. These factors play the most critical role in creating oxidative stress and developing inflammation after diving. Redox factors and oxygen-sensitive transcription factors cause inflammation. In other words, high PO_2 and oxidative

stress trigger these conditions[8-11]. As said above, vascular gas bubbles can also cause inflammation, and the greater the stress caused by decompression, the greater the severity of the inflammation[12]. Although some divers may get sick from the stress of diving, most divers are asymptomatic[1].

Researchers have proved that diving makes acute pathophysiological responses in the circulatory system. However, a few years ago, there was little known about permanent changes over time in healthy divers' circulatory systems[1]. Does diving repetition increase DCS risk by accumulating oxidative stress or creating protection through adaptation to new conditions[13,14]? However, this acclimatization has already been proven recently[15,16].

There is evidence that excessive diving without symptoms alters the pattern of genes expression involved in inflammation and oxidative stress. One such case was a study of healthy divers who had been diving for more than two months. Researchers observed that the interleukin-8 (IL-8) and lipocalin levels increased at the end of that study, and the secretory leukocyte protease inhibitor decreased[17]. These results showed pro-inflammatory and anti-inflammatory factors undergo a mild and balanced activation. This activation in diving supports circulatory homeostasis. Some animal studies have also proved that repeated attempts at high-pressure simulated diving reduce disease states in subsequent dives[2,18,19], and this is in line with previous results.

Current study also tried to determine whether the expression patterns of the inflammation and oxidative stress genes in asymptomatic healthy divers change in Iran. This study aimed to investigate genes and cells' behavior in healthy professional divers in steady-state compared to non-divers. In the current study, the expression activity of ten genes of disease-free divers was examined intending to determine these genes' behavior, biological pathways, and related cells disturbed by physiological stress in asymptomatic scuba diving.

Previous studies showed that before diving and in the steady-state, the genes related to apoptotic processes, innate immune responses, and inflammation are up-regulated[1,20-22]. This study aimed to identify some of the potentially long-lasting effects that appear in the body of asymptomatic professional divers as biomarkers.

The selected genes that were most involved in inflammation, apoptosis, and innate immune responses were: cytokines (Interleukins 6, 8, and 10 and tumor necrosis factor or *TNFα*), complement C3 (*C3α*), Tumor Necrosis Factor Receptor Type 1 - Associated Death Domain (*TRADD*), bradykinin receptor B2 (*BDKRB2*), renin (*REN*), arachidonate 5-lipoxygenase (*ALOX5* or *LOX5*), and prostaglandin-endoperoxide synthase 2 (*PTGS2*).

Materials and Methods

Research ethics

The human experimentation was done under the Islamic Azad University of Medical Sciences guidelines. The Committee on the Ethics of Human Experiments of the Islamic Azad University of Medical Sciences (Permit Number: 52-6106).

Control and study groups

The study group contained five experienced and certified male divers with diving experience of about 10 – 15 years. Their mean age was 30 years, and their weight was 80 kg. The control group consisted of 5 male non-divers with equal age, weight, and general physical features.

Sampling and RNA extraction

Blood samples for analysis were taken from the professional divers in steady-state and non-diving control persons. Venous blood (5 ml) drew into PAXgene tubes (PreAnalytix, Hombrechtikon, Switzerland). The RNA was extracted immediately after the sample collection.

According to the protocol of the manufacturer, the total RNA was extracted with the QIAamp RNA Blood Mini Kit (Qiagen). RNA samples were affected with DNaseI (Fermentas) at 37 °C for 30 min to eliminate contaminant DNA. Then, the DNaseI was inactivated and cleaned up the preparation with the guidance of the kit protocol.

Quantitative real-time RT-PCR and its data analysis

Total RNA was extracted separately from the control and study groups' blood samples. After that, 1 µg from each RNA was changed to cDNA by random hexamer primers (Fermentas) with RevertAid™ First-strand cDNA synthesis Kit (Fermentas). RT products were amplified using their specific primers (Table 1) and analyzed all PCR products by 2 % agarose gel electrophoresis to confirm the target genes' primers. All primers were designed within the regions with no homology with other known Gene Bank genes and analyzed using the Primer-BLAST from NCBI, Primer

Table 1 — Target genes and primers used in this study

Target gene	Primer name	Primer location (N)	Primer sequence (5′→3′)	Replicon size (bp)
TNFα[a]	TNF F TNF R	368 - 390 447 - 469	CCAGGGACCTCTCTCTAATCAGC CTTGAGGGTTTGCTACAACATGG	102
LOX5 [b]	LOX5 F LOX5 R	523 - 543 613 - 633	CTTGAGCATCGATGCCAAATG AACAGGTTCTCCATCGCTTTG	111
PTGS2 [c]	PTGS2 F PTGS2 R	172 - 192 245 - 268	GCTCAGCCATACAGCAAATCC GGTACAATCGCACTTATACTGGTC	97
C3α [d]	C3α F C3α R	4679 - 4702 4768 - 4788	GGAGTGGACTATGTGTACAAGACC ACCTCATCCGAGCCTGACTTG	110
TRADD [e]	TNFR F TNFR R	1306 - 1328 1371 - 1389	ACAGAGCCTAGACACTGATGACC CGCACGAATTCCTTCCAGC	84
BDKRB2 [f]	BDKRB2 F BDKRB2 R	189 - 210 268 - 288	TTCTGTCTGTTCGTGAGGACTC GCAAAGGTCCCGTTAAGAGTG	100
REN [g]	REN F REN R	430 - 451 527 - 547	TCTTCGATGCTTCGGATTCCTC ATTCCACCCACGGTGATGATG	118
IL6 [h]	IL6 F IL6 R	257 - 280 312 - 335	ACAACCTGAACCTTCCAAAGATGG TTCACCAGGCAAGTCTCCTCATTG	79
IL8 [i]	IL8 F IL8 R	76 - 99 191 - 216	CTTCCTGATTTCTGCAGCTCTGTG GGTCCACTCTCAATCACTCTCAGTTC	141
IL10 [j]	IL10 F IL10 R	442 - 463 512 - 533	GCTGTCATCGATTTCTTCCCTG GTAGATGCCTTTCTCTTGGAGC	92
β2M [k]	β2M F β2M R	156 - 177 88 - 108	CCCAGACACATAGCAATTCAGG GCTATCCAGCGTACTCCAAAG	90

[a] Tumor Necrosis Factor α, [b] arachidonate 5-lipoxygenase, *ALOX5*, [c] Prostaglandin-endoperoxide Synthase 2, [d] Complement C3a, [e] Tumor Necrosis Factor Receptor Type 1-Associated Death Domain, [f] bradykinin receptor B2, [g] Renin, [h] InterLeukin-6, [i] InterLeukin-8, [j] InterLeukin-10, [k] Beta-2-Microglobulin

Express software v.3.0 (Applied Biosystems), and Gene Runner software v.3.0 (Hastings Software, Inc). Bioneer Company (South Korea) synthesized the primers.

This study quantified the expression of target genes in the divers and non-diving control samples relative to the Beta-2-Microglobulin (β2M) housekeeping gene using specific primers. Samples for real-time PCR were separately prepared in 20 µl reactions containing 10 µl of 2X SYBR Green I master mix (Applied Biosystems), 1 µl of each RT product, and 100 nM of each primer. Then, by Applied Biosystems 7500 real-time PCR system, thermal cycling was performed using the following cycling conditions: 95 °C for 10 min, and 40 cycles at 95 °C for 15 s, 60 °C for 1 min. A dissociation stage followed each complete amplification stage; at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 sec. Using the $\Delta\Delta C_T$ method, each sample's relative expression level was measured[23]. From triplicate amplifications, the mean Threshold Cycle (mC_T) was obtained at three independent experiments and applied to calculate the number of transcripts using the 7500 Real-Time PCR Software v2.0.1 (Applied Biosystems).

Statistical analysis

The statistical analysis was conducted using Microsoft Excel 2016 (Microsoft corp.) and SPSS software, v.22 (SPSS Inc, USA). The *P*-value of < 0.01 was considered statistically significant for one-sample student's *t*-test analysis.

Results

Quantification of the inflammatory signaling pathways genes expression

Expression quantifying of the inflammatory signaling pathways genes, including *TNFα*, *LOX5*, *PTGS2*, and *C3α* in the divers and non-diving control samples, was determined relative to the β2M gene real-time RT-PCR. Gel electrophoresis (2 %) was used to assess the specificity of the real-time RT-PCR. The desired length of a single product was the result (Table 1). In addition, specific amplification during real-time RT-PCR was approved by the melting curve analysis (Fig. S1). $\Delta\Delta C_T$ method was used to determine the relative quantification of target genes compared to a reference gene. The expression level of the β2M gene was constant (with C_Ts

approximately equal) in the divers and non-diving control samples at different times. The TNFα and LOX5 mRNAs expression respectively increased on average 1.8 and 1.9-fold in the divers as compared to non-diving control samples (*t*-test; $P < 0.01$), while the PTGS2 and C3α mRNA expression didn't change in the divers as compared to non-diving control samples (Fig. 1).

Quantification of the apoptosis pathways genes expression

The expression quantification of the *TRADD*, one of the apoptosis signaling pathways genes, was determined relative to the β2M gene in the divers and non-diving control samples. Gel electrophoresis was used to assess the reaction's specificity, and the PCR product had the desired length of 110 bp. Also, specific amplification during real-time RT-PCR was shown using melting curve analysis (Fig. S1). The *TRADD* mRNA expression increased on average 1.9-fold in the divers as compared to non-diving control samples (*t*-test; $P < 0.01$) (Fig. 2).

Quantification of the vascular function genes expression

Expression of *BDKRB2* and *REN* from the vascular function genes was quantified relative to the *β2M* gene in the divers and non-diving control samples. The specificity of reactions determined with gel electrophoresis and PCR products had the desired length (Table 1). Specific amplification during real-time RT-PCR was shown using melting curve analysis (data Fig. S1). The *BDKRB2* mRNA expression decreased on average 2-fold in the divers as compared to non-diving control samples (*t*-test; $P < 0.01$), while the *REN* mRNA expression didn't change in the divers as compared to non-diving control samples (Fig. 3).

Quantification of the expression of the interleukins

Expression of the interleukins, including *IL-6*, *IL-8*, and *IL-10* in the divers and non-diving control

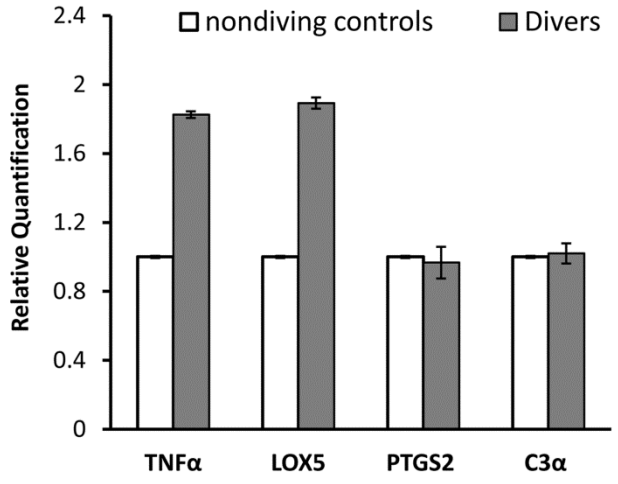

Fig. 1 — The relative expression level of *TNFα*, *LOX5*, *PTGS2*, and *C3α* genes in the divers and non-diving controls. For data normalization, the reference gene of β2M was used. Using the $\Delta\Delta C_T$ method, the relative expression levels of each sample were measured. The mean threshold cycle (mC_T) was obtained from triplicate amplifications and calculated transcripts. The student's *t*-test analysis estimated a significant difference (*P*-value of < 0.01). We have expressed the results as the mean ±SEM of three independent experiments

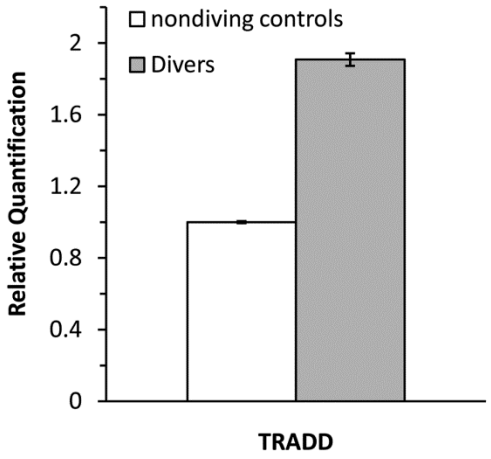

Fig. 2 — The relative expression level of the *TRADD* gene in the divers and non-diving control samples. The reference gene of β2M was used for data normalization. The student's *t*-test analysis calculated significant differences (*P*-value of < 0.01). We have expressed the results as the mean ±SEM of three independent experiments

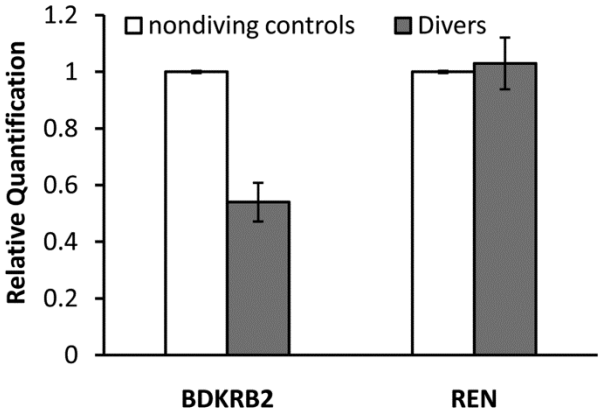

Fig. 3 — The relative expression level of *BDKRB2* and *REN* genes in the divers and non-diving control samples. The reference gene of *β2M* was used for data normalization. The student's *t*-test analysis calculated a significant difference (*P*-value of < 0.01). We have expressed the results as the mean ±SEM of three independent experiments

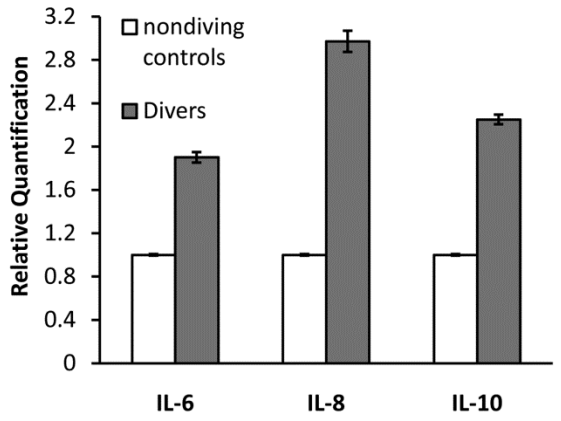

Fig. 4 — The relative expression level of *IL-6*, *IL-8*, and *IL-10* genes in the divers and non-diving control samples. The reference gene of *β2M* was used for data normalization. The student's *t*-test analysis calculated a significant difference (*P*-value of < 0.01). We have expressed the results as the mean ±SEM of three independent experiments

samples, were quantified relative to the β2M gene. Gel electrophoresis was used to determine the specificity of real-time RT-PCR that a single product with the desired length was resulted (Table 1). Specific amplification during real-time RT-PCR was assessed by melting curve analysis (Fig. S1). The *IL-6*, *IL-8*, and *IL-10* mRNAs expression increased on average 1.9, 3, and 2.2-fold, respectively in the divers as compared to non-diving control samples (*t*-test; $P < 0.01$) (Fig. 4).

Discussion

This study examined the potentially lasting effects on healthy divers' bodies after high scuba diving. For this purpose, the steady-state transcription levels of 10 genes for experienced divers and non-divers were measured and compared with each other. In this study, the divers were professional divers who had years of diving experience in the Persian Gulf at a depth of 10 to 40 msw (meter seawater). Also, the diving profiles produced in recreational diving may be similar; therefore, the results may be relevant to many people.

Of all the genes that play a role in innate immunity and inflammatory pathways, the four genes namely *TNFα*, *LOX5*, *PTGS2*, and *C3α* were studied and compared their expression in divers with non-divers. The two genes, *TNFα* and *LOX5*, significantly increased expression levels in divers. Contrariwise, in the two genes *PTGS2* and *C3α*, no significant differences were observed in divers' expression levels than non-divers.

TNFα is a pro-inflammatory cytokine that stimulates TRADD protein, which triggers the cascading activity of apoptosis. All of these activities are essential in cellular stress responses. TNF-α via TNFR1 (Tumor Necrosis Factor Receptor superfamily member 1A) and toll-like receptors activate the intracellular signaling pathways. These actions aligned with NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)-mediated transcription are necessary for cellular stress responses[24-26].

An increased expression level of the gene TRADD was also observed in this study. This gene encodes TRADD (death domain protein), an adaptor molecule that TNFR1 interacts with and mediates NF-κB activation and programmed cell death signaling[27]. NF-κB is a major transcription factor regulating the expression of genes involved in innate and adaptive immunity (such as interleukins 1 and 8)[28]. This study observed an increase in interleukins' expression, primarily IL-8, in divers.

The *LOX5* gene stimulates the pro-inflammatory response and is one of the contributing factors in Coronary Artery Disease (CAD), one of the world's significant problems in cardiovascular disease. CAD is a multifactorial disease for the involvement of genetic and environmental factors. The etiopathological relationship between these factors is not completely clear yet. Inflammation has been shown to play a crucial role in pathogenesis. It is one of the critical stimuli for developing atherosclerotic plugs, and 5-lipoxygenase is one of the triggers for this inflammation[29]. Therefore, it could probably be considered the increased expression of the *LOX5* gene as a precondition for cardiovascular disease.

The *PTGS2* gene is involved in inflammation and mitogenesis and causes inflammatory prostaglandins to be produced. The high expression level of *PTGS2* makes it to increased apoptosis resistance, cell adhesion, and phenotypic changes[30]. Increased expression of this enzyme (prostaglandin-endoperoxide synthase 2) can also indicate the onset and increase of inflammation in the body in response to cytokines. It has been shown that TNF stimulates prostaglandin biosynthesis in endothelial cells[31].

The *C3α* gene plays a role in the immune response and host defense. Types of this gene product's activities are involved in inducing pro-inflammatory and anti-inflammatory responses, activating macrophages, and so on, and in general, contributing to innate immune responses[32]. The increased

expression levels of *TNFα*, *TRADD*, and *LOX5* genes and no significant change in the expression levels of *PTGS2* and *C3α* genes could probably explain the existing regulatory factors that make homeostasis prevent oxidative stress development in healthy experienced divers.

Therenin-angiotensin and quinine-bradykinin system's genetic polymorphisms are crucial in protecting divers from vascular tensions[33]. Blood pressure, sodium-potassium balance, and fluid volume affect DCS's appearance regulated by the renin-angiotensin-aldosterone (RAAS) system, which has the most crucial influence[2]. Researchers have shown that plasma renin levels increase significantly after diving, causing persistent high blood pressure, renal damage, and cardiovascular disease in animal and human models[34,35]. This study aimed to see if this increased plasma renin level was stable in the blood plasma. However, any important changes in the gene *REN* expression level were not observed in the current study and attended a down-regulation of the gene *BDKRB2*. These genes are involved in DCS activation and vascular dysfunction overall. Therefore, not increasing the expression level of these genes in healthy divers can be a logical justification. However, the mechanisms involved behind the scenes and the effect of bradykinin on DCS deserve further investigation.

T lymphocytes and NK (Natural Killer) cells significantly affect innate and adaptive immunity[36]. Recent studies have shown that lymphocytes do not play an essential role in balanced physiological responses to oxidative stress. However, macrophages and dendritic cells are crucial in innate immunity. Oxidative stress also activates neutrophils[21]. Present study also gives the same results; however, the helper T cells' role in increasing interleukin-10 levels to maintain physiological homeostasis with TNF should not be ignored[37].

There are many problems with accessing essential biological materials in human studies, but peripheral blood is available and suitable for high or low throughput transcriptome analyses. Blood has interaction with all the human body's organs. So, the blood transcriptome can be considered as "an accessible window into the multiorgan transcriptome"[38].

One way to study gene expression is to look at the number of transcripts using high-throughput (such as microarray) or low-throughput (such as real-time PCR) transcriptomics technologies. However, in analyzing the data obtained from Real-time PCR and other transcriptome studies, it should be noted that gene expression and cell-related activities are potentially and indirectly predicted by measuring genes' transcription levels. Another essential point to note is the importance of low-throughput experiments, which continue to be the gold standard for validating high-throughput experimental results[39].

Conclusion

The genes and pathways most influential in the diver's stationary transcripts show a cellular state of conscious tolerance to external stress. These conditions can permanently affect before diving and indicate divers' physical health for subsequent dives. Sublethal oxidative stress changes genes' expression level after a long time scuba diving, causing lymphocytes to be suppressed, and the myeloid innate immune system is activated. As the last word, asymptomatic scuba diving affects the blood transcriptome significantly and stimulates innate immunity by activating macrophages, dendritic cells, and neutrophils. Researchers may use this study's results to identify healthy divers and people prone to some diving diseases as a prognosis. It should be also noted that these findings are currently in the hypothesis state, and further studies with larger sample sizes are needed to confirm these results.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at http://nopr.niscair.res.in/jinfo/ijms/IJMS_50(10)771-778_SupplData.pdf

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Conflicts of Interest

The authors of this manuscript, Ehsan Siami, Reza Mohammadi, and Vajiheh Zarrinpour, declare that they have no conflict of interest.

Author Contributions

ES did experiments and data analysis and wrote the manuscript; RM designed and supervised the study,

did data analysis, and edited the manuscript. VZ did data analysis and edited the manuscript.

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