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Oryzatensin-stimulated PBMCs Increase Cancer Progression In-vitro

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ABSTRACT

Oryzatensin (ORZ) can reduce potentially IFN-γ secretion by natural killer (NK) cells. Therefore, current study was designed to evaluate the effects of ORZ treatment on peripheral blood mono-nuclear cells (PBMCs) cytokine secretion, proliferation and also to evaluate vascular endothelial growth factor (VEGF) and Matrix Metalloproteinase 9 (MMP-9) expression in HEP-G2 cell line after culture with ORZ-stimulated PBMCs.

In this ex-vivo study, PBMCs from apparently healthy male volunteers (n=25) aged 20-30 were isolated by ficoll density gradient. Tetrazolium colorimetric test (MTT assay), ELISA test and real time PCR were performed to evaluate PBMCs proliferation, PBMCs cytokine secretion and the genes expression accordingly.

The results of MTT assay showed that ORZ significantly stimulated proliferation of the isolated PBMCs. The results also indicated that ORZ treatment significantly decrease and increase IFN-γ and IL-4 secretion by isolated PBMCs, respectively. Also, VEGF and MMP-9 expression significantly increased in HEP-G2 cells after culture with ORZ-stimulated PBMCs.

The previous studies have introduced ORZ-like peptide for pharmacological purpose and in this study we get to the conclusion that the administration of this peptide may change the immune system response and sensitize target populations to cancer.

Keywords: Bioactive peptide; Cell proliferation; Oryzatensin; Immune system; Peripheral blood mononuclear cell

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INTRODUCTION

Oryzatensin (ORZ) is a digestion refractory peptide extracted from rice with amino acid sequence of Gly-

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Tyr-Pro-Met-Tyr-Pro-Leu-Pro-Arg. The rice extracted bio-active peptide represents diverse physiologic functions such as activation of C3a receptor, contraction of the ileum and polymorphonuclear cells (PMN) stimulation.1,2 The carboxyl domain of ORZ activates C3a receptor (C3aR) and mimics C3a (one of the complement system components) function. The modification of ORZ was done to produce a digestion refractory peptide, ORZ-like peptide, with higher affinity to C3aR. The oral administration of the modified peptide induces potent physiologic functions such as anorexigenic, anti-analgesic, and anti-amnesic ones.[3,4]

Peripheral blood mononuclear cells (PBMCs) contain a mixture of natural killer (NK) cells, monocytes, T and B lymphocytes. NK cells can bind to complement system components by C3aR.[5] Activated receptors reduce IFN-γ secretion by these cells.[5,6] IFN-γ has been shown to have a critical role in Th1/Th2 skewing, so its reduction makes the milieu in favor of Th2 proliferation.[7,8] Th1 with IFN- γ and TNF-α can induce apoptosis in cancerous cells.[9]

Vascular endothelial growth factor (VEGF) is a regulator of angiogenesis during physiological and pathological conditions such as embryogenesis and tumor formation. It restores the oxygen supply to cells when blood circulation is inadequate. Matrix metalloproteinase 9 (MMP-9) belongs to MMPs family, which play a role in angiogenesis by extracellular matrix destruction. Both MMP-9 and VEGF have a pivotal role in cancer progression.[10,11]

To the best of our knowledge, there has been no study to evaluate the effects of immune response against ORZ on genes that have a key role in progression of cancer. Therefore, the present ex-vivo study was designed to investigate the effects of ORZ on PBMCs proliferation, PBMCs cytokine secretion as well as evaluation of VEGF and MMP-9 expression in Hep-G2 cell line after culture with PBMCs stimulated by ORZ.

MATERIALS AND METHODS

PBMCs Isolation, MTT Assay and Co-culture

Twenty five apparently healthy male volunteers aged 20-30 were involved in this study. The study protocol was approved by the ethic committee of Tabriz University of Medical Sciences (No. Tbzmed.rec-1393-3250) and written informed consent was obtained from each subject. In this study all tests on human participants were in accordance with the ethical standards of the institutional committee as well as the 1964 Helsinki declaration and its later amendments.

RPMI-1640 (Gibco, UK), DMSO (Gibco, UK), phytohemagglutinin (PHA) (Gibco, USA), fetal bovine serum (FBS) (Gibco, USA), ficoll-hypaque (GE healthcare, UK), penicillin/streptomycin solution (Gibco, USA), 96-well culture dishes (Sigma-Aldrich, USA), 25mm flask (Sigma-Aldrich, USA), Tetrazolium (MTT) powder (Sigma, Germany), heparinized tube (Greiner bio-one, UK) and ORZ with 98.8% purity (Biomatic, USA) were used in this study.

MTT test was performed in order to find the best ORZ concentration that could have the greatest effect on the PBMCs proliferation. Blood samples were collected in heparinized tubes and diluted with (1/2; v/v) RPMI-1640 containing antibiotics, then PBMCs were obtained by centrifuging in 1000 rpm for 25 minutes at 4°C on ficoll-hypaque. Isolated PBMCs were washed twice by RPMI-1640 containing antibiotic. Finally, the pellet was re-suspended in RPMI-1640 medium containing 10% FBS and the viability was assessed using 0.4% trypan blue staining. PBMCs were cultured in round-bottom 96-well plates. A number of 1×10^5 cells were seeded per well and incubated with different concentrations of ORZ (0, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} molar) and PHA (1.5%; v/v) for 24, 48 and 72 hours (Final volume: 200 µL per well). MTT solution was prepared in PBS (5 mg/mL) and then filtered through a 0.2 µm filter and at the end stored at 2-8°C. After each incubation period, 50 µL MTT solution was added and then re-incubation was done for 4 h then the plate was centrifuged in 3500 rpm for 5 minutes. The medium was removed and the formazan crystals were solubilized in 200 µL dimethyl sulfoxide (DMSO) and 25 µL sorenson buffer, which was then incubated in 37°C for 30 min. The absorbance was measured at 570 nm by ELISA reader (Bio Rad, USA). Triple measurements were performed for each subject and the average was used in data analysis.

After determining the best ORZ concentration that stimulates the PBMCs by MTT test, the blood samples were collected from the same subjects again and then PBMCs were isolated. The 4×10^6 PBMCs were cultured in exposure to ORZ (the final antigen concentration was 10^{-5} M). PBMCs were cultured without ORZ, as control group. PBMCs of both ORZ-

treated and control groups were separated from their medium after 72 h of incubation.

IFN-γ and IL-4 concentrations in separated media of both treatment and control groups were measured using ELISA kit according to manufacture instruction (Sanquin, Netherlands).

HEP-G2 cell line (a VGEF and MMP-9 high expressed cell line) was purchased from Pasteur Institute of Iran. In order to co-culture ORZ-treated PBMCs and HEP-G2 cells, 5×10^5 HEP-G2 cells were seeded in 25 mm flask and after 24 h of incubation, the cancerous cells were washed with PBS twice. Isolated PBMCs from both treatment and control groups were co-cultured with HEP-G2 cells (5/1 ratio). After 48 h incubation of cancerous cells with the PBMCs, the lymphocytes were removed then after The HEPG2 cells were detached using trypsin, the cells were stored at -70°C until RNA extraction.

RNA Extraction cDNA Synthesis and Real-Time PCR

Total RNA was extracted from harvested Hep-G2 cells at 48 h after incubation using RNA extraction Kit (Thermo Scientific, USA) according to the manufacturer's instruction. Extracted RNA was dissolved in nuclease-free water. RNA purification was assessed by Nano Drop Spectrophotometers (Isogen Life Science, Netherlands). A260/A280 and A260/A230 ratios higher than 1.8 and 2 were considered as acceptable purity, respectively. The integrity of RNA samples were assessed using 1% standard agarose gel and examining the ribosomal RNA bands.

Reverse transcription was carried out using the RevertAid first strand cDNA synthesis kit (K1622; Fermentas, Germany). Two µg RNA was used for the first-strand cDNA synthesis in a total volume of 20 µL according to the manufacturer's guidelines. Briefly, the reaction was cycled 40 times from 65°C for 10 min, 42°C for 60 min preceded by 70°C for 5 min and followed by 4°C for 5 min. For every reaction set, one RNA sample was prepared without RevertAid M-MuLV reverse transcriptase (RT reaction) to provide a negative control in the subsequent PCRs.

All PCRs were performed using the Corbett Rotor-Gene 6000 HRM (Corbett Research, Australia) in a total volume of 20 µL containing Power SYBR Green master mix (2x) (TaKaRa Ex Taq HS, Japan), Primer (0.4 nM), cDNA (20 ng/µl) plus nuclease free water. The mRNA-specific primers were designed using Oligo 7 v.7.52 software (Molecular Biology Insights, Inc, USA). The sequences are listed in Table 1. Each *β-actin*, VEGF and MMP-9 amplification were done in triplicates for each sample. *β-actin* was selected as an endogenous housekeeping gene. Forty five thermal cycles were performed in the following order: 2 min at 94ºC, 40 cycles, 94ºC for 15 sec and 59ºC for 1 min. Delta threshold cycle (CT) values were calculated in relation to *β-actin* CT values by the $2^{-\Delta\Delta CT}$ method, in which ΔCT represents the difference between the CT value of target genes and the CT value of *β-actin*.

Statistical Analysis

The data analysis was done using SPSS software version19.0 (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used to assess the distribution of quantitative variables. One Sample T test, Paired sample T test and repeated measure test were done for intra-groups statistical analysis. *p*-values less than 0.05 were considered statistically significant**.**

Table 1. Primers sequences used for defining genes expression by qPCR to evaluate the effect of oryzatensin-stimulated peripheral blood mononuclear cells increase cancer progression

Genes		Sequence (5' → 3')
***β*--actin**	Reverse	GTAGTTTCGTGGATGCCACA
	forward	TCCCTGGAGAAGAGCTACG
VEGF	Reverse	GTGGGTGGGTGTGTCTACAGGAA
	forward	CGCCACCACACCATCACCATC
MMP-9	Reverse	ATCCGGCAAACTGGCTCCTTC
	forward	ATTTCTGCCAGGACCGCTTCTAC

VEGF; Vascular endothelial growth factor, MMP-9; Matrix Metalloproteinase 9

RESULTS

The MTT Assay Results

The study results indicated that ORZ did not show statistically significant effects on PBMCs proliferation after 24 and 48 hours of incubation. ORZ at concentrations of 10-6 M and 10-5 M significantly increased the cells proliferation compared with the control group after 72 hours incubation. Therefore, the statistical analysis suggested that ORZ stimulate the PBMCs, time and dose dependently. The maximum proliferation response was seen after exposure of the PBMCs with ORZ in concentration of 10^{-5} M at the third time point (72 h) (p<0.05) (Figure 1).

The ELISA Results

IFN-γ levels in PBMCs soup of control and treatment groups were 8.50±1.1 and 0.61±0.21, respectively. Moreover, the IL-4 concentration in control and treatment groups was 50.11±11.23 and 80.41±12, respectively. ELISA results showed that ORZ treatment had resulted in significant decrease and increase in IFN-γ and IL-4 levels compared with the control group, respectively (p<0.05).

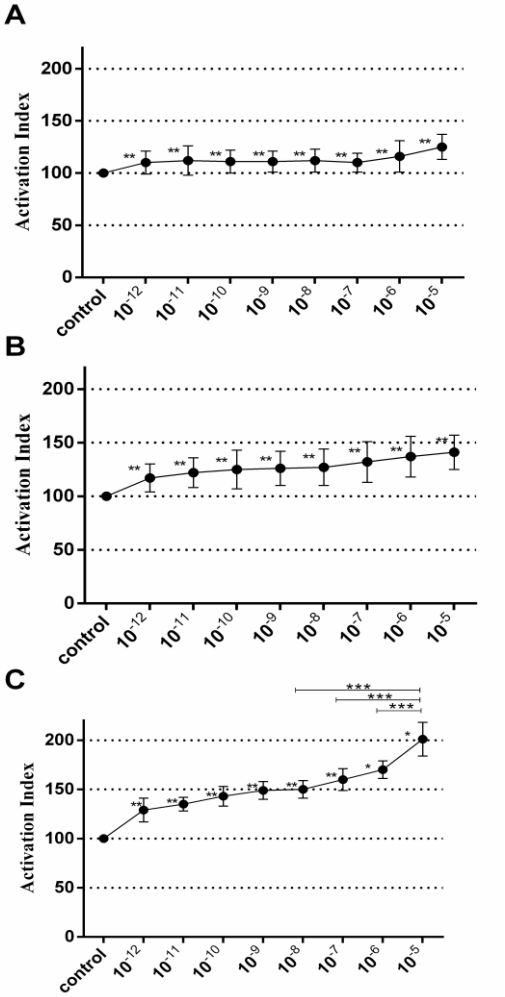

Figure 1. PBMCs proliferation assay using MTT test. (A) Proliferation after 24 hours of incubation. (B) Proliferation after 48 hours of incubation. (C) Proliferation after 72 hours of incubation. X-axis represents different ORZ concentrations by Molar and Y-axis shows activation index (activation index= [Proliferation of PBMC stimulated by PHA and ORZ / the proliferation of PBMC stimulated by PHA]*100). Statistical analysis was done by repeated measure test. Each point represent the mean± SEM. ** p> 0.05 vs control. * p<0.05 vs control. *** p< 0.05. PBMCs; peripheral blood mononuclear cells. MTT; tetrazolium. ORZ; oryzatensin. PHA; phytohemagglutinin.

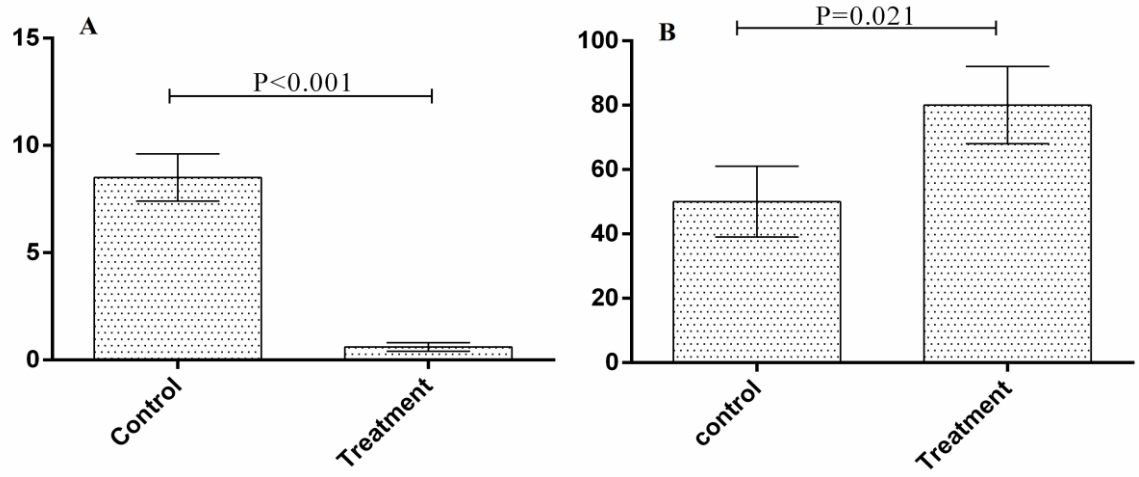

Figure 2. IL-4 and IFN-γ levels in PBMCs soup after ORZ treatment. (A) IFN-γ concentration (µgr/mL) in both ORZ-exposed and control PBMCs. (B) IL-4 concentration (ngr/mL) in both ORZ-exposed and control groups. X-axis represents treatment and control groups and Y-axis shows the concentration of IFN-γ and IL-4. Statistical analysis was done by paired sample T test. Each point represent the mean± SEM. PBMCs; peripheral blood mononuclear cells. ORZ; oryzatensin. IL-4; interleukin 4. IFN-γ; interferon-γ.

The Real Time Results

Co-culture of ORZ-stimulated PBMCs with HEPG2 cell line resulted in statistically insignificant over-expression of VEGF (Fold change=1.12±0.4) in HEP-G2 cells (p>0.05) but unlike VEGF the expression of MMP-9 significantly increased in cancerous cells (Fold change= 1.75±0.5) (Figure 3).

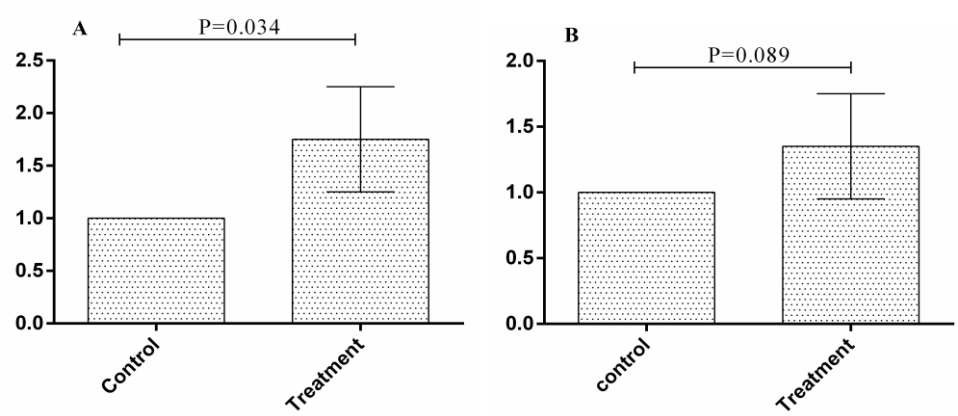

Figure 3. (A) The expression of MMP-9 in human HEP-G2 cell line co-cultured with ORZ-exposed PBMCs (B) The expression of VEGF in HEP-G2 cell line co-cultured with ORZ-exposed PBMCs. X-axis represents the study groups and Y-axis shows fold change of the genes. Statistical analysis was done by one sample T test. Each point represent the mean± SEM. VEGF; vascular endothelial growth factor, MMP-9; matrix metalloproteinase 9, PBMCs; peripheral blood mononuclear cells, ORZ; oryzatensin.

DISCUSSION

In the present study, we demonstrated that PBMCs from the healthy subjects (n=25) were stimulated by ORZ time and dose dependently. ORZ like C3a (a member of complement system) can bind and activate the C3a receptor.[1,2] C3a receptor is a G protein-coupled receptor known as C3aR1 and involved in the complement system. Recently it has been accepted that human C3aR1 is expressed on PMNs (basophils, mast cells, eosinophils). NK cells comprise up to 15% of PBMCs.[12,13] Min et al. revealed that NK cells express

the receptor to detect biologically active peptides like C3a and C5a. Moreover, their study demonstrated that C3 fragments reduce production of INF-γ by NK cells.[6] Our results showed that, ORZ significantly reduces the secretion of PBMCs-originated IFN-γ and increases the IL-4. This condition can cause presentation of ORZ to Th2 cells.[14-16]

Cell-mediated immunity is induced and maintained by Thl cells secreting IFN- γ. Patients with cancer often have impaired cellular immune responses associated with a switch from Thl to Th2. Th1 differentiation is driven mainly by IFN-γ. IFN-γ promotes the differentiation of CD4+ lymphocytes to the Th1 regulatory cells and inhibits the development of Th2 subset. Some inherited mutations in the IFN-γ or its receptors increase the risk of cancer. Th2 immune regulatory cells are the mediators of phagocyte-independent defense, in which PMNs have pivotal roles. IL-4 is the signature cytokine of the Th2 subset and functions as both an inducer and an effector cytokine of these cells.[17,19]

The study results proposed that the immune response against ORZ in 20-30 years old subjects may be a Th2-based response . The over expression of mentioned genes were seen in HEP-G2 cells. T helper cells play a main role in the regulation of the immune system responses. These cells subdivide to Th1 and Th2 based on the production of different types of cytokines.[20,21] A negative feed-back exists in the proliferation of Th1 and Th2. Th1 proliferation increases production of IFN-γ, which in turn has a positive effect on Th1 proliferation but a negative effect on Th2 proliferation.[6,22,23] Th1 cells and its related cytokine, i.e. IFN-γ, are involved in anticancer mechanisms while Th2 response enhance cancerous cells growth.[9,24]

Progression of different types of cancer depends on angiogenesis in a malignant tissue and the antiangiogenic therapy can delay tumor growth. VEGF play a central role in tumor angiogenesis; therefore, blockade of VEGF lead to inhibition of a tumor growth. The function of VEGF in cancer progression is not limited to angiogenesis. VEGF-mediated signaling occurs in cancerous stem cells also affects cancer progression by the mechanisms other than angiogenesis. MMPs are a family of endopeptidases with important functions in inflammation by extracellular matrix remodeling. The MMPs at the primary tumor site can also release some factors into blood stream that can facilitate cancer metastasis. MMP-9 is an MMP strongly associated with metastatic tumors, expression of which by tumor cells, mediates the invasion and metastasis.[10,25-27]

Our study has some limitations. Only 20- to30-year-old subjects were enrolled in this study and other age groups were not investigated. Immune system responses change during life span;[28] therefore, different results may ne obtained in other age groups. Although the ELISA results showed the immune response versus ORZ is a Th2 based response but the gold standard for assessment of Th1- or Th2-based immune response is the flow cytometry method.[29]

In conclusion regarding the significant decrease in INF-γ secretion and increase in IL-4 by ORZ-exposed PBMCs and also the increment of VEGF and MMP9 expression in HEPG2 cell line, which was co-cultured with ORZ-stimulated PBMCs, we propose that the immune response against ORZ is Th2-based, which increases cancerous cells growth. Hence, the pharmacological use of ORZ or ORZ-like peptide as an anti-analgesic, anti-amnesic and anorexigenic agents may change the immune response and sensitize target populations to cancer.

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