Vasoactive intestinal peptide, a promising agent for myopia?

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INTRODUCTION

Genetic and environmental factors are implicated in the etiology of myopia[1-2]. Studies have shown that a decrease or complete loss of image quality of an eye in the postnatal period results in an overgrowth that causes myopia[3]. This condition in the postnatal period of young animals is called form-deprivation myopia (FDM)[3]. FDM can be induced experimentally by the application of translucent occluders over an animal’s eyes[4]. Many candidate molecules, including vasoactive intestinal peptide (VIP), have been proposed to be involved in the pathophysiology of myopia[5-6,12-15]. Experimental investigations in animals have indicated that restricted vision affects eye growth and leads to an increase in axial length (AL). The increase in AL was associated with changes in neurotransmitters and growth factors, such as dopamine[7-8], retinoic acid[9], and glucagon[10].

VIP is a peptide hormone from glucagon family. VIP mainly plays a role in the gastrointestinal system as a smooth muscle relaxer, but it is also found in the brain and eyes where it functions as a neuromodulator[11]. Although a few studies have indicated that VIP, which exists mostly in the choroid tissue of the eye, impacts myopia[5-6], the results of some animal experiments contradict this finding[12]. Publications on the effects of VIP in FDM are lacking and have inconsistent results[3,6,12-15]. The ZENK transcription factor is a protein expressed in glucagon amacrine cells in the retina of the chick. The changes in the ZENK expression affect the ocular growth. Its upregulation has been shown to be related with the suppression of AL elongation[16-17]. The expressions of VIP receptors (also called VPAC₁ and VPAC₂) and the ZENK protein have been shown to be associated with FDM in several studies[5,17-19]. Our aim was to investigate the clinical effects of VIP and its effects on the expression of VIP receptors in experimental FDM.

MATERIALS AND METHODS

The study adhered to the ARVO Statement for the Use of
Assessment of Myopia  Ocular measurements were taken on P6 and P13. Two consecutive measurements per eye were performed by 2 ophthalmologists (Gursoy H and Cakmak A1), and the mean values were recorded. Cycloplegia was achieved 30min after instilling two drops of 1% cyclopentolate 5min apart. The refractive error measurements of both open and closed eyes were performed by streak retinoscopy under cycloplegia at a distance of 30 cm. Spherical equivalent (SEQ) was calculated by adding the spherical value and half of the cylindrical value. SEQ was used for analysis. The AL of the eyes was obtained transpalpebrally with B-scan ultrasonography (Advent AB, Accutome, Inc., Malvern, PA, USA).

Animals Sacrifice  All animals were sacrificed by an overdose of diethylether and cervical dislocation at the end of the deprivation period, P13. The left eyes of group 1 and the right eyes of all three groups were enucleated and immediately placed into a petri dish filled with ringer solution for immediate preparation. The eyes were perforated using a cannula and were opened by cutting around the iris with scissors. The anterior segment of the eye was discarded, and the vitreous gel was removed. Fixation was performed by immersion in 4% paraformaldehyde plus 3% sucrose in 0.1 mol/L phosphate buffer (pH 7.4) for 24h at room temperature. The eyes were hemisected equatorially with a razor blade, and the anterior portions were discarded along with the vitreous gel.

RNA Extraction and Real Time Polymerase Chain Reaction  The retinal tissues of left eyes of group 1 and the right eyes of all three groups were analyzed. The mRNA levels of VIP1, VIP2 and ZENK in relation to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were determined using real time polymerase chain reaction (RT-PCR) with Taqman prob. Total RNA was extracted from the retinal tissue using the RNA stabilization reagent (Qiagen, Germany) according to the manufacturer's instructions and was quantified by measuring its absorbance at 260 nm (Nanodrop1000; Thermo, Wilmington, DE, USA). Aliquots of 20 μL of RNA from each group were used to synthesize complementary DNA (cDNA). The newly synthesized cDNA, which was stored at -20°C, was used for the mRNA assay of the VIP1 receptors, VIP2 receptors, and ZENK protein. cDNA (5 μL) from each group was amplified in 20 μL of reaction mixture. RT-PCR was performed by real-time monitoring of the increase in the amount of Taqman prob using Rotor-Gene 6000 RT-PCR (Qiagen, Germany). The oligonucleotide sequences of the cDNA primers were designed at Gene Research Laboratories, UK. The following primers were used: rat VIP1, 5′-GAGAGAAAGACAGCTGGTGGTGG-3′ (sense) and 5′-CAGAAGGACCTGGTGTTGTTG-3′ (antisense); VIP2, 5′-GCAAGCTGACGCTCGTGTGGTGG-3′ (sense) and 5′-AGTTAGGGCCAGGAAACCTC-3′ (antisense); and 5′-AGGTAAGGCGGAGGAGCACC-3′ (antisense).

RNA Extraction and Real Time Polymerase Chain Reaction  The retinal tissues of left eyes of group 1 and the right eyes of all three groups were analyzed. The mRNA levels of VIP1, VIP2 and ZENK in relation to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were determined using real time polymerase chain reaction (RT-PCR) with Taqman prob. Total RNA was extracted from the retinal tissue using the RNA stabilization reagent (Qiagen, Germany) according to the manufacturer's instructions and was quantified by measuring its absorbance at 260 nm (Nanodrop1000; Thermo, Wilmington, DE, USA). Aliquots of 20 μL of RNA from each group were used to synthesize complementary DNA (cDNA). The newly synthesized cDNA, which was stored at -20°C, was used for the mRNA assay of the VIP1 receptors, VIP2 receptors, and ZENK protein. cDNA (5 μL) from each group was amplified in 20 μL of reaction mixture. RT-PCR was performed by real-time monitoring of the increase in the amount of Taqman prob using Rotor-Gene 6000 RT-PCR (Qiagen, Germany). The oligonucleotide sequences of the cDNA primers were designed at Gene Research Laboratories, UK. The following primers were used: rat VIP1, 5′-GAGAGAAAGACAGCTGGTGGTGG-3′ (sense) and 5′-CAGAAGGACCTGGTGTTGTTG-3′ (antisense); VIP2, 5′-GCAAGCTGACGCTCGTGTGGTGG-3′ (sense) and 5′-AGTTAGGGCCAGGAAACCTC-3′ (antisense); and 5′-AGGTAAGGCGGAGGAGCACC-3′ (antisense).
were compared to those on P13 using the Wilcoxon signed-
measurements of the right and left eyes for each group on P6
test and Tukey’s HSD multiple comparisons test. The initial
Kruskal-Wallis one-way analysis of variance by the ranks
compared among the three groups using the non-parametric
AL values of the right and left eyes on P6 and P13 were
for normality. Therefore, the SEq refractive error and the
Statistical Analysis
The data failed the Shapiro-Wilk test
for normality. Therefore, the SEq refractive error and the
AL values of the right and left eyes on P6 and P13 were
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were compared to those on P13 using the Wilcoxon signed-
rank test. Delta-delta Ct (ΔΔCt) assumes that the primers for
the unknown and reference genes have very similar efficiency
(i.e. reference gene amplification is nearly the same level
as that of the unknown gene). The ΔΔCt values for the VIP
receptors and the ZENK protein were compared among the
right eyes of groups 1, 2, and 3 and the left eyes of group
1 using non-parametric Kruskal-Wallis one-way analysis of
variance by the ranks test and Tukey’s HSD multiple
comparisons test. P-values <0.05 were considered to indicate
statistical significance. The statistical analyses were performed
using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS
One chick in each group was excluded from the statistical
analysis because of endophthalmitis development after the
third injection. Therefore, eight chicks in each group were
analyzed.
The comparisons of the SEq refractive errors and the AL
values of the right eyes on P6 and P13 among the three
groups are presented in Table 1. And the comparisons of the initial
SEq refractive errors and the AL values on P13 for the right
eyes of each group are shown in Table 1.
Multiple comparisons between groups were performed by
the Tukey’s HSD test. On P6, the right eyes in groups 1 and
3 were significantly more hyperopic than the right eyes in group
2. On P13, the eyes in groups 1 and 2 were significantly more
myopic than the VIP injected eyes (group 3). The eyes in
groups 1 and 2 had significantly higher AL values than the
eyes in group 3. The AL was significantly higher at the final
visit in all groups, so the eyes were significantly more myopic
at the final visit than at baseline.
The comparisons of the SEq refractive errors and the AL
values of the left eyes on P6 and P13 among the three
groups are presented in Table 2. And the comparisons of the initial
SEq refractive errors and AL values on P13 for the left
eyes of each group are shown in Table 2.
On P6, the left eyes in groups 1 and 3 were significantly more
hyperopic than the left eyes in group 2. The final SEq refraction
on P13 was similar among the three groups; however, Tukey’s
HSD multiple comparisons test showed that the AL values on
P13 were significantly higher in group 1 than in groups 2 and
3. All eyes were hyperopic, although the decrease in hyperopia
from baseline to P13 was significant in groups 1 and 3.
The mRNA levels of the VIP1 receptor were undetectable
in all groups. The comparisons of the ΔΔCt values for VIP2
 receptor and the ZENK protein among the right eyes of
groups 1, 2, and 3 and the left eyes of group 1 were compared in Table 3.
Multiple comparisons between groups were performed by
Tukey’s HSD test. The ΔΔCt values for VIP2 receptor were
significantly different between the right eyes in groups 1 and 3
(P=0.013), between the right eyes in groups 2 and 3 (P=0.002),

Table 1 The comparisons of the spherical equivalent refractive error and the axial length (AL) of the right eyes on P6 and P13 among the three groups

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spherical equivalent refractive error (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td>2.75 (2.25, 3.50)</td>
<td>1.25 (-0.25, 2.50)</td>
<td>2.25 (1.75, 2.75)</td>
<td>0.035*</td>
</tr>
<tr>
<td>P13</td>
<td>-13.75 (-16.00, -12.00)</td>
<td>-11.50 (-12.50, -7.50)</td>
<td>-1.50 (-4.75, -0.75)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>P</td>
<td>0.012*</td>
<td>0.012*</td>
<td>0.012*</td>
<td></td>
</tr>
<tr>
<td>Axial length (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td>9.00 (9.00, 9.05)</td>
<td>9.00 (8.95, 9.10)</td>
<td>9.00 (8.95, 9.00)</td>
<td>0.762</td>
</tr>
<tr>
<td>P13</td>
<td>10.65 (10.00, 11.10)</td>
<td>9.90 (9.70, 10.00)</td>
<td>9.20 (9.15, 9.25)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>P</td>
<td>0.012*</td>
<td>0.011*</td>
<td>0.027*</td>
<td></td>
</tr>
</tbody>
</table>

The 25th and 75th percentile values are given in parentheses along with the medians. *P<0.05.

ZENK 5'- ACTAACTCGTCACATTGCAGA -3' (sense) and
5'- TGCTGAGACCAGCTGCTGCT-3' (antisense). For
the housekeeping gene GAPDH the primer sequences were as
follows 5'-CTCTGACCACCCAGCCCAGCA-3' (sense) and
5'-TGTTATGGGGTGCTGCTGGGA-3' (antisense). The
RT-PCR thermal cycling conditions were as follows: 15min
at 42℃ and 10min at 4℃ for cDNA synthesis followed by
10min at 95℃ and then 50 cycles of 20s at 95℃, 30s at
55℃, and 20s at 72℃. RT-PCR data were collected using the
Rotor-Gene 6000 detection system. Cycle threshold (Ct) values
were determined by automated threshold analysis. Primer
quality (lack of primer-dimer amplification) was confirmed
by melting curve analysis. Relative quantification of gene
expression was performed using the standard curve method,
and the standard curves were constructed using serial dilutions
of control mRNA or RT-PCR amplicons. All experiments were
standardized with GAPDH (ratios of VIP1, VIP2 and ZENK to
GAPDH) to account for loading differences. Gene expression
levels (mRNA) were reported using the median as a point
estimator and the range of values.

Statistical Analysis
The data failed the Shapiro-Wilk test
for normality. Therefore, the SEq refractive error and the
AL values of the right and left eyes on P6 and P13 were
compared among the three groups using the non-parametric
Kruskal-Wallis one-way analysis of variance by the ranks
test and Tukey’s HSD multiple comparisons test. The initial
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VIP in myopia

The 25th and 75th percentile values are given in parentheses along with the medians. *P<0.05.

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<th>Group 3</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spherical equivalent refractive error (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td>3.25 (2.75, 3.75)</td>
<td>1.00 (0.50, 2.00)</td>
<td>2.25 (1.75, 2.50)</td>
<td>0.002*</td>
</tr>
<tr>
<td>P13</td>
<td>1.00 (0.50, 1.50)</td>
<td>1.25 (-1.75, 1.75)</td>
<td>1.25 (1.00, 1.75)</td>
<td>0.303</td>
</tr>
<tr>
<td>P</td>
<td>0.017*</td>
<td>0.574</td>
<td>0.017*</td>
<td></td>
</tr>
<tr>
<td>Axial length (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td>8.95 (8.90, 9.10)</td>
<td>9.00 (8.95, 9.10)</td>
<td>9.00 (8.95, 9.00)</td>
<td>0.827</td>
</tr>
<tr>
<td>P13</td>
<td>9.50 (9.20, 9.60)</td>
<td>9.20 (9.15, 9.35)</td>
<td>9.20 (9.15, 9.35)</td>
<td>0.005*</td>
</tr>
<tr>
<td>P</td>
<td>0.011*</td>
<td>0.011*</td>
<td>0.059</td>
<td></td>
</tr>
</tbody>
</table>

The 25th and 75th percentile values are given in parentheses along with the medians. *P<0.05.

<table>
<thead>
<tr>
<th>ΔΔC,</th>
<th>Right eyes</th>
<th>Left eyes</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIP2 receptors</td>
<td>Group 1</td>
<td>Group 2</td>
<td>Group 3</td>
</tr>
<tr>
<td></td>
<td>1.07 (0.82, 1.43)</td>
<td>1.22 (0.98, 1.65)</td>
<td>0.29 (0.22, 0.45)</td>
</tr>
<tr>
<td>ZENK protein</td>
<td>1.07 (0.63, 5.03)</td>
<td>3.55 (2.20, 5.55)</td>
<td>0 (Undetectable)</td>
</tr>
</tbody>
</table>

DISCUSSION

In the present experimental study, we observed a significant decrease in myopia after the intravitreal VIP injections. FDM was produced in all groups, but the intravitreal VIP injections significantly reduced the myopia observed at the final measurement. The final AL value was lowest in the group 3, and this finding was consistent with the fact that we observed the lowest myopia in that group. The expression of VIP receptors and the ZENK protein were analyzed to support our clinical findings. We showed that the intravitreal injections of VIP down-regulated the expressions of VIP2 receptor and the ZENK protein. However, we could not show a clear association between these mRNA findings and the possible mechanisms underlying the partial blockage of FDM by intravitreal injections of VIP.

The results of studies of the role of VIP in FDM have been inconsistent. It is claimed that VIP is involved both in the development of the refractive status and in the development of FDM in chicks[20]. Wiesel and Raviola[13] found that VIP level decreased in FDM, while Seltner and Stell[5] found that both VIP agonists and antagonists blocked FDM. Contrary to the report by Wiesel and Raviola[13], Stone et al[5] showed increased expression of VIP in FDM in monkeys. Wang et al[23] investigated the role of VIP antagonist in FDM and concluded that they decrease the development of FDM in chicks. In 1997, Basmak and Tuncel[22] obtained results consistent with those of the current study by injecting VIP into occluded chick eyes, but it was just a clinical trial without any PCR analysis. Dopamine is another neuro-peptide investigated for its role in myopia. In many studies, dopamine agonists blocked FDM[7-8]. It should be noted that VIP and dopamine appear to exert synergistic effects on retinal cAMP level[14]. This may be a common pathway through which dopamine agonists and VIP block FDM. In the present study, we tried to prevent FDM by injecting VIP based on previous reports by Wiesel and Raviola[13], Basmak and Tuncel[22].

Several reports have shown that the expression of VIP2 receptor was up-regulated in high myopia[16-17]. We found that the mRNA level of VIP1 receptor was undetectable in all groups, so we concluded that VIP1 receptor was not involved in ocular growth. On the other hand, the mRNA level of VIP2 receptor was significantly reduced in the occluded chick eyes after the intravitreal VIP injections (group 3), but the expression of VIP2 receptor was similar among the other three groups. Based on our findings, we conclude that FDM is not associated with any changes in VIP2 receptor expression but that exogenous intravitreal VIP injections can down-regulate the expression of this receptor. Contrary to our findings, Liu et al[18] found that VIP2 receptor was up-regulated in the eyes with FDM compared to the unoccluded eyes. We hypothesized that the partial suppression of FDM in the current study could be due to VIP agonistic and antagonistic effects. The antagonistic effect of intravitreal VIP injections on VIP receptor levels could be produced by the VIP fragments formed by hydrolysis in the chick’s eye[5,23]. The observed down-regulation of VIP2 receptor in the eyes of group 3 in our study supported our hypothesis because reduced receptor expression causes decreased agonistic activity of VIP. Similar to dopamine, VIP agonistic activity could prevent FDM through cAMP-dependent mechanisms[14].
The ZENK transcription factor (ZENK protein) regulates ocular growth. Schippert et al. showed that the ZENK knockout mice had more myopia than the mice in whom the ZENK gene was expressed. This protein induces the expression of many growth factors, such as fibroblast growth factor and platelet-derived growth factor. These factors suppress ocular growth, so a lower expression level of the ZENK protein was expected in the eyes with high DFM compared to the VIP injected eyes (group 3), as shown in several previous experimental studies. However, the most outstanding finding regarding the expression of the ZENK protein was the undetectable mRNA levels in the eyes of group 3 after the VIP injections. This could have led to an increased level of myopia in the third group. However, the partial blockage of FDM obtained after the intravitreal VIP injections was via other mechanisms that outweighed the possible consequences of a decreased ZENK mRNA level. The mRNA level of ZENK protein in the right and left eyes of group 1 was consistent with the fact that a decreased ZENK protein level is associated with FDM. Although the difference failed to reach statistical significance, the median ΔΔCt for the ZENK protein was 1.07 in the occluded eyes in group 1, while it was 1.89 in the unoccluded eyes in group 1. In the right eyes of the second group, the mRNA level of the ZENK protein was surprisingly up-regulated. This could have been associated with the prevention of FDM, but the second group received intravitreal saline injection, which is not thought to be involved in the prevention of FDM. The increase in the ZENK mRNA level could be a response to the simple trauma of intravitreal injection.

We hypothesized three possible explanations for the partial blockage of FDM after intravitreal VIP injection. We thought both agonistic and antagonistic activities played roles in the prevention of FDM. First, VIP could act like dopamine on a retinal cAMP level. This agonistic effect could partially block FDM in the same manner as dopamine agonists. Second, the antagonistic effects of the VIP fragments formed by hydrolysis could partially block FDM through unknown pathways. Some studies concluded that both VIP antagonists and down-regulation of VIP2 receptor expression prevent FDM. Several reports have shown that the expression of VIP is positively correlated with AL elongation. Finally, the down-regulation of the VIP2 receptor level augments the antagonistic effects of VIP fragments.

The strengths of the current study include its comparative design and that it was an experimental trial of the use of intravitreal VIP to block FDM. Our efforts to support the current clinical findings by performing PCR analysis of retinal tissues provide some clues into the mechanisms underlying the role of VIP in FDM. However, there are some limitations of this study, including limitations associated with the injection technique, the methodology, and the size of the study group. These limitations were unavoidable, except for the number of chicks studied. This study would be statistically stronger if we used a larger study group. We tried to inject the same doses of VIP in all chicks by using the same syringe at the same time of the day. However, it was not possible to obtain completely uniform VIP doses in all groups. We had to rely on our retinoscopy and ultrasonography measurements. However, the transpalpebral measurement of AL using contact B-scan ultrasonography was particularly subjective. We obtained a higher final AL in the first and second groups compared to the final AL in the third group in which intravitreal VIP injections were applied. These findings support our clinical findings by retinoscopy. However, the median AL was 9.20 mm in both the right and left eyes of group 3 despite the differences in refraction (-1.5 D myopia in the right eyes versus 1.5 D hyperopia in the left eyes). We concluded that this technique for measuring AL was not sufficiently sensitive to detect small differences in the AL values in the chick eyes. We measured refraction using retinoscopy under cycloplegia, but the accommodation of the examiner and the residual accommodation in the chick eyes could have affected the values we obtained. In the present study we analyzed the VIP1 and VIP2 receptors in the retinal tissues, but it has been reported that VIP also acts at PAC1 receptors. We could have gained more information regarding the mechanism of VIP in blocking FDM, if gene expression levels for PAC1 were also investigated.

In conclusion, in our study, intravitreal VIP injections partially blocked FDM in chicks. Both the agonistic and antagonistic effects of VIP could play a role in this prevention of FDM because the expressions of the ZENK protein and VIP2 receptor were down-regulated in the VIP-treated eyes. There is no single peptide, which has been shown previously to have definitive control of AL elongation and emmetropization. Several neuropeptides normally localized in the retina have been reported to be involved in the development of FDM. VIP is a neuropeptide, which seems to play an important role in the emmetropization process. VIP signaling pathway may be a promising target for myopia prevention. Further studies are required to clarify the mechanisms underlying the action of VIP in FDM.

ACKNOWLEDGEMENTS
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Conflicts of Interest

Cakmak AI, None; Basmak H, None; Gursoy H, None; Ozkurt M, None; Yildirim N, None; Erkasap N, None; Bilgec MD, None; Tuncel N, None; Colak E, None.

REFERENCES