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Review

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Higher incidence of nasopharyngeal carcinoma in some regions in the world confers for interplay between genetic factors and external stimuli

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Summary **Nasopharyngeal carcinoma (NPC) is a rare variety of head and neck cancers. The risk factors include three major causes: genetic factors, viral infection, and environmental and dietary factors. The types of NPC show strong ethnic and geographic variations. The keratinizing and non-keratinizing types are prevalent in the lower incidence regions like North America and Europe; whereas the undifferentiated type is mostly found in the regions with higher incidences like China, North Africa, Arctic, and Nagaland of North-East India. These suggest a possible major role of the internal genetic factors for generation and promotion of this disease. Viral infections might accelerate the process of carcinogenesis by helping in cellular proliferation and loss of apoptosis. Diet and other environmental factors promote these neoplastic processes and further progression of the disease occurs.**

Keywords: Nasopharyngeal carcinoma, incidence, ethnic variation, undifferentiated NPC, North-East India

1. Introduction

Nasopharyngeal carcinoma (NPC) is a rare cancer which starts at the mucosal epithelium of the nasopharynx and in the minor salivary glands present there. It is a box-like chamber located near the base of the skull and lying above the soft palate of the mouth; and it covers the upper region of the throat behind the nose (Figure 1A). According to WHO (World Health Organization), there are 3 types of NPC based on histopathological classification: keratinizing squamous cell carcinoma, non-keratinizing differentiated carcinoma, and undifferentiated carcinoma. The incidence of each of these types shows regional preferences; each specific type is seen more often in some areas of the world than in others. It has been reported that the keratinizing and non-keratinizing types are prevalent in the lower incidence regions, whereas the undifferentiated type is mostly found in the regions with higher and/or moderately higher incidences (*1*-*8*). The diagnosis, classification, and staging of NPC are done by microscopic examinations as well as by searching for the presence of distant metastases. In 2005, the updated edition of WHO classification of NPC describes three types of NPC: keratinizing squamous cell carcinoma, non-keratinizing carcinoma, and basaloid squamous cell carcinoma (*2*). The first variant is invasive type showing squamous differentiation with intercellular bridges and/or keratinisation over most of the tumor. Advanced tumors in local regions and low metastases to the lymph nodes are the key characteristics of this NPC. Lower association of EBV is also seen here. This type is further classified in well differentiated, moderately differentiated and poorly differentiated varieties. Non-keratinizing carcinoma of NPC is subdivided into the differentiated type of and undifferentiated type; of which the undifferentiated type is more prevalent in the high-incidence regions. Overall, this is the major type of NPC seen throughout the world. In the undifferentiated variety, the cells have vesicular nuclei, large nucleoli, and less distinct cell borders making the cell clusters look syncytial and overlapping. A huge amount of lymphocytes and plasma cells are found in these cell clusters; thereby disrupting the epithelial nature of the tumor. The differentiated variety shows cellular stratification and is uncommon in the high-incidence

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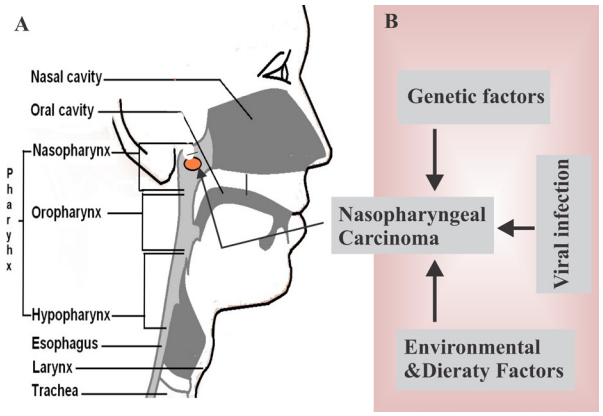

Figure 1. Origin and causes of nasopharyngeal carcinoma. (A). Location of nasopharynx and site of origin of nasopharyngeal carcinoma (NPC). Nasopharynx is a box-like chamber located near the base of the skull and lying above the soft palate of the mouth; and it covers the upper region of the throat behind the nose. NPC generally starts at the mucosal epithelium of it. **(B).** The risk factors of NPC include genetic factors like HLA antigens, viral infection with Epstein-Barr virus (EBV) or Human papilloma virus (HPV), and environmental and dietary factors like ill-ventilation and consumption of preserved foods.

regions. The third type, basaloid squamous cell carcinoma, is very rare and harbor closely packed small cells with hyperchromatic nuclei without nucleoli, and a small amount of cytoplasm (*2,6,9*). Stages differ by the depth of invasion of the soft tissue and bony structures at and near the nasopharynx, degree of presence of affected cranial nerves, and the involvement of local and regional lymph nodes of the head and neck (*1-3,9-11*).

One of the major problems associated with NPC is the detection of the disease at an early stage, so that it can be cured. It is one of the most confusing and poorly understood diseases; and so, is commonly misdiagnosed. In most of the times the disease is detected at a very late stage; thereby recurrence cannot be avoided. Metastases form secondary tumors at distant places making the condition even worse. In females, formation of secondary tumors in cervix due to early metastasis sometimes helps in diagnosis and treatment plan. The signs and symptoms of NPC include lump(s) in the neck, hearing loss, recurrent ear infection, stuffiness, headache, blurred vision, nosebleeds, *etc*. Microscopic examination is the primary type of detection method. This is complemented by other tests like physical examinations, endoscopic nasopharyngeal examinations, and computed tomography (CT) imaging or magnetic resonance imaging (MRI). But confirmation by biopsy is the "gold standard" for the diagnosis of NPC (*6,9,12-17*). Once diagnosed, NPC is treated by radiation therapy (RT) as well as chemotherapy drugs like cisplatin, carboplatin, 5-fluorouracil, docetaxel, paclitaxel, *etc*. Sometimes a tumor is removed by surgery and then RT and/or chemotherapy are followed. Novel treatment techniques like precision radiotherapy, endoscopic surgery, transoral robotic resection, immunotherapy, *etc*. are being practiced recently (*18-24*). Nowadays, the staging with any metastasis is diagnosed properly by the help of endoscopy, MRI, PET and CT scans; and then fusion images are formed from different modalities. These are then used for advanced targeted therapies like intensity-modulated RT (IMRT) and adjuvant chemotherapy. As NPC involves various signaling pathways inside the cells, a few inhibitors are being tested. For example, gefitinib and erlotinib for epidermal growth factor receptor (EGFR) and sorafenib for tyrosine-kinase are being tested. Recently, humanized monoclonal antibodies like nimotuzumab against EGFR and bevacizumab against vascular endothelial growth factor (VEGF) are also being tested in clinical trials (*22-24*).

The risk factors of this cancer includes *1*) family history and genetic factors (*25*); *2*) ethnicity, as the incidence is much higher in some populations compared to others (*4-7,25*); *3*) dietary factors, as consumption of saltine fish and meat is seen in affected populations (*6,7,12,25-27*); *4*) habits, like smoking and alcohol consumption (*25,28,29*); *5*) gender, as it is seen twice more often in males than females (*25,30-32*); *6*) and infection with EBV (Epstein-Barr virus) or HPV (human papilloma virus) (*25,29,33-34*) (Figure 1B). Globally, nasopharyngeal carcinoma (NPC) is an uncommon cancer accounting for about 0.7% of all cancers. In endemic areas like Southern China, Northern Africa, Alaska and Southeast Asia, the annual incidence is as high as 0.02-0.03% in males and about 0.01-0.015% in females, and the commonest form is undifferentiated carcinoma; whereas in North America and Europe, this incidence is much lower (0.001%) and the other forms of NPC are seen (*1-3,7*). In the present report, we will review the risk factors of NPC responsible for the higher incidence in some regions of the world and their consequences.

2. Dietary factors, lifestyle and environment contribute to the disease

NPC has a remarkable ethnic and geographical distribution; seen mainly in the Cantonese people in South East Asia, Eskimos in the Arctic, and Arabs in North Africa (*1-3,7,35,36*). The etiology of this disease involves a complex interaction of genetic factors, viral infection, and environmental factors including diet; of which, the environmental factors appear to have a relatively minor role contributing to the promotion of the disease (*35*). Diet, habits of smoking and/or alcohol consumption, and lifestyle contribute to the disease in the high-incidence races and each of these factors has gained experimental supports (*6,7,12,25-29*).

2.1. *Dietary factors*

Consumption of Cantonese salted fish has been suspected as a possible etiological factor in NPC. The

high incidence of this disease in Chinese population in China and Chinese migrated to other regions suggests that the dietary habit of these ethnic groups might play a contributory role in formation and/or promotion of this disease. Consumption of salt-preserved food, particularly at the time of weaning, is widely reported to be associated with NPC. Irrespective of the geographic location, two distinct peaks are seen: one in the early adolescence period (15-24 years), and the other in middle age (65-79 years). This suggests that internal genetic factors are the main players for the generation of the disease in these low-incidence populations. But in high-incidence regions, certain dietary materials and lifestyle add to the internal genetic factors, making the entire population much more susceptible to the disease (*4,7,8,10,25-35*). Children who were fed on salt-preserved fish and other foods starting at the time of weaning are more prone to get the disease at a younger age, generally within 14-19 years. On the other hand, children who were fed on fresh fruits and vegetables are less susceptible. In search for the reason behind such diet, presence of nitrosamines in Cantonese salted fish is suggested to be the main culprit as it can develop carcinoma of the nasal or paranasal cavities in rats fed on Cantonese salted fish (*37-40*). Continuous consumption of such nitrosamine containing foods helps accumulation of this chemical in the fat cells near nasopharynx. Later it promotes the detoxifying mechanisms of the cells of the body, particularly in the cells of the nasal and paranasal cavities as the smell of the food reaches these areas fast, and generation of procarcinogens are accelerated (*38-41*). Also, the inefficient process of salt preservation makes food items partially putrefied and such foods are consumed months later. *Staphylococci* and other bacteria including the nitrate-reducing bacteria grow during the process of salt preservation and turn the nitrates and nitrites of the salt to N-nitroso compounds like N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosopyrrolidine (NYPR), and N-nitrosopiperidine. Chemical nitrosation after consumption of salt-preserved fish may also occur under the acidic pH of the stomach. As a result, nitrosamines are again accumulated in high amounts (*42,43*). Salt-dried fish also contains various mutagens, genotoxins, and EBV-reactivating substances like 2-chloro-4-methylthiobutanoic acid (CMBA) and tyramine (*42-46*). In high-incidence populations in Tunisia, South-China and Greenland, a relatively high level of volatile nitrosamine has been detected in common food items (*47*). Similarly, presence of NDMA, NDEA, NYPR, and benzo (a) pyrene (BaP) in smoke-dried fish and meat may serve as a cause of NPC in the Nagas of North-East India and other populations. People of Nagaland of North-East India consume smoke-dried meat which is used as a preserved food. Usually meat to be dried is placed on a bamboo shelf hanging over the fire place used for cooking. This process is again very inefficient and makes the meat partially putrefied. Extract of smoke-dried meat has been reported to be mutagenic in *Salmonella typhimurium* and clastogenic in mammalian test system. It can cause skin papilloma and systemic tumours in mice (*48,49*). Other preserved food items, including eggs, fruits, and vegetables may also increase the risk of NPC. Also, childhood exposure of preserved food items at weaning is strongly correlated with this disease. It has been suggested that consumption of fresh fruits, vegetables and meat might help in prevention of NPC, particularly in children (*50,51*). A report based on Turkish population shows that consumption of French fries, fried meat, and herbal tea are associated with the elevated risk of it. Moreover, in rural areas, people with low-income groups hardly follow a healthy and regular meal pattern; such an irregular meal pattern increases the risk of getting this disease (*52*). Volatile and non-volatile nitroso compounds found in preserved foods thus play a vital role in the formation of NPC.

2.2. *Habits*

Habits like smoking and alcohol consumption are also tested as the risk factors of NPC. For alcohol consumption, there is no conclusive data. Some literatures report no correlation (*26*) and some report a strong correlation with heavier intake of alcohol and association of NPC (*26,52,53*). Smoking, on the other hand, has been reported widely as a strong risk factor for developing NPC (*52,53-55*). Another habit of use of herbal nasal medicine by the people of Nagaland in Northeast (NE) India has been reported recently as one of the risk-factors for NPC (*49*). Use of herbal tea in China has also been included as a risk factor of NPC (*27*). These herbal products contain natural carcinogens like pyrrolizidine alkaloids (PAs), tannins, safrole, *etc*. Each of these has been found to be mutagenic on bacterial, shrimp, and mouse models. Furthermore, most of the rural Naga people and many Africans in Kenya live in ill-ventilated houses, and they burn oak, pine and other wood for heating, lighting and/or cooking purposes throughout the day; thereby inhale the thick smoke continuously. Such living and dietary habits contribute to the etiology of this cancer (*27,52,56,57*).

2.3. *Environmental inhalants*

A number of environmental inhalants may play a significant role in the promotion of nasopharyngeal carcinoma. Formaldehyde is well known to cause heritable mutagenic effects on DNA of mice and deletions in human DNA, while point mutations are more common in *E. coli* DNA. Base-substitution and frame-shift mutations are also reported for formaldehyde. Deposition of cotton dust or wood dust in the nasal epithelium may activate the detoxifying processes and

might generate procarcinogens. Various reports show that inhalation of formaldehyde in printing industries, exposure to cotton dust and combustion in textile industries, exposure to wood dust, and working in ill-ventilated rooms increase the risk of the disease (*57-59*).

3. Infectious factors control the interplay between external and internal factors

Epstein-Barr virus and human pappillomavirus, both have been reported to be associated with nasopharyngeal carcinoma (*25,29,33,34,60*). Epstein-Barr virus (EBV), a human gammaherpesvirus, has been classified as a group I carcinogen by the International Agency for Research on Cancer because of its association with NPC (*61*). This virus was first detected in the African patients with Burkitt's lymphoma and infects B-cells and epithelial cells (*62*). This virus is very well known for causing infectious mononucleosis (glandular fever) and various types of cancers including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, and gastric carcinoma (*62-64*). Both Type-1 and Type-2 EBVs are seen in Africa and the Type-1 variety is prevalent in rest of the world (*62,63*). EBVs can persist latently in the B-cells for the lifetime of a person after initial infection. All latency programs may exist in B-cells and can move into lytic phase; eventually giving birth of infectious virions which may infect epithelial cells of the same or other person(s) depending upon the route of infection (*65,66*). After initial infection, the EBV nuclear antigens (EBNAs) are expressed along with some cellular proteins. Different latency programs of EBV give rise to different sets of expressed viral antigens (*67,68*). *EBNA-2* is one of the first genes expressed upon infection of B-cells and is essential for establishment of latent infection and cell growth transformation (*69-71*). Soon after the EBNA-2 expression, the EBV latent membrane proteins 1 and 2 (LMP1 and LMP2) expressed (*71*).

3.1. *EBV infection in NPC*

A study reports that EBV can be activated by aqueous extracts of salt-dried fish of China. Another study reports that the combination of salted fish and EBV shows a strong association with NPC (*44,72*). Also, herbal medicines used by Chinese and Naga people can help the proliferation of EBV and thus may help develop NPC. A number of reports show higher levels of antibodies (EBV-IgG) against the virus capsid protein (VCP) in these populations (*73,74*). Various EBNAs have also been detected in tumor cells of NPC (*75,76*). One subtype of the viral immediate-early gene *BRLF1*, *BR1-C*, and its gene product Rta may be associated with the tumorigenesis of NPC. The DNA binding and transactivation domains of Rta has been detected to have multiple mutations. Three CTL (cytotoxic T lymphocyte) epitopes, NAA, QKE and ERP, have been reported to have mutations (*77*). A 30-bp deletion in the *LMP1* gene and the loss of XhoI restriction site in *LMP1* gene have been detected in NPC tissues, whereas none of the non-malignant nasopharyngeal tissues harbour XhoI-loss and LMP1 30-bp deletion. This deletion is much frequent in Chinese population and is seen in undifferentiated NPC. Other studies have shown a higher proportion of LMP1 30-bp deletion is much more common in Inuit population (68%) rather than Chinese (30%) or Caucasians (20%). Distinct variations in C terminal, N terminal and transmembrane region in LMP-1 have been reported using molecular plylogenetic analysis of large panels of EBV isolated from southeast Asia, Papua New Guinea, Africa, and Australia where NPC is not endemic (*78,79*). The *BHRF1* gene, which is a homolog of the *bcl-2* oncogene, has been reported to express a particular variant of protein in NPC. This variant carries an L to V mutation in amino acid 88 and a no mutation in amino acid 79 (79V88V variant); thus contributing to the tumorigenesis (*80*). The *BZLF1* gene, which encodes the ZEBRA protein, has been reported to have a serine in place of alanine at the amino acid position 206 in NPC tissues (*78*). This may again explain the association of EBV with NPC. A high level of EBERs (96.67%) and LMP 1 (43.33%) expression and low amount of ZEBRA 1 (6.67%) expression have been detected in Spanish population. Variants of *EBER* genes have been identified in NPC in non-endemic regions, but its correlation with the formation of the disease has not been reported (81).

3.2. *HPV infection in NPC*

HPV is a DNA virus of the papillomavirus family and many of the types of this virus are transmitted sexually; thereby causing infections in the genital areas. Most such infections have no clinical symptoms and are resolved spontaneously. Some cause warts and precancerous lesions; which may eventually turn to cancers. Cervical cancer is the most common type of cancers caused by HPV; others are cancers of mouth and oropharynx, penis, anus, vulva, vagina, *etc*. Risk factors for HPV infection include early sexual experience, smoking, and poor immunity (*25,34*). Association of HPV with NPC usually results in overexpression of p16 and immunohistochemical methods are performed to detect this along with PCR of HPV DNA. In low-incidence areas, HPV-positive NPC cases are EBV-negative; but in endemic areas, co-infection by both of these viruses is common. Although the HPV-positive NPC cells generally show non-keratinizing morphotype, undifferentiated variety is less common and most cells are differentiated. The undifferentiated variety is seen in the endemic regions and in patients with EBV/HPV co-infection. The more infectious types of HPV, HPV16 and HPV18, are seen in NPC; though the HPV11 is also reported. Some of the HPV-positive NPCs actually originate from the oropharynx, but majority show the true nasopharyngeal

origin (*33,34,60*). The overall survival (OS) patterns are similar in HPV-positive, EBV-positive, and EBV-HPV co-infected NPCs. The involvement of viral gene(s) and interaction with the host genetic factors are to be examined in details and a long path is to be travelled to find out the mechanism of HPV infection in this rare disease.

4. Genetic factors render the body susceptible to the disease

As discussed earlier, a higher incidence of NPC is seen in some ethnic groups including Chinese people in China and Chinese migrated abroad. An intermediate incidence is seen in populations admixed with the Chinese. Therefore, this high incidence of NPC in specific ethnic groups indicates genetic factors controlling the disease. A number of reports have suggested the role of various genetic factors in association with NPC; particularly, polymorphisms in the histocompatibility locus antigens (HLA), cytochrome P450 2E1 (CYP2 E1), alteration of p53 codon 72 Arg>Pro, and some signaling pathways (*82-91*). It has been reported that human 8-oxoguanine DNA glycosylase 1 (hOGG1) gene and human MutY glycosylase homologue (hMUTYH) gene polymorphism is associated to the risk of NPC mainly among women in Chinese population (*92*).

4.1. *HLA antigens*

Studies on association of genetic factors with NPC have involved several NPC patients with different genetic systems. In all of those, only one group of antigens, the HLA, has been found to be associated with NPC (*85,86*). HLA genes express various proteins of the immune system and help it to process and present foreign antigens; thereby making those antigens vulnerable to immune lysis. In Chinese population, the HLA data reveal that the younger patients are genetically different from the older patients and may involve different mechanism for the onset of the disease. Susceptibility for NPC as well as survival after diagnosis, both are associated with the HLA antigens. Two different haplotypes, A2-C11-B46 and A33-C3-B58-DR3, have been diagnosed in NPC (*85,86,93*). Both these haplotypes are responsible for the susceptibility of the disease, and the A33-C3-B58-DR3 haplotype is associated with poor survival also. On the other hand, HLA-A2 without B46 or B58 and A2-B13 helps long-term survival. It has been inferred that HLA-A11, B13, and B22 are associated with lower risk for NPC development; whereas HLA-A2, C11, B14, B17, and B46 are associated with increased risk (*85-87,94,95*). In whites, HLA-A2 and A11 are found to be associated with lower risk of NPC (*95*). It has been suggested that in ethnic groups with higher incidence of NPC, HLA-A2, C11, B14, B17, and B46 show a reduced ability to process and present EBV antigens; thereby confer an increased risk of developing NPC in individuals carrying these alleles. In contrast, HLA-A11, B13, and B22 are efficient in processing and presenting EBV antigens; thus confer a decreased risk of getting NPC (*96*). It has been reported that HLA-DRB1 allele polymorphism is associated with NPC in Asian, Tunisian, and Caucasian population. DBR1 locus, the most ubiquitous one, encodes a number of variable gene products. Genotyping of HLA 1 region and its association studies among 20 NPC cases in Northestern India have revealed the association of microsatellite markers HL003 (allele 121) and D6S2704 (allele 218) in the HLA class I region with high risk of NPC; while a protective effect of HL003 (allele 127) and D6S2678 (allele 255) have been conferred (*97*).

4.2. *Tumor suppressors and oncogenes*

More than fifty percent of cancers contain mutation in the tumor-suppressor gene *p53* which controls the cell cycle check point, apoptotic pathways, metabolic changes, DNA repair, senescence, and genomic stability. In NPC, mutations in *p53* are less common, although some single nucleotide mutations, frame shift, deletion and duplication may occur. Rather, overexpression/ accumulation of p53 in NPCs of endemic areas is well documented (*89*). But it has been reported particularly that a certain type of single-nucleotide polymorphism of *p53* at codon72 increases the risk of NPC. Substitution of proline (Pro) in place of arginine (Arg) at codon 72 of the p53 product plays a role in disease susceptibility. Individuals with Arg/Arg genotype have a lower risk of getting NPC compared to the individuals with Arg/ Pro genotype; and those with Pro/Pro genotype have a much higher risk of acquiring NPC (*90*). Recently, codon 72 Arg>Pro polymorphism and the risk of NPC has been reported in Northestern India. Moreover, p53 codon72 polymorphism is affected by the mutations seen in some other genes, like PIN-1, TNF-α (tumor necrosis factor alpha), and GST. Further, both EBV and HPV can modulate the effects of p53 in controlling apoptosis. It has been suggested that EBV-LMP1 can induce the expression of antiapoptotic genes BCL-2 (B-cell lymphoma 2) and A20; and thus help prevent apoptosis by p53. Though EBV-positive NPC cells have shown a higher amount of BCL-2, its direct relation to EBV infection and LMP1 expression needs to be studied in detail. It is suggested that higher amount of BCL-2 poses a synergistic effect on LMP1 function and helps the NPC cells to grow rapidly and the aggressive nature of the cancer depends on it. Metastases to different body parts including the lymph nodes and poor prognoses with recurrence in NPC are thus explained. Patients having low levels of BCL-2 in NPC stages III and IV experience higher rate of disease-free 5-year survival. Therefore the importance of BCL-2 overexpression is established in NPC, though the detailed molecular

mechanism is ill-understood (*91*). On the other hand, EBNA3C can bind directly to p53 and inhibit its DNA binding property; thereby prevents apoptosis by p53 pathway. But the interrelation between EBV infection and the susceptibility of the alteration of *p53* codon72 Arg form or codon72 Pro form needs to be investigated thoroughly (*90*). The involvement of TNF-α and HSP-70 (heat shock protain-70) in tumor immunity and cancer biology have been investigated. TNF as an interleukin have multifunctional role in cell survival, proliferation, differentiation and death. High expression of interleukins has been inferred with development of many cancers. HSP-70 is a chaperone and act as an anti tumor immune recognition by cytotoxic T lymphocyte. *TNF* genes are located towards the telomeric position of *HSP* gene members. Recently TNF-α (– 308 G>A), TNFβ (+ 252 A>G), HSP 70-1 (+ 190 G>C) and HSP 70-hom (+ 2437 T>C) genes polymorphism have shown a high association with NPC in Northeastern Indian population (*98*).

4.3. *Signaling pathways*

Various signaling pathways have been studied for their contributions in NPC (*85*). The Wnt signaling pathway, which regulates the developmental processes of embryo and tissue homeostasis of adults, has been suggested to be involved in the development and maintenance of NPC. Prolonged Wnt signaling activates dishevelled (DSH) family proteins, which in turn, inhibit axin, glycogen synthase kinase-3β (GSK3β), and the adenomatous polyposis coli (APC) proteins. Downregulation of GSK3β results in cytoplasmic β catenin accumulation in the cytoplasm and translocation into the nucleus. Cytoplasmic β catenin can bind to various transcription factors and E-cadherin, thereby help in cellular proliferation, differentiation, and adhesion. β-Catenin can activate interleukin-8 (IL-8), c-Myc and cyclin D1 expression, all of which contribute to carcinogenesis by causing cellular proliferation and/or angiogenesis. Higher levels of β-catenin have been reported in NPC and these levels are inversely related to the survival rates. β-Catenin levels are found to higher in NPC cells infected with EBV. Moreover, Wnt inhibitory factor (WIF), which can downregulate the Wnt signaling pathway, has been found to be decreased in NPC. The levels of inactivation of WIF by promoter hypermethylation are found to be related to the TNM stages in NPC; the higher the stage, the higher the inactivation of WIF.

Uncontrolled expression of phosphoinositide 3-kinases (PI3K) activates a serine/threonine protein kinase B (Akt), which in turn, upregulates cell proliferation and prevents apoptosis. Significantly higher expression levels of Akt are seen in undifferentiated NPC and these levels are inversely related to the OS of patients (*91*). This pathway is also activated by EBV-LMP1 and thus activates several downstream pathways leading to various carcinogenic events like distant metastasis, lymph node involvement, advanced tumor stages, as well as worse prognosis. It has been found that NPC cells exhibit high level of Akt with low level of PTEN (phosphatase and tensin homolog protein). Inhibition of Akt with a PI3K inhibitor LY294002 results control of cell proliferation and inducing apoptosis. NPC metastasis by epithelial-mesenchymal transition occurs due to activated c-Src and Akt plays a vital role in this pathway. In NPC, Akt is also activated by the overexpression of UBE2T; thereby help proliferation, cell survival, invasion, and metastasis. The mitogen-activated protein kinase (MAPK) pathway, which regulates various cellular processes, has been shown to play an important role in cancer development. One of the MAPKs, the c-Jun N-terminal kinase (JNK), regulates cell survival. In NPC, constitutive activation of JNK results in p53 inactivation *via* phosphorylation and activation of DNA methyltransferase. This leads to inactivation of E-cadherin; thereby prevents apoptosis and alters cell adhesion. This finding also explains the loss of apoptosis *via* p53 even when the amount of p53 in NPC is mostly higher compared to normal cells. Another member of the MAPKs, the extracellular signal-related kinase (ERK), is found to be upregulated in NPC and it can induce transcription factors NF-κB, AP-1, and ETS. These, in turn, activate c-Fos, cyclin D1, and c-Myc resulting in cellular proliferation. The epidermal growth factor receptors (EGFRs) are also found to be upregulated in NPC and are associated with poor prognoses. Higher amounts of EGFRs then activate RAS/ERK signaling pathway leading to uncontrolled cell proliferation. Cytoplasmic interaction of cyclin D1 and cyclin E with EGFR helps in cellular proliferation by the induction of progression through G1/S phase. It has been shown that endocytosis and processing of EGFR is stimulated by EBV infection; thereby the translocated processed parts of EGFR act as transcription factors inside the nucleus and further activates signaling pathways that help cell proliferation (*22-24,91*). In latest studies, a few cellular micro RNAs like miR17 through miR92 and miR155, are reported to be upregulated in NPC. These miRs are known for their oncogenic properties and are involved in regulation of various genes in cellular signaling pathways like Wnt and apoptotic pathways. Downregulation of the tumor-suppressive miRs (like miR34 family, miR143, and miR145) may also regulate such pathways in NPC. Particularly, downregulation of miR29c, which targets genes for expression of extracellular matrix, helps in invasion and metastasis (*91*).

4.4. *Cytochrome P450 2E1*

Recently, the enzymes cytochrome P450 2E1 (CYP2E1) and glutathione S-transferase have been reported to be associated with NPC. Four varieties of cytochrome P450 (CYP1A1, CYP1A2, CYP2E1 and CYP3A4) can

activate procarcinogens which then produce reactive intermediates. These intermediates can damage DNA and play a vital role in chemical carcinogenesis. Case-control study and correlated study have shown that polymorphisms in CYP2E1 and CYP2A6 both contribute to the development of NPC in Thailand and South Chinese and Northeastern Indian population. Of these, CYP2E1 reacts with nitrosamine which is found in preserved foodstuffs. Inhaled xenobiotics and procarcinogens present in smoked foodstuffs may be activated by the oxidative and non-oxidative enzymes present in nasopharynx; thus contribute to the formation of NPC (*82,83,99,100*). The absence of glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) may also increase the risk. GSTs detoxify the body by helping glutathione (GSH) to bind to the xenobiotics and thus save the cellular proteins and nucleic acids. Therefore loss of GSTs may increase the risk of NPC as smoke and smoked foodstuffs contain various xenobiotics (*88*).

5. Conclusion

Studies on nasopharyngeal carcinoma (NPC) reveal three basic reasons of development of this rare disease: EBV infection (sometimes HPV), internal genetic factors, and environmental factors including diet. Incidence pattern and geographical distribution of NPC strongly suggest that the internal genetic factors are the main regulators for generation and maintenance of this cancer. Specific ethnic groups found in specific geographic regions support this phenomenon. Though environmental factors in those geographic regions are also suggested for their role in the disease, these might be minor players as it has been reported that Chinese people migrated to other regions show higher incidence; and the admixture of Chinese with others show moderately higher incidence levels. Thus, the genetic factors, that is, some specific allelic varieties found in susceptible ethnic groups might be the key regulators of NPC; EBV infections render these internal factors more active for causing the disease; finally, diet and environment stimulate the affected cells to promote the disease (Figure 2).

Different genes and gene clusters show distinct allelic variations among different ethnic groups. As for NPC, mainly Mongoloid people show higher incidence, as seen in Chinese of China and Nagas of North-East India; and higher or moderately higher incidences are seen in Eskimos and Arabians. Despite the geographic and ethnic variations, all studies show the involvement of the HLA genes and a few signaling pathways, as discussed

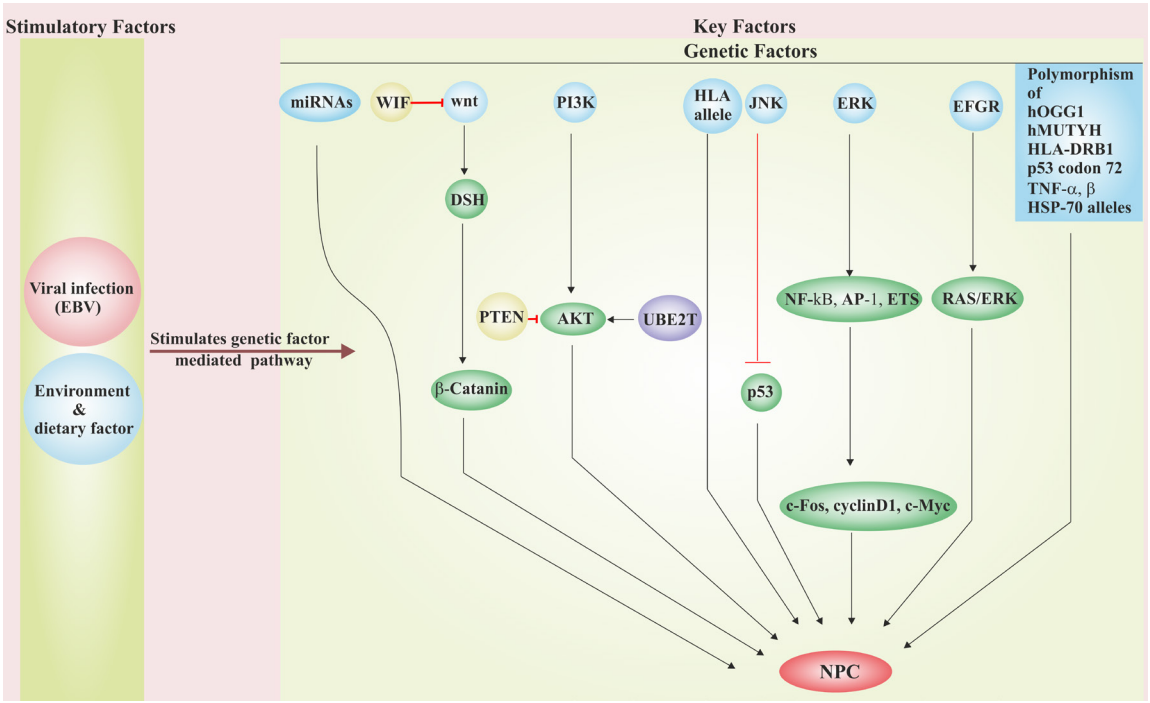

Figure 2. Mechanism of NPC formation. High incidence of nasopharyngeal carcinoma (NPC) in specific ethnic groups indicates internal genetic factors controlling the disease. Polymorphisms in the histocompatibility locus antigens (HLA), cytochrome P450 2E1 (CYP2E1), alteration of p53 codon 72 Arg>Pro, overexpression of BCL-2, and some signaling pathways like Akt, MAPK, and Wnt pathways are reported as the genetic players contributing to NPC. There are three basic reasons of development of NPC: EBV infection (or, sometimes infection with HPV), internal genetic factors, and environmental factors including diet. Specific alleles of HLA and alteration of the signaling pathway(s) render the body susceptible for getting the disease; some of the body cells in nasopharynx become neoplastic with uncontrolled proliferation. Carcinogenic and mutagenic agents, like nitroso compounds, present in the dietary and environmental factors add to the promotion of carcinogenesis as the nasal epithelium harbors a lot of detoxifying enzymes and can produce procarcinogens from those dietary agents. Infection with EBV and/or HPV makes the nasal epithelium more proliferative. Loss of activity of p53, overexpression of BCL-2, and activation of MAPK, Akt, and Wnt pathways lead to uncontrolled proliferation, angiogenesis, loss of apoptosis, and altered cell adhesion; and thus eventually help in invasion and metastasis.

Table 1. A global perspective of NPC distributions in respect to the incidence pattern (*1,5,10,12,32,56,72-75*)

Occurrence of NPC in respect to ethnic and geographical distribution		Factors responsible for NPC		
Geographical Distribution	Specific populations	Genetic Factors	Virus	Diet/Environment/lifestyle
Asian Continent	East and south Asian population/Arabian population	+	+	+
	Chinese population	+	+	+
	Naga Population in North East India	+	+	+
	Turkish Population	-	-	+
	Thailand population	+	-	-
Arctic continent	Arctic Eskimos/Inuit population	+	+	+
African Continent	North African population	+	+	+
	Population of Tunisia	+	-	+
	Population of Kenya	-	-	+
Australian Continent	Papua New Guinea	-	+	-
North-American Continent	Greenland	-	-	+
European Continent	Spanish population	-	+	-

+, incidence of a particular factor; –, no incidence of a particular factor.

earlier. The specific alleles of HLA and alteration of the signaling pathway render the body susceptible for getting the disease. The cells become neoplastic with uncontrolled proliferation and/or acquire EBV (or HPV) infection; which in turn, make the cells more proliferative. Carcinogenic and mutagenic agents present in the dietary and environmental factors add to the promotion of carcinogenesis, as the nasal cells can easily be infected with EBV and the nasal epithelium harbors a lot of detoxifying enzymes. People in poorly ventilated places are in continuous exposure to smoke which can deposit fine dust on nasal epithelium. This may then be detoxified by those enzymes and procarcinogens are generated in turn. Nitrosamines in smoke-dried food and salt-preserved food can contribute to the disease in the similar way. Global Distribution of NPC in respect to the incidence patterns is summarized in the Table 1.

The internal factors, that is, the genetic players, cannot be controlled. Infection with EBV is another factor which is almost uncontrollable. The only thing which can be controlled to prevent NPC is the third one. Environmental agents which may help carcinogenesis can be avoided, as well as the diet may be altered. Ventilation should be improved in houses, particularly in rural areas of Nagaland of North-East India and Africa. Proper ventilation in industries to reduce fumes, smokes and dusts may also decrease the risk of the disease. As the preserved food materials, mainly smoke-dried and salt-preserved foods, are well documented for causing NPC, these should be avoided and fresh fruits and vegetables should be consumed; particularly at the time of weaning. Delayed diagnosis and poor prognosis have made this disease a critical one; therefore intense research studies are needed to infer for the main causative factors and control measures to improve the treatment procedures. Based on present knowledge, novel therapies are being tested in three routes: one is the targeted regulation of signaling pathways to control cell proliferation, angiogenesis, and metastasis; the other one is immunotherapy, again in a targeted manner; and the third one is a combination of targeted IMRT and adjuvant chemotherapy. But preclinical trials of such targeted therapies face a major problem of finding an authentic undifferentiated cell line and animal model in which EBV infection may also be established. The cell line C666-1 is generally used with all its limitations, and a few xenografts from NPC patients are also being maintained to test these therapeutic promises; but there is a long way to go for treating NPC to assure a disease-free survival.

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Original Article

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Nanoemulsion: A suitable nanodelivery system of clove oil for anesthetizing Nile tilapia

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Summary **Clove oil ethanolic solution (CL-EtOH) have always been used for fish anesthesia. However, ethanol causes major side effect of fish hypersensivity. In this study, clove oil loaded nanoemulsion (CLN) was developed in order to enhance water miscibility of clove oil without using ethanol in the preparations. The obtained CLN was characterized in terms of droplet size, size distribution expressed as polydispersity index (PDI), and zeta potential. The anesthetic effect of CLN in comparison with CL-EtOH on *Oreochromis niloticus* (Nile tilapia) was investigated. The results showed that the best CLN was composed of 20% w/w clove oil and 15% w/w polysorbate 20. This CLN has internal droplet size of 63.2 ± 1.0 nm, PDI of 0.31 ± 0.04, and zeta potential of – 30.3 ± 8.1 mV. GC-MS analysis indicated that eugenol was the main compound in clove oil. It was found that the induction time to anesthesia for Nile tilapia that received this CLN was shorter than that received CL-EtOH at the same eugenol concentration. The results of this study showed the potential of nanoemulsion on water miscible and efficacy enhancing of clove oil without using ethanol. The obtained CLN from this study is a promising formulation for fish aquaculture where fish sedation is required.**

Keywords: Nanoemulsion, clove oil, fish anesthesia, Nile tilapia, *Oreochromis niloticus*

1. Introduction

Fish anesthesia is an important step before fish handling in aquaculture and veterinary research fields in order to ease handling and minimize stress and injury of fish (*1*). Ideal anesthetics for fish should have suitable properties such as water miscibility, convenience for use, effectiveness with low concentration, low physiological perturbation, and low cost, as well as safety for the fish, human, and environment (*2*). Chemical synthetic anesthetics are generally more expensive and toxic to environment than natural agents such as plant extracts. The fish exposed to chemical anesthetics require a long withdrawal period before human consumption (*3*). Clove oil has been used as food additive for human for a long time. It shows anesthetic activity to various kinds of fish species according to its bioactive compound, eugenol (*4-6*). This compound can depress medullary respiratory centers and reduce the gill ventilation with hypoxia leading to bradycardia and decrease blood flow in the gills (*7*). Clove oil can be easily obtained by hydrodistillation of buds of *Syzygium aromaticum*, therefore clove oil is characterized as natural anesthetic. It is also inexpensive compared with the chemical synthetic anesthetic agents. Clove oil is regarded as safe for both fish and human (*8*). Using clove oil for fish anesthesia does not require any withdrawal period for human consumption (*9*). However, clove oil is immiscible with water. Ethanol has always been used when diluting clove oil in water or in aqueous preparations in order to enhance water miscibility of clove oil. Clove oil ethanolic solution (CL-EtOH) is therefore available nowadays. However, hyperactivity of the fish was reported during anesthetizing with CL-EtOH (*10,11*). To avoid this side effect, the anesthetic preparations of clove oil without ethanol or any organic

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solvents are attempted to be developed.

Nanoemulsion is usually a dispersion of oil droplets in the aqueous system. Currently, nanoemulsion plays an important role on improving the properties of many hydrophobic active compounds for human and animals particularly on increasing water miscibility of those active compounds (*12*). The advantage of nanoemulsion is that it possesses high kinetic stability due to its extremely small droplet size of the internal phase, approximately 20-200 nm (*13*). This nanoformulation can be stable against creaming or sedimentation or phase separation. In this study, clove oil loaded nanoemulsions (CLN) were mechanically prepared using high-shear stirring and high-pressure homogenization in order to enhance water miscible of clove oil. The obtained CLN were characterized and tested for anesthetic effect in comparison with CL-EtOH using *Oreochromis niloticus* (Nile tilapia) as a fish model.

2. Materials and Methods

2.1. *Materials*

Clove oil was purchased from Thai-China Flavours & Fragrances Industry (Nonthaburi, Thailand). Polysorbate 20 and polysorbate 80 were from Sigma-Aldrich (Darmstadt, Germany). Dichloromethane was from (Merck Millipore, Germany). Ethanol was from Emsure (Darmstadt, Germany). Nile tilapia were purchased from a local farm in Chiang Mai, Thailand. The fish were allowed to acclimate at laboratory conditions for 2 weeks.

2.2. *Chemical analysis of clove oil*

Analysis of clove oil was performed using gas chromatography-mass spectrometry (GC-MS) on an Agilent 6890 gas chromatograph coupled to electron impact (EI, 70 eV) using a Hewlett Packard (HP) mass selective detector (MSD), model HP 5973-MSD (Agilent Technologies Inc, USA). The fused silica capillary column (HP5-MSI; 30.0 m × 0.25 mm *i.d.* × 0.25 µm film thickness) was used. The analytical conditions were as follows; carrier gas: helium (1.0 mL min^{-1}), injector temperature: 25°C, oven temperature: 3 min isothermal at 70°C, increased from 3°C min^{-1} to 188°C then from 20°C min^{-1} to 280°C and 3 min isothermal at 280°C. The detector temperature was 280°C. The oil sample was diluted with dichloromethane to 1:100 volume ratio. An exact portion of 1 µL of the diluted oil sample was injected. The identification of individual compound was based on their retention times relative to those of authentic samples and matching spectral peaks available in Wiley, NIST, and NBS mass spectral libraries.

2.3. *Preparation and characterization of CLN*

Two formulations of CLN (CLN-10 and CLN-20) composed of 10% and 20% w/w clove oil, respectively, were prepared. Polysorbate 20 and polysorbate 80 were used as surfactants for these formulations. The surfactant was firstly added to the aqueous phase to reach a final concentration in CLN of 15%. Then the oil phase was dispersed in the aqueous phase with stirring at 40-50°C. The mixture was emulsified by high shear homogenizer (Ultra-Turrax T25, IKA-WERKE, Germany) at 16,000 rpm for 3 min. Subsequently, this pre-emulsion was passed through a high pressure homogenizer (EmulsiFlex-C3, Avestin, Canada) for 10 cycles at the pressure of 1,000 bars. The homogenized nanoemulsions were cooled down to room temperature (about 30°C). The CLN formulations obtained were evaluated for droplet size, size distribution, and zeta potential by photon correlation spectroscopy (PCS) (Malvern Zetasizer Nano-ZS, Malvern, UK) once after preparation and at day 30 after storage. During storage, the physical appearance of the formulations was observed. The best CLN was selected for evaluation of anesthetic efficacy in Nile tilapia.

2.4. *Effect of CLN on fish anesthesia*

Samples of Nile tilapia with 42.85 ± 2.67 g body weight and 14.51 ± 0.54 cm length were randomly collected from the holding tanks (n = 20). The fish were fed twice daily with a commercial dry feed (INTEQC Feed, Thailand) and held in natural light conditions. These experimental methods were approved by the Animal Care Committee of the Faculty of Veterinary Medicine, Chiang Mai University (FVM-ACUC no. R19/2555). The content of eugenol in each formulation was calculated based on the percentage of eugenol in the clove oil obtained from the GC-MS results. An exact amount of the selected CLN was added to the induction tank (10 × 10 × 15.5 cm) to have a final eugenol concentration of 40 mg/L after adjusting the volume with water to 1 L. To a positive control tank, an exact amount of CL-EtOH having 1:9 volume ratio of eugenol to ethanol was added to have the same eugenol concentration as CLN after adjusting the volume with water to 1 L. Nile tilapia was individually transferred into these tanks (3 fish/tank). The effects of CLN or CL-EtOH on fish anesthesia were investigated by determining the induction time to anesthesia and recovery time from anesthesia. After reaching the desired stage of anesthesia, the fish were transferred into a recovery tank (15.5 × 25.5 × 18 cm) containing 5 L of oxygenated water. The fish behavior or mortality was observed until they are fully recovered to the desired recovery stage.

2.5. *Statistical analysis*

All experiments were done in triplicate. The data were presented as mean ± SD. Statistical evaluation of anesthesia induction and recovery times in Nile tilapia

was performed by *t*-test where $p < 0.05$ was considered to indicate the significant differences.

3. Results

3.1. *Chemical analysis of clove oil*

Clove oil appeared as a clear pale yellowish liquid. GC-MS chromatogram of clove oil showed the presence of 3 identifiable components (Table 1) which represented 98.08% of the total oil. It was found that the oil consisted of eugenol as a major component (96.11%). Caryophyllene and naphthalene were found as minor components.

3.2. *Preparation and characterization of CLN*

According to the difference of surfactants, polysorbate 20 and polysorbate 80, and of clove oil concentrations, 10% and 20%, used in the preparation of CLN, therefore 4 CLN formulations were obtained. It was found that CLN-10 and CLN-20 containing polysorbate 20 presented good appearance without any changes whereas those containing polysorbate 80 showed phase separation within 24 h. Therefore, only CLN formulations containing polysorbate 20 were selected for further investigation on droplet characteristics. The results were shown in Table 2. It was found that CLN-20 possessed significantly smaller droplet size than CLN-10. Both formulations presented narrow size distribution, expressed as polydispersity index (PDI) values. The size distributions of CLN-10 and CLN-20 were confirmed by the distribution curves shown in Figure 1 and Figure 2, respectively. From these figures, the peak intensity of CLN-10 was 98.2% and that of CLN-20 was 97.4%. Zeta potential of both CLN formulations was less than –30 mV. Keeping the formulations in room temperature (about 30°C) for 3 days, the appearance of phase separation occurred in CLN-10 whereas no phase separation was observed in CLN-20 even keeping for 30 days. CLN-20 containing polysorbate 20 therefore was concluded to be the best formulation suitable for further study because it demonstrated the highest stability, smallest droplet size, lowest PDI, and the optimum zeta potential.

3.3. *Effect of CLN on fish anesthesia*

In this experiment, CLN-20 containing polysorbate 20 was selected to compare the anesthetic effects with CL-EtOH on Nile tilapia. The induction to anesthesia in fish was divided into various stages according to the depth of anesthesia (*14*) as shown in Table 3. Stage 4 or a final stage of anesthesia was confirmed by checking pain reflex at a tail near a fish caudal fin. The fish recovery from anesthesia was also divided into various stages according to the level of recovery (*15*), as shown in Table 4. The effects of CLN in comparison with CL-EtOH on anesthesia of Nile tilapia was shown in

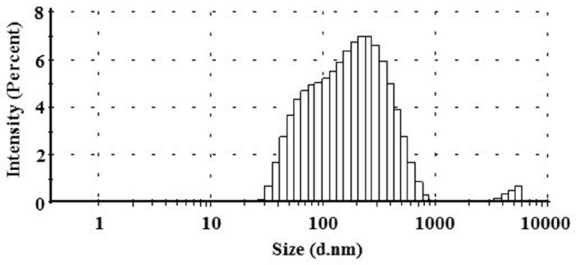

Figure 1. PCS analysis of CLN-10.

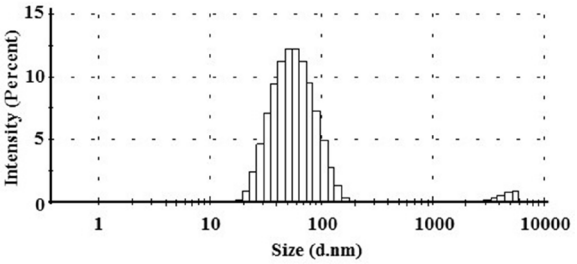

Figure 2. PCS analysis of CLN-20.

Table 1. GC-MS analysis of clove oil

Components	Retention time (min)	Area (%)
Eugenol	19.96	96.11
Caryophyllene	21.59	1.34
Naphthalene	22.90	0.63

Table 2. Internal droplet characterization of CLN by PCS

Characteristics	Formulations	
	CLN-10	CLN-20
Size (nm)	222.4 ± 9.8	63.2 ± 1.0
PDI	0.34 ± 0.08	0.31 ± 0.04
Zeta potential (mV)	–31.0 ± 3.3	–30.3 ± 8.1

Table 3. Stages of induction of fish anesthesia

Stages	Description of anesthesia
2	Partial loss of equilibrium
3a	Total loss of equilibrium but retain swimming ability
3b	Swimming ability stops
4	No responds to pressure on the caudal peduncle

Table 4. Stages of recovery from fish anesthesia

Stages	Description of recovery
1	Body immobilized but opercular movements just starting
2	Regular opercular movements and gross body movements beginning
3	Fish have normal equilibrium and normal swimming ability

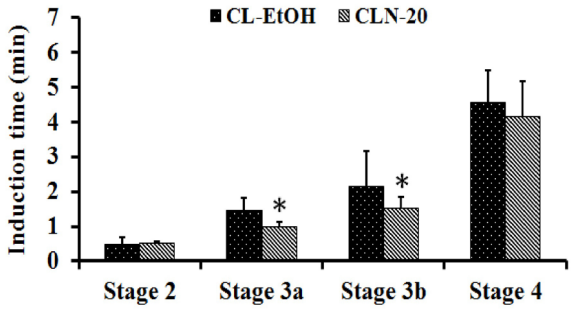

Figure 3. Times required for induction to each stage of anesthesia in Nile tilapia (n = 20) after exposure to CLN-20 and CL-EtOH. Asterisk (*) represents a significant difference ($p < 0.05$).

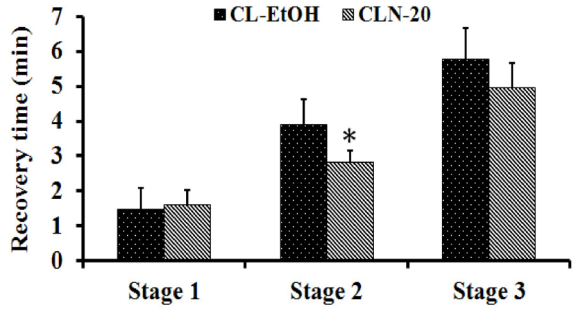

Figure 4. Times required for each stage of recovery from anesthesia in the anesthetized Nile tilapia (n = 20) caused by CLN-20 and CL-EtOH. Asterisk (*) represents a significant difference ($p < 0.05$).

Figure 3. CLN caused the fish to enter stage 3a and 3b of anesthesia within 1.0 ± 0.1 and 1.5 ± 0.3 min, respectively, which was significantly faster than CL-EtOH. The fish anesthetized with CLN-20 reached stage 2 of recovery from anesthesia within 2.8 ± 0.3 min, significantly shorter than those anesthetized with CL-EtOH, as shown in Figure 4.

4. Discussion

The common form of emulsion always shows physical instability such as aggregation of internal oil droplets or phase separation upon storage for a period of time. According to this, nanoemulsion is developed in the current study because it is much stable than the common emulsion. Nanoemulsion can be formulated by using suitable surfactant. Even there are many types of surfactant, the nonionic type such as polysorbates are the most popular for pharmaceutical preparations because of their compatibility with various active compounds. In the systems containing oil and water, nonionic surfactants can rapidly adsorb at oil/water interface to reduce the oil/water interfacial tension and provide steric or electrostatic hindrance to the dispersed internal droplets to prevent droplet aggregation (*16*). Previous literature has reported the developed nanoemulsions of clove oil using polysorbate 80 for enhancing antibacterial activity of clove oil (*17*). However, their formulations contained low amount of clove oil and used high concentration of surfactant. In the present study, polysorbate 20 and polysorbate 80 were compared for forming CLN with high loading of clove oil. The hydrophilic-lipophilic balance (HLB) of polysorbate 20 and polysorbate 80 are obviously different so that it is interesting to use both of them in the current study in order to investigate the effects of the HLB on loading efficiency of clove oil. The results demonstrate that those CLN containing polysorbate 20 have higher efficiency on loading clove oil than those containing polysorbate 80 indicating the effects of HLB on loading this oil in the nanoemulsions. Interestingly, CLN-20 which the concentration of clove oil is 2 times higher than CLN-10 showed better characteristics and higher stability than CLN-10. This result indicates that to prepare the best nanoemulsion, the ratio of oil, surfactant, and water should be in optimum. Excess surfactant can lead to a phase separation. Low PDI values indicate monodispersed systems (*18*) and can be related to high stability on storage for nanoemulsions (*19*). CLN-20 possesses significantly smaller droplet size and lower PDI values than CLN-10, therefore the stability of CLN-20 is higher than CLN-10. The zeta potential indicates the degree of electrostatic repulsion between particles in a dispersion. A high zeta potential value provides high stability to the dispersion and prevents aggregation (*20*). The high zeta potential in CLN is considered to be due to the interaction between the hydrophilic parts of the surfactant and the water molecules during the process of nanoemulsion formulation to form potentially negative charges. These negative charges can be absorbed to the emulsifier layer of oil/water interface and electric double layer similar to that of ionic form (*21,22*).

The efficacy of clove oil on anesthesia of Nile tilapia has been previously evaluated (*23,24*). However, those studies evaluated clove oil or synthetic eugenol in ethanolic solutions. The present study showed that clove oil loading nanoformulation like CLN is much better than the ethanolic solution like CL-EtOH. Besides no side effect of ethanol, CLN can cause faster induction of fish anesthesia. Nanoformulations can enhance the water miscibility of insoluble drugs (*25,26*). Due to the extremely small size with large interfacial area of internal oil droplets surrounding with the suitable surfactant in CLN, the miscibility of clove oil in aqueous systems can be enhanced. This leads to the rapid absorption of clove oil via the fish gills and skin, depression at medullary respiratory centers as a consequence, and efficient delivery of the active eugenol (in clove oil) to the sites of action with rapid onset. In conclusion, nanoemulsions can improve the aqueous miscibility and efficacy of clove oil suitable utility for fish anesthesia. CLN composed of 20% w/w of clove oil and 15% w/w of polysorbate 20 is the best formulation for this purpose.

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Effects of *Alpinia galanga* oil on anesthesia and stress reduction in *Oreochromis niloticus*

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Summary ***Oreochromis niloticus*** **(Nile tilapia) is one widely cultured fish in Thailand. Handling processes and transportation causes high stress in Nile tilapia. This study explores anesthetic effect and stress reduction of *Alpinia galanga oil* (AGO) on Nile tilapia. The anesthetic activity was evaluated by the time for fish induction to anesthesia and full recovery. It was found that the suitable dose of AGO that caused desirable anesthesia of Nile tilapia was 700 mg/L. This dose gave induction and recovery times of approximately 257 and 438 sec, respectively. Blood glucose and plasma cortisol of the fish anesthetized with AGO showed nearly normal levels indicating that the fish stress during handling was not increased. Study on loading densities of fish mimicked general fish transportation and showed that loading density of fish was a crucial factor on fish stress. The highest water quality was found in the lowest loading density of fish. Water containing AGO at a concentration of 150 mg/L showed significantly higher potential for reducing fish activity and water improvement than without AGO. Therefore, AGO is a promising natural edible plant oil for anesthesia in Nile tilapia.**

Keywords: *Alpinia galanga*, plant oil, anesthesia, stress, *Oreochromis niloticus*

1. Introduction

The rapidly growing human population causes raising global demand for food consumption. Aquatic animals have become the fast-growing in the food-production sector globally. Several processes such as netting, weighing, sorting, vaccination, and transportation in which fish are subjected to handling and confinement can induce stress in fish (*1*). In addition, exposure to environmental stressors (aquatic pollutants) also stimulates the hypothalamic-pituitary-adrenal axis (HPA) resulting in elevated plasma cortisol levels (*2-4*). A prolonged elevation of cortisol due to stress has been considered harmful due to increased susceptibility to disease and suppression of reproductive processes (*5-7*).

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Chemical agents such as benzocaine, tricaine methansulfonate (MS-222), metomidate hydrochloride, isoeugenol, 2-phenoxyethanol and quinaldine are routinely used as anesthetics in aquaculture to minimize stress associated with handling (*8*). However, undesirable side effects such as hypersecretion of fish mucus and skin irritation have been observed when using these compounds (*9*). Clove oil is a natural anesthetic that has been used in aquaculture for a long time (*10*). The effectiveness of clove oil as anesthetic for many fish species; *Cyprinus carpio* (*11*), *Siganus guttutus* (*12*), *Siganus lineatus* (*13*) has been reported.

However, some fish species exposed to clove oil showed an elevation of blood cortisol and glucose whereas blood lymphocytes were decreased (*14-16*). Those side effects from clove oil on fish indicate that stress still occurs when clove oil is used. Moreover, eugenol, the main active compound of clove oil, has been reported that it might damage hepatic tissue in mice (*17*). It has been reported that the essential oils from *Oleum malaleucae* (*18*), *Ocimum gratissimum* (*19*), and *Lippia alba* (*20*) have anesthetic activity in fish. The

desirable properties of anesthetic agents in fish should be highly effective and safe. The extracts from inedible plants might contain some toxic compounds (*21*).

Alpinia galanga, an edible plant in family Zingiberaceae, is widely grown throughout Southeast Asian countries (*22,23*). The plant is well-known in Thai and Chinese traditional medicine and used as anti-inflammatory, antipyretic, carminative, antiflatulent, and anti-itching (*24,25*). Its rhizomes and flowers have been widely used for centuries in Asian food remedies. According to our best knowledge, no information is available in the literature about anesthetic activity of *Alpinia galanga oil* (AGO) on fish, particularly on those cultured for human food.

Oreochromis niloticus (Nile tilapia) is an important source of protein and source of human income. It is one of the most popular fish in Thai cuisine. Trends of Nile tilapia culture have increased from small to large scale production in the Americas (*26*). In addition, Nile tilapia culture also has quickly expanded due to overconsumption (*27*). Using anesthetics in fish grown for human food should be carefully done because chemical anesthetics may be harmful to humans due to their possible carcinogenic and mutagenic effects. Humans have a long history of consuming *A. galanga* without any toxicity, AGO is of interest to investigate for its effectiveness on anesthesia and stress reduction in food fish like Nile tilapia. Safety can be sure even if there is some AGO residue left in the anesthetized fish. The present study also explores the advantage of AGO for water conditions for fish transportation.

2. Materials and Methods

2.1. *Fish materials*

Juvenile and adult Nile tilapia were purchased from a local ornamental fish shop, Chiang Mai province and acclimated to laboratory conditions at Faculty of Veterinary Medicine, Chiang Mai University. The water parameters for fish maintenance are the following; temperature, 25°C; pH, 7.4; total hardness, 110 ppm; alkalinity, 90 ppm; and total ammonia and nitrite were negative. All *in vivo* experiments were conducted according to permission from the animal care and use committee of the Faculty of Veterinary Medicine, Chiang Mai University (FVM-ACUC) (process no. R3/2555).

2.2. *Plant materials*

Fresh rhizomes of *A. galanga* were collected from a local garden in Chiang Mai province of Thailand. The plant was identified and the voucher specimen (No. 009245) was deposited at the Herbarium, Northern Research Center for Medicinal Plants, Faculty of Pharmacy, Chiang Mai University.

2.3. *Chemical materials*

Absolute ethanol, anhydrous sodium sulphate, and sodium bicarbonate were of analytical grade from Merck Millipore (Darmstadt, Germany). MS-222 and Drabkin's reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of the highest grade available.

2.4. *Extraction and component determination of AGO*

Fresh rhizomes of *A. galanga* were washed and cut into small pieces and subjected to hydro-distillation using a Clevenger apparatus for 3 h. The AGO obtained was kept in a light-resistant container at –20°C until further use.

For component determination, gas chromatography-mass spectrometry (GC-MS) on an Agilent 6890 gas chromatograph with a fused silica capillary column (HP5-MSI; 30.0 m × 0.25 mm i.d. × 0.25 µm film thickness) was used. AGO was diluted with dichloromethane to a ratio of 1:100 (v/v) before injection. The identification of each compound was based on their retention times relative to those of authentic samples and matching spectral peaks available in the mass spectral libraries.

2.5. *Anesthetic activity study*

Stock solution of AGO was first prepared by diluting AGO with absolute ethanol to obtain an AGO ethanolic solution (AGO-EtOH) with a concentration of 100 mg/mL. Various volumes of AGO stock solution were added to the induction tank to obtain AGO final concentrations of 100-900 mg/L and the volume was adjusted using dechlorinated water. Juvenile Nile tilapia (average weight; 40.8 ± 1.2 g and total length; 13.6 ± 0.2 cm) were used in this experiment. The stages of fish anesthesia and recovery were modified from criteria outlined by Zahl *et al*. (*1*) using the following details. Stage 1; fish are disoriented and reduce swimming activity, stage 2; the fish show partial loss of equilibrium. When the fish stopped their swimming activity, presented a total loss of equilibrium and no responsiveness at all, they were in surgical anesthesia stage 3. In this study, anesthetic activity was intensively studied on anesthesia stage 3 because this stage is normally suitable for fish surgery, clinical examination, or treatment. The time that the fish reached the desired anesthetic stage was determined. Then, the fish were moved to the recovery tank containing only dechlorinated water and monitored until fully recovered. The recovery time was recorded. After that, the recovered fish were placed in another tank for one week and fed with pellet feed in order to observe mortality.

2.6. *Transportation simulation study and water quality analysis*

Juvenile Nile tilapia (average weight; 1.34 ± 0.07 g and total length; 4.25 ± 0.22 cm) at various loading density (100, 200, and 300 fish/plastic bag) were used in this study. In a control group, the plastic bag contained 4 L of dechlorinated water and 12 L of pure oxygen. In a treatment group, AGO alcoholic solution prepared as mentioned in Section 2.5 was added to the plastic bag and the volume was adjusted to 4 L with dechlorinated water to have a final AGO concentration of 150 mg/L, and then 12 L oxygen was added. The plastic bags were sealed after the fish were completely transferred inside to protect against leakage of oxygen gas. There were triplicates for each loading density. Transportation simulation was created by vertical and horizontal movement of a plastic bag every 15-20 min. Water quality parameters were analyzed before and after transportation. Dissolved oxygen (DO) and temperature were measured with a YSI oxygen meter (Model Y550A, USA). The pH was determined by pH meter (CyberScan 500, Singapore). Total ammonia nitrogen (TAN) levels were measured according to the method of Grasshoff *et al*. (*28*). Un-ionized ammonia (NH_3) levels were calculated according to the method of Emerson *et al*. (*29*). The experiment was done in triplicate. After the experiment, the fish were placed in another tank and fed with pellet feed for two weeks in order to observe mortality.

2.7. *Stress study*

The effect of AGO on fish stress was investigated by determination of hematological parameters, blood glucose, and plasma cortisol of fish after being anesthetized with AGO at 700 mg/L. MS-222 at 130 mg/L was used as a control. Adult Nile tilapia (average weight; 545.2 ± 9.2 g and length; 29.2 ± 0.5 cm) were fasted 1 day prior to use. The fish were divided into 10 groups, each group contained 10 fish and was cultured in an aquarium filled with 5 L of dechlorinated water. AGO alcoholic solution prepared as mentioned in Section 2.5 was added to five aquaria and MS-222 aqueous and solution pH was adjusted using sodium bicarbonate added to the other five aquaria. Blood was collected from anesthetized fish via a caudal vessel at time intervals of 0, 30, 60, 90, and 120 min and each fish was subjected to blood collection only at one-time point.

A portion of the blood was analyzed for hematocrit (Hct) by a standard microhematocrit method (*30,31*), hemoglobin (Hb) by a colorimetric cyanomethemoglobin method (*32*), and total red blood cells (RBCs) and white blood cells (WBCs) by counting (*33,34*), as well as glucose using a glucometer (Accu-Chek®, Roche, Australia). The remainder was centrifuged at 3,000 rpm for 10 min (Beckman Coulter Inc., USA) for plasma separation. Plasma cortisol was determined using single direct antibody competitive enzyme immunoassays previously described by Brown *et al*. (*35*).

2.8. *Statistical analysis*

The data presented as mean ± S.E.M. were analyzed by independent *t*-test or a one-way ANOVA followed by Tukey's post hoc test. Kolmogorov-Smirnov's test was used as normality of data evaluation. The statistical significance was considered as p-value < 0.05.

3. Results

3.1. *Extraction and component determination of AGO*

A. galanga rhizomes yielded 0.11% AGO. The obtained AGO appeared as a clear pale yellowish liquid. The GC chromatogram of AGO as shown in Figure 1 demonstrated that AGO contained many components. It was found that 1,8-cineole (retention time at 6.6 min) and 4-allylphenyl acetate (retention time at 18.9 min) were the major compounds whereas eugenol (retention time at 19.2 min) and its derivatives *i.e*., methyl eugenol (retention time at 21.4 min) and eugenyl acetate (retention time at 26.0 min) were the minor compounds in AGO.

3.2. *Anesthetic activity study*

AGO at a concentration of 100-600 mg/L showed sedative and anesthetic effects but did not induce Nile tilapia to anesthesia stage 3 during a 20 min evaluation period whereas 700-900 mg/L AGO was capable of inducing fish to anesthesia stage 1, 2, and 3 within 83-26, 163-126, and 257-151 sec, respectively (Figure 2). The induction and recovery times for fish anesthetized to all stages showed significant differences among concentrations (p < 0.05), but there was no significant difference of stage 1, 2, and recovery time between 800-900 mg/L AGO. An elevation of concentration caused an induction time decrease. In terms of recovery, high concentration of AGO caused longer recovery times than low concentrations. No mortality was observed after one week of monitoring.

3.3. *Transportation simulation study*

Transported fish in the AGO group showed slow movement whereas the fish in the control group showed

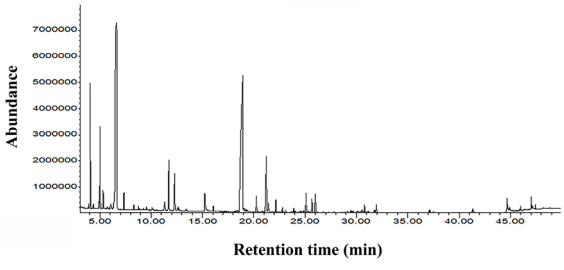

Figure 1. GC chromatogram of AGO

normal activity. After transport, an elevation of loading density significantly affected water parameters. TAN and NH_3 levels were found to be increased but DO level and the pH value were decreased ($p < 0.05$) (Table 1). The content in the plastic bags of the groups with the highest loading density exhibited the lowest DO and pH but highest TAN and NH_3. Mortality of 4.2 ± 1.2% was found in the highest density in the control group while no mortality was found in the AGO group. DO levels in the control group were significantly higher than those in the AGO group in the high loading density (200 and 300 fish/4L). In the AGO group, the pH values of all loading densities was similar. In addition, the TAN and NH_3 levels were significantly higher in the control group than in AGO groups at all loading densities except NH_3 in the groups with the highest loading density ($p < 0.05$).

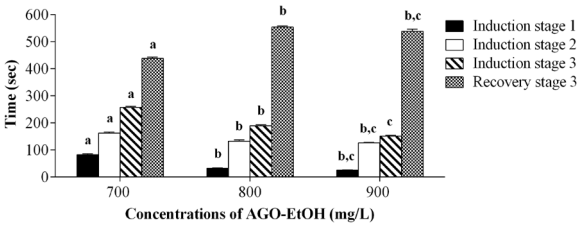

Figure 2. Induction and recovery times for Nile tilapia (n = 20) exposed to AGO-EtOH in dose-dependent manner. Different lowercase letters indicate significant differences of induction time and recovery time between concentrations based on one-way ANOVA and Tukey's test ($p < 0.05$).

3.4. *Stress study*

Blood analysis of fish exposed to AGO showed 13 g/dL Hb and 33% Hct whereas the concentrations of RBC and WBC were 1×10^6 and 19×10^3 cells/µL, respectively. The blood of those exposed to MS-222 exhibited approximately 13 g/dL Hb, 35% Hct, 2×10^6 cells/µL RBC, and 20×10^3 cells/µL WBC (Figure 3). For the anesthesia study, the results showed a significant difference among the treatments over the period of study except at two time points (30 and 60 min) of exposure (Figure 4a). The blood cortisol level of Nile tilapia anesthetized with MS-222 was

Table 1. Effect of AGO on water parameters before and after 4-h transportation in plastic bags

Water parameters	Before transport	End of transport simulation					
		n = 100		n = 200		n = 300	
		Control	AGO	Control	AGO	Control	AGO
DO (mg/L)	8.51 ± 0.24	5.92 ± 0.16	6.14 ± 0.27	4.49 ± 0.20	4.82 ± 0.10*	3.91 ± 0.39	4.41 ± 0.27*
pH	7.41 ± 0.03	6.47 ± 0.20	6.61 ± 0.10	6.67 ± 0.20	6.72 ± 0.26	6.40 ± 0.09	6.57 ± 0.35
TAN (mg/L)	0.01 ± 0.01	0.96 ± 0.08	0.75 ± 0.08*	1.46 ± 0.13	1.30 ± 0.07*	2.22 ± 0.20	1.94 ± 0.20*
NH_3 (mg/L)	0.00 ± 0.01	0.002 ± 0.01	0.001 ± 0.01*	0.003 ± 0.01	0.002 ± 0.01*	0.004 ± 0.01	0.004 ± 0.01

*significant differences between group in the rows using AGO and control after transportation ($p < 0.05$).

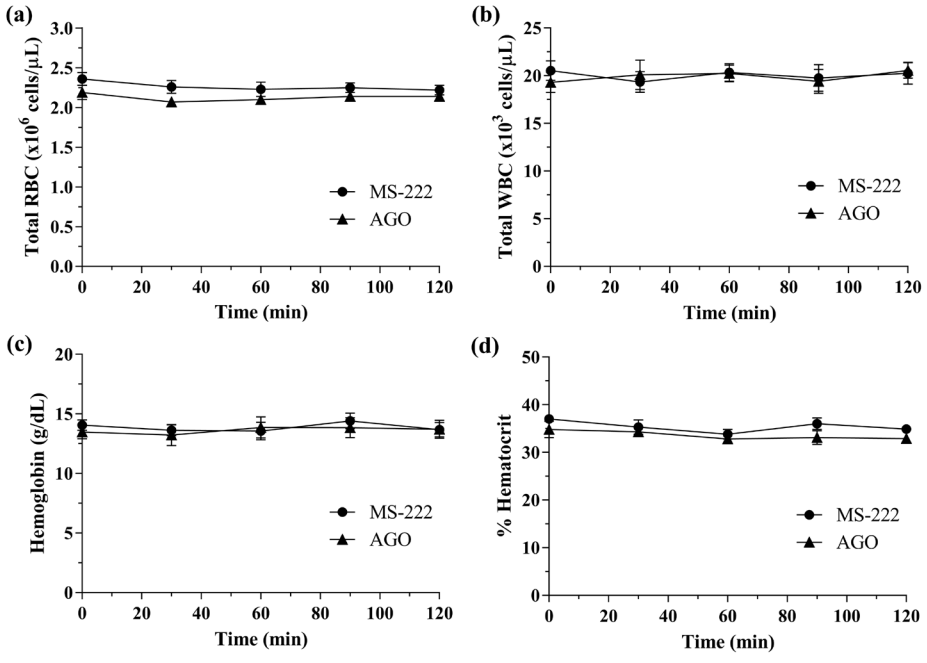

Figure 3. Comparison of total RBC (a), WBC (b), Hb (c) and Hct (d) in Nile tilapia after exposure to AGO and MS-222.

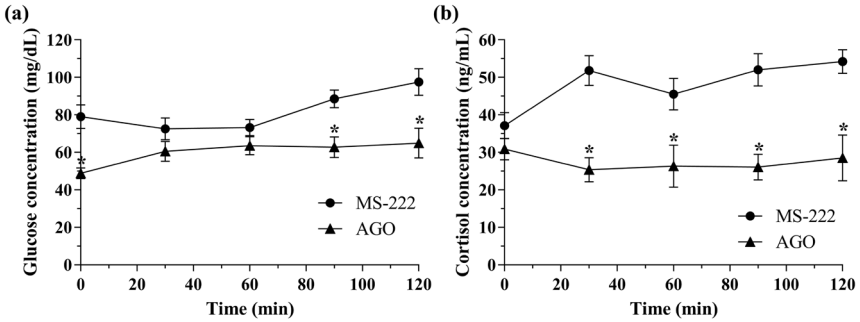

Figure 4. Comparison of glucose (a) and cortisol (b) levels in fish after exposure to 700 mg/L AGO and 130 mg/L MS-222. Asterisks (*) indicate significant differences between treatments at a given time point ($p < 0.05$).

significantly higher than those anesthetized with AGO after 30 min of exposure (Figure 4b). It was found that the cortisol level of the fish anesthetized with MS-222 increased rapidly up to 52 ng/mL within 30 min and did not return to normal levels within 120 min.

4. Discussion

Eugenol is known as a bioactive compound that can induce fish anesthesia. Plant oils composed of eugenol are expected to have potential for anesthesia of fish. However, analysis of AGO in the present study shows that this oil contains not more than 6% of eugenol and eugenol derivatives whereas 1,8-cineole and 4-allylphenyl acetate are found in the oil at a very high concentration. Therefore, these two compounds are considered to also have anesthetic activity on the fish.

According to the criteria of the ideal fish anesthetic, an anesthetic activity is determined by its ability to allow fish to reach surgical anesthesia stage 3 with an induction time of 3 or 5 min with a recovery time that should not be more than 2 fold of the induction time (*36*). In this study, AGO at a concentration of 700 mg/L was an appropriate concentration for Nile tilapia because of its induction and recovery time. We previously reported that a concentration of 300 mg/L of AGO was most suitable to anesthetize *Cyprinus carpio*. These dose differences were considered to be mainly due to species difference of the fish. Different fish species have different characteristics such as surface area of the body especially the surface area of the gill, which is the most important organ. The gill is the main entrance for anesthetics to the vascular system and to the target receptors. Therefore, the gill surface and the thickness of gill epithelium plays an important role for anesthesia (*37*). Generally the gill surface area decreases when the body weight increases (*38*). Moreover, body design, enzymes, lipid content or metabolic rate are different according to fish species difference (*39*). Therefore, the suitable dose for different fish species is different.

Fish transportation is a common procedure for fish farms and is considered to be a stressor that could negatively affect fish health (*3*). Stress during fish transport is due to handling and aquatic pollutants such as low oxygen and acidified water (*40*). In the present study, the transportation simulation was built to reproduce vertical and horizontal movement of a vehicle on a road, thus mimicking a commercial operation. Usually the farmers decrease the cost of fish transportation by increasing fish loading density. However, the results in the current study show that when fish are transported at high loading density, particularly without anesthetics, it can cause more stress than those transported at low loading density. Fish stress can be indicated by increased levels of DO, TAN, and NH_3. Stress also affects the respiratory system by an increase in oxygen uptake rate, ventilation rate, oxygen transport capacity of the blood (*41*), and results in an increase of Hct, Hb, RBCs and causes variation of WBCs (*42*). The results of the present study demonstrate that TAN and NH_3 levels in the water of the AGO group were significantly lower than the control group. These results indicate that AGO has a significant effect on reducing activity in fish. It was reported that low ammonia excretion indicated a decreased metabolic rate for fish (*43*). Therefore, it is considered that AGO may decrease the metabolic rate of Nile tilapia. This was the reason explained why DO level consumed by the fish was higher in the AGO group than in the control group.

Stress response of the fish after exposure to AGO was intensively studied compared to those using MS-222. The recommended dose of MS-222 on Nile tilapia was 130 mg/L (*4*) while the suitable dose of AGO was 700 mg/L. Generally, the hematological parameters; Hb, Hct, RBC, and WBC of resting fish are approximately 6 g/dL, 26%, 1×10^6 cells/μL, and 15×10^3 cells/μL, respectively (*44,45*). In the present study, the hematological parameters of anesthetized Nile tilapia with AGO and MS-222 were higher than the normal level. In addition, blood glucose and plasma cortisol levels are important stress indicators (*46*). The blood glucose and plasma cortisol levels of resting Nile tilapia are approximately 50-70 mg/dL (*47*) and 20-30 ng/mL (*48*). Surprisingly, the glucose and cortisol

levels in Nile tilapia exposed to AGO were quite constant at normal levels while the fish exposed to MS-222 showed higher levels of blood glucose and plasma cortisol than the resting fish at all-time points. These results suggested that AGO has potential for reducing stress in fish, which showed blood glucose and plasma cortisol levels of Nile tilapia to be close to normal. In conclusion, the current study indicates that AGO is a promising natural agent suitable for anesthesia in Nile tilapia.

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Original Article

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T-786C variation in the promoter sequence of human *eNOS* gene markedly influences its expression level

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Summary **This study investigated the role of the T-786C polymorphism (SNP) in the 5'-flanking sequence of the endothelial nitric oxide synthase gene (*eNOS*) on its expression level in vascular endothelium with the ultimate goal of shedding more light on the mechanisms by which genetic variations of *eNOS* might affect the vascular level of nitric oxide (NO). Sequences in the 5'-flanking region of *eNOS* gene were PCR-amplified using genomic DNA templates isolated from blood samples collected from cardiovascular disease (CVD) patients. Two sequence-versions carrying the same SNP site were used; a short (345 bp) and an extended one (1,594 bp), numbered relative to the translational start site. All sequences were cloned into a promoter-less vector (pGL3-basic), which carries the firefly luciferase gene as a reporter. Genotyping of the T-786C polymorphism was performed using Sanger sequencing of the insert region. Luminescence levels were then recorded 24-48 h after transfecting human endothelial cell line (EA.hy926). Three genotypes were identified in the subject samples; TT, TC, or CC. The highest expression levels associated with the TT genotype, followed by the TC genotype, then the CC genotype. The extended sequence version produced higher expression levels compared to the shorter version. Our results provide evidence that the T allele at the T-786C SNP site of the *eNOS* gene results in increased expression of the enzyme, and consequently might provide a protective mechanism from CVD. The extended promoter sequence of *eNOS* resulted in higher expression of the gene, suggesting the presence of some essential binding sites for transcription enhancing proteins.**

Keywords: T-786C polymorphism, *eNOS* expression, luciferase, reporter gene

1. Introduction

Nitric oxide (NO) is a soluble gas produced in the vascular endothelium from L-arginine by the constitutive calcium-calmodulin-dependent enzyme endothelial nitric oxide synthase (eNOS). NO plays a crucial role in retaining the normal endothelium function and maintaining vascular homeostasis. NO maintains the vasodilatation tone of the vascular endothelium, regulates its cell growth, and protects it from injuries caused by platelet aggregation (*1*). Previous investigations on the production of endogenous NO suggested that vascular endothelium diseases probably result from reduced production of endogenous NO. Several factors are known to affect the release of endothelium NO including: down-regulation of eNOS, increased bioavailability of Asymmetric dimethylarginine (ADMA) that down-regulates the activity of eNOS, decreased bioavailability of Tetrahydrobiopterin (BH4), which is an essential co-factor for NOS activity, and the availability of reactive oxygen species (ROS), *e.g.* superoxide (O^{2-}) (*2,3*).

The *eNOS* gene (located on chromosome 7 at the 7q36.1 region) contains 26 exons and 25 introns spanning ~23.5 kb of genomic DNA. Although the gene is known to encode an mRNA of 4,052 nucleotides, some 10 splice variants of the gene have been reported (The Gene Cards human gene database index, ensemble). The gene has been the focus of intensive research to identify potentially functional polymorphisms or mutations that might affect mRNA

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transcription and gene expression. To date more than 1,730 polymorphic sites have been identified in, or in the vicinity of the gene (*http://www.ncbi.nlm.nih.gov/snp*). However, no clear correlation could be detected between the presence of SNPs in the coding and/or non-coding (*e.g*. promoter) sequences of the *eNOS* gene and NO production, despite the fact that several SNPs have been associated with cardiovascular disease (CVD) (*4*).

One of the most studied clinically relevant genetic variants is a SNP in the promoter region (T-786C; rs2070744), frequently associated with the development of coronary artery disease (CAD) (*5*). A statistically significant decrease in average eNOS expression has been reported for the T-786C polymorphism and was suggested to contribute to the vascular, contractile, and autonomic responses in failing human myocardium (*6*). Another study provided evidence that the same SNP is associated with the presence and severity of angiographically defined CAD in the Italian population (*7*).

We have previously investigated the distribution of several reported SNPs in a number of genes involved in the NO signaling pathway, including DDAH2 SNP1 (−1151 C/A, rs805304) and SNP2 (−449 C/G, rs805305) (*8*), eNOS (Glu298Asp polymorphism) (*9*), NADPH oxidase (C242T polymorphic site of p22 phox gene) (*10*) and paraoxonase (PON1 Q192R) (*11*), in a large number of DNA samples extracted from Egyptian healthy subjects and myocardial infarction (MI) patients. In these studies, however, no clear correlation could be detected between the reported SNPs and other relevant biochemical parameters.

Thus, the main goal of the present study is to investigate the effect of the T-786C polymorphism in the 5'-flanking promoter region of the *eNOS* gene on the gene expression level in the vascular endothelium, using a sample of Egyptian cardiovascular patients. To accomplish this goal, promoter sequences of *eNOS* carrying three reported SNPs were extracted from Egyptian cardiovascular patients before being cloned into a promoter-less vector (pGL3-basic) carrying the luciferase gene as a reporter. Luminescence levels were then recorded after transfecting human endothelial cell line (EA.hy926).

2. Materials and Methods

2.1. *Samples*

The T-786C SNP was examined in blood samples collected from acute myocardial infarction patients recruited for the study from the intensive care unit of the National Heart Institute, Imbaba, Giza and El Demerdash Hospital, Cairo, Egypt. Written informed consent were obtained from all participants in the study. The study protocol was approved by the German University in Cairo ethics committee and complied with Declaration of Helsinki. The main target is to classify the samples into TT, TC, or CC according to the genotype distribution of the *eNOS* T-786C SNP.

Blood samples were collected into EDTA coated vacuum tubes and stored at 4°C until DNA extractions were performed. Extracted DNA was used as a template for PCR amplification of the *eNOS* gene.

2.2. *Purification and amplification of DNA by polymerase chain reaction (PCR)*

DNA purification was done using Thermo Scientific GeneJET Whole Blood Genomic DNA Purification Mini Kit (Rockford, USA). The purified DNA was used directly in the PCR. For PCR amplification of DNA, oligonucleotide primers were designed to flank either short or extended fragments upstream of the translation start site. An extended fragment represents the complete *eNOS* promoter (1,594 bp), while the short fragment (345 bp) is a basal central part of the promoter. Recognition sequences for restriction endonucleases were inserted at both ends of the corresponding oligonucleotide (Table 1). All PCR amplifications mixtures were carried out in a total volume of 100 µL containing 50 µL EmeraldAmp GT PCR Master Mix (TAKARA, Japan), 2 µg genomic DNA as a template, 40 pmol final concentrations from each of the forward and reverse primers, and the volume was completed to 100 µL using nuclease-free sterile water. The PCR amplification programs for the long and short fragments are listed in Tables 2 and 3, respectively. The amplified PCR products were purified using PureLink PCR Purification Kit (Thermo Fisher

Table 1. Primer sequences

Primer name	Sequence (Underlined Restriction Sites)	Amplicon size
Forward *eNOS* primer – extended insert	GAAGATCTATCTGATGCTGCCTGTCACCTTGACCCTGAG (*Bgl* II)	1,612bp
Reverse *eNOS* primer – extended insert	ATTAAGCTTTGCCTGCTCCAGCAGAGCCCTGGCCTTTTC (*Hind* III)	
Forward *eNOS* primer – extended insert	GAAGATCTCTGATGCTGC CTGTCACCTT (*Bgl* II)	1,594bp
Reverse *eNOS* primer – extended insert	ATTAAGCTTCCAGCAGAG CCCTGGCCTT (*Hind* III)	
Forward *eNOS* primer – short insert (primers use for sequencing)	GAAGATCTAGATGGC ACAGAACTACAAACC (*Bgl* II)	345bp
Reverse *eNOS* primer – short (primers use for sequencing)	ATTAAGCTTGTCCTT GAGTCTGACATTAGG (*Hind* III)	

Table 2. PCR thermo-cycling conditions for the long insert (1,612 bp)

Protocol step	Temperature	Time	Number of cycles
Initial step	95°C	10 min	1
Denaturation step	95°C	30 sec	35
Annealing step	60°C	30 sec	
Extension step	72°C	2 min	
Final elongation	72°C	10 min	1
Final hold	4°C	Pause	hold

Table 3. PCR thermo-cycling conditions for the short insert (345 bp)

Protocol step	Temperature	Time	Number of cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	35
Annealing	57°C	30 sec	
Extension	72°C	1 min	
Final elongation	72°C	10 min	1
Pause	4°C	Hold	

Scientific, Rockford, USA) and their concentrations estimated using Qubit dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific). The amplified fragments were then digested using FastDigest *Bgl* II and *Hin*d III (Thermo Fisher Scientific, Rockford, USA) before being ligated into a pGL3-basic vector (Promega, Madison, USA); Luciferase reporter vector (E1741, Promega, USA) previously restricted using the same endonucleases. After an overnight incubation at 23°C, the ligation mixtures were transformed into competent *E. coli* XL1 cells (Stratagene, USA). All recombinant plasmid sequences were confirmed by Sanger chain termination sequencing.

2.3. *Transfection of EA.hy926 cells using the polyethylenimine (PEI) reagent*

Procedures were done following the protocols of a previous study (*12*). Basically 600 ng of plasmid DNA (*e-NOS* TT SNP, *e-NOS* TC SNP, *e-NOS* CC SNP long, or *e-NOS* CC SNP short, or the control plasmids pGL3-basic or pSV-β-galactosidase) were added to 1.8 µg of PEI reagent and completed to 40 µL using Dulbecco's Modified Eagle's Medium (DMEM) without serum. Transfection mixtures were incubated at room temperature for 15 minutes. Each transfection mixture (40 µL) was transferred to 24-well plate previously seeded with 10^5 cells/well; where each well contained 400 µL of total liquid volume (360 µL of complete medium and 40 µL of transfection mixture). The plate was incubated at 37°C for 48 hours.

Cell lysis and luminescence assays were performed using 1× Glo Lysis Buffer, Steady-Glo, and Beta-Glo Luciferase Assay Systems (Promega, Madison, USA). Luminescence was measured using GloMax®-Multi Detection System (Promega, Madison, USA).

2.4. *Statistical analysis*

Average luminescence levels were determined for three independent measurements and differences were analyzed using analysis of variance (ANOVA) using GraphPad Prism v5.

3. Results

3.1. *Effect of T-786C SNP on luciferase expression using pGL3 eNOS promoters*

EA.hy926 cells were transfected with plasmid constructs carrying the different promoter fragments. pSV-β-galactosidase was co-transfected into the cells as a control for transfection efficiency. Both luminescence intensity and β-galactosidase activities were measured in cell lysates 48 hours post-transfection. Luminescence levels were normalized to β-galactosidase activities. Luminescence levels for EA.hy926 cells harboring the CC_{short}-786 *eNOS* promoter constructs were not significantly different ($p = 0.5903$) from those obtained for cells transfected with empty plasmid vectors. On the other hand, the CT_{short} and TT_{short} SNP genotypes resulted in luminescence levels that were 1.6 and 1.8-fold higher than their respective controls ($p < 0.0001$). Luminescence results obtained for constructs carrying CC_{short}-786, CT_{short}, TT_{short} were significantly different from each other's as shown in Figure 1.

3.2. *Effect of eNOS promoter sequence length on luciferase expression*

Luminescence results obtained for EA.hy926 cells transfected with constructs carrying the extended version of *eNOS* promoter ($eNOS_{long}$) with the CC_{-786} genotype

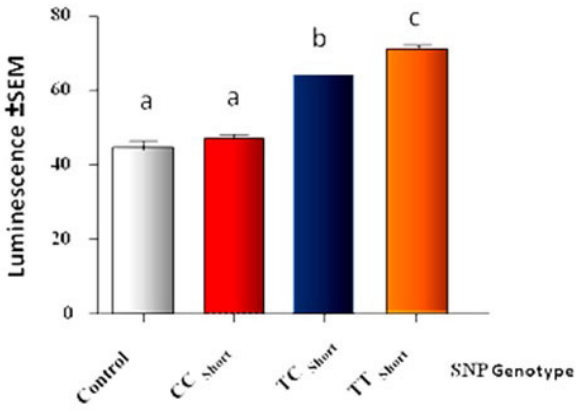

Figure 1. Comparison between luminescence levels obtained for EA.hy926 cells transfected with plasmid constructs of short *eNOS* promoter sequences carrying the three different SNPs. Control luminescence is the Luminescence produced by EA.hy926 cells transfected with the empty plasmid vector pGL3-Basic. Differences in luminescence emission were analyzed using independent *t* test, where different letters (a, b, or c on bars) indicate significant differences at ***$p < 0.0001$. Error bars represent standard error of the mean (SEM).

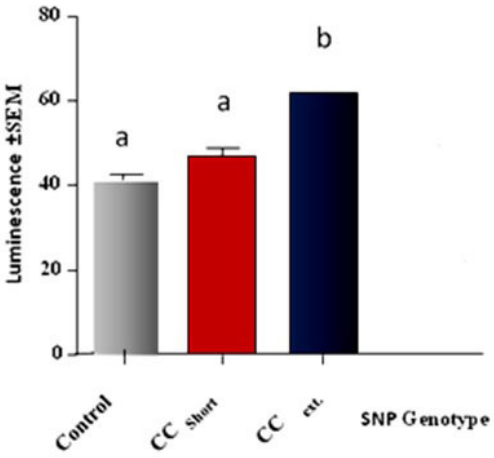

Figure 2. Luminescence levels obtained using constructs carrying the CC genotype on the entire, or shorter *eNOS* promoter sequences. Control luminescence is the Luminescence produced by EA.hy926 cells transfected with the empty plasmid vector pGL3-Basic. Differences in luminescence emission were analyzed using independent t test, where different letters indicate significant differences at $^{***}p <$ 0.0001. Error bars represent standard error of the mean (SEM).

(CC_{long} = 1,594 bp) were significantly higher than those obtained for cells transfected with constructs of the shorter promoter version (CC_{short}) that carries the same SNP site (Figure 2).

4. Discussion

To date more than 1,730 polymorphic sites have been identified in, or in the vicinity of the *eNOS* gene (*http://www.ncbi.nlm.nih.gov/snp*). However, no clear correlation could be detected between the presence of SNPs in the coding or non-coding sequences of the gene and the amount of NO produced to reflect the activity of the encoded enzyme.

Here, we extended our studies on *eNOS* by performing *in vitro* gene expression analyses of the T-786C polymorphism in the promoter region of the *eNOS* gene, with the ultimate goal of shedding more light on the mechanisms by which genetic variations of *eNOS* might affect the expression of eNOS and therefore the vascular level of NO.

DNA sequencing of amplified regions of the *eNOS* gene revealed the presence of three different genotypes; T/T, T/C, and C/C. Our results show significantly higher expression levels of *eNOS* for the T/T genotype compared to the other two genotypes; a T to C mutation resulted in 1.7~1.9-fold lower expression levels of the gene. In agreement with our findings, earlier *in vitro* observations suggested that the replacement of a T at position – 786 by a C resulted in ~50% lower transcriptional activity (*13,14*).

In harmony, Cattaruzza *et al*. (2004) investigated the effect of T-786C SNP on the shear stress-induced NO release from endothelial cells isolated from umbilical cords. They found that shear stress-induced *eNOS* mRNA and protein expression were present in TT and CT genotype cells but absent in cells with CC genotype. They concluded that the T-786C SNP of *eNOS* gene constitutes a genetic risk factor for CAD, presumably due to binding of an inhibitory transcription factor to the C-type promoter blocking shear stress-dependent maintenance of *eNOS* expression (*15*).

In their trials to elucidate the molecular mechanism behind the reduced *eNOS* gene expression associated with the T-786C mutation, Miyamoto *et al*. were able to identify and purify a protein called replication protein A1 (RPA1) that contributes to the diminished *eNOS* expression in T-786C placenta cells. Diminished *eNOS* expression was revealed by the finding that serum nitrite/nitrate levels among individuals carrying the T-786C SNP were significantly lower than among the T variant subjects. They concluded that RPA1 apparently functions as a repressor protein in the T-786C polymorphism-related reduction of *eNOS* gene transcription associated with the development of CAD (*14*).

AliReza *et al*. investigated the frequency of T-786C polymorphism of the *eNOS* gene in non-smoker and non-diabetic CAD patients in North West Iran. Compared to the healthy control group, the *eNOS* T-786C genotype was associated with reduced serum levels of NO (*16*). In a sample of Japanese population, Nakayama *et al*. assessed the influence of the same polymorphism on coronary spasm development. They found that the T-786C mutation resulted in a significant reduction in *eNOS* gene promoter activity that reduces the endothelial NO synthesis and predisposes the patients to coronary spasm (*13*). Moreover, Popov *et al.* demonstrated that T-786C polymorphism contributes to a higher prevalence of postoperative mortality after emergency cardiac surgery. Thus, the *eNOS* T-786C polymorphism could serve to differentiate high risk subgroups in individuals with cardiac disease who need cardiac surgery with cardiopulmonary bypass (*17*).

In contrast, however, Kincl *et al*. found no impact of T-786C polymorphism on CAD prognosis (death or AMI, unstable angina, necessity of percutaneous or coronary artery bypass graft, heart failure, or cardioverter/defibrillator implantation) (*18*). Similarly, Jaramillo *et al*. and Alp *et al*. observed that T-786C polymorphism of the *eNOS* gene was not associated with CAD in a studied sample of Chilean and Turkish individuals, respectively (*19,20*). To our knowledge, no parallel investigations were performed on Egyptian population regarding the association of T-786C polymorphism of *eNOS* with the incidence of CAD.

Two stretches of *eNOS* promoter were tested in the study; a short sequence (345 bp) carrying the CC genotype and a longer promoter sequence (1,594 bp) carrying the same genotype. Luminescence levels obtained for EA.hy926 cells transfected with constructs carrying the longer promoter version were significantly higher than those obtained from cells bearing the shorter

inserts ($p < 0.0001$).

A study made by Xing *et al*. using DNA sequence deletion, concluded that 68% of the basal activity of the *eNOS* promoter was controlled by the region from – 1 to – 166 bp and the rest was dependent on the region from – 1,033 to – 1,600 bp (*21*). The study strongly suggested that the main functional region of *eNOS* promoter is from – 1 to – 166 bp that binds to the upstream activator transcription factor 1 (AP1). This explains the increased gene expression using the extended sequence for *eNOS* promoter (long version = 1,594 bp) observed in our study as compared to the gene expression using the short version (345 bp) that lacks the binding site to transcription factor AP1.

In conclusion, our study suggests that a T allele at the – 786 site of *eNOS* gene increases genetic expression of the enzyme, while the CC genotype at the same site could be a predisposing factor to CVD through marked down expression of *eNOS*.

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Original Article

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Genotyping of *CYP2C9* and *VKORC1* polymorphisms predicts south Indian patients with deep vein thrombosis as fast metabolizers of warfarin/acenocoumarin

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Summary **Deep vein thrombosis (DVT) is a life-threatening disease. Warfarin and acenocoumarol are anticoagulants used to treat DVT and vary among individuals in terms of treatment response/toxicity. Single nucleotide polymorphisms (SNPs) in *CYP2C9* and *VKORC1* play a role in the pharmacokinetics and dynamics of warfarin and acenocoumarol and they determine the efficacy of treatment by controlling drug clearance in treated individuals. The aim of the current study was to genotype the critical SNPs of *CYP2C9* and *VKORC1* genes in a south Indian population in order to understand the metabolizer phenotype of patients with DVT. *CYP2C9* (rs1799853, rs1057910, rs1057909, rs28371686) and *VKORC1* (rs9923231) SNPs were genotyped in 124 cases of DVT. Genomic regions of these SNPs from genomic DNA were amplified with PCR and directly sequenced using Sanger sequencing except for the SNP rs1799853, which was detected using Sau96I restriction endonuclease-based digestion of variant alleles. Among south Indian patients with DVT, 6.5% (8/124) had the rs1799853 SNP of *CYP2C9* and 11% (14/124) had the rs1057910 SNP while 16% (20/124) had the rs9923231 SNP of *VKORC1* which were associated with the response to warfarin treatment. None of the patients tested positive for poor drug metabolizing genotypes of the *CYP2C9* gene and only 1.6% of the south Indian population was sensitive to warfarin treatment. Genotyping results suggest that a relatively greater amount of the therapeutic drug is required to achieve/maintain the international normalized ratio (INR) in south Indian patients with DVT.**

Keywords: Warfarin, deep vein thrombosis, anti-coagulants, SNP, pharmacogenetics, CYP2C9, VKORC1

1. Introduction

Deep vein thrombosis (DVT) often occurs in large veins such as the femoral or popliteal vein and is one of the leading causes of mortality and morbidity (*1*). Though both genes and environment are widely considered as two major risk factors that cause DVT, numerous genetic analyses of various populations have revealed a close association among genetic factors responsible for the risk of DVT/thromboembolism (*2*). Coumarins are widely used in therapeutics as anti-coagulants with a narrow range because of variability among individuals in terms of pharmacokinetics and pharmacodynamics due to genetic and environmental factors (*3*). A patient's response to coumarin is influenced by various factors such as age, sex, vitamin K intake, and medication taken (*1,3*). In addition, defective alleles of certain genes that render individuals to be poor metabolizers of

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therapeutic drugs result in internal bleeding and stroke during clinical practice (*4*).

Genetic variations in *CYP2C9* (cytochrome P450 2C9, drug-metabolizing enzyme) and *VKORC1* (vitamin K-epoxide reductase 1, drug target) have been found to account for 30-50% of the variability in the drug dose (*5*). Warfarin is a widely used first-generation anti-coagulant and its derivative acenocoumarol is a second-generation anti-coagulant, and both share the same metabolizing gene pathways and result in similar pattern adverse reactions in patients (*6-8*). Genome-wide association studies have verified that these genes are associated with the metabolism of the drugs (*9*). Recently several studies have identified polymorphisms in these two genes (*10,11*).

CYP2C9 ranks amongst the most important drug-metabolizing enzymes in humans. Since several of the drugs (notably warfarin) are metabolized by CYP2C9, the activity of this enzyme is an important factor for therapeutic response, clearance, and toxicity in an individual. Therefore, genetic polymorphisms that affect the enzyme efficiency need to be studied in order to prescribe the right dose of drugs and avoid drug overdosing based on individual genetic variations. Various single nucleotide polymorphisms (SNPs) such as *CYP2C9*2* (rs1799853), **3* (rs1057910), **4* (rs1057909), and **5* (rs28371686) have been identified in individuals with impaired CYP2C9-mediated metabolism and lower warfarin dose requirements (*10*). Compared to individuals with the *CYP2C9*1/*1* wild-type allele, individuals with the *CYP2C9*1/*2*, *CYP2C9*1/*3*, *CYP2C9*2/*2*, *CYP2C9*2/*3*, and *CYP2C9*3/*3* variants require lower warfarin doses (*12*). *CYP2C9*2* and **3* were well-documented variant alleles that have been found to play major roles in drug clearance (*13*). *In vitro* studies have also revealed that individuals with *CYP2C9*2* have 12% less catalytic activity and those with **3* have 5% less catalytic activity than individuals with the wild-type allele (*14,15*). *CYP2C9*2* and **3* have also been associated with an increased risk of excessive anticoagulation and bleeding events among patients treated with warfarin (*16*).

Similar to the effect of SNPs on *CYP2C9* gene efficiency, SNPs in the non-coding region of *VKORC1* gene are associated with sensitivity to coumarin derivatives (*17*). The presence of non-coding variants in *VKORC1* results in differential expression of the VKOR protein that determines the drug dosage in patients. In Asians, *VKORC1* polymorphisms have been associated with warfarin response, accounting for 11% to 32% of the variability in dose response when compared to the wild-type allele (*18*). The *VKORC1* SNP, –1639 G>A (rs9923231) has been found to be an important tag for low-dose haplotypes (variant allele) and high-dose haplotypes (wild allele) (*19*).

Together, the non-coding SNPs in *VKORC1* and *CYP2C9*2* and **3* variants are linked to a reduced dose requirement for warfarin, and *CYP2C9*3* has a similar effect on acenocoumarol (*20,21*). The combined effect of the *CYP2C9* and *VKORC1* alleles on the required dose of warfarin and acenocoumarol has been intensively studied in several populations and the therapeutic dose has been determined depending on the patient's allelic variant (*22,23*). However, the allelic status of *CYP2C9* and *VKORC1* and their distribution in south Indian patients with DVT was not definitively ascertained. Doing so would help to determine the optimal drug dose based on the genotype. The current study analyzed the frequency of allelic variants of the *CYP2C9* and *VKORC1* genes in the south Indian population with DVT; among them many were found to be fast metabolizers. In addition, a meta-analysis of several individual/consortium studies was performed to ascertain the distribution of the *CYP2C9* and *VKORC1* genotype frequency in the current study population in comparison to other populations.

2. Materials and Methods

2.1. *Collection of patient blood samples and extraction of genomic DNA*

Potential subjects were outpatients/inpatients ≥ 18 years of age with a stable anticoagulation status who were being monitored. No exclusion criteria were used. This study was approved by the Institutional Ethics Committee, Rajiv Gandhi Government General Hospital and Madras Medical College, Chennai (approval No. 04092010), and this study was conducted within the ethical framework of the Dr. ALM PG Institute of Basic Medical Sciences, Chennai. Informed consent was obtained from all participants in this study. Patients (*n* = 124) on long-term maintenance therapy (July 2011 – March 2013) with acenocoumarol were recruited from Rajiv Gandhi Government General Hospital, Chennai, India, and data on age, gender, and weight were collected for each patient. Blood samples were collected with EDTA and blood was drawn irrespective of drug dosage and period of administration since this study sought to determine the allelic distribution of drug-metabolizing genes. Genomic DNA was extracted using standard phenol:chloroform extraction. The quality and quantity of the genomic DNA were respectively checked using 0.7% agarose gel electrophoresis and NanoDrop (Thermo Inc., USA).

2.2. *Determination of allelic variants using Sanger sequencing*

A polymerase chain reaction (PCR) amplicon of 473 bp covering rs1799853, which is referred as *CYP2C9*2*, was amplified using a forward primer: 5'-CATGGCTGCCCAGTGTCAGC-3' and a reverse primer: 5'-TCCCATGTTCTCCTGAACTTTGCT-3'

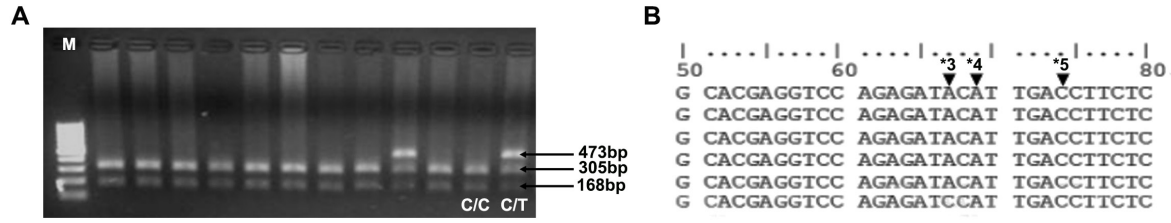

Figure 1. Genotyping gel electrophoresis for *CYP2C9*2* (rs1799853) by restriction digestion (A). The PCR fragment (473 bp) was digested with the restriction endonuclease Sau96I when the patient carried the wild-type allele (CC) and yielded two fragments 305 bp and 168 bp in size; M, DNA 100 bp ladder. **Multiple sequence alignment of *CYP2C9* indicating the nucleotide position of 3 SNPs in *CYP2C9* (*CYP2C9*3* (rs1057910), **4* (rs1057909), and **5* (rs28371686)) (B).

and digested with Sau96I restriction endonuclease, which digested the fragment when the PCR amplicon carried the wild-type allele (C allele). Two fragments 305 bp and 168 bp in size were yielded. Other *CYP2C9* variants **3* (rs1057910), **4* (rs1057909) and *5 (rs28371686) were amplified using a forward primer: 5'-GTGTGATTGGCAGAAACCGGAGC-3' and a reverse primer: 5'-TCTCACCCGGTGATGGTAGAGG -3' with an amplicon size of 256 bp. The SNP rs9923231 (–1639 G>A) located in the promoter region of *VKORC1* was amplified with PCR using a forward primer: 5'-GTTCCAGGGATTCATGCAGGGACA-3' and a reverse primer: 5'-TTGCCCTGACACCTAGTGG CTG-3' with a fragment length of 579 bp. All of the PCR reactions were carried out in 20 µL with the following temperature cycles: 94ºC 2 min for initial denaturation, followed by 40 cycles of 94ºC for 30 sec, 59ºC for 30 sec and 72ºC for 30 sec, and 7 min of final extension at 72ºC. Amplified PCR fragments were purified and sequenced using the internal reverse primer 5'-GATACTATGAATTTGGGGACTTCG-3' for *CYP2C9* and a PCR reverse primer for *VKORC1*.

2.3. *Statistical analysis and meta-analyses*

A confidence interval for proportion was calculated using the online software Stats Calculator at *www.allto.co.uk/tools/statistic-calculators*. The URL for the data presented herein: Online Mendelian Inheritance in Man (OMIM), *http://ncbi.nlm.nih.gov/Omim/* (for *CYP2C9* and *VKORC1*-dependent blood clotting deficiency disorders). Studies with complete genotype data were selected for meta-analysis. MeSH terms like CYP2C, VKORC1, SNPs, Warfarin, Acenocoumarol, Anti-coagulant, and Pharmacogenetics were used to screen publications in the NCBI – PubMed public database. The most relevant studies were selected. Studies featuring only one of the *CYP2C9* variants (**2* or **3*) were excluded, and studies that analyzed both variants were selected for meta-analysis to avoid allelic frequency bias. Studies on the *VKORC1* non-coding variant that featured both the coding and non-coding forms or the non-coding form alone were considered acceptable since sensitivity to the drug is highly dependent on the level of the *VKORC1* gene product, which is based on the transcription efficacy determined by the promoter polymorphism. Meta-analysis was performed using comprehensive meta-analysis software (Biostat, USA). A binary random-effects model with no control group was used and the impact of heterogeneity among studies was estimated using I^2 testing. Heterogeneity was regarded as statistically significant with $p < 0.05$ or $I^2 > 50\%$.

Table 1. Genotype frequency of *CYP2C9* variants

Items	*CYP2C9* variants			
	rs1799853	rs1057910	rs1057909	rs28371686
Wild type	93.5% (116)	89% (110)	100% (124)	100% (124)
Heterozygous	6.5% (8)	11% (14)	-	-
Mutant	-	-	-	-

3. Results

3.1. *Identification of frequencies of variant alleles of CYP2C9 in south Indian patients with DVT*

Variant alleles of *CYP2C9* (*CYP2C9*2*, **3*, **4*, and **5*) were screened for in south Indian patients with DVT (n = 124) and only the *CYP2C9*2* and **3* variants were found in south Indian patients with DVT (Figure 1). The homozygous wild type allele (CC) of the *CYP2C9*2* variant (rs1799853) was present in 93.5% patients (116/124) and the CT heterozygous allele was present in 6.5% patients (8/124). Similarly, the homozygous wild-type allele AA for rs1057910 was present in 89% (110/124) patients and the heterozygous allele (AC) was present in 11% (14/124) patients with DVT. *CYP2C9*2* and **3* homozygous mutant alleles were not found among subjects (Table 1).

The frequencies of *CYP2C9* genotypes were analyzed among all of the 124 south Indian patients with DVT, and warfarin and acenocoumarol metabolizer status was categorized by combining the various allelic frequencies identified in the SNPs of *CYP2C9* summarized in Table 2. Data revealed that 82% (102/124) of south Indian patients with DVT were normal metabolizers, 18% (22/124) were intermediate metabolizers, and none were poor metabolizers.

Table 2. Predicted metabolizer frequency (*n* = 124) in south Indian patients with DVT

Metabolizer category	Genotypes (*n*)			Frequency (%)			95% CI
	Male	Female		Male	Female		
NM (*1/*1)	53	49	102	51	49	82	75.24 - 88.76 (± 6.76)
IM (*1/*2, *1/*3, *1/*5)	12	10	22	55	45	18	11.24 - 24.76 (± 6.76)
PM (*2/*2, *2/*3, *3/*3)	-	-	-	-	-	-	-

NM, normal metabolizers; IM, intermediate metabolizers; PM, poor metabolizers.

Table 3. Genotype and allele frequency of *VKORC1*

Genotypes	Number of subjects*		Frequency % (*n*)	95% CI
	Male	Female		
GG	54	50	84 (104)	77.55 - 90.45 (± 6.45)
GA	9	9	14.5 (18)	8.3 - 20.7 (± 6.2)
AA	2	-	1.5 (2)	-0.64 - 3.64 (± 2.14)
Allele	Number of alleles		Frequency %	95% CI
G	226		91	87.44 - 94.56 (± 3.56)
A	22		9	5.44 - 12.56 (± 3.56)

* In total, 124 subjects (males 65 and females 59) were genotyped for the *VKORC1* –1639 G>A allele.

Table 4. Prevalence of genotype frequency with respect to warfarin sensitivity

Genotype combinations		Prevalence *n* (%)	Warfarin sensitivity
VKORC1 –1639 G>A	*CYP2C9*		
A/A	*1/*3, *2/*2, *2/* 3, *3/*3	1 (0.8)	Very high
G/A	*3/*3	-	
A/A	*1/*1	1 (0.8)	High
G/A	*2/*3	-	
G/G	*3/*3	-	
A/A	*1/*1	-	Moderate
G/A	*1/*2, *1/*3, *2/*2	5 (4)	
G/G	*2/*3	-	
G/G	*1/*2, *1/*3, *2/*2	16 (13)	Mild
G/A	*1/*1	13 (10.4)	Normal
G/G	*1/*1	88 (77)	Less than normal
Total		124 (100)	

3.2. *Allele and genotype frequencies of VKORC1 alleles in south Indian patients with DVT*

The *VKORC1* –1639 G>A promoter polymorphism (rs9923231) was found in 16% (20/124) of the south Indian patients with DVT. Among 124 patients, 84% (104/124) were homozygous for the wild-type allele (GG), 14.5% (18/124) were heterozygous for the mutant allele (GA), and only 1.5% (2/124) were homozygous for the mutant allele (AA) (Table 3).

3.3. *Analyses of a combination of CYP2C9 and VKORC1 genotypes associated with warfarin sensitivity in South Indian patients with DVT*

The genotype combinations of *CYP2C9* with *VKORC1* variants were analyzed to classify warfarin/acenocoumarol sensitivity in the study population. Only 1.6% (2/124) of the patients with DVT were highly sensitive to warfarin treatment, 4% (5/124) were carriers with moderate sensitivity, and 13% (16/124) were carriers with mild sensitivity. Interestingly, about 81.4% (101/124) of the study population carried allelic combinations that would classify them as normal metabolizers and suggest they were unlikely to be sensitive to warfarin treatment (Table 4).

3.4. *Meta-analysis of CYP2C9 and VKORC1 genotypes in various populations*

A meta-analysis of the minor allele frequency (MAF) of *CYP2C9* (rs1799853 and rs1057910) SNPs and the

VKORC1 SNP (rs9923231) was performed in different populations from several individual/consortium studies (Table 5). The MAF of respective SNPs and confidence interval have been graphically presented as a forest plot (Figure 2). A binary random-effects model with no control group was used and a significant heterogeneity for *CYP2C9*2* (I^2 = 99.3%, $p < 0.001$), *CYP2C9*3* (I^2 = 91.3%, $p < 0.001$) and *VKORC1* rs9923231 (I^2 = 99.3%, $p < 0.001$) was observed. The meta-analysis revealed that MAFs were less frequent in the south Indian population than in other populations, and a similar frequency was reported for the north Indian population. Interestingly, the southwest Chinese population had a lower frequency of the variant allele *CYP2C9*2* compared to the study population but the same was not true for *CYP2C9*3* and *VKORC1*. Similarly, Swedes and a mixed European population studied by the International Warfarin Pharmacogenetics Consortium had a lower frequency of the variant allele in *CYP2C9* gene (**3*) than the study population. However, most studies reported a higher frequency of the polymorphic allele (the *VKORC1* non-coding variant) compared to populations on the Indian sub-continent (north and south Indians).

Table 5. Minor allele frequencies of *VKORC1* –1639 G>A and *CYP2C9*2* observed in the south Indian population (current study) and in other populations

SNP	Study	Population	MAF
VKORC1			
rs9923231 (G/A)	Current study	South Indian	0.088
	1000Genomes	European	0.387
		African	0.054
		American	0.410
		South Asian	0.145
	Hapmap	European	0.398
		African	0.022
		Han Chinese	0.951
		Japanese	0.901
		GIUS	0.193
	Rathore SS, *et al*.	North Indian	0.142
	Takeuchi F, *et al*.	Swedish	0.402
	Bodin L, *et al*.	French	0.420
	IWPC	Mixed	0.514
	Gu Q, *et al*.	SWC	0.917
	Oner Ozgon G, *et al*.	Turkish	0.500
	Anton A, *et al*.	Spanish	0.408
	Borobia AM, *et al*.	Spanish	0.372
	Scott, *et al*.	AJ	0.533
		SJ	0.500
CYP2C9			
rs1799853 (C/T)	Current study	South Indian	0.016
	1000 Genomes	European	0.124
		African	0.008
		American	0.099
		South Asian	0.034
		Caucasians	0.129
		Hispanic	0.065
	Hapmap	European	0.103
		African	-
		Han Chinese	-
		Japanese	-
	Rathore SS, *et al*.	North Indian	0.049
	Takeuchi F, *et al*.	Swedish	0.109
	IWPC	Mixed	0.080
	Gu Q, *et al*.	SWC	0.000
	Oner Ozgon G, *et al*.	Turkish	0.130
	Anton A, *et al*.	Spanish	0.165
	Borobia AM, *et al*.	Spanish	0.163
	Scott, *et al*.	AJ	0.127
		SJ	0.194
rs1057910 (A/C)	Current study	South Indian	0.028
	1000 Genomes	European	0.072
		African	0.002
		American	0.037
		South Asian	0.109
		Caucasians	-
		Hispanic	-
	Hapmap	European	0.058
		African	0.000
		Han Chinese	0.044
		Japanese	0.033
	Rathore SS, *et al*.	North Indian	0.039
	Takeuchi F, *et al*.	Swedish	0.109
	IWPC	Mixed	0.040
	Gu Q, *et al*.	SWC	0.098
	Oner Ozgon G, *et al*.	Turkish	0.100
	Anton A, *et al*.	Spanish	0.070
	Borobia AM, *et al*.	Spanish	0.081
	Scott, *et al*.	AJ	0.159
		SJ	0.138

1000 Genomes and Hapmap results were obtained from NCBI dbSNP. IWPC, International Warfarin Pharmacogenetics Consortium; GIUS, Gujarati Indians in the US; SWC, southwest Chinese; AJ, Ashkenazi Jews; SJ, Sephardi Jews.

4. Discussion

DVT is a potentially life-threatening condition due to numerous risk factors such as age, sex, vitamin K intake, and medications. The annual global incidence of DVT, and especially that in veins of the leg, is estimated to be 1.6 per 1,000 with a 10-year recurrence rate of 30% (*24,25*). Although certain risk factors have been identified, DVT is mainly caused by acquired factors including age, hospitalization, pregnancy, hormone therapy, cancer and surgery, and genetic risk factors including mutations and SNPs present in the genes actively involved in drug transport and metabolism. A number of various family and twin studies have revealed that genetic factors account for more than 60% of the risk for developing DVT (*26,27*). Regardless of ideal traditional treatment strategies with anticoagulants, a post-thrombotic syndrome often develops in one in four patients within a year while DVT recurs in one-third of patients within five years (*28,29*).

Although warfarin and acenocoumarol are widely used anticoagulants, they have a narrow range of therapeutic use because of variability among individuals. The combined action of *CYP2C9* and *VKORC1* are essential for the clearance of the anticoagulant drug given to patients with DVT. Several individual studies and GWAS have established the importance of genetic variants in the efficiency with which anticoagulants such as s-warfarin and acenocoumarol are cleared from the circulation (*9*). The dosage requirements of the anticoagulants are determined based on different combinations of alleles present in *CYP2C9* and *VKORC1*

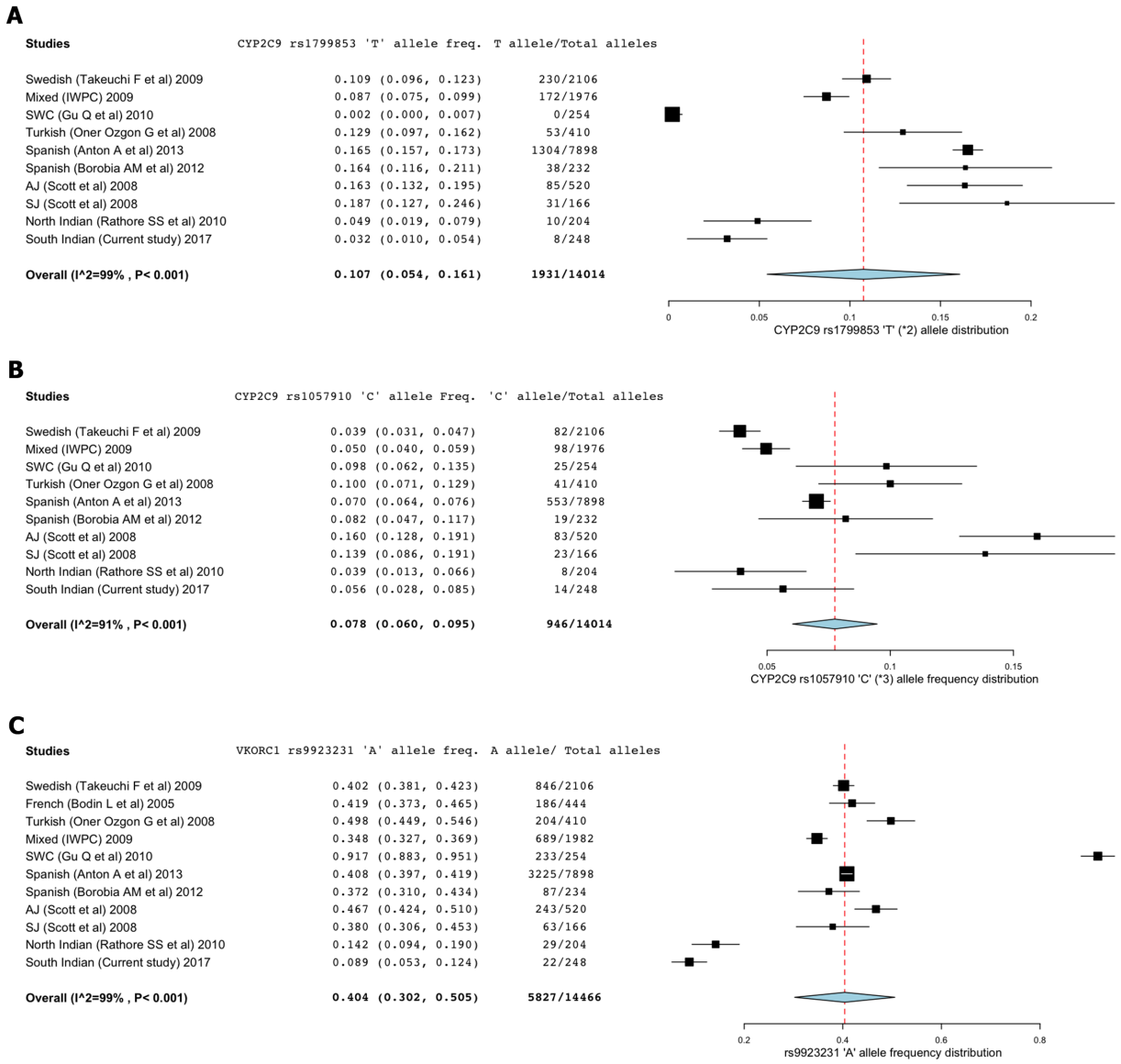

Figure 2. Forest plot of *CYP2C*2* (A), *CYP2C*3* (B), and *VKORC1* promoter SNP (C) among the south Indian population with DVT and other populations. A forest plot generated with comprehensive meta-analysis software with allelic proportion models was analyzed using a random-effects model. IWPC, International Warfarin Pharmacogenetics Consortium, SWC; Southwest Chinese; AJ, Ashkenazi Jews; SJ, Sephardi Jews.

that influence the rate of drug clearance.

The current study determined the allele frequency of *CYP2C9* polymorphisms (rs1799853, rs1057910, rs1057909, and rs28371686) and the *VKORC1* promoter polymorphism (rs9923231) in 124 south Indian patients with DVT. Results indicated that 93.5% of south Indian patients carry homozygous wild alleles in *CYP2C9*, suggesting that they are normal metabolizers. A relatively lower frequency of the mutant alleles of this gene (4%) has previously been reported in the north Indian population (*30*). The *CYP2C9*2* variant is reported to be more frequent among Caucasian populations, with ~1% of the population being homozygous carriers while 22% are heterozygous; corresponding figures for the *CYP2C9*3* allele are 0.4% and 15%, respectively (*31*). In contrast, only 6.5% of the population in the current study was heterozygous for the *CYP2C9*2* variant and 11% was heterozygous for the **3* variant, and none of the population was homozygous for variant alleles. The *CYP2C9*4* and **5* variants were not noted, suggesting that these variants are very rare in the south Indian population. The current results indicate that most south Indian patients with DVT have a *CYP2C9* wild-type allele. Similarly, 84% of the south Indian patients with DVT had the normal allele (GG) for *VKORC1* rs9923231 and 16% had a mutant allele (GA, AA). Likewise, Rathore *et al.* reported that 14% of north Indian patients with DVT had a mutant allele (GA and AA) for VKORC1 (*30*). Results of a meta-analysis also indicated that Indian patients with DVT have a lower frequency of minor alleles than other populations.

In conclusion, combined genotype analyses of both *CYP2C9* and *VKORC1* genetic variants suggested that most south Indian patients with DVT examined in this study were normal metabolizers. None of the patients in the current study exhibited a poor drug-metabolizing genotype with regard to *CYP2C9*. Only 1.6% of the south Indian population is sensitive to warfarin treatment. Combined genotype analysis of *CYP2C9* and *VKORC1* polymorphisms from the south Indian population with DVT suggested that an increase in the anti-coagulant drug dose may be necessary for Indian patients with DVT to achieve/maintain the international normalized ratio (INR). Overall, the results of the current study suggest that the drug therapy should be personalized for South Indian patients since this group displays a distinct genotype.

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Original Article

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The α7 nicotinic acetylcholine receptor positive allosteric modulator attenuates lipopolysaccharide-induced activation of hippocampal *IκB* and *CD11b* gene expression in mice

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Summary **We have reported that 3a,4,5,9b-tetrahydro-4-(1-naphthalenyl)-3*H*-cyclopentan[c]quinoline-8-sulfonamide (TQS), α7 nicotinic acetylcholine receptor (nAChR) positive allosteric modulator (PAM) reduces lipopolysaccharide (LPS)-induced hyperalgesia and allodynia in mice. The objective of the present study was to determine the effects of TQS on LPS-induced activation of hippocampal inhibitor of κB (*IκB*) and cluster of differentiation 11b (*CD11b*) gene expression involving hyperalgesia and allodynia in mice. We also examined the effects of TQS on microglial phenotype following LPS administration. Pretreatment of TQS (4 mg/kg) reduced the expressions of IκB and CD11b mRNA. Pretreatment of methyllycaconitine (3 mg/kg), an α7 nAChR antagonist, reversed TQS-induced decrease in IκB and CD11b mRNA expressions in the hippocampus indicating the involvement of α7 nAChR. In addition, TQS (4 mg/kg) reversed the LPS-induced microglial morphological changes. These results suggest that TQS reduces LPS-induced *IκB* and *CD11b* gene expression and microglial activation associated with hyperalgesia and allodynia by targeting microglial α7 nAChR in the hippocampus.**

Keywords: Nicotinic receptor, α7 positive allosteric modulator, microglia, hippocampus, pain, mice

1. Introduction

The α7 nicotinic acetylcholine receptors (nAChRs) are composed of α subunits that assemble to form homo-pentomers. These receptors have been identified to be highly expressed on microglia within hippocampus (*1,2*). Moreover, brain nicotinic cholinergic pathway has been proposed to regulate microglial activation involving α7 nAChRs (*3*). The α7 agonists have been shown to reduce hyperalgesia and allodynia in a number of animal models (*4-6*). However, these receptors undergo rapid desensitization upon agonist binding (*7*), and adopt stable non-conducting (desensitized) conformations (*8*). One alternative approach to target α7 nAChRs, is the use of type II positive allosteric modulators (PAMs) that can efficaciously prevent normal desensitization and can even reactivate desensitized α7 nAChR (*8,9*). The α7 nAChR type II PAMs were found to decrease pain sensitivity in animal models in the spinal cord (*10,11*). However, the antinociceptive effects of α7 nAChR type II PAMs on pain sensitivity in the hippocampus remain unknown.

Emerging evidence indicates that hippocampus plays a critical role in pain perception and processing (*12,13*) and this limbic structure is densely populated with microglia cells compared to other brain regions (*14*). This suggests a potential role of hippocampal microglia during hyperalgesia and allodynia. Furthermore, recent studies indicate that microglial cells are important regulators in the development and maintenance of pain-like symptoms (*15*). Furthermore, ramified "resting state" microglial cells do not exhibit activation for inflammatory signaling pathway reflecting in binding of inhibitor of κB (IκB)

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to nuclear factor-κB (NF-κB) within cytoplasm (*16,17*). However, IκB becomes phosphorylated and exposed to proteolytic degradation upon an appropriate stimulation (*18*). The degradation of IκB protein unmasks NF-κB from inactive to active state and the active NF-κB is then translocated to nucleus and positively regulates the transcription of various pain mediating genes, including IκB mRNA (*19,20*). A growing body of evidence has shown the involvement of increased IκB mRNA expression in the mediation of pain-like symptoms (*6,21*). Therefore, the evidence indicates that increased IκB mRNA and cluster of differentiation 11b (CD11b) mRNA, a microglial activation marker in the brain, expressions occur simultaneously during hyperalgesia and allodynia (*6,15,22*).

Recently, we have shown that the α7 PAM 3a,4,5,9b-tetrahydro-4-(1-naphthalenyl)-3H-cyclopentan[*c*] quinoline-8-sulfonamide (TQS) (*9*) reduced lipopolysaccharide (LPS)-induced hyperalgesia and allodynia due to decreased microglial activation in the hippocampus involving α7 nAChRs (*23*). However, the effects of TQS on IκB remain unknown during LPS-induced hyperalgesia and allodynia in the hippocampus. In the present study, we have examined the effects of TQS on IκB mRNA and CD11b mRNA expressions and microglial phenotype in the hippocampus following LPS administration in mice. Additionally, we have determined the effects of methyllycaconitine (MLA), an α7 nAChR antagonist, on IκB mRNA and CD11b mRNA expressions in the hippocampus.

2. Materials and Methods

2.1. *Animals*

Male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The animals were housed four per cage (29 × 18 × 12 cm), under standard laboratory conditions (22 ± 2°C, relative humidity 50-60%) with a 12-h light/dark cycle (lights on from 06:00 AM to 6:00 PM) having unlimited access to food and water. Mice were 10-12 weeks of age at the beginning of the experiment. All procedures were in compliance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at South Dakota State University. Good Laboratory Practice and ARRIVE guidelines were obeyed. All efforts were attempted to ensure limited animals suffering.

2.2. *Drugs*

The LPS (055:B5) and MLA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The TQS was purchased from Tocris Bioscience (Ellisville, MO, USA) and was dissolved in normal saline (0.9% NaCl) containing 0.5% tween 80 and 1% dimethyl sulfoxide (DMSO). LPS and MLA were dissolved in normal saline. All drugs were administered intraperitoneally in a volume of 10 mL/kg of body weight.

2.3. *Experimental timeline*

LPS was injected as described previously (*23*) and TQS was administered 0.5 h before LPS administration. The MLA was given 10 min before TQS injection. Brain tissue was collected for quantitative real-time polymerase chain reaction and immunohistochemistry six h after LPS administration.

2.4. *RNA isolation and cDNA synthesis*

Total RNA was isolated and cDNA was synthesized as described previously with minor modifications (*6*). Briefly, mice were sacrificed six h after LPS administration and their hippocampi and brain stems were dissected out, frozen on dry ice, and stored in – 80°C refrigerator until further analysis. RNA was extracted from the tissue by using chloroform and isopropanol after homogenizing the tissue in the presence of Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNA samples were stored at – 80°C until further analysis. RNA was then reverse transcribed into first-strand cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) in Master Cycler Personal (Eppendorf, Hauppauge, NY, USA). The reaction mixture consisted of RT random hexamer primer, dNTP Mix, multiscribe reverse transcriptase, RNase inhibitor, RT buffer, and nuclease free water to a total volume of 20 µL. The mixture was then incubated at 25°C for 10 min, at 37°C for 2 h, and at 85°C for 5 min for deactivating the enzyme. All cDNA was stored at – 80°C until quantitative real-time PCR was performed.

2.5. *Quantitative real-time polymerase chain reaction*

Quantitative real-time PCR was performed as described previously with minor modifications (*6*). Briefly, cDNA template was used for PCR reaction. Primer sequences for IκB, CD11b, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were obtained from Integrated DNA Technologies (Coralville, Iowa, USA). Amplification of cDNA was performed using SYBR Select PCR Master Mix Kit (Applied Biosystems by life technologies, Austin, TX, USA) in StepOnePlus quantitative real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The reaction mixture (20 µL) was composed of SYBR Select PCR Master Mix, 200 nM of prime primer, nuclease free water, and cDNA template. GAPDH primer was used as housekeeping gene. Reactions were carried out using 96 wells qPCR plate (Life Technologies, Grand Island, NY, USA). Cycling parameters were as followed; 50°C for 2 min once;

95°C for 2 min once; then 95°C for 15 s (denaturation), 60°C for 30 s (annealing), and 72°C for 1 min and 30 s (extension) for 40 cycles ending with a melting curve analysis to control amplification. The cycle threshold (Ct) obtained form PCR was used for analysis. The Ct value was determined for each gene and relative expression of each gene was calculated using delta-delta Ct method. The level of the target mRNA was quantified relative to GAPDH and presented as percentage of vehicle control.

2.6. *Immunohistochemistry*

Immunohistochemistry was performed as described previously with some modification (*17*). After sacrificing mice six h after LPS administration, their brains were harvested and fixed with 4% paraformaldehyde (Acros organics, New Jersey, USA) for 24 h. Mice brains were then cryoprotedcted at 4°C in 30% sucrose (Sigma-Aldrich, St. Louis, MO, USA) until brains sank. The 40 µm coronal sections were cut using Leica cryostat. Free floating sections were then placed in water bath maintained at 90°C having boiling 0.01 M citrate buffer (pH 6.0) for 10 min, followed by quenching in 0.3% hydrogen peroxide (Acros Organics, New Jersey, USA) in methanol for 5-10 min. Hippocampal tissue sections were then blocked in 1.5% normal goat serum (Santa Crus Biotech, Dallas, Texas, USA) at room temperature for 1 h. The sections were then incubated with primary antibody (goat anti-rabbit-Iba-1, 2.25:1000, Wako, Osaka, Japan) at room temperature for 1.5 h. The sections were then incubated in secondary antibody (biotinylated goat anti-rabbit; 5:1000, Santa Crus Biotech, Dallas, Texas, USA) at room temperature for 30 min followed by staining with avidin-biotin complex, diaminobenzidine, and mounted on superfrost plus microscope slides (Fisher Scientific, USA). Images were taken three to four random fields in the hippocampus at 20× after overnight drying with bright field microscopy. Quantification of % area occupied by microglial cells was carried out using Image J software.

2.7. *Data analysis*

Data were analyzed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons or Student's *t*-test for MLA data using GraphPad Prism 5.0 (GraphPad Inc., San Diego, CA, USA). The difference between treatments was considered significant at $p < 0.05$. Results are expressed as mean ± S.E.M.

3. Results

3.1. *Effects of TQS on LPS-induced activation of IκB mRNA expression in the hippocampus*

One-way ANOVA revealed that TQS significantly decreased the expression of IκB mRNA following LPS administration (Figure 1, F3,20 = 23.93; $p < 0.0001$). Multiple comparisons of means indicated that LPS (1 mg/kg) significantly ($p < 0.001$) increased the expression of IκB mRNA. Furthermore, TQS (4 mg/kg) significantly ($p < 0.001$) decreased IκB mRNA expression during LPS-induced hyperalgesia and allodynia in the hippocampus. Moreover, LPS did not significantly alter the expression of IκB mRNA in brain stem (control: 100 ± 7.0 *vs*. LPS: 115 ± 2.7).

3.2. *Effects of MLA on TQS-induced decrease in IκB mRNA expression in the hippocampus*

To examine the involvement of α7 nAChR, MLA, an α7 nAChR antagonist, was administered along with TQS. Student's *t*-test indicated that MLA significantly ($p < 0.01$) reversed decrease in IκB mRNA expression (Figure 2).

3.3. *Effects of TQS on LPS-induced activation of CD11b mRNA expression in the hippocampus*

One-way ANOVA indicated that TQS significantly

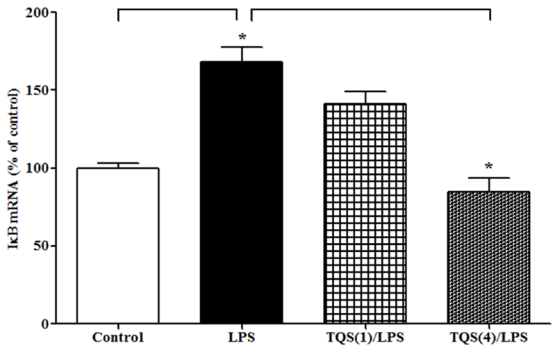

Figure 1. Effects of TQS on LPS-induced activation of IκB mRNA expression in the hippocampus in mice. Mice (n = 6) were administered TQS (1 or 4 mg/kg, *i.p.*) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Data are expressed as mean ± S.E.M. * $p < 0.001$.

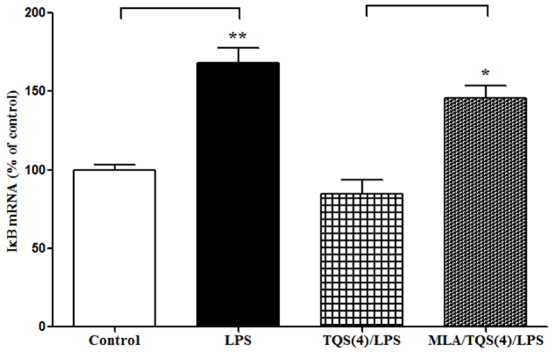

Figure 2. Effects of MLA on TQS-induced decrease in IκB mRNA expression in the hippocampus. Mice (n = 4-6) were administered TQS (4 mg/kg, *i.p.*) 0.5 h before LPS administration. MLA (3 mg/kg, *i.p.*) was given 10 min before TQS injection. Control animals received equal volume of vehicle. Data are expressed as mean ± S.E.M. *$p < 0.01$; **$p <$ 0.001.

reduced the expression of CD11b mRNA following LPS administration (Figure 3; F3,14 = 10.36; $p < 0.001$). Multiple comparisons of means revealed that LPS significantly ($p < 0.01$) increased CD11b mRNA expression. Furthermore, TQS (4 mg/kg) significantly ($p < 0.01$) reduced CD11b mRNA expression in the hippocampus. Moreover, LPS did not alter the expression of CD11b mRNA in brain stem (control: 100 ± 9.4 *vs*. LPS: 104 ± 14.9).

3.4. *Effects of MLA on TQS-induced decrease in CD11b mRNA expression in the hippocampus*

To characterize the involvement of α7 nAChR during microglial activation, MLA was administered along with TQS. Student's *t*-test indicated that MLA significantly ($p < 0.001$) reversed reduction in CD11b mRNA expression (Figure 4).

3.5. *Effects of TQS on microglial cells morphology following LPS administration in the hippocampus*

One-way ANOVA revealed that TQS pretreatment significantly affected microglial activation in the hippocampus (Figure 5; F3,19 = 11.83; $p < 0.001$). Multiple comparisons of means revealed that LPS (1 mg/kg) significantly ($p < 0.001$) increased microglial activation by increasing microglial morphology in the hippocampus as compared to control. Furthermore, TQS (4 mg/kg) significantly ($p < 0.05$) decreased microglial activation due to reduced LPS-induced morphological changes in the hippocampus compared to LPS treated group.

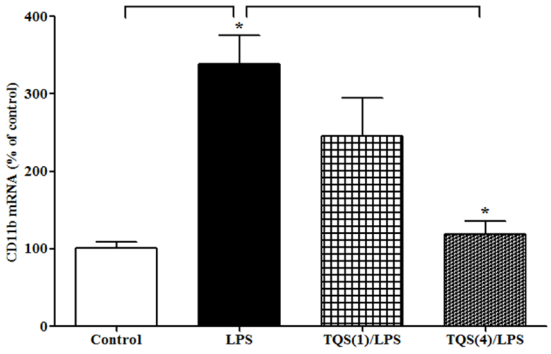

Figure 3. Effects of TQS on LPS-induced activation of CD11b mRNA expression in the hippocampus in mice. Mice (n = 4-5) were administered TQS (1 or 4 mg/kg, *i.p.*) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Data are expressed as mean ± S.E.M. $^{*}p < 0.01$.

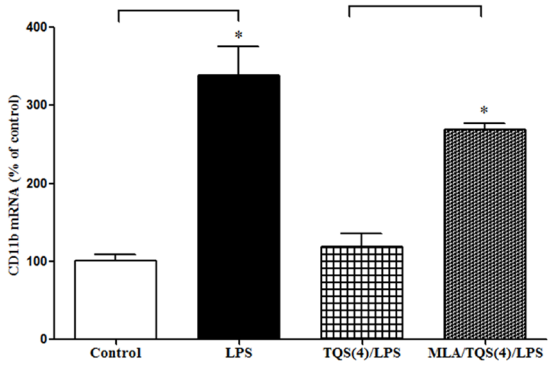

Figure 4. Effects of MLA on TQS-induced decrease in CD11b mRNA expression in the hippocampus in mice. Mice (n = 4-5) were administered TQS (4 mg/kg, *i.p.*) 0.5 h before LPS administration. MLA (3 mg/kg, i.*p*.) was given 10 min before TQS injection. Control animals received equal volume of vehicle. Data are expressed as mean ± S.E.M. $^{*}p < 0.001$.

4. Discussion

In the present study, we have demonstrated that pretreatment of TQS reduced the expression of LPS-induced IκB mRNA, CD11b mRNA and regulated microglial morphological changes in the hippocampus. Furthermore, we have found that pretreatment of methyllycaconitine, an α7 nAChR antagonist, reversed TQS-induced decrease in IκB mRNA and CD11b mRNA expressions in the hippocampus indicating the involvement of α7 nAChR during LPS-induced hyperalgesia and allodynia in mice. The data are consistent with microglial mechanisms involving LPS-induced hyperalgesia and allodynia (*23*).

Elevated level of IκB mRNA, associated with increased NF-κB activity in parallel and used as an index for increased NF-κB activation, in the CNS is associated with occurrence of pain-like symptoms (*6*). Moreover, the activation of NF-κB associated with pain hypersensitivity has been identified in other pain models (*24,25*) and is likely involved in pain hypersensitivity. Given the evidence, we propose a

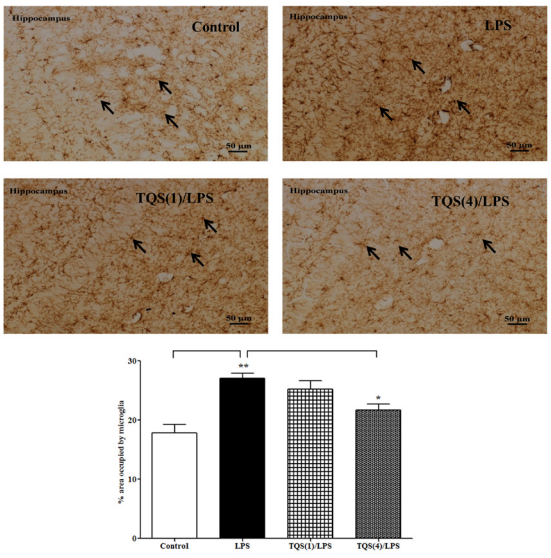

Figure 5. Effects of TQS on microglial cells morphology following LPS administration in the hippocampus in mice. Representative immunoreactivity for microglial morphology in hippocampus (top panel). Mice (n = 5-6) received TQS (1 or 4 mg/kg, *i.p.*) 0.5 h before LPS administration. Control animals received equal volume of vehicle. Data are expressed as mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$.

link between increased IκB mRNA expression and NF-κB activation involving LPS-induced hyperalgesia and allodynia in mice as previously reported (*23*). The pretreatment of MLA, an α7 nAChR antagonist, reversed the TQS-induced decrease in I-κB mRNA expression in the hippocampus supporting the involvement of α7 nAChRs during NF-κB inactivation. To the best of our knowledge, this is the first report which indicates that the effects of α7 nAChR PAM on the hippocampal microglia results in microglial deactivation by reducing NF-κB activation associated with LPS-induced hyperalgesia and allodynia.

It is noteworthy to mention that increased level of IκB mRNA expression is associated with NF-κB activation in the hippocampus. This activation is likely regulated by increased microglial activation as evidenced by elevated expression of CD11b mRNA expression during LPS-induced hyperalgesia and allodynia. Therefore, it is conceivable that the effects of α7 nAChR PAM in the hippocampus are mediated through microglia as LPS-induced increased expression of CD11b mRNA was significantly attenuated by TQS. On the other hand, pretreatment of MLA reversed the TQS-induced decrease in CD11b mRNA expression suggesting an activation of α7 nAChR that in turn reduces microglial activation in the hippocampus. The notion regarding microglial activation is further supported by the finding is that pretreatment of TQS also reduces LPS-induced increase in hippocampal ionized calcium-binding adapter molecule 1 (Iba-1), another microglial marker, expression in mice (*23*), suggesting an involvement of α7 nAChR. Furthermore, microglial cells adopt resting phenotype in inactivated state but undergo rapid activation in response to acute insults. This leads to change in their morphological phenotype characterized by retracted processes, hypertrophy and amoeboid morphology under strongly pathological conditions (*17,22,26*). Our results show that TQS-mediated antiallodynic and antihyperalgesic effects (*23*) are due to reduced microglial activation as indicated by reduced morphological changes in the hippocampus. It is important to mention that active NF-κB within the microglia increases expression of proinflammatory cytokines and other mediators through NF-κB mediated gene expression that plays a critical role in pain facilitation (*6,27*). Therefore, further studies are essential to examine the involvement of proinflammatory cytokines and other mediators, including brain-derived neurotrophic factor during LPS-induced hyperalgesia and allodynia in the hippocampus.

In conclusion, this study demonstrated that α7 nAChR PAM decreases *IκB* and *CD11b* gene expression and microglial activation associated with hyperalgesia and allodynia by targeting microglial α7 nAChR in the hippocampus. Therefore, α7 nAChR PAM may represent a new class of treatment for neuroinflammatory pain.

Acknowledgements

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Brief Report

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Sushi repeat-containing protein X-linked 2 promotes angiogenesis through the urokinase-type plasminogen activator receptor dependent integrin αvβ3/focal adhesion kinase pathways

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Summary **Sushi repeat-containing protein X-linked 2 (SRPX2) is a newly identified chondroitin sulfate proteoglycan that is markedly elevated in multiple solid tumors. It is also suggested that SRPX2 is associated with angiogenesis. A conditioned medium of SRPX2 overexpressing colorectal cancer (CRC) cells and SRPX2 recombinant protein was used to evaluate the effect of secretory SRPX2 on the angiogenesis ability of human umbilical vein endothelial cells (HUVECs) and the involved molecular mechanisms. It was revealed that the activity of SRPX2 is dependent on the urokinase-type plasminogen activator receptor and cooperation of the integrin αvβ3 co-receptor. Subsequent studies showed that both PI3K/Akt and Ras/MAPK pathways and phosphorylation of focal adhesion kinase is involved in the intracellular signaling pathway of SRPX2/uPAR. This study suggests that SRPX2 promotes angiogenesis of HUVECs through the cooperation of the uPAR and integrin/FAK pathway.**

Keywords: SRPX2, extracellular matrix, angiogenesis, proteoglycan

1. Introduction

Proteoglycans represent a large and diverse family of macromolecules composed of a specific core protein with covalently linked glycosaminoglycan (GAG) chains as important components of extracellular matrix (ECM). Proteoglycans have important effects on various aspects of tumor angiogenesis. For example, the V2 isoform of versican, a member of chondroitin sulfate proteoglycan (CSPG), promotes the adhesion of glioblastoma cells to endothelial cells and facilitates the formation of tube-like structures (*1*); NG2, a member of CSPG, is involved in the interaction of pericytes with endothelial cells, and the knockdown of NG2 in pericytes leads to increased permeability of endothelial cell layers (*2*); YKL-40, a secreted heparin-binding glycoprotein, promotes angiogenesis ability in both breast cancer and colorectal cancer tumor cells through syndecan 1 and integrin αvβ3 (*3*) and upregulates VEGF expression in glioblastoma cells (*3,4*).

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Sushi repeat-containing protein X-linked 2 (SRPX2) is a newly demonstrated secretory CSPG (5) that is markedly increased in multiple cancers, such as gastric cancer, colorectal cancer (CRC), pancreatic cancer, and glioblastoma and correlates with a poor outcome or advanced tumor stage (*5-10*). SRPX2 is also believed to have a role in angiogenesis. Miljkovic-Licina *et al*. (*14*) found that *SRPX2* is a markedly upregulated gene in the mice angiogenic phenotype t.End.1V cell line, and transfection of siRNAs against *SRPX2* markedly inhibits the migration and angiogenic sprout formation of these cells. However, the exact effect of molecular mechanism of human SRPX2 in tumor angiogenesis is still elusive.

In this study, the angiogenic ability of SRPX2 in human umbilical endothelial cells (HUVECs) was investigated. First, conditioned medium (CM) of colorectal cancer (CRC) SW480 cells were prepared and the effect of secretory SRPX2 from SW480 on HUVECs was examined. Next, SRPX2 recombinant protein was used to explore the signaling pathways involved. Our findings provide new insights into the role of proteoglycans in ECM in tumor angiogenesis and sheds light on the development of new anti-tumor angiogenesis therapy.

2. Materials and Methods

2.1. *Cell culture*

Human CRC cell lines SW480 purchased from ATCC (Manassas, VA, USA) was maintained in a humidified atmosphere of 5.0% CO_2 at 37°C with Dulbecco's modified Eagle medium/F12 medium (Neuronbc Co., Ltd., Beijing, China). HUVEC, a gift from the Institute of Materia Medica, Chinese Academy of Medical Sciences, was maintained in HuMedia-EG2 (KURABO, Tokyo, Japan) medium with 1.0% fetal bovine serum (FBS) with epidermal growth factor and fibroblast growth factor.

2.2. *SRPX2 recombinant protein and SRPX2-conditioned medium*

SRPX2 human recombinant protein prepared in an *in vitro* wheat germ expression system was purchased from Abnova (H00027286-P01), Taipei, Taiwan. The overexpression vector of pcDNA3.1(+)-SRPX2 plasmid and empty plasmid vector as control were constructed and transfected into SW480 (ATCC, Manassas, VA, USA) as previously described (*8*). SRPX2-CM was prepared according to Tanaka *et al*. (*10*). The medium in which subconfluent SW480 cells were being cultured was replaced with an Opti-MEM reduced serum medium (Gibco Life Technologies Inc., Grand Island, New York, USA), after which the cells were cultured for an additional 24 h and the CM collected. The CM was centrifuged, filtered, and stored at – 80°C.

2.3. *Cell proliferation assay*

HUVECs were cultured with prepared CM from pcDNA 3.1(+)-SRPX2 plasmid-introduced SW480 (SRPX2-CM) or control plasmid-introduced SW480 (negative control, NC) for 72 h and incubated with Cell Counting Kit (CCK)-8 for 4.0 h. Cell viability was measured at the designated time by the amount of absorbance at 450 nm.

2.4. *Transwell migration assay*

In Transwell assays, 5.0×10^3 HUVECs were planted into the top chamber of a Transwell chamber (Corning Cabelcon, Vordingborg, Denmark) lined with a non-coated membrane. SRPX2 human recombinant protein (50 ng/μL) or SW480 cells transfected with SRPX2 overexpressing plasmid were added to the lower chamber. After incubation at 37°C in 5.0% CO_2 for 24 h, the cells remaining in the top chamber were removed and those attached to the underside of the membrane were fixed and stained with 0.1% crystal violet. Each insert was counted using a microscope (Olympus Corp., Tokyo, Japan) on three random fields.

2.5. *Wound-healing assay*

In wound-healing assays, HUVECs were seeded on 24-well plates at a density of 1×10^5 cells/well. After adhesion to the plate, the cell monolayer was scratched with a 10-μL tip. After 24 h at 37°C, photos were taken with an Olympus camera (Olympus Corporation, Tokyo, Japan) under a light microscope (40×) at 0, 6, and 12 h, and analyzed using Image J (National Institudes of Health, Bethesda, MD, USA).

2.6. *Tube formation assay*

Fifty microliters of Matrigel (50 mg/L, BD Biosciences, San Jose, CA, USA) were added to 96-well plates and incubated at 37°C for 1.0 h. HUVECs (5.0×10^5) were seeded into a 96-well plate precoated with Matrigel at a density of 3.0×10^4 cells/well. Capillary tube structures were observed and the representative images were captured using an inverted microscope (4×) equipped with a camera. The tube-forming-structures were counted and analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

2.7. *Neutralizing antibodies and small molecular inhibitors*

Before investigating the intracellular signaling pathway, urokinase plasminogen activator receptor (uPAR) neutralizing antibody (20 μg/mL, MAB807, R&D Systems, Minnesota, USA), integrin αvβ3 neutralizing antibody (20 μg/mL, MAB3050, R&D Systems, Minnesota, USA), or immunoglobulin G (20 μg/mL, MAB002, R&D Systems, Minnesota, USA) as a control were added to HUVECs for 2.0 h. To evaluate the role of the PI3K/Akt and MAPK pathways, 10 μM PI3K inhibitor LY294002 and 10 μM MEK inhibitor PD98059 (both Selleck Chemicals, LLC, Houston, TX, USA) in dimethyl sulfoxide (DMSO) or blank DMSO were added to the HUVECs for 2.0 h.

2.8. *Western blots*

Western blots were performed as previously described (*8*). The primary antibodies involved were as following: 1:1,000 phospho-Akt (Ser473, 4060), 1:1,000 Akt (9272), 1:1,000 phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199, 4228), 1:1,000 PI3 Kinase p85 (42571), 1:1,000 phospho-ERK1/2 (Thr202/Tyr204, 43771), 1:1,000 ERK1/2 (4695), and the focal adhesion kinase (FAK) antibody kit (9330) including phospho-FAK (Tyr576/577) antibody, phospho-FAK (Tyr925) antibody, phospho-FAK (Tyr397) 1:2,000 antibody, FAK antibody, and 1:10,000 glyceraldehyde 3-phosphate dehydrogenase (5174). All antibodies were purchased from Cell Signaling Technology, Danvers, MA, USA.

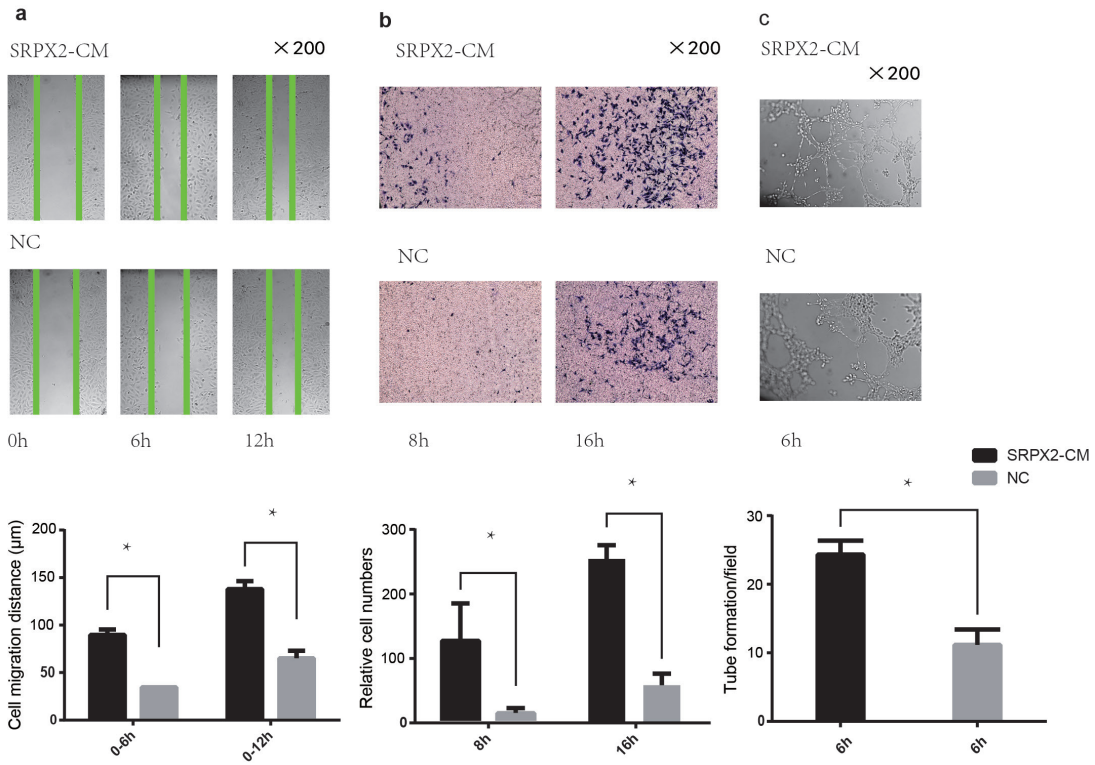

Figure 1. SRPX2 conditional media (CM) significantly promoted migration of HUVECs in scratching assays (a) and Transwell assays (b) respectively compared with negative control (NC) group; (c) in matrigel assays, tube formation ability of HUVECs was also markedly promoted in SRPX2-CM group. *$p < 0.001$.

2.9. *Statistical analyses*

Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All data were expressed as the means ± SEM. Statistical analyses were done using the Student's *t*-test or one-way analysis of variance. The differences were considered statistically significant when $p < 0.05$.

3. Results and Discussion

Since *SRPX2* has three sushi domains encoding three sushi motifs and one hyaline domain mediating specific protein-protein and protein-carbohydrate interaction (*7*), it was predicted that SRPX2 has a role in cellular motility. Hence, the effect of SRPX2 on the migration of HUVECs was first investigated. In wound-healing assays, SRPX2-CM obviously accelerated scratch healing at both 6 and 12 h (both $p < 0.001$; Figure 1a). Similarly, in Transwell migration assays, SRPX2-CM markedly increased the number of HUVECs migrating through the membrane at both 8 and 16 h (both $p < 0.001$; Figure 1b). In addition, the number of tube-like structures per field on Matrigel was also significantly increased by SRPX2-CM ($p < 0.001$, Figure 1c). However, in CCK-8 assays, SRPX2-CM did not promote the proliferation of HUVECs, suggesting that cell proliferation was not involved in the pro-angiogenic effects of SRPX2 on HUVECs. Taken together, we demonstrated that a conditioned medium of SRPX2 from SRPX2 overexpressing SW480 cells significantly increases the angiogenic ability of HUVECs.

The detailed molecular mechanism of the proangiogenic effect of SRPX2 remains unknown. As referenced above, Miljkovic-Licina *et al*. (*11*) proved that mouse SRPX2 directly binds to vascular uPAR on t.End.1V cells. SRPX2 is a proven ligand of cell surface uPAR located on the cell membranes of both tumor cells and HUVECs (*12*); therefore, the role of uPAR in the pro-angiogenic effects of SRPX2 in HUVECs was evaluated using SRPX2 recombinant protein and uPAR-neutralizing antibody. As shown in Figure 2a, in Transwell assays, the number of HUVECs that migrated through the membrane per field was markedly increased in the SRPX2 group compared with that of the control group ($p < 0.001$), while treating HUVECs with uPAR-neutralizing antibody before the Transwell assay significantly attenuated the number of migrating cells compared with the SRPX2 group ($p < 0.01$). Similarly, this effect was also observed in tube-formation assays (Figure 2b). The number of tube-formation structures was significantly increased in the SRPX2 group compared with control group ($p < 0.001$), while significantly decreased after pretreatment with uPAR-neutralizing antibody ($p < 0.001$). These results suggest that the binding of SRPX2 to uPAR is crucial for the proangiogenic effect of SRPX2 on HUVECs.

As is known, uPAR is a GPI-anchored receptor

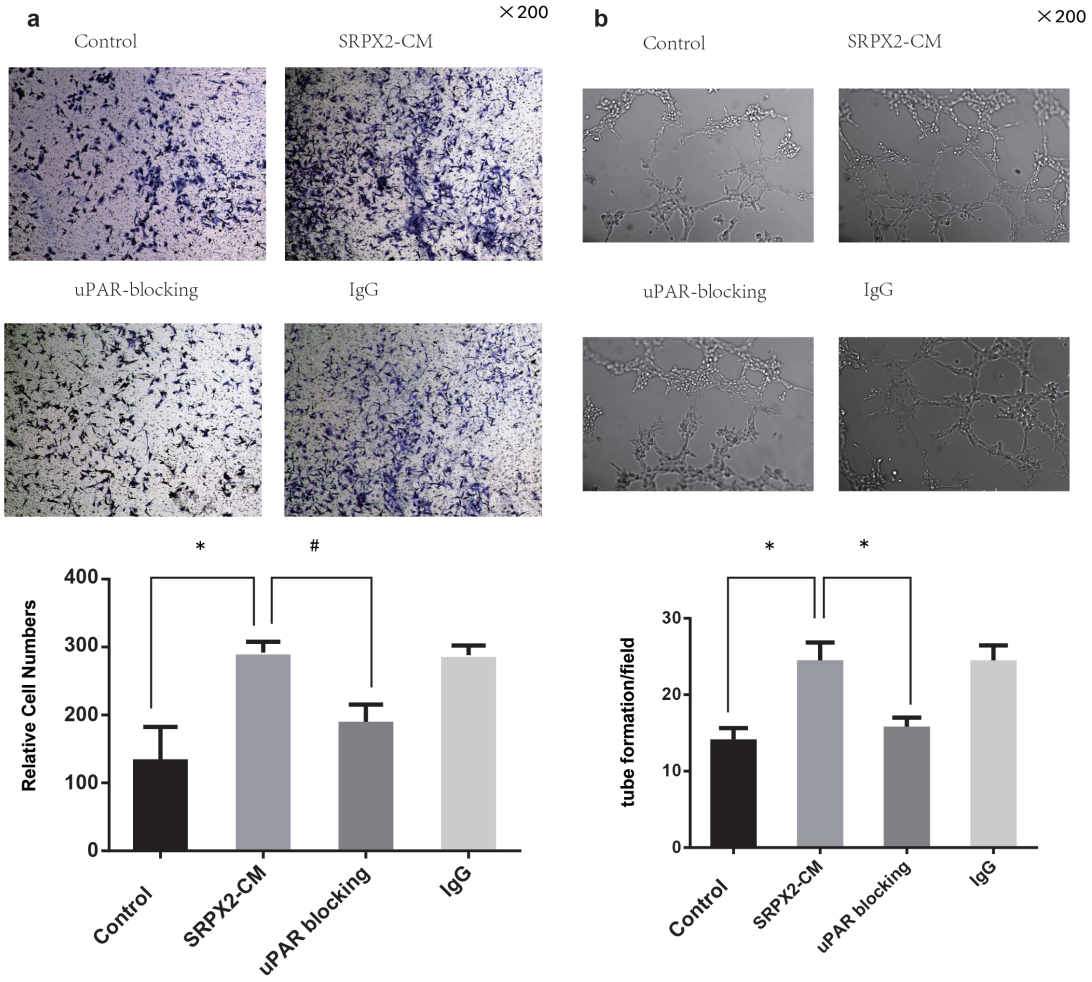

Figure 2. SRPX2 recombinant protein promoted both migration through Transwell chamber and tube structure formation of HUVECs on matrigel compared to control group, while pretreatment of uPAR neutralizing antibody in HUVECs (uPAR blocking group) markedly blocked this effect both in Transwell assay (a) and matrigel tube formation assay (b). IgG group:pretreatment of HUVECs with control mouse IgG antibody .*$p < 0.001$, #$p < 0.01$.

lacking transmembrane and intracellular domains, intracellular signaling of uPAR is dependent on the interaction of uPAR and multiple integrin molecules as co-receptors (*13*). Integrin receptors, an important family of ECM receptors essential in cancer biology, are major co-receptors of uPAR (*14,15*). Because integrin αvβ3 is strongly implicated in uPAR signaling (*16*) and also involved in YKL-40/S1 ligand-receptor interaction mediating angiogenesis (*3*), integrin αvβ3-neutralizing antibody was used to demonstrate that cooperation of uPAR and integrin αvβ3 is necessary for the SRPX2/uPAR complex. In tube-formation assays, the number of tube-like structures of HUVECs in the SRPX2 group was significantly increased compared to control group ($p < 0.001$), while pretreatment with the integrin αvβ3-neutralizing antibody significantly inhibited this effect ($p < 0.001$, Figure 3a), confirming integrin αvβ3 is a crucial mediator for the SRPX2/uPAR complex.

In addition, FAK is a cytoplasmic tyrosine kinase that plays a critical role in integrin-mediated signal transductions as well as in angiogenesis and tumor progression (*17,18*). Tanaka *et al*. (*7*) reported that SRPX2 increases FAK phosphorylation levels in several gastric cancer cells. Gao *et al*. (*9*) also found that the increase in SRPX2 is associated with FAK phosphorylation in pancreatic cancer tissue. In this study, FAK phosphorylation in HUVECs after addition of SRPX2 recombinant protein for 4.0 h was investigated. Similarly, compared to the control group, FAK phosphorylation (Tyr 397 and Tyr 576/577) in the SRPX2 group was significantly increased, while Tyr 925 was not affected (Figure 3b). These results demonstrated that the integrin αvβ3/FAK pathway is involved in the pro-angiogenic effects of SRPX2 on HUVECs and Tyr397 on FAK is indeed a crucial component in angiogenesis (*19-21*).

Because the PI3K/Akt and Ras/MAPK pathways are known as downstream signaling pathways of FAK (*3,19,20,22*), PI3K inhibitor LY294002 and MEK inhibitor PD98059 were used to further clarify the intracellular signaling of SRPX2/uPAR in HUVECs. In tube-formation assays, tube like–structure formation of HUVECs promoted by SRPX2 was significantly inhibited after pretreatment of LY294002 and PD98059 (both $p < 0.001$). Furthermore, phosphorylation of PI3K/Akt and ERK1/2 in HUVECs after addition of SRPX2

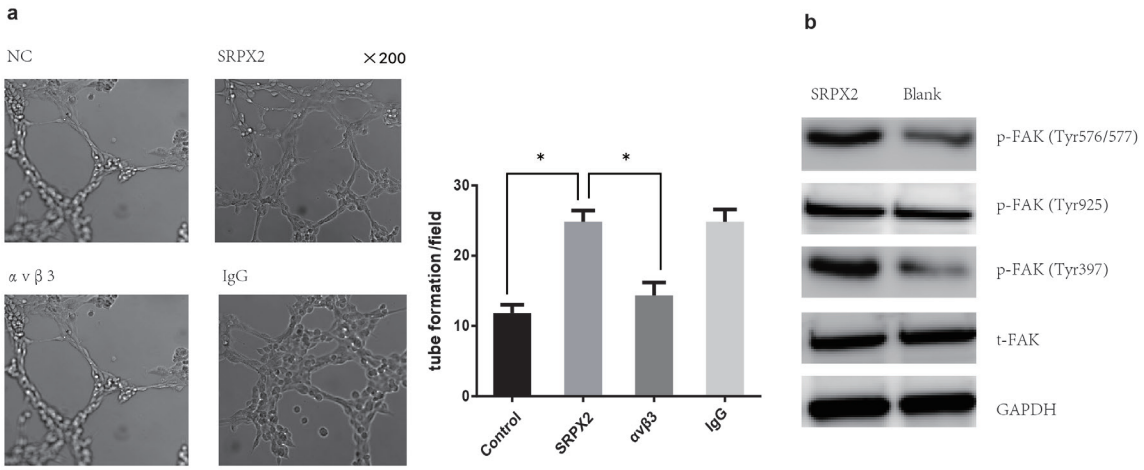

Figure 3. SRPX2 recombinant protein promoted tube formation of HUVECs on matrigel and pretreatment of integrinαvβ3 neutralizing antibody in HUVECs (αvβ3 group) significantly attenuated this effect (a). IgG group: pretreatment of HUVECs with control mouse IgG antibody. **SRPX2 recombinant protein increased phosphorylation of FAK at sites of Tyr 397 and Tyr 576/577 in HUVECS after 4h culture compared with NC and Blank group (b).** *$p < 0.001$.

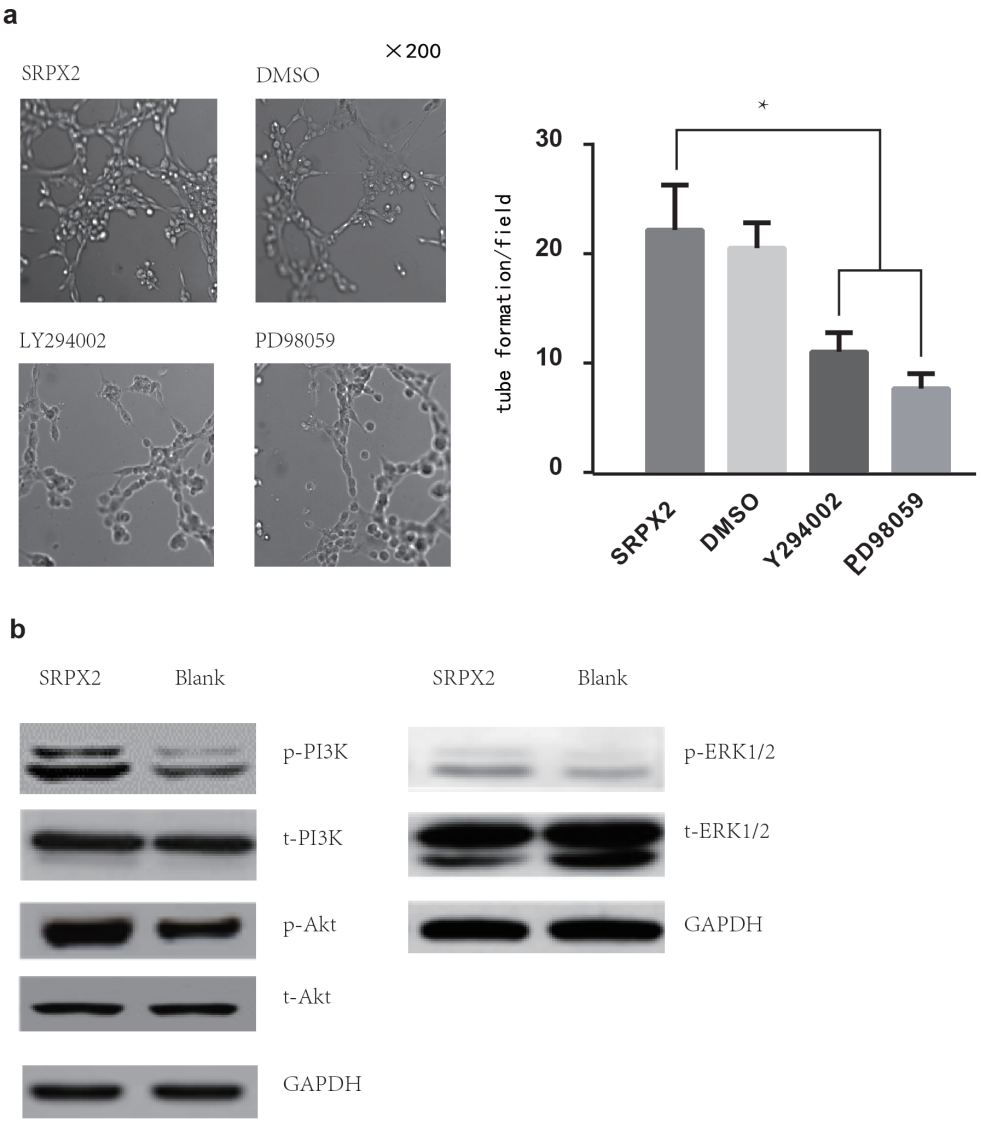

Figure 4. Pretreatment wiht PI3K inhibitor LY294002 and MEK inhibitor PD98059 in HUVECs both significantly inhibited the effect of enhancing tube formation of HUVECs in matrigel by SRPX2 recombinant protein (SRPX2 group) compared with DMSO group (only DMSO was added to HUVECs as control of small molecular inhibitors) (a). *$p < 0.001$. **SRPX2 recombinant protein activated phosphrylation of PI3K, Akt and ERK1/2 in HUVECs compared with blank group (b).**

recombinant protein for 4.0 h was increased compared with control group. All of these findings supported the role of the ERK and PI3K pathways in the angiogenesis effect of SRPX2 on HUVECs.

In this study, we found that SRPX2 from CRC cells promoted migration and tube formation of HUVECs and further molecular mechanisms were investigated. As far as we know, this is the first study to explore the effects and molecular mechanisms of secretory SRPX2 in human vascular endothelial cells. However, in this study, only the direct effect of SRPX2 on HUVECs was investigated. As a secretory proteoglycan in ECM, the interaction of SRPX2 with other growth factors, such as vascular endothelial growth factor and hepatocyte growth factor, both of which are also involved in tumor angiogenesis, can be presumed. Further research should focus on the complex interactions between SRPX2 and other ECM components in tumor angiogenesis. The clarification of the function of SRPX2 in tumor angiogenesis might hold promise for new therapeutic agents targeting overexpressed SRPX2 in various tumors.

Acknowledgements

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Case Report

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Successful treatment of three patients with organizing pneumonia associated with rheumatoid arthritis using clarithromycin and prednisolone

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Summary **Macrolides have anti-inflammatory effects and have been used to treat diffuse panbronchiolitis, bronchiectasis, and cystic fibrosis. Lately, several cases of cryptogenic organizing pneumonia (COP) and radiotherapy-related organizing pneumonia (OP) that were successfully treated with macrolides considering their anti-inflammatory effects were reported. We report three cases of OP associated with rheumatoid arthritis (RA) successfully treated with clarithromycin (CAM) and prednisolone (PSL). Case 1: A 70-year-old woman suffering from RA was admitted with cough and severe dyspnea. She was diagnosed with OP associated with RA on the basis of computed tomography (CT) findings and transbronchial lung biopsy results. She was successfully treated with PSL and cyclosporine A. At the exacerbation of OP, she was successfully treated with CAM and PSL. Case 2: A 74-year-old man suffering from COP visited our department with arthralgia and articular swellings. He was diagnosed with RA, which was thought to be associated with OP. He was successfully treated with CAM and PSL. Case 3: A 54-year-old man suffering from RA presented with an exacerbation of arthralgia and articular swellings and cough. He was diagnosed with OP associated with RA on the basis of CT findings. He was successfully treated with CAM and PSL. The present cases suggest that CAM and PSL treatment may be effective in some cases of OP associated with RA.**

Keywords: Rheumatoid arthritis, organizing pneumonia, clarithromycin, prednisolone

1. Introduction

Bronchiolitis obliterans organizing pneumonia (BOOP) was described in 1985 as a distinct entity, with different clinical, radiographic, and prognostic features, compared with obliterative bronchiolitis and the usual interstitial pneumonia/idiopathic pulmonary fibrosis (*1*). Most patients with the above features were diagnosed with idiopathic BOOP, now called cryptogenic organizing pneumonia (COP), but there are several known causes of organizing pneumonia (OP). One of the causes is collagen vascular disease, such as rheumatoid arthritis (RA), systemic lupus erythematosus and Sjogren's syndrome. In addition, radiotherapy and some drugs are known to cause OP (*2*). In general, treatment for COP commonly includes corticosteroids (CSs) with or without another immunosuppressive agent. Macrolides have anti-inflammatory effects and have been used to treat diffuse panbronchiolitis (DPB), bronchiectasis, and cystic fibrosis (*3-5*). Several cases of COP and radiotherapy-related OP that were successfully treated with macrolides considering their anti-inflammatory effects were reported (*6-9*). Herein, we report three cases of OP associated with RA successfully treated using prednisolone (PSL) and clarithromycin (CAM), a macrolide.

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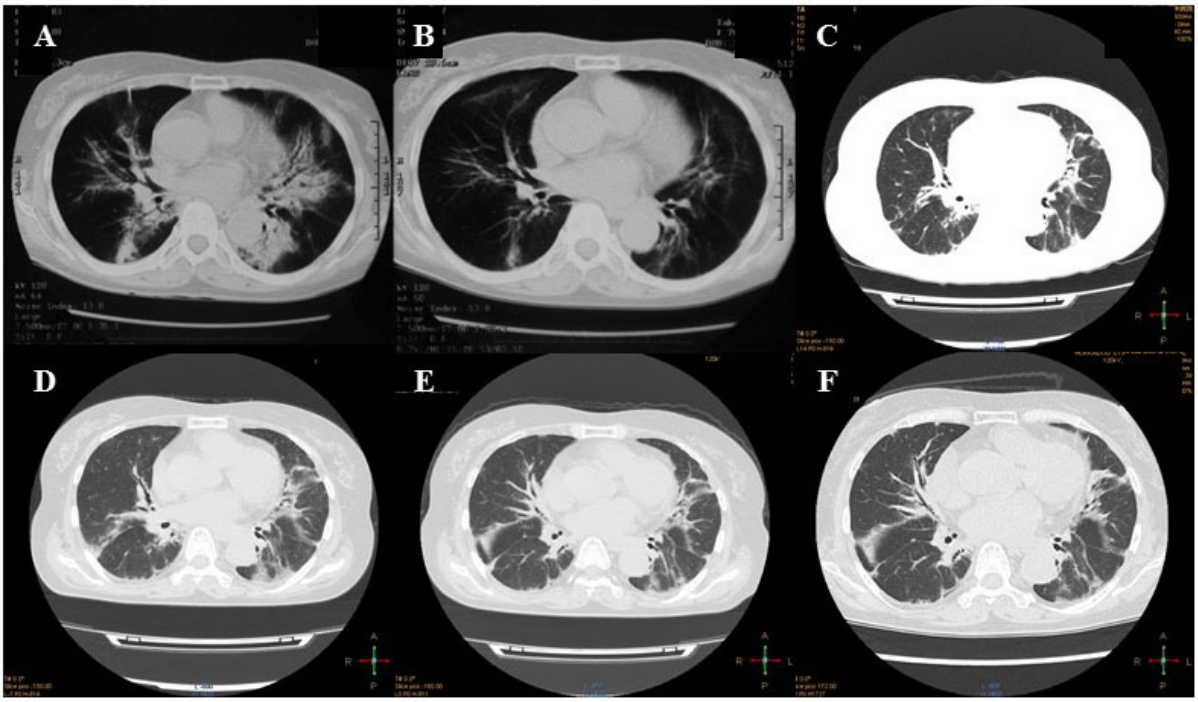

Figure 1. Computed tomography images. (A) Chest computed tomography (CT) reveals airspace consolidation and ground-glass opacity with a peribronchovascular distribution in bilateral lung fields on admission. **(B)** Chest CT reveals considerable improvement of organizing pneumonia (OP) shadows 1 month after prednisolone (PSL) (40 mg/day) in combination with cyclosporine A (100 mg/day) treatment. **(C)** High-resolution CT reveals non-exacerbated OP shadows on PSL (5 mg/day) treatment. **(D)** High-resolution CT reveals exacerbated OP shadows at 78 years of age when the PSL dosage was 5 mg/day. **(E)** High-resolution CT reveals a slight improvement in OP shadows 1 month after clarithromycin (400 mg/day) in combination with PSL (5 mg/day) treatment. **(F)** High-resolution CT reveals further improvement in OP shadows 6 months after PSL (8 mg/day) treatment.

2. Case Report

2.1. *Case 1*

A 70-year-old woman was admitted with cough and severe dyspnea. She had been diagnosed with RA at 68 years of age and had been administered salazosulfapyridine (SASP). For 2 years, her general condition including arthralgia and articular swellings had been stable under this treatment. On this admission, coarse crackles were heard in bilateral lower lung fields. Laboratory findings were as follows: white blood cell (WBC) count, 6,430/μL (neutrophils, 61.1%; lymphocytes, 25.7%); aspartate aminotransferase level, 33 IU/L; alanine aminotransferase level, 25 IU/L; lactate dehydrogenase (LDH) level, 366 IU/L (normal range, 115-245 IU/L); C-reactive protein (CRP) level, 1.07 mg/dL; and rheumatoid factor (RF), 5 IU/mL (normal value, < 15.0 IU/mL). The anti-nuclear antibody titer was 40× with a speckled pattern, and the anti-SS-A antibody was positive. Negative results were obtained for anti-SS-B, anti-RNP, anti-DNA, and myeloperoxidase anti-neutrophil cytoplasmic antibodies. Arterial blood gas analysis showed pH, 7.412; $PaCO_2$, 30.7 Torr; PaO_2, 55.8 Torr; and HCO_3, 22.1 mmol/L. Chest roentgenogram showed interstitial shadows in bilateral lower lung fields. Chest computed tomography (CT) revealed airspace consolidation and ground-glass opacity with a peribronchovascular distribution in bilateral lung fields (Figure 1A). The Shirmer's test and chewing gum test were negative. A transbronchial lung biopsy was performed. The lung biopsy showed intra-alveolar organization, alveolar thickening, and mononuclear cell infiltration (Figure 2). The histology results were compatible with OP. We diagnosed the patient with OP associated with RA. During hospitalization, dyspnea and OP shadows grew worse; therefore, the patient was treated with

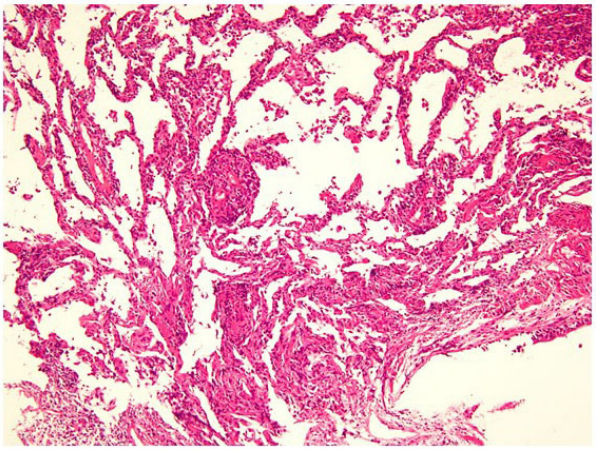

Figure 2. Lung biopsy specimen. Lung biopsy specimen reveals intra-alveolar organization, alveolar thickening, and mononuclear cell infiltration (H&E stain, 100×).

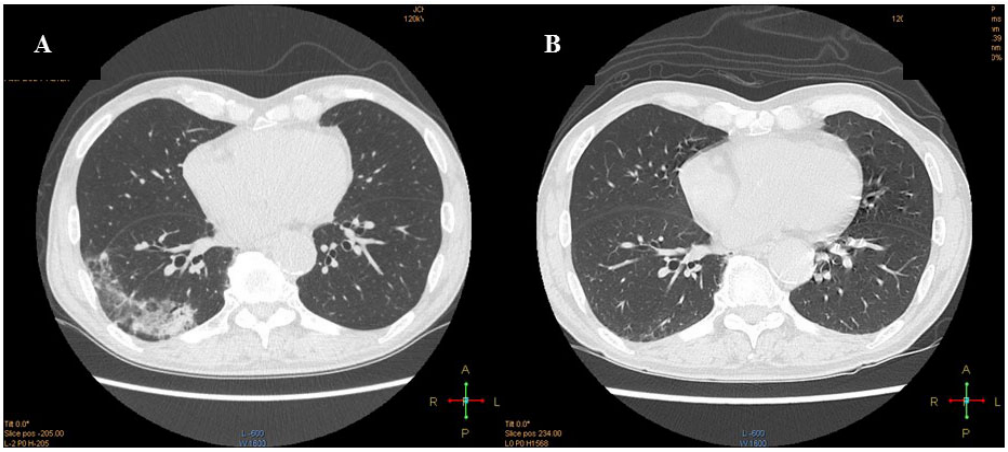

Figure 3. Computed tomography images. (A) High-resolution computed tomography (CT) reveals airspace consolidation and ground-glass opacity in the right lower lung field. **(B)** High-resolution CT reveals an improvement in OP shadows 6 months after clarithromycin (400 mg/day) in combination with prednisolone (5 mg/day) treatment.

PSL (40 mg/day) in combination with cyclosporine A (CyA) (100 mg/day). One month after initiating this treatment, chest CT revealed considerable improvement of OP shadows (Figure 1B). The PSL dosage could be gradually reduced to 5 mg/day over 7 months. On the other hand, CyA could be discontinued in 19 months. OP shadows had been stable under PSL in combination with CyA treatment and subsequent PSL (5 mg/day) treatment. At 72 years of age, OP shadows exacerbated on PSL (5 mg/day); therefore, the PSL dosage was increased to 20 mg/day. Because OP shadows gradually improved, the PSL dosage could be gradually decreased again to 5 mg/day without an exacerbation of OP shadows on high-resolution CT findings (Figure 1C). The long-term use of PSL induced diabetes mellitus and osteoporosis. At 78 years of age when the PSL dosage was 5 mg/day, OP shadows on high-resolution CT findings grew worse (Figure 1D). Laboratory findings were as follows: WBC count, 5,600/μL (neutrophils, 60.3%; lymphocytes, 24.5%); LDH level, 237 IU/L; and CRP level, 0.56 mg/dL. Instead of increasing the PSL dosage, we added CAM (800 mg/day) to PSL (5 mg/day) considering its anti-inflammatory effects. One month after initiating CAM, a slight improvement was found in OP shadows on high-resolution CT findings, with CRP level decreasing to 0.30 mg/dL (Figure 1E). Because the patient complained of severe diarrhea, CAM was ceased. The PSL dosage was increased to 8 mg/day. Six months after initiating PSL (8 mg/day), OP shadows on high-resolution CT findings further improved slightly (Figure 1F).

2.2. *Case 2*

A 74-year-old man suffering from COP visited our department with arthralgia accompanied by partial swellings of proximal interphalangeal and metacarpophalangeal joints with morning stiffness. COP was diagnosed at another hospital 2 months before the episode of articular symptoms during a routine examination of his chest roentgenogram for hypertension. Because the patient did not complain of respiratory symptoms, he did not receive any treatment at the time. On this visit to our department, a slight fine crackle was heard in the right lower lung field. Laboratory findings were as follows: WBC count, 6,280/μL; CRP level, 6.05 mg/dL; and RF, 83 IU/mL. Bone roentgenogram revealed periarticular osteopenia in the bilateral knees and wrist joints. High-resolution CT revealed airspace consolidation and ground-glass opacity in the right lower lung field compatible with OP (Figure 3A). Because of symmetric polyarthritis of the small joints in both hands and feet along with morning stiffness and a positive RF, a diagnosis of RA was made according to the American College of Rheumatology criteria. It was thought that OP was associated with RA. Because the above-mentioned opacities gradually exacerbated, the patient was treated with PSL (5 mg/day) and CAM (400 mg/day) considering their anti-inflammatory effects on RA as well as OP (*10*). After 6 months of treatment with PSL and CAM, OP shadows on high-resolution CT findings, as well as arthralgia and articular swellings improved considerably (Figure 3B).

2.3. *Case 3*

A 54-year-old man suffering from RA presented with an exacerbation of arthralgia and articular swellings and cough. He had been diagnosed with RA at 45 years of age and had been administered SASP and subsequently SASP in combination with PSL (5 mg/day). His general condition including arthralgia and articular swellings had been stable under this treatment about for 1 year; however, arthralgia and articular swellings had worsened. SASP was changed to methotrexate (MTX) (6 mg/week), and his symptoms improved. At 47 years of age, the patient presented with an exacerbation of arthralgia and articular swellings and dyspnea. Chest

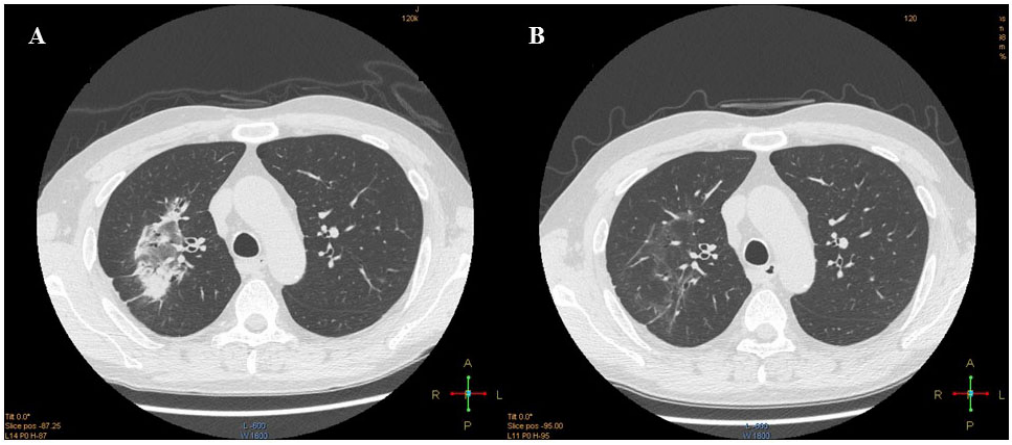

Figure 4. Computed tomography images. (A) High-resolution computed tomography (CT) reveals peculiar airspace consolidation and ground-glass opacity (reversed halo sign) in the right lung field. **(B)** High-resolution CT reveals an improvement in OP shadows 3 months after clarithromycin (800 mg/day) in combination with prednisolone (10 mg/day) treatment.

CT demonstrated pleural and pericardial effusions. The patient was diagnosed with pleuritis and pericarditis associated with RA. He was treated with methyl PSL (1 g/day) for 3 days and subsequently PSL (60 mg/day) in combination with cyclophosphamide (CPM) (100 mg/day). After pleuritis and pericarditis improved, the PSL dosage was gradually decreased to 5 mg/day over 3 years. On the other hand, CPM was ceased in 8 months. For about 4 years, his general condition including arthralgia and articular swellings had been stable under PSL (5 mg/day) treatment. On this visit, his laboratory findings were as follows: WBC count, 6,020/μL (neutrophils, 42.2%; lymphocytes, 27.9%); LDH level, 152 IU/L; and CRP level, 0.41 mg/dL. High-resolution CT revealed peculiar airspace consolidation and ground-glass opacity (reversed halo sign), compatible with OP (*11*) (Figure 4A). The patient was diagnosed with OP associated with RA. Because he was suffering from diabetes mellitus, the PSL dosage was increased from 5 mg/day to 10 mg/day only. Additionally, we prescribed CAM (800 mg/day) considering its anti-inflammatory effects. Three months after initiating CAM, OP shadows on high-resolution CT findings improved considerably (Figure 4B). CAM was ceased in 3 months and the PSL dosage could be gradually decreased to 5 mg/day over 4 months.

3. Discussion

In general, the treatment for COP commonly includes CSs with or without other immunosuppressive agents. CSs, with their potent anti-inflammatory property, continue to be recommended as the first-line treatment for patients with COP with symptomatic and progressive disease. Minimal data have been reported regarding alternative immunosuppressive agents in cases of CS-refractory COP. Some reports suggest that early treatment with CPM can be effective, particularly in patients who fail to respond to treatment with CS alone (*12*). The administration of CyA together with a CS in a fulminant patient with COP resulted in a successful outcome (*13*). In Case 1, because OP shadows and hypoxemia were severe and grew worse during hospitalization, the patient was successfully treated with PSL (40 mg/day) in combination with CyA.

Macrolides have not only antibacterial effects but also anti-inflammatory effects. Anti-inflammatory effects of macrolides in DPB were thought to reduce the influx of polymorphonuclear cells in the lung and decrease the production of cytokines involved in the inflammatory cascade, such as interleukin (IL)-8 and IL-1β (*14*). Apart from anti-inflammatory effects on polymorphonuclear cells or neutrophils, macrolides exhibit anti-inflammatory effects on T-cells by inhibiting cytokine gene expression (*15*).

As to macrolide treatment for COP, Ichikawa *et al*. reported a successful case using erythromycin (*6*). Stover *et al*. reported three cases of COP and three cases of radiation-related OP, which improved with CAM (*7*). Lee *et al*. successfully prescribed the macrolide roxithromycin (300 mg/day) in combination with CyA and CS for rapidly progressive COP (*8*). Oh *et al*. reported two cases of COP treated with CAM and only one responded to CAM (*9*).

According to a review of macrolide therapy in COP in which 29 patients were investigated, 20 patients initially received a macrolide as a single agent. Sixteen cases were cured with the medication after 3-14 months; however, the improvement took a longer time than that taken with glucocorticoids (GCs). It usually took 2-3 weeks for symptom improvement and 1 month for radiological improvement in chest images. Four patients had no improvement following macrolide treatment for 1 month and had to switch to GCs or a combination treatment with GCs. After that, the disease was well-controlled. The remaining nine patients were later treated with macrolides due to poor efficacy or side effects of GCs (*16*). It was speculated that the beneficial

effects of macrolides may be due to not only their immunosuppressive effect on polymorphonuclear cells and their products but also their influence on T-cells (*9*).

According to a case-based review, the PSL dosage for OP in patients with RA was 25-60 mg/day or 1-1.5 mg/kg/day (*17*). In Case 1, although CAM was ceased due to its adverse side effect of diarrhea, when OP shadows grew worse with PSL (5 mg/day) treatment, CAM was successfully added to the treatment. Case 2 was successfully treated with PSL (5 mg/day) in combination with CAM (400 mg/day). Case 3 was also successfully treated with PSL (10 mg/day) in combination with CAM (800 mg/day). Based on these findings, CAM treatment in OP associated with RA was thought to be effective as in COP and reduced a conventional dosage of PSL.

Elderly patients as those in Cases 1 and 2 tend to suffer from chronic diseases, such as diabetes mellitus, osteoporosis, infections and hypertension that are exacerbated by a CS, CAM treatment may therefore represent a useful option for elderly patients with OP associated with RA. Before CAM treatment can be adopted, patients who cannot accept a full dose of CS must be recruited and CAM treatment must be carried out after obtaining informed consent.

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Case Report

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Successful treatment with clarithromycin and/or tacrolimus for two patients with polymyalgia rheumatica

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Summary **Polymyalgia rheumatica (PMR) is an inflammatory rheumatic disease in the elderly. Glucocorticoids (GCs) remain the mainstay of treatment. GC therapy usually dramatically improves the clinical picture, but approximately one-third of patients experience disease recurrence when the dose is reduced. Moreover, long-term use of GCs causes adverse reactions. Macrolide antibiotics have anti-inflammatory action. Several recent studies have reported the successful treatment of rheumatoid arthritis (RA) and PMR treated using clarithromycin (CAM), a macrolide, because of its anti-inflammatory action. Tacrolimus (TAC) has been indicated as a treatment for RA in patients who failed to respond to methotrexate. Recently, a case of RA was successfully treated using CAM and TAC according to one report. Reported here are two cases of PMR treated using CAM and/or TAC. Case 1: A 73-year-old man suffering from PMR was successfully treated with prednisolone (PSL) and CAM. Because his muscle pain disappeared, CAM was discontinued. However, the pain returned after that discontinuation, so CAM was successfully administered again. Case 2: An 83-year-old man suffering from PMR was successfully treated with PSL and CAM. Because muscle pain disappeared, the CAM dosage was halved. The pain returned after the dosage was reduced, so the CAM dosage was successfully resumed and the PSL dosage was reduced. When the PSL dosage was reduced, muscle pain recurred. Because the PSL and CAM dosages were not successfully increased, TAC was also administered and was found to be effective at treating muscle pain. These two cases suggest that CAM and/or TAC are effective at treating PMR.**

Keywords: Polymyalgia rheumatica, clarithromycin, tacrolimus

1. Introduction

Macrolide antibiotics (MACs) such as clarithromycin (CAM) have anti-inflammatory action as well as antibacterial activity. Several recent studies reported the successful treatment of rheumatoid arthritis (RA) (*1*) and polymyalgia rheumatica (PMR) through use of CAM as an anti-inflammatory drug (*2*). A recent study has indicated that tacrolimus (TAC), a Japanese domestic disease-modifying antirheumatic drug, is a treatment for active RA patients who fail to respond to methotrexate (*3*). Recently, a case of RA was successfully treated with CAM and TAC (*4*). Reported here are a case of PMR treated with CAM and a case of PMR treated with CAM and TAC.

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2. Case Reports

2.1. *Case 1*

A 73-year-old man presented with subacute onset of severe muscle pain in his neck, both shoulders, his lower back, and both thighs. Muscle tenderness was noted in all of the areas in question. However, swelling and deformity of joints were not noted. Laboratory results were a white blood cell count (WBC) of 9,090 cells/μL (normal range, 4,000 to 9,800 /μL), a C-reactive protein (CRP) level of 7.14 mg/dL (normal value, < 0.3

mg/dL), an erythrocyte sedimentation rate of 95 mm/h (normal range, 1 to 10 mm/h), a rheumatoid factor concentration of < 15 IU/mL (normal value, < 15 IU/mL), and an antinuclear antibody (ANA) titer of 40× (normal value, < 40×). Tests for myeloperoxidase and proteinase-3 antineutrophil cytoplasmic antibodies were negative. Ultimately, the patient was diagnosed with PMR and treated with prednisolone (PSL) (15 mg/day). According to a previous report (*2*) CAM has anti-inflammatory action, so the current patient was also treated with CAM (400 mg/day). After 1 week of this treatment, muscle pain disappeared, and CRP decreased to 0.20 mg/dL. The PSL dosage was gradually decreased; when PSL was administered at a dosage of 10 mg/day, CAM was discontinued. The PSL dosage was gradually decreased further to 6 mg/day without any muscle pain. The patient was then administered a dosage of 5 mg/day. After 2 weeks of this treatment, muscle pain recurred, and CRP increased to 1.24 mg/dL (first relapse). Instead of increasing the PSL dosage, CAM (400 mg/day) was administered again. Two weeks after restarting CAM treatment, muscle pain disappeared, and CRP decreased to 0.21 mg/dL. CAM was then discontinued again. Two weeks after discontinuing CAM, muscle pain recurred, and CRP increased to 1.98 mg/dL (second relapse). CAM was administered again at a dose of 400 mg/day. Two weeks after restarting CAM treatment, muscle pain disappeared, and CRP decreased to 0.20 mg/dL. For 8 weeks, the patient was successfully treated with PSL (5 mg/day) and CAM (400 mg/day), so the PSL dosage was successfully decreased to 4 mg/day.

2.2. *Case 2*

An 83-year-old man presented with subacute onset of severe muscle pain in his neck, both shoulders, his lower back, his hip girdle, and both thighs. Muscle tenderness was noted in all of the areas in question. However, swelling and deformity of the joints were not noted. Laboratory results were a WBC of 7,180 cells/μL, a CRP level of 11.59 mg/dL, an anti-cyclic citrullinated peptide antibody titer of 0.6 U/mL (normal value, < 4.5 U/mL), and an ANA titer of 40×. Tests for myeloperoxidase and proteinase-3 antineutrophil cytoplasmic antibodies were negative. Ultimately, the patient was diagnosed with PMR. The PSL dosage needed to be minimized in light of osteoporosis and hyperglycemia, so the patient was treated with PSL at a dosage of 10 mg/day instead of 15 mg/day. According to a previous report (*2*) CAM has anti-inflammatory action, so the current patient was also treated with CAM (400 mg/day). One week after this treatment, muscle pain disappeared, and CRP decreased to 0.67 mg/dL. The PSL dosage was gradually decreased to 7 mg/day without any muscle pain. Since muscle pain disappeared, the CAM dosage was decreased to 200 mg/day while the PSL dosage was 6 mg/day. Two weeks after starting CAM (200 mg/day) treatment, muscle pain recurred, and CRP increased to 1.31 mg/dL (first relapse). Treatment with CAM (400 mg/day) was therefore restarted. Four weeks after resuming CAM (400 mg/day) treatment, muscle pain disappeared, and CRP decreased to 0.21 mg/dL. The PSL dosage was gradually decreased to 4 mg/day. The patient was then treated with PSL (3 mg/day) and CAM (400 mg/day). Four weeks after starting this treatment, slight muscle pain developed but this was tolerated by the patient. Eight weeks after starting this treatment, muscle pain worsened, and CRP increased to 12.97 mg/dL (second relapse). The PSL dosage was increased to 10 mg/day and the CAM dosage was increased to 800 mg/day. For 1 week, this treatment alleviated muscle pain to a certain extent, but CRP remained high (7.90-9.04 mg/dL); therefore, the PSL dosage was successfully increased to 15 mg/day while the CAM dosage was decreased to 400 mg/day. The PSL dosage was gradually decreased to 10 mg/day without muscle pain. Although muscle pain substantially disappeared, CRP remained high at approximately 1.9 mg/mL while the patient received PSL (10 mg/day) and CAM (400 mg/day). The PSL dosage could not be decreased any further for fear of recurrent muscle pain, so TAC (0.5 mg/day) was also administered. One week after the addition of TAC treatment, CRP decreased to 0.55 mg/dL. The trough level of TAC was 3.3 ng/mL. The PSL dosage was gradually decreased to 4 mg/day, and CRP remained at 0.30 mg/dL without recurrent muscle pain.

3. Discussion

Glucocorticoids (GCs) are the mainstay of PMR treatment. GC therapy usually dramatically improves the clinical picture within a few days, but approximately one-third of patients experience disease recurrence when the dose is reduced. Long-term use of GC causes adverse reactions in up to 60% patients.

MACs have anti-inflammatory action as well as antibacterial activity. MACs exhibit anti-inflammatory action by affecting several pathways of the inflammatory process, such as the production of proinflammatory cytokines. In fact, CAM inhibits the production of interleukin-6 (IL-6) (*5*), which is associated with the clinical features of RA. Similarly, TAC has been reported to suppress the production of IL-6 (*6*). Serum IL-6 levels are reported to increase and are closely associated with disease activity in PMR (*7*), so the efficacy of treatment with CAM and/or TAC in the current cases might be due to the anti-inflammatory action of CAM and/or TAC with regard to the suppression of IL-6 production. In Case 1, CAM was effective in treating both relapses. In Case 2, however, CAM was effective in treating the first relapse but not in treating the second relapse. This necessitated

the addition of TAC. CAM was ineffective in treating the second relapse in Case 2 presumably because inflammation was more severe during the second relapse than during the first relapse or because long-term use of CAM induced drug tolerance. In light of the current findings, treatment with CAM and/or TAC for PMR was effective. Moreover, TAC appeared to be superior to CAM in terms of anti-inflammatory action. Studies have noted pharmacokinetic interaction between CAM and TAC since CAM causes an increase in the concentration of TAC in the blood by inhibiting its metabolism (*8*). Research has shown that TAC is primarily metabolized by cytochrome P450 (CYP)3A; when TAC is administered orally, its concentration in the blood tends to widely vary between individuals. CAM is a potent CYP3A inhibitor, so combined administration of both CAM and TAC leads to pharmacokinetic interactions. As a result, CAM increases the concentration of TAC in the blood by inhibiting its TAC metabolism via inhibition of CYP3A (*8*). Recent cases of lupus nephritis and adult-onset Still's disease were successfully treated using TAC; in those cases, CAM was administered in order to increase the concentration of TAC in the blood (*9,10*). Suzuki et al. measured the concentration of TAC in the blood of patients with RA, and they found that the concentration of TAC in the blood was 2.96 ng/mL in patients receiving a dose of 1 mg/day, 4.29 ng/mL in patients receiving a dose of 2 mg/day, and 8.32 ng/mL in patients receiving a dose of 3 mg/day (*11*). However, the concentration varied widely in individual patients in those three groups. The concentration of TAC (0.5 mg/day) in the blood was 3.3 ng/mL in Case 2, so pharmacokinetic interaction between CAM and TAC presumably increased the concentration of TAC in the blood, resulting in a reduction in the dose of TAC, which is quite expensive. Therefore, treatment of refractory PMR with CAM and TAC, as in the second relapse in Case 2, may be recommended from an economic perspective. PMR is one of the most common chronic inflammatory syndromes in elderly individuals. Elderly patients tend to suffer from chronic diseases that are exacerbated by the use of GCs, such as diabetes mellitus, osteoporosis, and hypertension, so treatment with CAM and/or TAC may be helpful in reducing conventional GS dosages.

Because only two cases have been reported here, more research is necessary before this treatment with CAM and/or TAC can be adopted on a wider basis.

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Case Report

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Visual field improvement in non-arteritic posterior ischemic optic neuropathy in a patient treated with intravenous prostaglandin E1 and steroids

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Summary **Non-arteritic posterior ischemic optic neuropathy (NA-PION) is a disorder of reduced blood flow to the retrobulbar optic nerve. There is usually an acute loss of visual acuity and field. Previous studies have noted an improvement in visual acuity and in ocular and retrobulbar blood flow with the use of a potent vasodilator of the microcirculation, prostaglandin E1 (PGE1), and steroids. The current report describes immediate improvement in the visual fields and visual acuity in a patient with NA-PION treated with intravenous PGE1 and steroids 66 hours after onset. An 89-year-old white female was first seen in December 2016 with a sudden loss of vision in the right eye. After a complete eye exam and visual fields, the patient was diagnosed with NA-PION. Treatment was immediately started with steroids and intravenous PGE1. This was repeated once again the next morning. Visual acuity in the right eye improved from 1/10 + 1 to 7/10 + 3 at 5 days. The mean deviation of the visual field improved from – 7.10 decibels (dB) with a central scotoma of – 22 dB to – 2.97 dB with a central scotoma of – 19 dB. After 2 weeks, her visual acuity was 7/10 + 1 and visual field testing of the right eye revealed a mean deviation of – 2.54 dB with a central scotoma of – 9 dB. The left eye was unchanged. In cases of NA-PION, PGE1 and steroids should be considered to immediately restore blood flow to help improve visual acuity and visual fields.**

Keywords: Non-arteritic posterior ischemic optic neuropathy, prostaglandin E1, visual field, central scotoma

1. Introduction

Posterior ischemic optic neuropathy is a disorder of reduced blood flow to the retrobulbar optic nerve, usually of acute onset. This condition can be classified as surgical, arteritic or non-arteritic (*1,2*). Use of high-dose systemic steroids to treat non-arteritic posterior ischemic optic neuropathy (NA-PION) results in improved visual acuity (*2*). Steroid therapy is not universally successful.

Prostaglandin E1 (PGE1), a powerful vasodilator of the microcirculation, improves ocular blood flow in the presence of peripheral vascular disease and diabetes (*3*). In cases of NA-PION, PGE1 was given intravenously at a dose of 1 μg/kg with steroids and was found to improve visual acuity as well as ocular and retrobulbar blood flow (*4,5*). In the current case, visual fields as well as visual acuity were improved in a patient with NA-PION treated with intravenous PGE1 and steroids 66 hours after onset.

2. Case Report

An 89-year-old white female was seen in December 2016. She had dry age-related macular degeneration in the right eye and a neovascular membrane in the left eye. One morning three days prior, she underwent ocular surgery with the Limoli retinal restoration technique on her right eye. This technique involves placing a cellular autograft in an equatorial scleral pocket of the eye (*6*). Eight hours after surgery while

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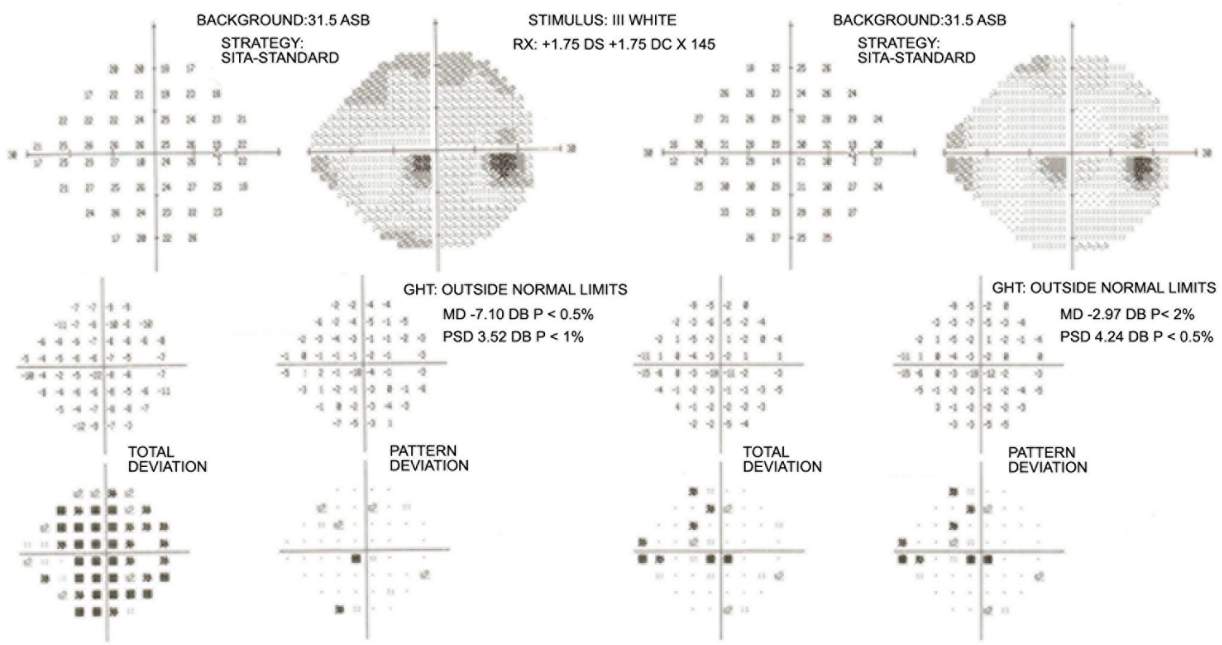

Figure 1. Visual field of the right eye on the left side before treatment and the visual field of the right eye on the right side 5 days post-treatment.

watching television in the evening, she had a sudden decrease in the visual acuity in the right eye (the eye that underwent surgery). She was examined 63 hours later. She was pseudophakic in both eyes with a visual acuity of 1/10 + 1 in the right eye according to the Early Treatment Diabetic Retinopathy Study chart and a visual acuity of 1/30 in the left eye. The intraocular pressures were normal with no signs of secondary cataracts. There were no clinical signs of giant cell arteritis. Her blood work from surgery did not indicate signs of diabetes or giant cell arteritis. A fundus exam revealed dry age-related macular degeneration in the right eye with no signs of retinal hemorrhage, inflammation or edema. Edema was present in the fundus of the left eye. The optic nerve heads were normal in both eyes with no pallor or edema. Optical coherence tomography revealed no signs of fluid in the macula in the right eye and an optical coherence tomography angiography revealed no signs of a neovascular membrane in the right eye. A neovascular membrane was present in the left eye. Humphrey central 24-2 visual field testing was immediately done. In the right eye, there was a mean deviation of – 7.10 decibels (dB) with a central scotoma of – 22 dB (Figure 1 and Table 1). The visual field in the left eye could not be tested due to lack of central fixation.

The patient was diagnosed with NA-PION. The experimental nature of the treatment was explained and after written consent was obtained, treatment was immediately begun 66 hours after onset. PGE1 comes as a liquid in vials of 20 µg and is given intravenously in physiologic solution at a dose of 1 µg/kg. The patient weighed 68 kilograms and was started on 60 µg of PGE1 administered intravenously over 1 hour. Subjectively, she noted a gradual return in the visual acuity in the right eye the same evening after treatment. The following morning, PGE1 was administered again intravenously and the patient was started on 25 mg of prednisone once a day for 5 days. Five days after the first treatment, she was examined. Visual acuity in the right eye was 7/10 + 3. There was no change in the left eye. Other results of the exam, including the fundus, remained the same. Visual field testing of the right eye revealed a mean deviation of – 2.97 dB with a central scotoma of – 19 dB (Figure 1 and Table 1). Two weeks later, visual acuity was 7/10 + 1 and visual field testing of the right eye revealed a mean deviation of – 2.54 dB with a central scotoma of – 9 dB (Figure 2 and Table 1). The patient was continued on 60 µg of PGE1 in the form of a skin cream for systemic absorption every 2 weeks. One, 4 and 7 months (at the time of this report), her vision was stable and she continued to use PGE1 skin cream every 2 weeks.

Table 1. Visual acuity and Humphrey 24.2 visual fields of the right eye before and after treatment with prostaglandin E1 (PGE1)

Date	Visual Acuity	Mean Deviation	Central Scotoma
Initial visit (T0)	1/10 + 1	– 7.10 dB	– 22 dB
After 5 days (T5)	7/10 + 3	– 2.97 dB	– 19 dB
After 2 weeks (T2ws)	7/10 + 1	– 2.54 dB	– 9 dB

dB: decibels.

3. Discussion

The purpose of this case report is to present further evidence, which includes improvement in visual fields,

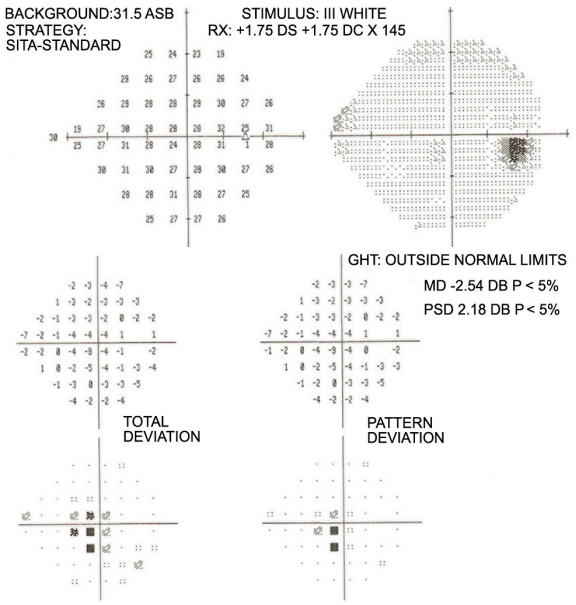

Figure 2. Visual field of the right eye 2 weeks post-treatment.

for the use of intravenous PGE1 and steroids in the treatment of NA-PION. The authors also intend to describe how that treatment, even though it was delayed up to 66 hours after onset, was still beneficial.

NA-PION is a disorder of reduced blood flow to the retrobulbar optic nerve (*1,2,5*). The use of a potent vasodilator, PGE1, to immediately re-establish blood flow could be important (*3,7*). PGE1 given intravenously at a dose of 1 µg/kg together with steroids has been successfully used to treat cases of NA-PION and acute non-arteritic anterior ischemic optic neuropathy (NA-AION) (*4,5,8*). PGE1 has helped in the treatment of acute arteritic anterior ischemic optic neuropathy when given with high-dose steroids (*9*). In the current case, intravenous PGE1 and steroids were immediately started as soon as the patient was diagnosed with NA-PION, resulting in an improvement in visual fields and visual acuity. This is the first report of improvement in visual fields in NA-PION and is further evidence for this therapy. Based on the authors' clinical experience with cases of NA-AION, PGE1 must be given immediately, *i.e*. within 12 hours, to be effective. In the current case involving NA-PION, the therapy was effective even after 66 hours, so this therapy should be attempted. One intravenous infusion of PGE1 improves peripheral blood flow for up to 4 weeks in patients with peripheral vascular disease (*3,7*). Because of this, PGE1 was given only 2 times over 2 days and was effective. Repeated treatments could lead to systemic hypotension. The dosage is 1 µg/kg in physiologic solution over 1 hour. Higher dosages could also lead to systemic hypotension. PGE1 is well-tolerated and causes few adverse reactions, so it can be used in patients who are hypotensive. Systemic blood pressure needs to be monitored frequently during its intravenous administration (*3,7*). The main mechanism of action of PGE1 is *via* vasodilatation of the microcirculation. PGE1 has a direct action on the smooth muscle of the vascular wall, leading to a vascular dilatation and increased flow. PGE1 is rapidly metabolized by oxidation during passage through the pulmonary circulation. It is excreted in the urine as metabolites within about 24 hours. This rapid elimination also contributes to its safety (*10*).

The current patient had poor vision in the left eye because of a neovascular membrane. Ischemic episodes in NA-AION and NA-PION are known to reoccur in the affected eye or in the other eye (*5,11*). To avoid another episode in the good eye, the authors wanted to maintain vasodilatation of the microcirculation with PGE1 without resorting to intravenous treatments. Accordingly, the patient continued to receive 60 µg of PGE1 in a skin cream. The PGE1 concentration in this cream is 20 µg/cc. Three cc of cream, for a total dosage of 60 µg of PGE1, is applied in repeated individual doses over 2 hours to the inner surface of the forearm, spread and allowed to dry. This allows for systemic absorption over 2 hours. This is done every 2 weeks. This is a compound pharmacy preparation that has been patented by one of the authors (RDS).

Steroids, in the form of prednisone, were given orally to the current patient for 5 days. Systemic steroids have been used to treat NA-PION and NA-AION (*2,12*). Steroid therapy is, however, not universally accepted. A more recent study using high-dose systemic steroid treatment in acute NA-AION noted no visual or anatomic benefit and some serious complications due to steroids (*13*). However, the current authors used steroids for another reason: to try to reduce ischemia-reperfusion injury. Ischemia leads to tissue hypoxia, depletion of energy-rich phosphates, accumulation of metabolic waste products including reactive oxygen species and cellular edema, all of which may cause cellular injury (*14,15*). The immediate resumption of blood flow is necessary to prevent further tissue damage but the reperfusion itself may cause further tissue damage *via* reperfusion injury. Infiltrating leukocytes are thought to play a major role in this ischemia-reperfusion injury (*14,15*), and this was why the current authors administered 25 mg of prednisone along with PGE1 during the first 5 days.

Surgery with the Limoli retinal restoration technique was done 8 hours prior to the loss of vision. This technique involves fashioning a scleral pocket at the equator of the eye filled with adipose stromal cells derived from orbital fat, platelets from platelet-rich plasma and adipose-derived stem cells from abdominal adipose tissue. This is meant to bring nutrition to the choroid and retina (*6*). Upon examination of the patient after the visual loss, there were no signs of unusual ocular or orbital inflammation due to the surgery. The authors do not think that the surgery was the cause of NA-PION.

In the current case, visual field testing was possible before treatment without an unusual delay in treatment, so this testing was done immediately. The Humphrey visual field improved after treatment, with a reduction in the mean deviation from – 7.10 to – 2.54 dB. The central scotoma improved from – 22 to – 9 dB (Table 1 and Figures 1 and 2). This paralleled the improvement in the visual acuity from 1/10- to 7/10 + 1, which is consistent with her age-related macular degeneration. In another case of NA-PION, vision improved to 11/10 with no central scotoma on visual fields tested only after treatment (*5*). In that case, treatment was administered within 24 hours and there was no age-related macular degeneration. In the current case, improvement in visual fields and the central scotoma are evidence of the benefit of therapy even though it started 66 hours after onset.

This case report describes the beneficial use of intravenous PGE1 and steroids in the treatment of NA-PION even though that treatment started 66 hours after onset. The addition of steroids may help to reduce damage from ischemia-reperfusion injury, but this point needs to be evaluated further. In order to provide the complete clinical picture, use of PGE1 was continued in the form of a skin cream every 2 weeks to avoid recurrence; however, this approach also needs to be properly studied.

Conflicts of interest

One of the authors, RDS, has a financial interest in the prostaglandin E1 skin cream mentioned in this case report. The other authors have no proprietary or financial interests in or conflicts of interest with regard to the products mentioned in this study. The authors alone are responsible for the contents and writing of the paper.

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