Korean J. Plant Res. 21(1) : 96～102(2008)

Effect of *Nelumbo nucifera* on Proliferation, Migration and Expression of MMP-2 and MMP-9 of rSMC, A431 and MDA-MB-231

Rajendra Karki, Dong-Young Rhyu and Dong-Wook Kim*
Department of Medicinal Plant Resources, College of Natural Science, Mokpo National University, Korea

Abstract - *Nelumbo nucifera* Gaertn. (family Nymphaeaceae) has been used for summer heat syndrome as home remedy in Japan, China and Korea. Although whole plant parts are edible, root is commonly consumed. It has been reported that rhizome extract showed anti-diabetic and anti-inflammatory effects. However, in spite of usefulness for treatment of various diseases, the effect of *Nelumbo nucifera* rhizome (NNR) on proliferation, migration and matrix degrading enzymes-matrix metalloproteinsases (MMPs), the expression of which degrades extracellular matrix (ECM) leading to metastasis, has not been fully elucidated. We examined the effect of hot water extract of NNR on the proliferation, migration and secretion of MMP-2 and MMP-9 in rat smooth muscle cells (rSMC), epidermoid cancer cells (A431) and breast cancer cells (MDA-MB-231). The proliferation assay was carried out using MTT assay, the principle of which depends upon the conversion of MTT by mitochondrial dehydrogensases of viable cells to formazan crystals. The effect of NNR on migration of cells was examined using wound healing assay. Our results showed that there was inhibition in the proliferation, migration and expression of MMP-2 and MMP-9 in dose dependent fashion in all the cells used. Thus, we concluded that NNR could be used as traditional medicine in the treatment of various diseases where proliferation, migration and MMPs' expression plays a pathological role like in restenosis and metastasis.

Key words - *Nelumbo nucifera* rhizome, MMPs, Proliferation, Migration

Introduction

Nelumbo nucifera Gaertn. (Nymphaeaceae) is a large aquatic herb widely found in the native of India, China, Japan and Korea. Traditional medicinal systems advocate several different therapeutic effects of this herb. The lotus rhizome is considered to maintain homeostasis and people consume it as foodstuff. The rhizome is considered to be nutritive, demulcent, diuretic and cholagogue and is used to treat piles, dyspepsia and diarrhoea (Kirtikar and Basu, 1975). Different pharmacological and antomicrobial activities of the methanol extract of the rhizomes, including antidiarrhoeal, psychopharmacological, diuretic, antipyretic, antibacterial and antifungal (Mukherjee *et al.,* 1996) potentials have been reported previously. The matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases which collectively are capable of degrading all components of the extracellular matrix (ECM). These enzymes appear to be important in the normal turnover of connective tissue macromolecules in embryogenesis, postnatal remodeling and wound healing (Woessner J. F., 1991). Currently the MMP family is known to contain at least 20 members, loosely classified into four groups: (1) interstitial collagenase (MMPs 1, 8 and 13); (2) type-IV collagenase (MMPs 2 and 9); (3) stromelysins (MMPs 3, 7, 10, 11 and 18); and (4) membrane-type MMPs (MMPs 14, 15, 16, 17, 24 and 25) (Curran S. and Murray G. I., 2000). MMP-9 is an important collagenase contributing to the digestion of collagen type IV, the primary component of basement membranes. MMP-9 expression was previously shown to correlate with an invasive phenotype in rat embryo cells as well as a number of malignant tumors (Iwata H. *et al.,* 1996). In the normal mammary gland, constitutive expression of MMPs is low except during times of development, pregnancy, and involution (Curran S. and Murray G. I., 2000). However, in pathologic states such as breast cancer, increased levels of MMPs have been reported in breast tumor cells as well as in the surrounding noncancerous breast tissue. Iwata *et al.,* 1996 showed MMPs 1, 2 and 9 to be highly expressed in human breast carcinoma cells. Garbett *et al.* (2000) showed MMPs 1, 2, 3, 9 and 11 to have a higher frequency of expression in breast cancer as opposed to normal breast tissue or benign breast disease. Furthermore, other investigators found that MMPs 1, 2, 9, 13 and 16 were expressed specifically in malignant or benign breast tissue (Hanemaaijer R. *et al.,* 2000). The harmful effects of UV irradiation are immuno-

*Corresponding author. E-mail : dbkim@mokpo.ac.kr

suppression, photoaging and above all skin carcinogenesis (Matsumura Y. and Ananthaswamy H. N. *et al.,* 2002). There is increasing evidence for the generation of reactive oxygen species (ROS) in skin upon UV exposure. Increased ROS generation can overwhelm the antioxidant-defense mechanism, resulting in oxidative stress and oxidative photodamage of proteins and other macro-molecules in the skin (Sander C. S. *et al.,* 2002). Oxidative stress is thought to play a central role in initiating and driving the signaling events that lead to the cell response following exposure to UV irradiation (Rittie L. and Fisher G. J., 2002). It has also been reported that exposure of human or mouse skin to UV irradiation upregulates the synthesis of the matrix-degrading enzymes (MMP), such as MMP-1, -2, -7, -8, -9 and -12 (Fisher *et al,* 2001; Fisher and Voorhees, 1998; Inomata *et al,* 2003). The initial observation of the importance of MMPs in cancer biology was that the ability of tumour cells to invade the surrounding tissue correlated with increased MMP levels (Kerkela E. and Sarrialho-kere U., 2003). Basal cell carcinomas (BCCs) are generally only locally aggressive, whereas squamous cell carcinomas (SCCs) have high metastatic potential. The reduced expression of collagen IV, the major component of basal lamina, combined with an increased expression of both MMP-2 and MMP-9, could account for the high invasive potential of SCC relative to that of BCC. Human squamous cell carcinomas frequently display an increase in the expression of the EGF receptor (EGFR), first demonstrated for A431 cells (Kawamoto T. *et al.,* 1983). Increased EGFR expression renders the proliferation of A431 cells less dependent on an exogenous source of EGF (Fan Z. *et al.,* 1994). While recoil and remodeling of the vessel which occur mainly after percutaneous transluminal angioplasty (PTCA), there is overabundant proliferation, migration and ECM production of vascular smooth muscle cells (SMCs) (Fischman D. L. *et al.,* 1994). The mechanism of neointimal hyperplasia is controlled by a magnitude of mediators. Of special importance among these are basic fibroblast growth factors (FGF) (Linder V. and Reidy M. A. *et al.,* 1991) and platelet derived growth factor (PDGF) (Bell L. and Madri J. A. *et al.,* 1989) which stimulate SMC proliferation. Because SMCs in the large vessels are usually surrounded by and embedded in extracellular matrix proteins, the migration of SMCs and the remodeling of tissues during the intimal thickening require controlled degradation of the ECM (Bendeck M. P. *et al.,* 1994). It is known that SMCs can produce proteolytic enzymes such as matrix metalloproteinases (MMPs), which are a family of zinc-dependent proteinases involved in the degradation and remodeling of the connective tissue (Kusuya M. *et al.,* 2003). Among MMPs, MMP-2 is most frequently overexpressed in the carotid artery after injury, and that MMP-2 deficiency impaired neointimal formation (Bendeck M. P. *et al.,* 1994; Kusuya M. *et al.,* 2003). The activation of MMP-2 is in fact one of the crucial steps of the enzymatic cascade leading to ECM degradation. In this paper we tried to examine the effect of NNR on proliferation, migration and expression of MMP-2 and MMP-9 of MDA-MB-231, A431 and rSMC cells.

Materials and Methods

Preparation of water extract of NNR

The NNR was boiled in 2000 ml of distilled water for 3 hours. The residues were removed by filtration using cotton wool and Whatman paper. The extract thus obtained was evaporated and freeze dried. The yield was about 20% of the starting material. In the typical experiment, the powder thus obtained was dissolved in distilled water to make desired concentrations. All other chemicals and solvents used were of analytical and HPLC grade.

Cell culture

MDA-MB-231, A431 and rSMC cells were grown in Dulbecco Modified Eagle Medium (Cambrex Inc., USA) supplemented with an antibiotic mixture (penicillin 100 units/ml and streptomycin 100 μg/ml) and 10% fetal bovine serum (Cambrex Inc., USA) and incubated at 37℃ in a humidified atmosphere with 5% CO_2.

Proliferation assay

MDA-MB-231, A431 and rSMC cells were seeded at a concentration of 5×10^5 cells/well in 100μl of DMEM containing 0.1% of FBS in 96 well plates (Iwaki, Japan). After 5 hours incubation at 37℃ and 5% CO_2, the complete media was replaced with DMEM containing 10% FBS and treated with hot water extract of NNR (HWE) at concentrations of 0, 1, 10, 100, 500 and 1000μg/ml for 24 hours. The assay was dependent on the reduction of Tetrazolium salt (MTT) by mitochondrial dehydrogenase of viable cells into dark violet formazan product which was then solubilised in 100μl of Dimethyl sulphoxide (DMSO). Viability of the cells was determined using a colorimetric assay at 540nm.

Wound healing assay

Wound healing assay was used to examine the migration of cells.

MDA-MB-231, A431 and rSMC cells were seeded at a concentration of 6×10^5 cells/ml in 6 well plates (Iwaki, Japan). After overnight incubation at 37℃ and 5% CO_2, each monolayer was scratched with the use of 200μl-pipette tip which was followed by washing with phosphate buffered saline (PBS). The cells were allowed to migrate into the wound in the presence of 10% FBS with HWE at concentrations of 100μg/ml for 24 and 48 hours. Phase contrast images were taken and distance of each scratch closure was measured using software.

Gelatin zymography

The secretion and acitivity of MMP-2 and MMP-9 in the conditioned media was assayed by gelatin zymography. Briefly, MDA-MB-231, A431 and rSMC cells were seeded at a concentration of 1×10^5 cells/ml in in 6 well plates (Iwaki, Japan). The cells were incubated for 48 hours in the presence of HWE at concentrations of 0, 1, 10, 100, 500 and 1000μg/ml. Then the conditioned media were collected and subjected to gel electrophoresis with 10% running gels containing 0.1% gelatin. The gels were washed in 2.5% Triton X-100 for 30 minutes followed by incubation for 18 hours at 37℃ in incubation buffer containing 50mM Tris-HCl (pH 7.5), 0.2M NaCl, 5mM CaCl2 and 0.02% brij detergent. The gels were stained for 2 hours in 0.5% Coomassie brilliant blue, destained with destaining solution (30% methanol and 10% acetic acid) and photographed. Densitometric analyses were performed using National Institutes of Health (NIH, Rockville, MD) Image 1.61.

Statistical analysis

The results are expressed as mean ± standard deviation of independent experiments. The statistical significance between sample treated groups and control group was determined using student' s t test analysis of variation. A probability value of $p < 0.05$ was considered significant.

Results

Effects of HWE on proliferation

The effects of HWE on the proliferation of MDA-MB-231, A431 and rSMC cells are as shown in Fig. 1. In case of MDA-MB-231, at the concentration of 1000μg/ml, HWE possessed the significant inhibition of 20% ($p < 0.01$) while at the concentration of 500μg/ml, it possessed the inhibition of 17% ($p < 0.05$). Similarly, in case of A431, HWE at a concentration of 1000μg/ml possessed the significant inhibition of 27% ($p < 0.001$) while it possessed the inhibition of 12% ($p < 0.05$) at 500μg/ml. Also, in case of rSMC, HWE at concentrations of 1000μg/ml and 500μg/ml showed the significant inhibitions with $p < 0.01$ and $p < 0.05$ respectively.

Effects of HWE on migration

The effects of HWE on migration of MDA-MB-231, A431 and rSMC cells are as shown in Fig. 2 and their respect photographs are shown in Fig. 3. When added to MDA-MB-231 at a concentration of 100μg/ml for 48 hours, HWE significantly inhibited the migration ($p < 0.05$), while 24 hours treatment did not show significant inhibition

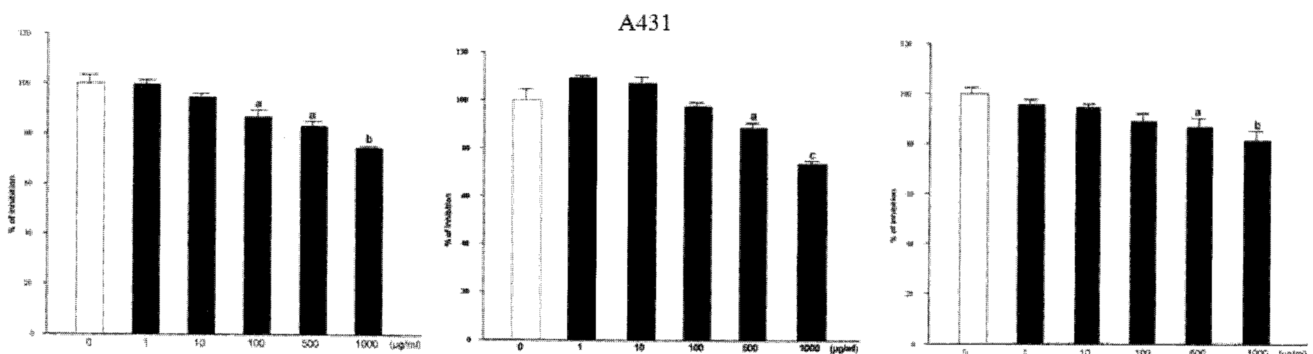

Fig. 1. Effect of *Nelumbo nucifera* roots on viability of rSMC, A431 and MDA-MB-231.
MDA-MB-231, A431 and rSMC cells were seeded at a concentration of 5×10^5 cells/well in 100μl of DMEM containing 0.1% of FBS in 96 well plates. After 5 hours incubation at 37℃ and 5% CO_2, the complete media was replaced with DMEM containing 10% FBS and treated with hot water extract of NNR (HWE) at concentrations of 0, 1, 10, 100, 500 and 1000μg/ml for 24 hours. The formazan crystals thus formed were solubilised in DMSO and viability of the cells was determined using a colorimetric assay at 540 nm. Statistical significances: $^a p < 0.05$, $^b p < 0.01$ and $^c p < 0.001$

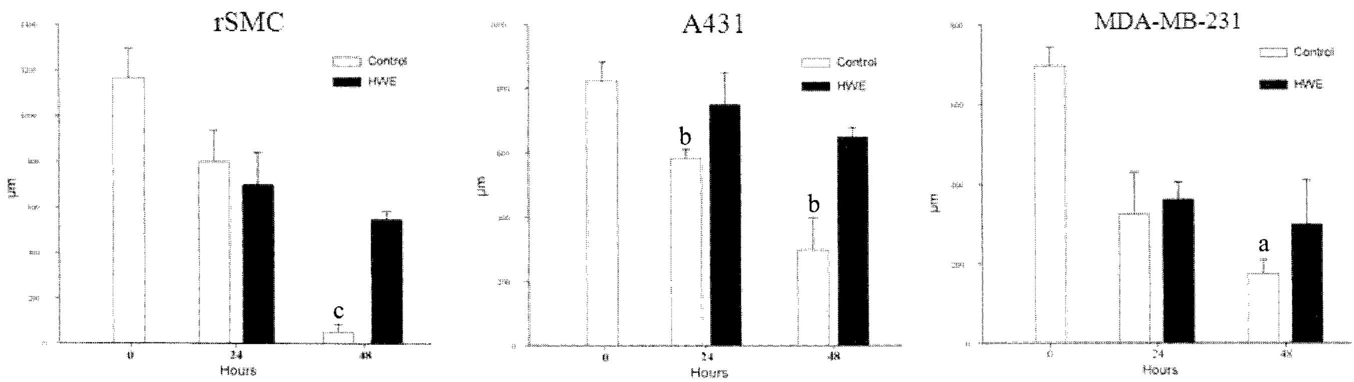

Fig. 2. Effect of *Nelumbo nuficera* roots on wound healing of rSMC, A431 and MDA-MB-231.
MDA-MB-231, A431 and rSMC cells were seeded at a concentration of 6×10^5cells/ml in 6 well plates. After overnight incubation at 37℃ and 5% CO_2, each monolayer was scratched with the use of 200μl-pipette tip which was followed by washing with phosphate buffered saline (PBS). The cells were allowed to migrate into the wound in the presence of 10% FBS with HWE at a concentration of 100μg/ml for 24 and 48hours. Phase contrast images were taken and distance of each scratch closure was measured using software. Statistically significant value compared with non-treatment group (control) data by T test ([a]p<0.05, [b]p<0.01, [c]p<0.001).

1) rSMC

a) b) c) d) e)

2) A431

a) b) c) d) e)

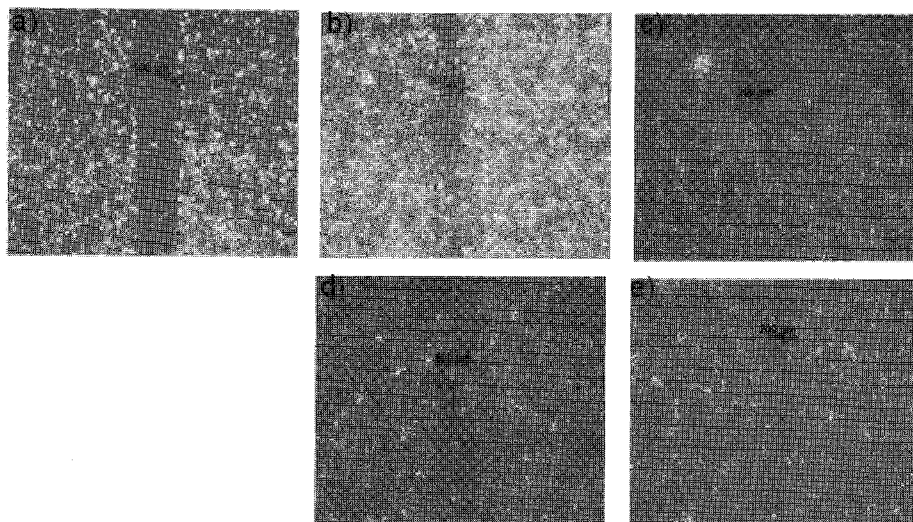

Fig. 3. The respective photographs showing the effect of NNR on wound healing of rSMC, A431 and MDA-MB-231.
a) at the time of scratching, b) after 24 hours of scratching, c) after 48 hours of scratching, d) 24 hours treatment of 100㎍/ml of HWE, e) 48 hours treatment of 100㎍/ml of HWE

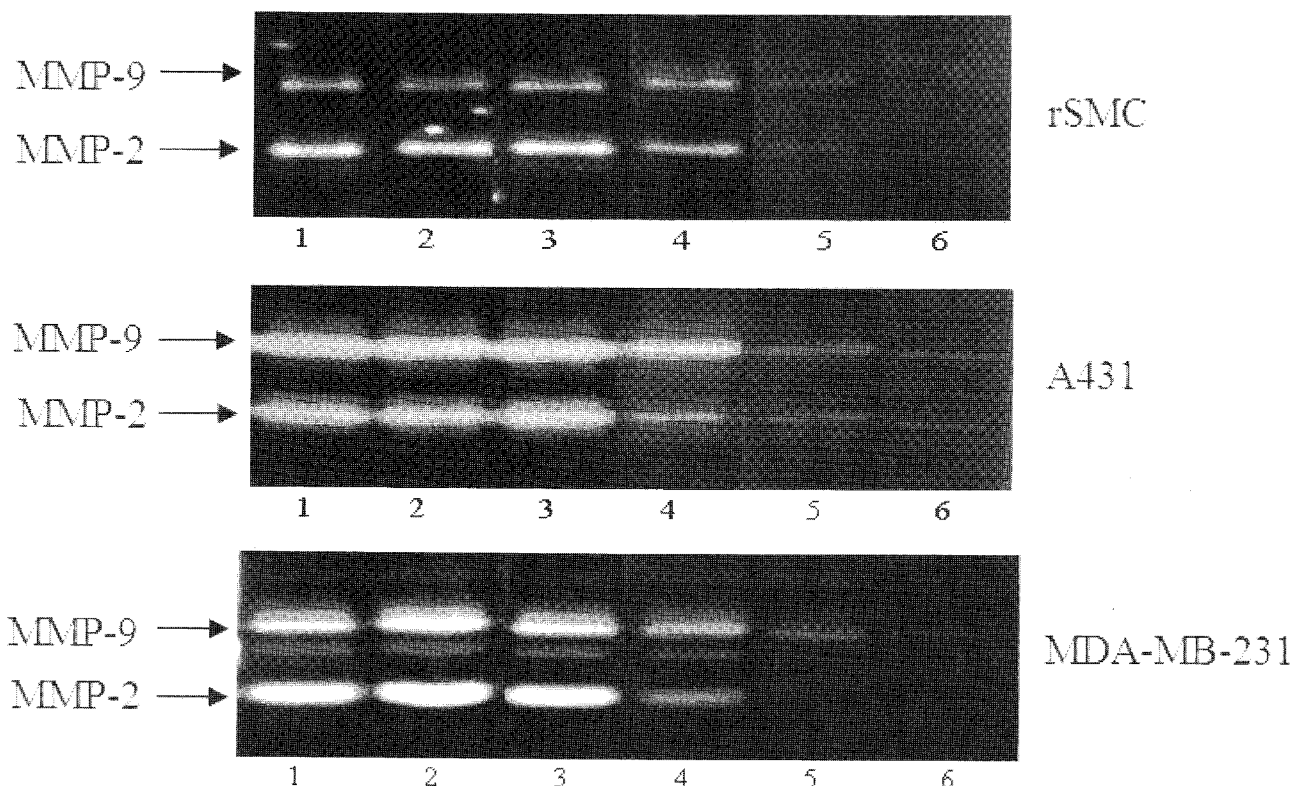

Fig. 4. Effect of *Nelumbo nucifera* on MMP-9 and MMP-2 expression of rSMC, A431 and MDA-MB-231.
MDA-MB-231, A431 and rSMC cells were seeded at a concentration of 1×10^5cells/ml in 6 well plates. The cells were incubated for 48 hours in the presence of HWE at concentrations of 0, 1, 10, 100, 500 and 1000㎍/ml. Then the conditioned media were collected and subjected to gel electrophoresis with 10% running gels containing 0.1% gelatin. The gels were washed in 2.5% Triton X-100 for 30 minutes followed by incubation for 18 hours at 37℃ in incubation buffer containing 50mM Tris-HCl (pH 7.5), 0.2M NaCl, 5mM $CaCl_2$ and 0.02% brij detergent. The gels were stained for 2 hours in 0.5% Coomassie brilliant blue, destained with destaining solution (30% methanol and 10% acetic acid) and photographed. Lanes- 1: control 2: 1㎍/ml 3: 10㎍/ml 4: 100㎍/ml 5: 500㎍/ml and 6: 1000㎍/ml.

in the migration of MDA-MB-231. Similarly, when added to A431 at a concentration of 100μg/ml for 24 and 48 hours, HWE significantly inhibited the migration ($P<0.01$). But in case of rSMC, 24 hours treatment of 100μg/ml of HWE didn' t inhibit the migration while 48 hours treatment significantly inhibited the migration of rSMC ($P<0.001$).

Effects of HWE on the expression of MMP-2 and MMP-9

The effects of HWE on the expression of MMP-2 and MMP-9 by MDA-MB-231, A431 and rSMC cells were as shown in figure 3. When added to the cells at concentrations of 0, 1, 10, 100, 500 and 1000μg/ml for 48 hours, HWE inhibited the expression of MMP-2 and MMP-9 in a dose dependant fashion.

Discussion

The use of herbal products in the prevention and/ or treatment of several chronic diseases have been practiced traditionally in various ethnic societies worldwide (Li and Wang, 2005). However, the restraining effect and involved mechanisms to the cancer metastasis and restenosis of herbal products have not been cleared yet. *Nelumbo nucifera* root possesses different pharmacological effect. Oral administration of the ethanolic extract of rhizomes of *Nelumbo nucifera* markedly reduced the blood sugar level of normal, glucose-fed hyperglycemic and streptozotocin-induced diabetic rats, when compared with control animals (Mukherjee P. K. *et al.,* 1997). The methanolic extract of the *Nelumbo nucifera* root showed antipyretic effect and psychopharmacological actions in rats and mice (Mukherjee P. K. *et al.,* 1996). Gelatinase A (MMP-2, 72 kDa gelatinase) is expressed in a variety of normal and transformed cells, including fibroblasts, keratinocytes, endothelial cells and chondrocytes. Gelatinase B (MMP-9, 92 kDa gelatinase) is produced by keratinocytes, monocytes, macrophages and many malignant cells (Kerkela and Saarialho-kere, 2003). UV irradiation of skin increases the concentration of hydrogen peroxide and other oxygen species (ROS) (Jurkiewicz and Buettner, 1994). ROS can activate mitogen-activated protein kinases that phosphorylate transcription factor activator protein 1, which in turn results in upregulation of MMPs (Rittie and Fisher, 2002). Photoaging is characterized degradation of collagen and accumulation of abnormal elastin in the superficial dermis; several matrix MMPs have been implicated in this process (Fisher *et al,* 2002). Simultaneous expression of MMP-2, -3, -7 and -9 could lead to degradation of non-collagenous extracellular matrix, including the basement membrane and proteoglaycans (Kahari and Sarrialho-Kere, 1997; Fisher and Voorhees, 1998). Various attempts have been made to prevent restenosis by selective inhibition of PDGF (25), FGF (26) and TGFβ (27). In this study we used 10% FBS, the proliferative action of which was inhibited by HWE of NNR in dose dependent fashion. A number of studies have shown that among MMPs, MMP-9 is expressed in injured arterial walls only in early phase after injury that MMP-2 is expressed constitutively in a latent form in the arterial wall and overexpressed in an activated form during the period from 5 to 14 days after injury, and that MMP-2 deficiency significantly reduced neointimal hyperplasia. (Bendeck M. P. *et al.,* 1994 and Kuzuya M. *et al.,* 2003). In the present work we observed that MMP-2 but not MMP-9 was released in the conditioned medium. However, the data from our and other groups' studies indicated that MMP-9 may be namely secreted by infiltrated inflammatory cells at early period after the injury and plays an important role for the atherosclerotic lesion and neointimal formation (Kuzuya M. *et al.,* 2003). It has been shown that MMPs are expressed in increased levels in breast tumors as opposed to their expression in normal breast tissues (Garbett *et al.,* 2000). The tumor line MDA-MB-231 was shown by RT-PCR to constitutively express MMPs 1, 2, 7-11 and 13-16 (John *et al.,* 2002). In our study by gelatin zymography, MDA-MB-231 expressed MMP-2 and MMP-9, the expressions of which were inhibited by HWE of NNR in dose dependent fashion. This study revealed that HWE of NNR could significantly inhibit the proliferation, migration and expression of MMP-2 and MMP-9 of rSMC, A431 and MDA-MB-231, therefore, NNR could be a potential agent for the prevention of epidermoid cancer, breast cancer and restenosis. Furthermore, to our knowledge, this is the first report to demonstrate the inhibitory effects of NNR on epidermoid cancer, breast cancer and restenosis via inhibition of proliferation, migration and expression of MMP-2 and MMP-9.

Acknowledgements

This study was supported by Technology Development Program for Agriculture and Forestry (TDPAF), Ministry of Agriculture and Forestry, Republic of Korea.

Literature Cited

Bell L. and J.A. Madri. 1989. Effect of platelet factors on migration

of cultured bovine aortic endothelial and smooth muscle cells. Cir. Res. 65: 1057-1065.

Bendeck M. P., N. Zempo, A.W. Clowes, R.E. Galardy and M.A. Reidy. 1994. Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. Circ Res. 75: 539-545.

Curran S. and G.I. Murray. 2000. Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis. Eur. J. Cancer 36: 1621.

Fan Z., Y. Lu, X. Wu and J. Mendelsonn. 1994. Antibodyinduced epidermal growth factor receptor dimerization mediates inhibition of autocrine proliferation of A431 squamous carcinoma cells. J. Biol. Chem. 269: 27595-27602.

Fisher G.J. and J.J. Voorhees. 1998. Molecular mechanisms of photoaging and its prevention by retinoic acid: Ultraviolet irradiation induces MAP kinase signal transduction cascades that induce AP-1-regulated matrix metalloproteinases that degrade human skin in vivo. J. Investig Dermatol. Symp. Proc. 3: 61-68.

Fisher G.J., H.C. Choi and Z. Bata-Csorgo *et al.,* 2001. Ultraviolet irradiation increases matrix metalloproteinase-8 protein in human skin in vivo. J. Invest Dermatol 117: 219-226.

Fisher G.J., S. Kang, J. Varani, Z. Bata-Csorgo, Y. Wan, S. Datta and J.J. Voorhees. 2002. Mechanisms of photoaging and chronological skin aging. Arch Dermatol 138: 1462-1470

Fischman D.L., M.B. Leon and D.S. Baim *et al.,* 1994. A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. N. Engl. J. Med. 331: 496-501.

Garbett E.A., M.W. Reed, T.J. Stephenson and N.J. Brown. 2000. Proteolysis in human breast cancer. Mol. Pathol. 53: 99-105.

Hanemaaijer R., J.H. Verheijen, T.M. Maguire, H. Visser, K. Toet, E. McDermott, N. O' Higgins and M.J. Duffy. 2000. Increased gelatinase-A and gelatinase-B activities in malignant vs. benign breast tumors. Int. J. Cancer 86: 204-210.

Iwata H., S. Kobayashi, H. Iwase, A. Masaoka, N. Fujimoto and Y. Okada. 1996. Production of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human breast carcinomas. Jpn. J. Cancer Res. 87: 602-609.

John E.B., D.S. Edgar and E.A. Hubert. 2003. Matrix Metalloproteinase Expression in Breast Cancer. Journal of Surgical Research 110: 383-392.

Jurkiewicz B.A. and G.R. Buettner. 1994. Ultraviolet light-induced free radical formation in skin: an electron paramagnetic resonance study. Photochem. Photobiol. 59: 1-4.

Kawamoto T., J.D. Sato, A. Le, J. Polikoff, G.H. Sato and J. Mendelsonn. 1983. Growth stimulation of A431 cells by epidermal growth factor: Identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. Proc. Natl. Acad. Sci. USA 80: 1337-1341.

Kerkela E. and U. Saarialho-Kere. 2003. Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer. Exp. Dermatol. 12: 109-125.

Kirtikar K.R. and B.D. Basu. 1975. Indian medicinal plants, Deharadun, India. pp. 116-120.

Kuzuya M., S. Kanda, T. Sasaki, N. Tamaya-Mori, X.W. Cheng, T. Itoh, S. Itohara and A. Iguchi. 2003. Deficiency of gelatinase a suppresses smooth muscle cell invasion and development of experimental intimal hyperplasia. Circulation. 108: 1375-1381.

Li X. and H. Wang. 2005. Chinese herbal medicine in the treatment of chronic kidney disease. Adv. Chronic Kidney Dis. 12(3): 276-281.

Lindner V. and M.A. Reidy. 1991. Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. Proc. Natl. Acad. Sci. USA. 88: 3739-3743.

Matsumura Y. and H.N. Ananthaswamy. 2002. Short-term and long-term cellular and molecular events following UV irradiation of skin: implications for molecular medicine. Exp. Rev. Mol. Med. 2002: 1-22.

Mukherjee P.K., J. Das, K. Saha, S.N. Giri, M. Pal and B.P. Saha. 1996. Antipyretic activity of *Nelumbo nucifera* rhizome extract. Indian J. Exp. Biol. 34(3): 275-276.

Mukherjee P.K., K. Saha, M. Pal and B.P. Saha. 1997. Effect of *Nelumbo nucifera* rhizome extract on blood sugar level in rats. Journal of Ethnopharmacology 58: 207-213.

Rittie L. and G.J. Fisher. 2002. UV-light-induced signal cascades and skin aging. Ageing Res. Rev. 1: 705-720.

Sander C.S., H. Chang, S. Salzmann, C.S. Muller, S. Ekanayake-Mudiyanselage, P. Elsner and J.J. Thiele. 2002. Photoaging is associated with protein oxidation in human skin *in vivo*. J. Invest. Dermatol. 118: 618-625.

Woessner J.F. 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 5: 2145-2154.

(Received 5 February 2008 ; Accepted 15 February 2008)