ANTICANCER RESEARCH *40*: 2657-2665 (2020)
doi:10.21873/anticanres.14236

High Nitric Oxide Adaptation in Isogenic Primary and Metastatic Head and Neck Cancer Cells

KUSUMAWADEE UTISPAN[1] and SITTICHAI KOONTONGKAEW[1,2]

[1]Oral Biology Research Unit, Faculty of Dentistry, Thammasat University (Rangsit Campus), Pathum Thani, Thailand; [2]Walailak University, International College of Dentistry, Bangkok, Thailand

Abstract. *Background/Aim: Nitric oxide (NO) functions have been studied in many cancer types, but rarely in head and neck squamous cell carcinoma (HNSCC). This study aimed to investigate the behavior of HNSCC cells following exposure to high NO (HNO). Materials and Methods: Two pairs of isogenic HNSCC cell lines (HN18/HN17, HN30/HN31) were used, and were treated with a NO donor for 72 h. Cell viability, cell cycle, apoptosis, invasion, and MMP activity were determined using MTT, flow cytometry, Matrigel invasion, and gelatinase zymography assays, respectively. Results: HNO induced HN18 and HN31 cell cycle progression in S and G_2/M phases. Anti-invasion, MMP-2 inhibition, and apoptosis induction were observed in certain HNO-adapted cell lines. High NO did not affect MMP-9 activity in all cell lines. Conclusion: NO enhanced cell cycle progression and apoptosis but inhibited cell invasion in HNSCC cells.*

There is an incidence of a total of 600,000 cases of head and neck squamous cell carcinoma (HNSCC) per year worldwide and the mortality rate is 40-50% (1). HNSCC has broad etiology, symptoms, treatment, and prognosis. Hypoxia within the tumor is a common feature that leads to recurrence (2). Nitric oxide (NO) has an important role in the response to hypoxia by inducing angiogenesis and leading to cancer cell survival (3). NO is generated through the activity of three isoforms of NO synthase (NOS): (i) neuronal (NOS1 or nNOS), (ii) inducible (NOS2 or iNOS) and (iii) endothelial (NOS3 or eNOS). NO is a short-lived molecule, which affects cellular functions. NO affects cellular functions in a bimodal fashion with regard to its concentration. NO behaves as a signal transducer at low concentration and plays important role in various physiological processes such as iron homeostasis, blood flow, and neurotransmitter regulation. In contrast, at high concentrations, NO may have beneficial effects against tumors and pathogens (4, 5).

Because NO exhibits mutagenic behavior, long-term exposure of cells to high NO concentrations, resulting from iNOS induction during chronic inflammation, may serve an active role in carcinogenesis (6, 7). A tumor microenvironment (TME) contains cancer cells, host-derived cells including endothelial cells and tumor-infiltrating leukocytes. These cells may express any of the active NOS isoforms, serving as a source of NO in the tumor microenvironment. Tumor-derived NO regulates cancer cell proliferation, survival, migration, invasion and angiogenesis (8). NO regulates behaviors of the human hepatocellular carcinoma cell line (HepG2) in short term exposure models. Treatment with a NO donor for 24 h induces arrest at the G_0/G_1 phase of the cell cycle and apoptosis, and inhibition of migration and invasion in a dose-dependent manner (9). Previous studies, which investigated property changes of high NO (HNO)-adapted cancer cells found that adaptation of breast (10), and head and neck cancer (11) cell lines to HNO results in a faster growth compared to the parental cells. Moreover, HNO-adapted human lung adenocarcinoma cell (A549) proliferation was inhibited by activating antiapoptotic pathways. In addition, slow progression of HNO-adapted A549 cells through the S phase will allow time for DNA repair. Immunohistochemical staining demonstrated that iNOS expression levels were orderly increased from normal, through precancer, to oral squamous cell carcinoma (OSCC) (12). Hence, iNOS could play a role in epithelial transformation and OSCC formation.

According to the recent studies, NO has diverse effects on several cancer cells; however, its effect on HNSCC remains unclear (9, 10, 13, 14). The aim of the present study was to investigate the effects of HNO on HNSCC cell viability, cell

Correspondence to: Kusumawadee Utispan, Faculty of Dentistry, Thammasat University (Rangsit campus), Pathum Thani, Thailand. Tel: +66 29869213, Fax: +66 25165385, e-mail: kusumawadee.utipan@gmail.com

Key Words: Nitric oxide, HNSCC human head and neck cell, isogenic cell lines, cell proliferation, apoptosis, invasiveness, matrix metalloproteinases.

cycle status, apoptosis, invasion, and matrix metalloproteinases (MMPs) activity using a short term HNO adaptation model.

Materials and Methods

Cell culture. Two pairs of isogenic HNSCC cell lines, which derived from primary and metastatic lesions from the same patients were used in this study (15). HN18 cells were obtained from primary tongue lesions and HN17 cells were obtained from neck dissections (T2N2M0 stage). HN30 cells were taken from primary pharynx lesions and HN31 cells were taken from lymph node metastases (T3N0M0 stage). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Grand Island, NY, USA) with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and antimycotic. The cells were cultured at 37˚C and in a 5% CO_2 incubator and they were sub-cultured using 0.25% trypsin-EDTA when confluency reached about 90-100%.

Induction of HNSCC cells towards HNO-adapted cells. HNSCC cell lines were seeded in 96-well plates (5,000 cells/well) and incubated at 37˚C. The cells were treated with the NO donor DEA-NONOate (Sigma, St Louis, MO, USA) at concentrations 5, 6.5, 7.5, 8.5 and 10 mM for 72 h. Following incubation, the number of viable cells in each group was determined using the thiazolyl blue tetrazolium bromide (MTT, Sigma) assay. The medium was removed and 150 μl fresh medium was added followed by the addition of 2 mg/ml MTT solution (50 μl/well). The culture plates were incubated at 37˚C for 4 h. The formazan crystals were solubilized in DMSO (200 μl/well). The absorbance (Abs) of the final solution was measured at 570 nm by a microplate reader (Tecan Trading, Salzburg, Austria). Cell viability (%) was calculated using the formula: cell viability (%)=(mean $Abs570_{treated\ cells}$ - mean $Abs570_{blank}$)/(mean $Abs570_{parental\ cells}$ - mean $Abs570_{blank}$) ×100. High nitric oxide (HNO)-adapted cells were defined as those survived treatment that resulted in less than 10% cell viability. HNO-adapted cells were harvested and grown in complete medium before using them in subsequent experiments.

Cell cycle analysis by flow cytometry. HNO-adapted or parental cells (5×10^5/well in 6-well plates) were synchronized at the G_1 phase of the cell cycle by culturing them in serum-free DMEM for 24 h. The G_1 phase-arrested cells were treated with complete medium for 24 h. The cells were collected, centrifuged at 200xg for 5 min, and washed with phosphate-buffered saline (PBS) twice. The pellets of the cells were fixed in 70% ethanol and incubated for 15 min at –20˚C and then washed with cold PBS twice, centrifuged at 200 × *g* for 5 min, and incubated with 10 μg/μl DNase-free RNaseA (USB Corporation, Cleveland, OH, USA) at 37˚C for 30 min to eliminate the RNA. After washing twice with PBS and centrifuging at 200 × *g* for 5 min, the cells were incubated at 4˚C in the dark with 1 mg/ml propidium iodide (Sigma) for 15 min. The number of cells in each stage of the cell cycle was determined using a flow cytometer and the CyteExpert Software (Cytoflex®, Beckman Coulter, Indianapolis, IN, USA). The percentage of cells in the S phase of the cell cycle was measured and compared between HNO-adapted and parental cells. Cell cycle analyses were independently performed three times.

Apoptosis assessment by flow cytometry. HNO-adapted or parental cells (5×10^5/well in 6-well plates) were cultured in complete medium for 24 h and then collected, centrifuged at 200 × *g* for 5 min, and washed twice with PBS. The apoptotic cells were assessed using BD Annexin V FITC Assay (BD Biosciences, CA, USA) following the manufacturer's instructions. The number of apoptotic cells were calculated in a flow cytometer using the CyteExpert Software (Cytoflex®, Beckman Coulter). The percentage of apoptotic cells was measured and compared between HNO-adapted and parental cells. Apoptosis was assessed three times in independent experiments.

Invasion assay. An invasion assay through Matrigel was performed using a blind-well Boyden chemotaxis chamber (Neuro Probe, Gaithersburg, MD, USA) as previously described (16). At first, the upper surface of the 13 μm pore polycarbonate filters (Fisher Scientific, Ontario, Canada) was coated with Matrigel, a reconstituted basement membrane gel (Corning, Tewksbury, MA, USA) and placed between the upper and lower well plates of a blind-well Boyden chemotaxis chamber. In the lower chamber, growth medium was used as a source of chemoattractants. HNO-adapted or parental cells (8×10^4 cells) were resuspended in DMEM containing 0.1% BSA and seeded into the upper well of the chamber. The non-migrating cells on the upper surface of the filter were removed after incubation at 37˚C for 5 h. The cells were counted under a microscope at 200× magnification at the lower surface of the filters at 5 randomly selected fields per group. Two investigators performed the cell counting. The invasion assays were performed in three independent experiments.

Conditioned medium preparation and zymography. HNO-adapted or parental cells (2×10^6 cells) were cultured in 6-well plates and incubated at 37˚C for 24 h. Then, the wells were washed with PBS and switched to DMEM containing 0.1% BSA for 48 h. Conditioned medium (CM) was collected and centrifuged at 1,000 × *g* at 4˚C for 10 min. Protein concentration in the CM was measured using the Protein Assay Kit (Pierce™ BCA, Thermo Fisher Scientific, Waltham, MA, USA).

MMP-2 and MMP-9 activity in the CM were determined using gelatin zymography as previously described (17). Briefly, 0.2% gelatin (bloom 300, Sigma) was added to a 10% acrylamide separating gel. Equal amounts of total protein from each sample were mixed with non-reducing sample buffer and added to the gel. The gels were washed in 2.5% Triton X-100 for 30 min following electrophoresis, incubated at 37˚C overnight in developing buffer, stained with 0.5% Coomassie blue G250 in a 30% methanol and 10% glacial acetic acid solution for 30 min and destained in the same solution without Coomassie blue. The gelatin-degrading enzymes were identified as clear bands against the blue background of the stained gel. Images of the stained gels were acquired under illumination using a G:BOX gel documentation system (Syngene, Frederick, MD, USA). The gelatinolytic bands were quantified using GeneTools software (Syngene). The experiments were repeated three times independently.

Statistical analysis. Descriptive and inferential statistical analyses were carried out in the present study. Results of numerical measurements are presented as means and standard error of the mean (SEM). The Student's *t*-test (two-tailed, independent) was used to identify the significance of study parameters on a numerical scale between two groups. The statistical analysis was performed with Prism GraphPad 7.0 (GraphPad Software, La Jolla, CA, USA). The significance level was set at 0.05.

Table I. *Adaptation of HNSCC cell lines to high nitric oxide (HNO) concentration.*

HNSCC cell lines	Cell viability (%)						*HNO concentration (mM)
	DEA-NONOate concentrations (mM)						
	0	5	6.5	7.5	8.5	10	
HN18	100	15.44	8.45	7.13	0.22	0.34	6.5
HN17	100	32.69	21.06	14.95	5.55	0.17	8.5
HN30	100	27.94	20.04	13.09	1.09	0.3	8.5
HN31	100	34.78	28.87	12.73	6.68	0.17	8.5

*Cell viability less than 10%.

Results

Adaptation of HNSCC cells to NO. The results showed that the viability of HN18 cells was decreased to 8.45% at 6.5 mM DEA-NONOate (Table I). At 8.5 mM DEA-NONOate, the viability of HN17, HN30 and HN31 cells was decreased to 5.55, 1.09 and 6.68%, respectively. Thus, the HNO-adaption concentration for HN18 cells was 6.5 mM and the HNO-adaption concentrations for HN17, HN30 and HN31 cells were 8.5 mM. All HNO-adapted cell lines were harvested and grown in the growth medium before use in the subsequent experiments.

Alteration of the cell cycle status of HNO-adapted HNSCC cell lines. HNO-adapted HN18 and HN31 cells exhibited significantly increased DNA content in the S phase compared with the parental cells ($p<0.05$) (Figures 1A and D). However, the DNA content during the S phase of HNO-adapted HN17 and HN30 cells did not change compared with the parental cells ($p>0.05$) (Figures 1B and C).

Apoptotic activity in HNO-adapted HNSCC cell lines. The percentage of apoptotic HNO-adapted HN18, HN17 and HN30 cells showed no significant difference compared with the parental cells ($p>0.05$) (Figures 2A-C). In contrast, HNO-adapted HN31 cells exhibited significantly increased apoptotic activity compared with the parental cells ($p<0.05$) (Figure 2D).

Reduction of invasiveness activity of HNO-adapted HNSCC cell lines. All HNO-adapted HNSCC cell lines showed significantly decreased invasiveness compared with the parental cells ($p<0.05$) (Figure 3).

Alteration of MMPs activity in HNO-adapted HNSCC cell lines. MMP-2 and 9 activity was studied in HNO-adapted cells and the results are illustrated in Figure 4A. The MMPs activity in gelatin zymography was converted to an arbitrary number of intensities. The MMP-9 activity in all HNO-adapted HNSCC cell lines did not significantly differ compared with the parental cells ($p>0.05$) (Figure 4B). Interestingly, MMP-2 activity was significantly increased in HNO-adapted HN18 and HN30 cells compared with the parental cells ($p<0.05$) (Figure 4C). However, the MMP-2 activity was significantly decreased in HNO-adapted HN31 cells compared with the parental cells ($p<0.05$).

Discussion

NO is involved in various physiological and pathological processes. NO could generate differential effects depending on its concentration, leading to "NO-sensitive" and "NO-resistant" tumor cells. At low concentrations, NO promotes tumor growth. Expression of a tumor suppressor gene, p53 has commonly been found to be upregulated by high concentrations of NO and this might lead to tumor growth arrest. In contrast, "NO-resistant" or "HNO adapted" tumor cells with mutated p53 support tumor progression (18). Nevertheless, studies on the effects of NO on HNSCC cells are rare. Our findings agree with a previous study (11) which demonstrated that HNSCC cells were adapted to high levels of NO. However, all our HNSCC cell lines can adapt at relatively high NO concentration (6.5-8.5 mM DEA-NONOate) in contrast with the previous study (0.6 mM DEA-NONOate).

Our results suggest that the process of NO adaptation seems to be universal across HNSCC, irrespective of the metastatic behavior and tumor stage. HNSCC cell lines in our study showed comparable results; increasing NO concentrations resulted in increased cell death. However, variations in the adaptation of tumor cells in low and high NO concentrations were demonstrated in the present study. Stage 2 metastatic HN17 cells exhibited more resistance to NO compared with the corresponding primary tumor cell line (HN18). In contrast, there was no difference in NO resistance between stage 3 metastatic HN31 cells and their primary cancer cells (HN30).

The mechanism explaining NO adaptation has been previously reported. Aqil *et al*. (14) have demonstrated that

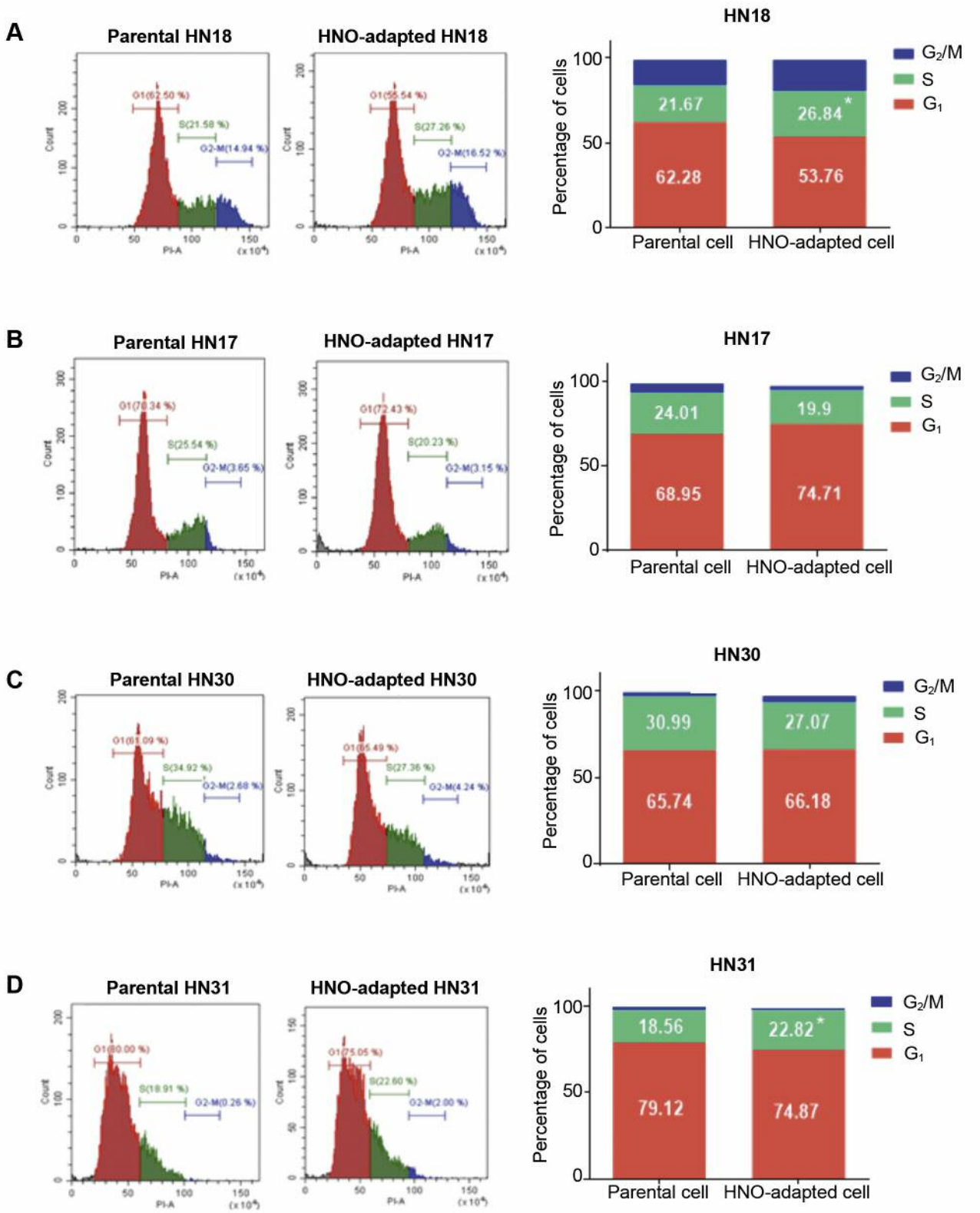

Figure 1. *Cell cycle status of HNO-adapted HNSCC cell lines. The cell cycle distribution of all HNO-adapted and the corresponding parent cells are shown in the histograms. The percentage of cells in each phase of the cell cycle was calculated in the parental and HNO-adapted HN18 (A), HN17 (B), HN30 (C) and HN31 (D) cells. *p<0.05 compared with the parental cells.*

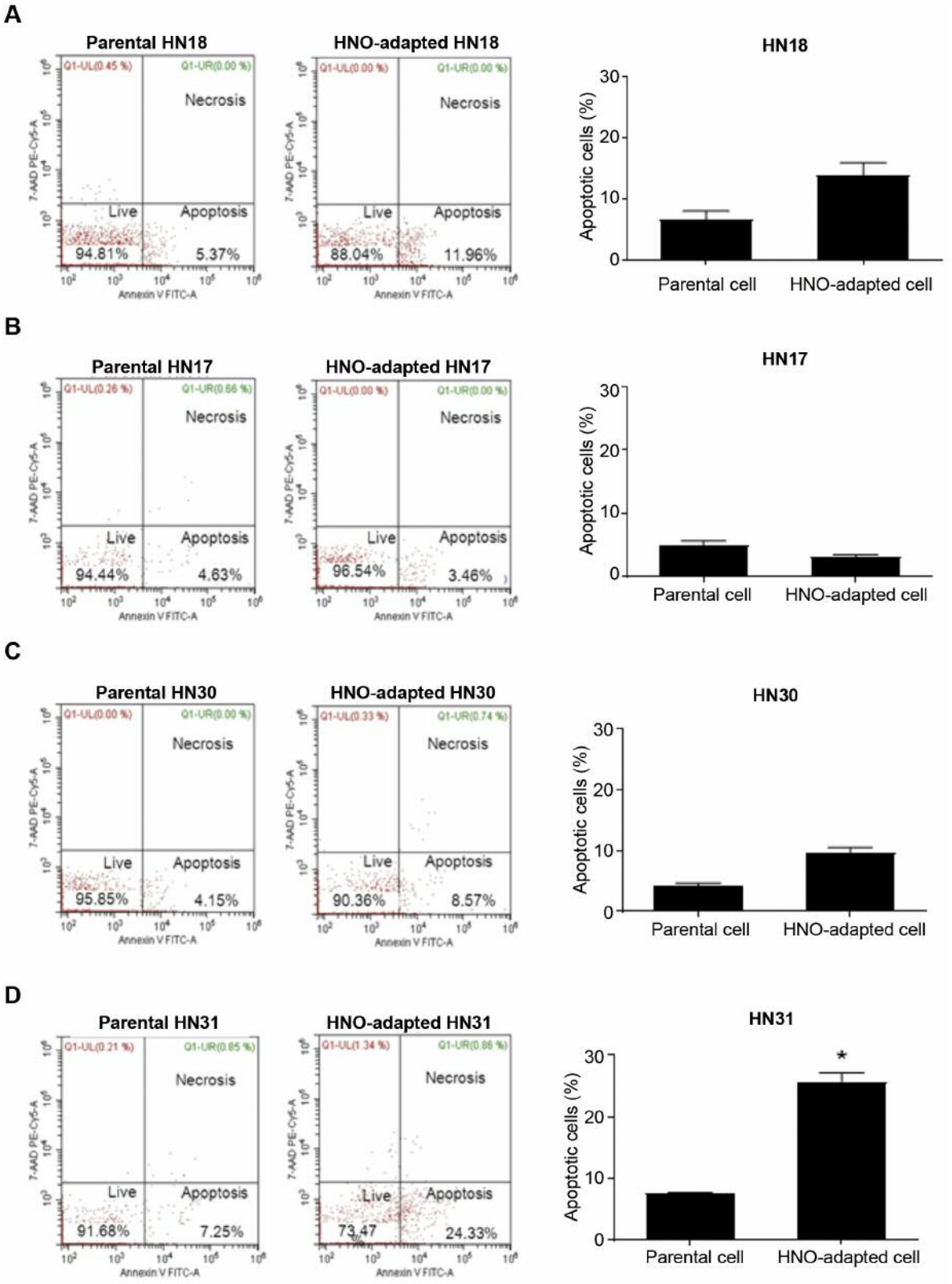

Figure 2. *Apoptotic activity in HNO-adapted HNSCC cell lines. The percentage of apoptotic cells in all HNO-adapted and the corresponding parent cells are shown in the histograms. The percentage of apoptotic cell was calculated in the parental and HNO-adapted HN18 (A), HN17 (B), HN30 (C) and HN31 (D) cells. Bars represent mean±SEM of the percentage of apoptotic cells (n=3). *p<0.05 compared with the parental cells.*

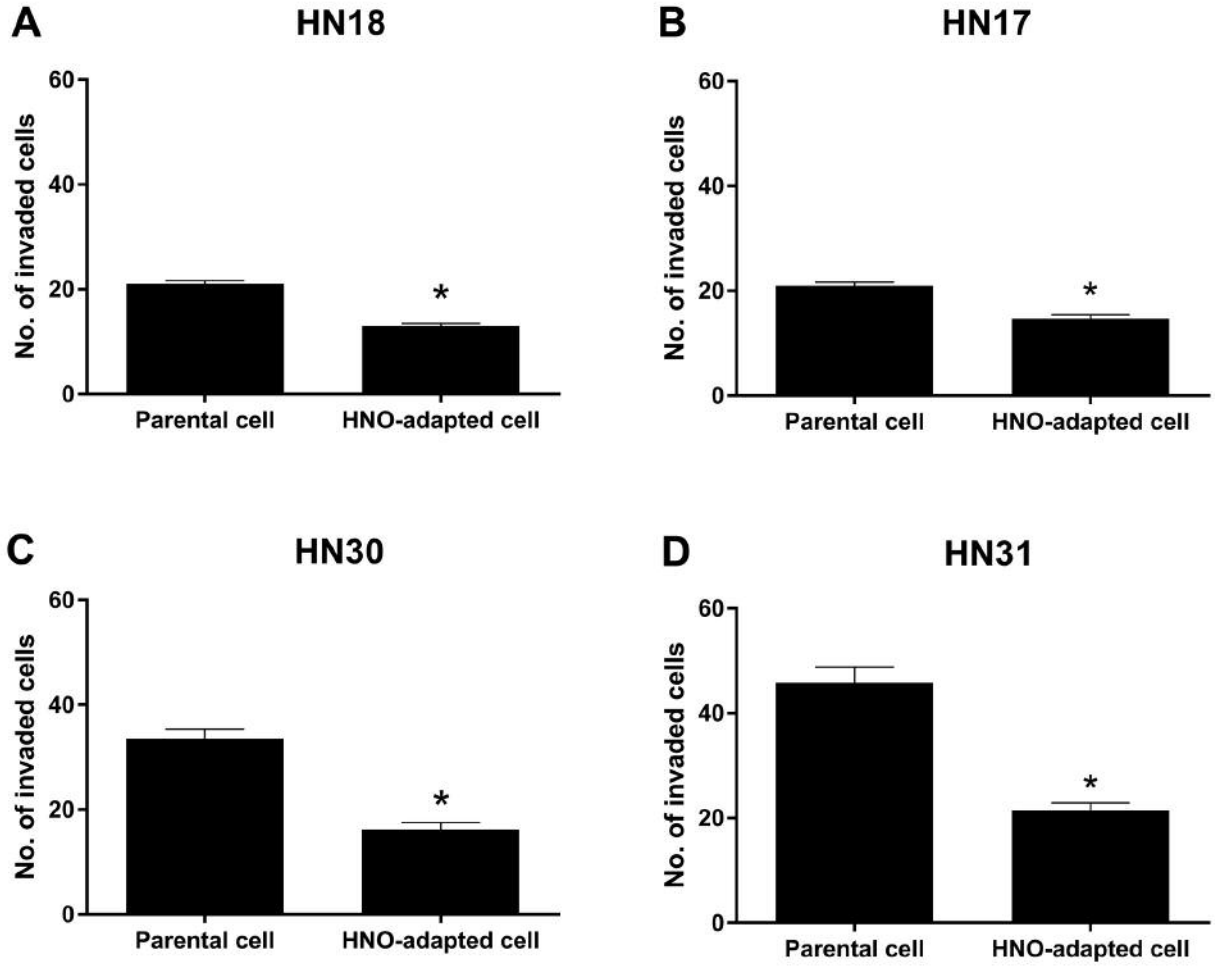

Figure 3. *Invasiveness activity of HNO-adapted HNSCC cell lines. Invasion of all HNO-adapted and the corresponding parental cells were evaluated. The number of invaded cells of the parental and HNO-adapted HN18 (A), HN17 (B), HN30 (C) and HN31 (D) cells were counted under a light microscope at magnification 200x. Bars represent mean±SEM invaded cell number (n=3). *p<0.05 compared with the parental cells.*

modifications in DNA damage and anti-apoptosis signaling might aid to high NO resistance of tumor cells. This ultimately affects the cell cycle and tumor cells stay in the S phase for longer, before proceeding to the G_2/M phase (11). In this study, the stimulatory action of HNO on cell proliferation was observed as an increase in cell number in the S and G_2/M phases in HNO-adapted HN18 and HN31 cells, but not in HNO-adapted HN17 and HN30 cells. Probably, up-regulation of genes promoting cell multiplication and downregulation of genes inhibiting multiplication might occur in the HNO-adapted cancer cells (13). Notably, our findings suggest that the effect of high NO concentrations on cell cycle progression does not depend on the metastatic properties and disease stage. The results in our study are not in line with a related study reporting that the effects of NO on the progression of cell cycle in colon cancer depended on NO concentrations and disease stages (19).

The present study also analyzed the effects of high NO on apoptosis. Related studies in head and neck cancer cells (11) and lung cancer cells (14, 20) have demonstrated that a decrease of apoptosis occurred in HNO-adapted cancer cells, in opposition to our data indicating pro-apoptotic or no effects of high NO.

The apoptotic effects of NO depend on its production rate, redox state of cells, and cell type. High levels of NO have pro-apoptotic effects, whereas low levels of NO can inhibit apoptotic cell death either by direct or indirect interaction with molecules of the apoptotic-signaling cascade (21, 22). In the present study, a significant increase in NO-induced apoptotic cell death was observed in HN31 cells. In addition, the number of apoptotic HNO-adapted HN18 and HN30 cells was relative higher, compared with that of their parental cells although this did not reach statistical significance. Therefore, it appears reasonable to suggest that high NO may, at least

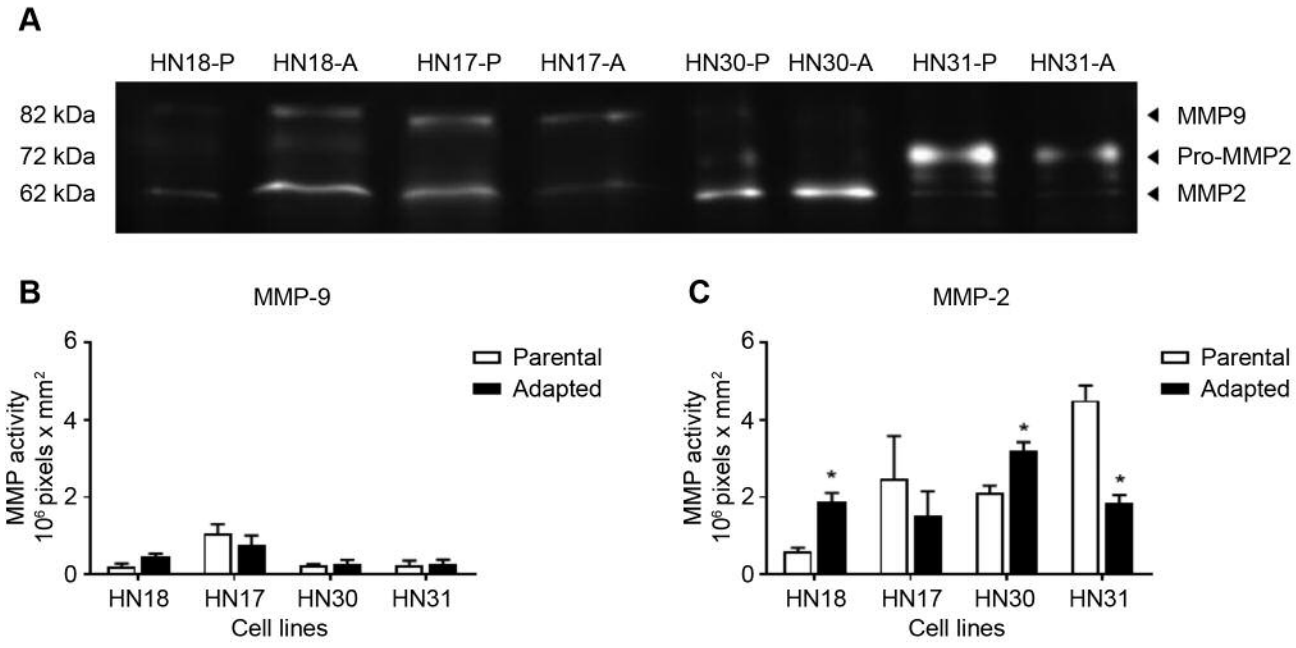

Figure 4. *MMPs activity in HNO-adapted HNSCC cell lines. MMP-9 and 2 activity in all HNO-adapted and the corresponding parental cells are shown in a zymographic gel (A). The intensity of gelatinolytic bands in all HNO-adapted and the parental cells were converted to activity of MMP-9 (B) and MMP-2 (C). Bars represent mean±SEM MMPs activity (n=3). *p<0.05 compared with the parental cells.*

in part, increase susceptibility to apoptosis (23). Our results are discordant to those of a related study demonstrating activation of p53 and apoptosis inhibition in a human NO-adapted lung adenocarcinoma cell line (A549) (14). This discrepancy may be due to the differences in the duration of NO exposure and cell types. We exposed HNSCC cell lines with DEA-NONOate at 6.5-8.5 mM for 72 h whereas the related report performed long term (65 days) treatment of A549-adapted cells with 0.6 mM DEA-NONOate. Our findings could lead to the inference that high levels of NO play no role or probably some other factors are involved in the apoptosis of tumor cells.

In this present study, *in vitro* cell invasion and MMPs activities were examined to determine the effect of NO adaptation on the invasion ability of HNSCC cells and found that 6.5-8.5 mM DEA-NONOate, significantly decreased the invasion of adapted HNO-HNSCC cell lines through Matrigel. The results were similar with other studies, in which they found that the NO suppressed *in vitro* invasion of hepatocellular carcinoma cell lines (9) and ovarian cancer cell lines (24). However, these reports are discordant to another human study demonstrating that 7- or 14-days treatment with NO increased significantly the invasion of lung cancer cell lines (H460 cells) (25). These conflicting results can be clarified by the observation that the activity of high NO in cancer cell behaviors depends on the microenvironment, such as cell types, redox reaction, final intracellular concentration, and duration of exposure to NO (26).

MMP-2 and MMP-9 play major roles in the metastasis of cancer cells and, recently, have been studied extensively (27). Regarding to tumor cell invasion and MMPs activation, our results show the disparities of MMP-2 and MMP-9 activities in HNO adaptation. MMP-2 and MMP-9 activities were obviously non-coordinated and were differentially affected in HNO-adapted HNSCC cell lines. In our study, gelatinase zymography showed no significance difference in MMP-9 activities between HNO-adapted cells and their parental cells. Our findings are different from those of a related study reporting up-regulation of MMP-9 release in ovarian cancer cells that were treated with NO (24). Mechanistically, the inhibition of MMP-9 overexpression might be explained by the possibility of MMP-9 degradation in the very high levels of NO in our study (28). Surprisingly, differential effects of HNO on MMP-2 activities in HNSCC cell lines were observed in this study. HNO inhibited MMP-2 activity in HNO-adapted HN 31 cells while an increased MMP-2 activity was observed in HNO-adapted HN18 and HN30 cells. Furthermore, there was no significant difference regarding the MMP-2 activity between HNO-adapted HN 17 cells and their parental cells. This indicated that the cell lines have different susceptibility to NO donors. The inhibition of MMP-2 activity by exogenously applied NO donors has been reported previously in ovarian cancer cells (24). In addition, it should be noted that neither the expression of MMP-2 nor that of MMP-9 in HNO-adapted HNSCC cells was correlated with cell invasion in our studies. However, it has been

reported that the increase in the levels of MMPs does not essentially relate to the invasive phenotype (29, 30). Due to the conflicting results, the tumor invasion activity of NO needs to be further investigated.

Some limitations of the present study need to be discussed. First, in this study we carried out short time stimulation of HNSCC cells with HNO. Nevertheless, our data show that this was able to produce NO adaptation because changes in biological activities were observed in HNO-adapted cells in our experiments. Second, the action of high NO on cell cycle regulation and apoptosis were not confirmed by gene expression. Third, in this study, we investigated the evidence for the roles of NO in particular focusing on tumor epithelial cells. Although NO is a multifunctional signaling molecule with potentially high bioavailability in TME but, the interactions between tumor and stromal cells such as tumor associated macrophages were not conducted in the present study (31). Therefore, the diverse role of NO in tumor and TME needs to be further investigated in more detail. However, the strong point of this study was the use isogenic primary and metastatic cancer cell lines obtained from various tumor stages. According to our knowledge, no study has been conducted using isogenic primary and metastatic cancer cell lines in NO adaptation experiments. The benefit of this model is that it clearly reveals only the differences that are due to treatments and not to differences in the biological system, since isogenic cancer cell lines are used.

Conclusion

We found that DEA-NONOate enhanced cell cycle progression of primary and metastatic HNSCC cells. However, the tested isogenic cancer cell lines showed differential sensitivity to NO donors. High NO induced apoptosis in HNO-adapted cancer cells. Reduced cell invasion was observed in HNO-adapted cancer cells. However, certain NO-adapted cancer cells showed decreased MMP-2 release or no MMP-9 release. However, considering this discrepancy, our findings confirm that NO donors may behave as anti-metastatic and pro-metastatic agents, and their actions depend on many factors such as the type of cancer cells, redox reaction, final intracellular concentration, and duration of exposure to NO.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

Kusumawadee Utispan: funding, design, data acquisition, data analysis, preparation and revision of manuscript; Sittichai Koontongkaew: funding, design, manuscript editing.

Acknowledgements

This study was supported by TU-Grant 2561, Thammasat University, Thailand. The Authors would like to thank Dr Nattisa Niyomthan (a postdoctoral fellow); and Drs Benjawan Sangkaewsantisuk, Nutthawat Taokhampu, Artit Swangrungruangkit, Vitharinee Srikenkhan (the former undergraduate students at Faculty of Dentistry, Thammasat University) for their help in acquiring the preliminary data by cell-culturing and preparing the methods and experimental settings. Finally, the Authors would like to thank Dr Dinesh Rokaya, Walailak University for English editing.

References

1 Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in globocan 2012. Int J Cancer *136(5)*: E359-386, 2015. PMID: 25220842. DOI: 10.1002/ijc.29210

2 Janssen HL, Haustermans KM, Balm AJ and Begg AC: Hypoxia in head and neck cancer: How much, how important? Head Neck *27(7)*: 622-638, 2005. PMID: 15952198. DOI: 10.1002/hed.20223

3 Wongvaranon P, Pongrakhananon V, Chunhacha P and Chanvorachote P: Acquired resistance to chemotherapy in lung cancer cells mediated by prolonged nitric oxide exposure. Anticancer Res *33(12)*: 5433-5444, 2013. PMID: 24324079.

4 Burke AJ, Sullivan FJ, Giles FJ and Glynn SA: The yin and yang of nitric oxide in cancer progression. Carcinogenesis *34(3)*: 503-512, 2013. PMID: 23354310. DOI: 10.1093/carcin/bgt034

5 Ruggiero M, Ward E, Smith R, Branca JJ, Noakes D, Morucci G, Taubmann M, Thyer L and Pacini S: Oleic acid, deglycosylated vitamin D-binding protein, nitric oxide: A molecular triad made lethal to cancer. Anticancer Res *34(7)*: 3569-3578, 2014. PMID: 24982371.

6 Calmels S, Hainaut P and Ohshima H: Nitric oxide induces conformational and functional modifications of wild-type p53 tumor suppressor protein. Cancer Res *57(16)*: 3365-3369, 1997. PMID: 9269997.

7 Laval F and Wink DA: Inhibition by nitric oxide of the repair protein, O^6-methylguanine-DNA-methyltransferase. Carcinogenesis *15(3)*: 443-447, 1994. PMID: 8118926. DOI: 10.1093/carcin/15.3.443

8 Lala PK and Chakraborty C: Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol *2(3)*: 149-156, 2001. PMID: 11902565. DOI: 10.1016/s1470-2045(00)00256-4

9 Zhou L, Zhang H and Wu J: Effects of nitric oxide on the biological behavior of HepG2 human hepatocellular carcinoma cells. Exp Ther Med *11(5)*: 1875-1880, 2016. PMID: 27168820. DOI: 10.3892/etm.2016.3128

10 Vesper BJ, Elseth KM, Tarjan G, Haines GK, 3rd and Radosevich JA: Long-term adaptation of breast tumor cell lines to high concentrations of nitric oxide. Tumour Biol *31(4)*: 267-275, 2010. PMID: 20480412. DOI: 10.1007/s13277-010-0028-6

11 Yarmolyuk YR, Vesper BJ, Paradise WA, Elseth KM, Tarjan G, Haines GKand Radosevich JA: Part I. Development of a model system for studying nitric oxide in tumors: High nitric oxide-adapted head and neck squamous cell carcinoma cell lines. Tumour Biol *32(1)*: 77-85, 2011. PMID: 20972659. DOI: 10.1007/s13277-010-0101-1

12 Varghese SS, Sunil PM and Madhavan RN: Expression of inducible nitric oxide synthase (iNOS) in oral precancer and oral squamous cell carcinoma: An immunohistochemical study. Cancer Biomark *8(3)*: 155-160, 2010. PMID: 22012771. DOI: 10.3233/CBM-2011-0207

13 Aqil M, Deliu Z, Elseth KM, Shen G, Xue J and Radosevich JA: Part II-mechanism of adaptation: A549 cells adapt to high concentration of nitric oxide through bypass of cell cycle checkpoints. Tumour Biol *35(3)*: 2417-2425, 2014. PMID: 24241959. DOI: 10.1007/s13277-013-1319-5

14 Aqil M, Elseth KM, Vesper BJ, Deliu Z, Aydogan B, Xue J and Radosevich JA: Part I-mechanism of adaptation: High nitric oxide adapted A549 cells show enhanced DNA damage response and activation of antiapoptotic pathways. Tumour Biol *35(3)*: 2403-2415, 2014. PMID: 24241898. DOI: 10.1007/s13277-013-1318-6

15 Cardinali M, Pietraszkiewicz H, Ensley JF and Robbins KC: Tyrosine phosphorylation as a marker for aberrantly regulated growth-promoting pathways in cell lines derived from head and neck malignancies. Int J Cancer *61(1)*: 98-103, 1995. PMID: 7705939. DOI: 10.1002/ijc.2910610117

16 Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM and McEwan RN: A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res *47(12)*: 3239-3245, 1987. PMID: 2438036.

17 Thomas GJ, Lewis MP, Hart IR, Marshall JF and Speight PM: AlphaVbeta6 integrin promotes invasion of squamous carcinoma cells through up-regulation of matrix metalloproteinase-9. Int J Cancer *92(5)*: 641-650, 2001. PMID: 11340566. DOI: 10.1002/1097-0215(20010601)92:5<641::aid-ijc1243>3.0.co;2-p

18 Williams EL and Djamgoz MB: Nitric oxide and metastatic cell behaviour. Bioessays *27(12)*: 1228-1238, 2005. PMID: 16299735. DOI: 10.1002/bies.20324

19 Jiang H, Tang Y, Garg HK, Parthasarathy DK, Torregrossa AC, Hord NG and Bryan NS: Concentration- and stage-specific effects of nitrite on colon cancer cell lines. Nitric Oxide *26(4)*: 267-273, 2012. PMID: 22504070. DOI: 10.1016/j.niox.2012.03.010

20 Sawachi K, Shimada Y, Taniguchi H, Hirota K, Inagawa H, Kohchi C, Soma G, Makino K and Terada H: Cytotoxic effects of activated alveolar macrophages on lung carcinoma cells via cell-to-cell contact and nitric oxide. Anticancer Res *30(8)*: 3135-3141, 2010. PMID: 20871032.

21 Choi BM, Pae HO, Jang S, Kim YM and Chung HT: Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. J Biochem Mol Biol *35(1)*: 116-126, 2002. PMID: 16248976. DOI: 10.5483/bmbrep.2002.35.1.116

22 Vahora H, Khan MA, Alalami U and Hussain A: The potential role of nitric oxide in halting cancer progression through chemoprevention. J Cancer Prev *21(1)*: 1-12, 2016. PMID: 27051643. DOI: 10.15430/JCP.2016.21.1.1

23 Bonavida B and Garban H: Nitric oxide-mediated sensitization of resistant tumor cells to apoptosis by chemo-immunotherapeutics. Redox Biol *6:* 486-494, 2015. PMID: 26432660. DOI: 10.1016/j.redox.2015.08.013

24 Kielbik M, Szulc I, Brzezinska M, Bednarska K, Przygodzka P, Sulowska Z, Nowak M and Klink M: Nitric oxide donors reduce the invasion ability of ovarian cancer cells *in vitro*. Anticancer Drugs *25(10)*: 1141-1151, 2014. PMID: 25035962. DOI: 10.1097/CAD.0000000000000149

25 Sanuphan A, Chunhacha P, Pongrakhananon V and Chanvorachote P: Long-term nitric oxide exposure enhances lung cancer cell migration. Biomed Res Int *2013*: 186972, 2013. PMID: 23984323. DOI: 10.1155/2013/186972

26 Huerta S, Chilka S and Bonavida B: Nitric oxide donors: Novel cancer therapeutics (review). Int J Oncol *3(5):* 909-927, 2008. PMID: 18949354. DOI: 10.3892/ijo_00000079

27 Koontongkaew S: The tumor microenvironment contribution to development, growth, invasion and metastasis of head and neck squamous cell carcinomas. J Cancer *4(1)*: 66-83, 2013. PMID: 23386906. DOI: 10.7150/jca.5112

28 Akool el S, Kleinert H, Hamada FM, Abdelwahab MH, Forstermann U, Pfeilschifter J and Eberhardt W: Nitric oxide increases the decay of matrix metalloproteinase 9 mRNA by inhibiting the expression of mRNA-stabilizing factor HuR. Mol Cell Biol *23(14)*: 4901-4916, 2003. PMID: 12832476. DOI: 10.1128/mcb.23.14.4901-4916.2003

29 Cepeda MA, Evered CL, Pelling JJH and Damjanovski S: Inhibition of MT1-MMP proteolytic function and ERK1/2 signalling influences cell migration and invasion through changes in MMP-2 and MMP-9 levels. J Cell Commun Signal *11(2)*: 167-179, 2017. PMID: 28070797. DOI: 10.1007/s12079-016-0373-3

30 Lichtinghagen R, Musholt P, Lein M, Romer R, Rudolph B, Kristiansen G, Hauptmann S, Schnorr D, Loening S and Jung K: Different mRNA and protein expression of matrix metalloproteinases2 and 9 and tissue inhibitor of metalloproteinase 1 in benign and malignant prostate tissue. Eur Urol *42(4):* 398-406, 2002. PMID: 12361907. DOI: 10.1016/s0302-2838(02)00324-x

31 Ryan AE, Burke AJ, Giles FJ, Sullivan FJ and Glynn SA: Mechanisms of nitric oxide-dependent regulation of tumor invasion and metastasis. *In*: Nitric oxide and cancer: pathogenesis and therapy. Bonavida B (ed.). Springer International Publishing, Switzerland, pp. 49-63, 2015.

Received April 4, 2020
Revised April 10, 2020
Accepted April 13 2020