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Nisin induces apoptosis in cervical cancer cells via reactive oxygen species generation and mitochondrial membrane potential changes

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Abstract

Nisin, an antimicrobial peptide produced by *Lactococcus lactis*, is widely used as a safe food preservative and has been recently attracting the attention of many researchers as a potential anticancer agent. The cytotoxicity of nisin against HeLa, OVCAR-3, SK-OV-3, and HUVEC cells was evaluated using MTT assay. The apoptotic effect of nisin was identified by Annexin-V/propidium iodide assay, and then it was further confirmed by western blotting analysis, mitochondrial membrane potential ($\Delta\Psi_m$) analysis, and reactive oxygen species (ROS) assay. The MTT assay showed concentration-dependent cytotoxicity of nisin towards cancer cell lines, with the IC_{50} values of 11.5-23 μ M, but less toxicity against normal endothelial cells. Furthermore, treatment of cervical cancer cells with 12 μ M nisin significantly ($P<0.05$) increased the Bax/Bcl-2 ratio (4.9-fold), reduced $\Delta\Psi_m$ (70%), and elevated ROS levels (1.7-fold). These findings indicated that nisin might have anticancer and apoptogenic activities through mitochondrial dysfunction and oxidative stress damage in cervical cancer cells.

Keywords: Nisin, Apoptosis, Mitochondrial membrane potential, Reactive oxygen species, Uterine cervical neoplasms, Ovarian neoplasms

Introduction

Gynecologic cancers (*e.g.*, cervical and ovarian cancer) are common cancer with high mortality among women worldwide. Cervical cancer was the fourth most common cancer among women and was also the fourth leading cause of death due to cancer in women according to the Global Cancer Observatory 2018 database (Arbyn et al., 2020). In 2021, 21,410 new ovarian cancer cases will be diagnosed and an estimated 13,770 women will die from this cancer in the United States (Siegel et al., 2021). Acquired (*e.g.*, chronic human papillomavirus or HPV infection) and genetic (*e.g.*, family history) susceptibility factors are the major cause of cervical cancer (Tan and Ankathil, 2015, Chan et al., 2019). Recent studies have explored that vaginal dysbacteriosis or vaginal microbiota imbalance also plays an important role in the development of reproduction system cancers including cervical cancer in women (Brusselaers et al., 2019). Different therapeutic approaches including surgery, radiation therapy, and pharmacotherapy are currently applied for the treatment of cervical cancer. However, most patients experience adverse effects which have long-term effects on their quality of life (Pfaendler et al., 2015). Therefore, the development of novel anticancer agents is still required to reduce side effects and improve the effectiveness of therapeutic approaches for the management of cervical cancer. Recent evidence has shown that probiotics and their metabolites (*e.g.*, antimicrobial peptides) can rebalance the host's microbiome, improve the immune system, and reduce inflammation (Zheng et al., 2020); therefore, these agents have potential preventive, therapeutic, or diagnostic applications in different malignancies including gynecologic cancers (Perisić et al., 2011, Cha et al., 2012, Linn et al., 2019). Nisin is a cationic antimicrobial peptide produced by *Lactococcus lactis*, the first living transgenic bacteria used for the treatment of human disease (Baat et al., 2006). This

antimicrobial peptide is widely used as a food preservative and is generally regarded as safe (GRAS) for human usage even at high doses (2021). Nisin exhibits various pharmacological activities including antimicrobial/biofilm, immunomodulatory and antitumor properties (Shin et al., 2016). Anticancer and cytotoxic effects of nisin have been investigated in several cancer cell lines including head and neck squamous carcinoma cells (HNSCC) (Kamarajan et al., 2015), breast cancer cells (*e.g.*, MCF-7) (Avand et al., 2018), skin cancer cells (*e.g.*, A375) (Lewies et al., 2018) and colorectal cancer cells (*e.g.*, SW480) (Hosseini et al., 2020).

To the best of our knowledge, there is no report on the cytotoxic effect of nisin against ovarian and cervix cancer cell lines. In this study, we aimed to evaluate the anticancer effect of nisin against cervical and ovarian cancer cell lines (HeLa, OVCAR-3, and SK-OV-3) using the cell viability assay. We also performed flow cytometric analysis, western blot analysis, mitochondrial membrane potential analysis, and reactive oxygen species (ROS) assay to gain mechanistic insights into the nisin-induced cervical cancer cell death.

Materials and Methods

Chemicals and reagents

High glucose Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute 1640 medium (RPMI), fetal bovine serum (FBS), penicillin-streptomycin were obtained from Bioidea (Iran). Nisin, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were provided from Sigma-Aldrich (USA). Mouse anti-Bcl-2 (sc-7382), mouse anti- β -actin (sc-47778), mouse anti-Bax (sc-20067), and goat anti-mouse IgG-HRP (sc-2031) were provided from Santa Cruz Biotechnology (USA). All other reagents were purchased from Sigma Aldrich (USA) and were of analytical grade.

78 *Cell lines*

79 The human cervical cancer cell line (HeLa), human ovarian carcinoma cell lines (OVCAR-3 and
80 SK-OV-3), and human umbilical vein endothelial cells (HUVEC) were purchased from the
81 Pasture Institute (Tehran, Iran). The cells were incubated in a humidified atmosphere containing
82 5 % CO₂ at 37 °C. HeLa, OVCAR-3 and SK-OV-3 were fed with RPMI supplemented with 10 %
83 FBS, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. HUVEC cells were cultured in
84 high glucose DMEM supplemented with 10 % FBS and penicillin-streptomycin (100 IU/ml and
85 100 µg/ml, respectively).

86 *Cell viability assay*

87 To evaluate the cytotoxic effect of nisin on the OVCAR-3, SK-OV-3, HeLa, and HUVEC cells,
88 the MTT assay was performed as described previously (Safaeian et al., 2016). Briefly, the cells
89 were seeded at a density of 1×10^4 cells/well in 96-well plates and incubated overnight. Based on
90 the IC₅₀ values reported in the previous studies (Avand et al., 2018, Rana et al., 2019), the 0.75-
91 75 µM concentration range was chosen for the present work. The cells were incubated with
92 different concentrations of nisin (0.75–75 µM). Every plate had four wells with cells treated with
93 phosphate buffered saline (PBS) as the negative control. After 48 h of incubation, 20 µl of MTT
94 solution (5 mg/ml in PBS) was added to each well and incubated at 37 °C for 4 h. Then, the
95 medium was removed and 150 µl DMSO was added to each well to dissolve insoluble formazan
96 crystals. Finally, the absorbance values were measured at 570 nm by a plate reader (BioTek
97 Instruments, USA). The percentage of cell viability was determined according to the following
98 equation:

$$\begin{aligned} &99 \quad \% \text{ Cell viability} = (\text{Absorbance of treated wells} - \text{Absorbance of blank}) / (\text{Absorbance of} \\ &100 \quad \text{negative control} - \text{Absorbance of blank}) \times 100 \end{aligned}$$

Apoptosis assay via Annexin-V/PI staining

Induction of apoptosis by nisin was evaluated using Apoptosis Detection Kit (Annexin V-FLUOS Staining kit, Roach, USA). HeLa cells (2×10^5 cells/well) were seeded in a 12-well plate and incubated with various concentrations of nisin (3, 6, 12 and 24 μ M) and the negative control for 16 and 24 h. Then, the cells were harvested and washed with PBS by centrifugation at $500 \times g$ for 5 min at 4 °C. Next, the cells were co-stained with FITC conjugated Annexin V (Annexin-V) and propidium iodide (PI) according to the manufacturer's instruction and analyzed by flow cytometry (BD Biosciences, USA).

Western blot analysis

To evaluate the effect of nisin on the expression of apoptotic and anti-apoptotic proteins, western blot analysis was performed as described previously (Shahrestanaki et al., 2019). Briefly, HeLa cells were grown on cell culture dishes and treated with different concentrations of nisin (6, 12, and 24 μ M) and the negative control for 48 h. The cells were harvested using a sterile cell scraper and resuspended in RIPA buffer containing protease inhibitors (0.5 mM PMSF, and 0.5% protease and phosphatase inhibitor cocktails) and then subjected to probe-sonication (Hielscher, Germany) on ice to disrupt cells. The cell debris was removed by centrifugation at $12,000 \times g$ for 10 min at 4 °C and the total protein content of the supernatant was determined using the Bradford method. An equal amount of each protein sample was separated by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) 2 h at 25 °C and then probed with anti-human Bax, Bcl-2, and β -actin antibodies at 4 °C overnight. After three washes, the membrane was incubated with a peroxidase-conjugated secondary antibody for 2 h at 25 °C. Then, the membrane was washed three times

with TBST and the blots were visualized on an X-ray film using an ECL kit (Cell signaling, USA). The density of each band was quantified by ImageJ software (version 1.52, National Institutes of Health, Bethesda, USA).

Mitochondrial membrane potential analysis

The mitochondrial membrane potential ($\Delta\Psi_m$) was determined using a mitochondrial probe, JC-1, as described previously (Shahali et al., 2018). Briefly, HeLa cells (1×10^3 cells/well) were seeded in a 384 well plate and were treated with nisin (6, 12, 24 μM) and the negative control for 24 h. The medium was replaced with 100 μl per well of HEPES buffer and incubated for 30 min at 37 °C. The plate was washed once more with 100 μl per well of HEPES buffer and then cells were stained with 2.5 mM JC-1 in the dark. Fluorescence was measured for each well at every 15 min for 2 h at excitation/emission 485/530 nm (green) and excitation/emission 530/580 nm (red) using a microplate reader (Synergy H1 Hybrid Multi-Mode; Bio-Tek). Changes in the ratio of red to green fluorescence were determined and then normalized to untreated control cells.

Reactive oxygen species (ROS) assay

The generation of ROS was determined using a Live Cell Fluorescent Reactive Oxygen Species detection kit (Marker Gene Technologies, USA) as described previously (Shahali et al., 2018). In brief, HeLa cells were seeded in a 384-well plate at 1×10^3 cells per well overnight, and incubated with nisin (6, 12, 24 μM) for 48 h. The medium was removed and cells were stained with 20 μM 2',7'-dichlorofluorescein diacetate (DCFDA) at 25 °C for 1 h in the dark. Fluorescence was determined for each well at excitation/emission 485/528 nm using a microplate reader (Synergy H1 Hybrid Multi-Mode; Bio-Tek). The ROS levels were expressed as a fold of change to the control.

Statistical analysis

The IC₅₀ was calculated with the Prism 9 software (GraphPad Prism Software Inc., San Diego, CA). Data from at least three independent experiments were reported as mean ± standard deviation (SD). Significant differences between groups were determined by analysis of variance (ANOVA) using SPSS (Statistical Package for the Social Sciences) software program (Chicago, IL, USA), version 20 followed by a Tukey post hoc test. The *p* values <0.05 were considered significant.

Results

In vitro cytotoxicity of nisin

To determine the cytotoxicity of nisin, HUVCE, HeLa, OVCAR-3, and SK-OV-3 cell lines were incubated with nisin at different concentrations for 48 h and an MTT assay was performed to evaluate the cell viability. As presented in Figure 1, nisin revealed a low level of cytotoxicity for non-cancerous cells, HUVEC; cell viability was slightly reduced at higher concentrations of nisin (6 and 12 µM) and the IC₅₀ value was 63.2 µM. In contrast, nisin exhibited significant cytotoxicity (*P*<0.05) on HeLa and OVCAR-3 cells, and the IC₅₀ value was 11.5 µM and 14.6 µM, respectively. Interestingly, SK-OV-3, a more invasive ovarian cancer cell line, was less affected by nisin and the IC₅₀ was obtained 22.9 µM. Our findings indicate that the HeLa cell line was the most sensitive to nisin. Therefore, we decide to proceed with HeLa cells in the following experiments.

Nisin induces apoptosis in HeLa cells

Annexin-V/PI staining was performed to evaluate the effect of nisin on the induction of apoptosis in HeLa cells. In flow cytometric analysis, Annexin-V/PI-negative cells indicate live cells; Annexin-V-positive and PI-negative cells refer to early apoptotic cells, Annexin-V-negative and PI-positive cells indicate primary necrotic cells and Annexin-V/PI-positive cells

show late apoptotic cells or secondary necrosis. As shown in Figure 2 (A and B), with enhancing nisin concentration both early apoptotic and late apoptotic or necrotic cells were increased in a concentration-dependent manner. A significant increase ($P < 0.001$) in early apoptosis and late apoptosis were observed after treatment with nisin (12 and 24 μM) for 16 and 24 h when compared to the control (Figure 2.C). Incubation of HeLa cells with nisin at a concentration near IC_{50} (12 μM) for 16 h led to $19.33 \pm 3.05\%$, $20 \pm 3.46\%$, and $3.39 \pm 0.43\%$ of early apoptosis, late apoptosis or secondary stage of necrosis and primary necrotic stage, respectively. In comparison, longer incubation time (24 h) with 12 μM of nisin showed $11.97 \pm 3.63\%$, $40.64 \pm 4.27\%$, and $6.20 \pm 0.35\%$ of early apoptotic cells, the late apoptotic or secondary necrotic cells and the primary necrotic cells, respectively.

Nisin increases Bax/Bcl-2 ratio in HeLa cells

Western blotting analysis was performed to evaluate the effect of nisin on the expression of a pro-apoptotic protein, Bax, and an anti-apoptotic protein, Bcl-2. As represented in Figure 3.A, the expression of Bax was increased while the expression of Bcl-2 was decreased after treatment with nisin in a concentration-dependent manner. Quantification of the intensity of bands by ImageJ densitometer showed (Figure 3.B) that following treatment of cells with 6, 12 and 24 μM of nisin, the Bax/Bcl-2 ratio was remarkably enhanced (3, 4.9, and 9.9 times, respectively) as compared with the control group (untreated cells).

Nisin decreases $\Delta\Psi_m$ levels in HeLa cells

The changes in $\Delta\Psi_m$ levels in HeLa cells treated with nisin were evaluated using JC-1 fluorescent dye which selectively internalized by mitochondria. When cells have an intact mitochondrial membrane and high $\Delta\Psi_m$ level, JC-1 polymerizes to J-aggregates and shows red fluorescence. In the case of mitochondrial depolarization, it remains as a JC-1 monomer and

produces green fluorescence. The red/green fluorescence intensity ratio was used to determine the level of $\Delta\Psi_m$ (Figure 4). Our finding showed that in comparison with untreated control cells, $\Delta\Psi_m$ levels decrease 44%, 70% and 82% after treatment with 6, 12 and 24 μM of nisin, respectively ($P<0.05$).

Nisin increases ROS levels in HeLa cells

To investigate the effect of nisin on induction of oxidative stress in HeLa cells, DCFDA fluorogenic dye was applied, which produces green fluorescence after oxidation. As shown in Figure 5, treatment with 6, 12 and 24 of nisin led to an increase in the intracellular ROS of about 1.36, 1.70, and 2.06 fold, respectively, compared to the untreated control ($P<0.05$).

Discussion

Chorionic vaginal dysbacteriosis and outgrowth of some groups of anaerobic bacteria releasing toxic metabolites (*e.g.*, nitrous acid) can promote gynecological malignancies including ovarian and cervical cancers (Sowjanya et al., 2016). Probiotic microorganisms and their metabolites including bacteriocins can recover the vaginal microbiome balance, reduce the HPV-induced cytological alterations and inhibit the proliferation of cervical cancer cells (Wang et al., 2018, Verhoeven et al., 2013). Nisin, an FDA-approved bacteriocin, has been shown to have anti-tumor and apoptotic effects on several cancer cell lines. Here, we found that nisin exhibits significant cytotoxic effects on ovarian and cervical cancer cell lines and induces apoptotic cell death mainly via mitochondrial membrane potential disruption and ROS generation.

Our results showed that nisin remarkably inhibited the growth of three cancer cell lines with IC_{50} values of 9-21 μM . Another research group reported that nisin showed the cytotoxic effect on A431 human skin carcinoma cell line with IC_{50} at 9.5 μM (Rana et al., 2019). In another investigation, nisin displayed significant cytotoxicity to the intestinal epithelial cells, HT29 and

216 Caco-2, with IC₅₀ values of 89.9 and 115.0 μ M, respectively, which is much higher than that of
217 the present study (Maher and McClean, 2006). Differences in the sensitivity of different cell
218 lines to nisin can be explained based on membrane variations. The main interactions between
219 nisin and cancer cell membrane components are electrostatic interactions. Based on our previous
220 study, the cytotoxic activity of nisin as a cationic peptide can be improved by decreasing the pH
221 (Avand et al., 2018). Furthermore, nisin is more soluble and stable at low pH which is
222 compatible with the pH of vaginal and cervical mucosa. Therefore, nisin might have better
223 activity in the genital tract. However, more *in vitro* and *in vivo* investigations are still required to
224 evaluate its efficacy and safety in gynecologic cancers.

225 Notably, nisin exhibited much less cytotoxicity against normal cells, HUVEC. Our findings are
226 consistent with previous works reporting the high selectivity of nisin for malignant HNSCC and
227 A375 cells without affecting normal keratinocytes (Lewies et al., 2018, Kamarajan et al., 2015).
228 The plasma membrane of tumor cells is composed of more negatively charged molecules (*e.g.*,
229 anionic phospholipids) and thereby can more interact electrostatically with cationic peptides such
230 as nisin (Ran et al., 2002). Furthermore, the surface of cancer cells contains many more
231 microvilli than that of normal cells which may provide more surface area for interaction with
232 nisin (Chan et al., 1998).

233 Annexin-V/PI flow cytometry analysis showed that nisin can induce significant apoptotic cell
234 death in the cervical cancer cell line, HeLa, in a concentration-dependent manner. A reduction in
235 $\Delta\Psi_m$ and enhanced levels of the Bax/Bcl-2 ratio further confirm that the cytotoxic effect of nisin
236 towards cervical cancer cells is mainly due to the activation of an apoptotic pathway.

237 BCL-2 family plays a critical role in the regulation of the mitochondrial apoptotic pathway. The
238 ratio of pro- to anti-apoptotic members of the BCL-2 family significantly influences the

susceptibility of cells to undergo apoptotic death (Hata et al., 2015). In agreement with our findings, it was reported that 30-60 μ M of nisin up-regulated Bax expression and down-regulated the expression of Bcl-2, which led to elevated Bax/Bcl-2 ratio both at mRNA and protein levels in SW480 colon cancer cells (Ahmadi et al., 2017).

Recent evidence indicates that nisin interaction can modify the tumor cell membrane properties such as fluidity and dipole potential resulting in the death of malignant cells (Kamarajan et al., 2015, Prince et al., 2019). Nisin contains a pore-forming domain which may lead to a reduction in $\Delta\Psi_m$. Similar to our results, another work reported that nisin can depolarize the mitochondrial membrane of melanoma cancer cells which negatively affects glycolysis metabolism and mitochondrion respiration (Lewies et al., 2018).

Intracellular ROS plays an important role in oxidative stress and can affect cellular metabolism, differentiation and survival (Zhang et al., 2016). Mitochondria are one of the main sources of cellular ROS generation. The excess levels of ROS can result in cell death via promoting cell signaling pathways triggering apoptosis. Cancer cells usually have lower levels of antioxidants than healthy cells, and thus are more susceptible to ROS-induced cell death (Zaidieh et al., 2019). In the present study, treatment of HeLa cells with nisin led to increased intracellular ROS levels, suggesting its role in apoptosis induction. Furthermore, ROS generation can indirectly induce apoptosis through more disruption of mitochondrial membrane and subsequently complete mitochondrial depolarization as reported in this work. Similarly, another study showed the involvement of mitochondrial dysfunction and ROS overproduction in the apoptosis induced by nisin in melanoma cells (Lewies et al., 2018).

Conclusion

261 In summary, here we have shown that nisin exhibits great cytotoxicity HeLa, OVCAR-3 and SK-
262 OV-3 cell lines and low toxic effect against normal endothelial cells. Nisin induces apoptosis in
263 cervical cancer cells through regulation of BCL-2 family proteins, depletion of mitochondrial
264 potential and ROS accumulation. Our findings provide preliminary evidence for the potential
265 application of nisin alone or in combination with other anticancer agents for the treatment of
266 cervical malignancies. However, further investigations are still required to confirm anti-tumor
267 effectiveness and safety of nisin in experimental *in vivo* cervical cancer models.

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Competing interests

The authors declare there are no competing interests.

Author contribution

V.A. is the principal investigator of the project; M.A. and H.S. planned for the project; H.S., M.A and V.A. performed the required experiments; V.A. wrote the manuscript in consultation with M.A. and H.S. All authors discussed the results and contributed to the final form of the manuscript.

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Figure 1. Cytotoxicity of nisin on the HUVEC, SK-OV-3, OVCAR-3 and HeLa cell lines. Cells were incubated different concentrations of nisin for 48 h and the cell viability was determined by MTT assay. (mean±SD, n=3)

Figure 2. Representative flow cytometric analysis for apoptosis in HeLa cells after treatments with nisin (3, 6, 12 and 24 µM) for 16 h (A) and 24 h (B). The percentages indicated in the lower left, the lower right, the upper right and the upper left quadrants refer to viable cells, early apoptotic cells, late apoptotic cells or secondary necrotic cells and primary necrotic, respectively. C) The results are shown as bar chart. (mean±SD, n=3)

Figure 3. A) Representative western blot analysis of Bax and Bcl-2 expression levels in HeLa cells after treatment with nisin (6, 12 and 24 µM). B) The graph showing the level of Bax and Bcl-2 compared with the untreated control group. Quantification of the intensity of bands was performed using ImageJ software and the protein level of Bax and Bcl-2 were normalized to the β-actin level. (mean±SD, n=3)

Figure 4. The effect of nisin on mitochondrial membrane potential ($\Delta\Psi_m$) of HeLa cells. Cells were treated with nisin (6, 12 and 24 µM) and then stained with JC-1 probe. The red/green fluorescence intensity ratio or $\Delta\Psi_m$ was measured by spectrofluorometry and normalized to untreated control cells (mean±SD, n=3). Asterisks indicate the means which were significantly different ($P < 0.05$) from the control.

Figure 5. The effect of nisin on intracellular ROS generation of HeLa cells. Cells were treated with nisin (6, 12 and 24 μ M) and then loaded with DCFDA. The fluorescence intensity or ROS was determined by spectrofluorometry and normalized to untreated control cells (mean $\pm$ SD, n=3). Asterisks indicate the means which were significantly different ($P < 0.05$) from the control.

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Figure 1. Cytotoxicity of nisin on the HUVEC, SK-OV-3, OVCAR-3 and HeLa cell lines. Cells were incubated different concentrations of nisin for 48 h and the cell viability was determined by MTT assay. (mean±SD, n=3)

140x98mm (150 x 150 DPI)

Figure 2. Representative flow cytometric analysis for apoptosis in HeLa cells after treatments with nisin (3, 6, 12 and 24 μ M) for 16 h (A) and 24 h (B). The percentages indicated in the lower left, the lower right, the upper right and the upper left quadrants refer to viable cells, early apoptotic cells, late apoptotic cells or secondary necrotic cells and primary necrotic, respectively. C) The results are shown as bar chart. (mean $\pm$ SD, n=3)

134x271mm (150 x 150 DPI)

Figure 3. A) Representative western blot analysis of Bax and Bcl-2 expression levels in HeLa cells after treatment with nisin (6, 12 and 24 μ M). B) The graph showing the level of Bax and Bcl-2 compared with the untreated control group. Quantification of the intensity of bands was performed using ImageJ software and the protein level of Bax and Bcl-2 were normalized to the β -actin level. (mean $\pm$ SD, n=3)

122x125mm (150 x 150 DPI)

Figure 4. The effect of nisin on mitochondrial membrane potential ($\Delta\Psi_m$) of HeLa cells. Cells were treated with nisin (6, 12 and 24 μ M) and then stained with JC-1 probe. The red/green fluorescence intensity ratio or $\Delta\Psi_m$ was measured by spectrofluorometry and normalized to untreated control cells (mean $\pm$ SD, n=3). Asterisks indicate the means which were significantly different ($P < 0.05$) from the control.

127x106mm (150 x 150 DPI)

Figure 5. The effect of nisin on intracellular ROS generation of HeLa cells. Cells were treated with nisin (6, 12 and 24 µM) and then loaded with DCFDA. The fluorescence intensity or ROS was determined by spectrofluorometry and normalized to untreated control cells (mean±SD, n=3). Asterisks indicate the means which were significantly different ($P < 0.05$) from the control.

127x106mm (150 x 150 DPI)