

Effect of Iron Superoxide and Nickel Oxide Nanoparticles Alone and Combined with Coenzyme Q10 on hsa_circ_0001518 Expression in Breast Tumor-bearing BALB/c Mice

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ABSTRACT

Background: Breast cancer is considered the second prevailed type of cancer among women. Circular RNAs are a group of non-coding RNAs that play a significant role in tumorigenesis and the progression of malignancies. This study aimed to determine the inhibitory concentration (IC₅₀) of iron superoxide (Fe₂O₃) and nickel oxide (NiO) nanoparticles and coenzyme Q10 (Q10) antioxidant against 4T1 cell line and their single and combination treatment effect on hsa_circ_0001518 expression in healthy and breast tumor mice.

Methods: The 4T1 cell line was cultivated and treated with Fe₂O₃ (50, 100, 150, and 200 µg/mL) and NiO (10, 20, 30, and 40 µg/mL) nanoparticles, and Q10 antioxidant (20, 60, 80, and 100 µg/mL) for 48 hours. Cell viability was measured using the MTT assay. The expression of hsa_circ_0001518 after treatment with the half-maximal inhibitory concentration (IC₅₀) of all single and combined antioxidant and NPs in healthy and breast tumor-bearing mice were evaluated by qRT-PCR.

Results: The IC₅₀ of Fe₂O₃ and NiO nanoparticles and Q10 antioxidant after 48h was 92.42 µg/mL, 21.49 µg/mL, and 83.47 µg/mL, respectively. The qRT-PCR results showed that combined treatment with antioxidant and NPs caused a more significant downregulation in the expression of has_circ1518 in breast tumor-bearing mice than either agent alone. In addition, combined treatment with antioxidant and NPs caused a more significant downregulation in the expression of has_circ1518 in breast tumor-bearing mice than either agent alone.

Conclusion: This study suggests that the combined treatment of Fe₂O₃ and NiO nanoparticles with Q10 could be exploited as a potential source for developing novel drugs against breast cancer.

Keywords: Breast Cancer; circRNA, Iron Superoxide Nanoparticles; Nickel Oxide Nanoparticles; Q10 antioxidant

INTRODUCTION:

Breast cancer is the most prevailed aggressive inhomogeneous malignancy and is considered one of the essential cancers regarding women's health worldwide (1). With the identification of 2.3 million new cases and 685000 deaths worldwide in 2020, this type of cancer is the second prevailed type of cancer after lung cancer and the fifth leading cause of cancer death among women (2). Breast cancer is sometimes diagnosed after symptoms appear, whereas, in many women, it develops with no symptoms (3,4). Despite many years of research and considerable progress made in treatment strategies, especially in surgical and chemotherapy methods, the development of new cancer treatment methods with the lowest damage imposed on natural tissues and improved patients' lives has turned into a challenging topic. In recent years, nanoparticle- (NP) based therapeutic strategies have attracted much attention as a carrier system for drug delivery, hyperthermia, and imaging purposes and have overcome limitations associated with conventional methods such as non-targeted effects, resistance to treatment, and late diagnosis. Metal oxide NPs such as nickel oxide (NiO), zinc oxide (ZnO), titanium dioxide (TiO₂), etc., as well as inorganic NPs such as iron peroxide (Fe₂O₃), have attracted considerable attention due to their anticancer activity (5). Many studies on the use of NPs in the treatment of breast cancer have investigated the effect of NPs on cellular mechanisms such as DNA degradation, production of ROS compounds, and apoptosis. However, the interaction of NPs and their effectiveness on molecular and regulatory mechanisms involved in cancer development and progression is still unknown. Growing evidence has shown that the occurrence and progress of breast cancer are adjusted in complex form by various types of ncRNAs, including long non-coding RNAs (lncRNA), micro RNAs (miRNA), and circular RNAs (circRNA) (6). Circular RNAs are a new class of single-stranded non-coding RNAs, covalently closed without the usual end structures of 5', caps, and 3' Poly(A) tails (7, 8). Recently, many studies have proved that the dysregulated expression of circRNAs is involved

in multiple pathological processes of breast cancer. For example, a study by Shi et al. to construct a general circRNA expression profile of breast cancer has shown an increase and decrease in 715 and 440 circRNA expression, respectively (9). Another study suggested the role of CDR1as (CiRS-7) and circ-FOXO3 as miRNA sponges in the development of breast cancer. Several studies showed that elevated expression of Circ-Amotl1 in breast cancer tissues and cell lines with function on the C-myc transcription factor is involved in cancer-associated biological processes and prognosis (10).

Hsa_circ_0001518 is encoded by the FBXL17 gene (F-Box and Leucine-Rich Repeat Protein 17) and located on chromosome 5. In this study, the circBase bioinformatics database (<http://www.circbase.org>) predicted that hsa_circ_0001518 was associated with breast cancer through affecting the Bcl-2 gene and modulating the activity of various miRNAs. In the present study, we aim to examine the effect of Fe₂O₃, NiO NPs, and Q10 antioxidant and their combination on the expression level of hsa_circ_0001518 in breast tumor-bearing BALB/c mice compared to healthy mice.

Material and methods**Animals**

This experimental study has been performed on 60 BALB/c female mice (6-7 weeks old) obtained from the Animal Breeding Stock Facility of the Pasteur Institute, Karaj, Iran. Animals were maintained under standard light-dark cycle at 23±2°C and relative humidity of 50±10% to become compatible with lab conditions. The animals received filtered water and food ad libitum. All experiments involving animals were approved by the Ethical Committee of Islamic Azad University, Tehran Medical Sciences branch (IR.IAU.TNB.REC.1399.024) and carried out following the relevant guidelines and regulations.

Experimental Design

The 4T1 breast cancer cell line was purchased from the National Cell Bank of Iran, Pasteur Institute of Iran, to determine IC₅₀ of Fe₂O₃, NiO NPs, and Q10 and create breast tumor-bearing models. The Fe₂O₃ and NiO NPs

(purity >95%, particle size: 50 nm) and Q10 antioxidant (product number: C9538) used in this study were purchased from Sigma-Aldrich (USA). Cells were cultivated in RPMI-1640 culture medium (Gibco-Invitrogen) containing 10% fetal bovine serum (FBS) (Gibco-Invitrogen) and 1% of penicillin and streptomycin antibiotics in a 37°C incubator containing 5% CO₂. The tumor implantation model was performed through subcutaneous injection of 1x10⁶ 4T1 cells resuspended in 0.1 ml PBS into the mammary fat pad (Fadpad#8) of mice in Shahid Beheshti Research Center (11). Then, healthy and tumor groups were divided into six subgroups (n = 10 per subgroup) and treated with IC₅₀ concentrations of NPs and antioxidants. Subgroup 1 received normal saline solution as a control. The experimental subgroups 2, 3, and 4 received Fe₂O₃, NiO NPs, and Q10, respectively. The experimental subgroup 5 received Fe₂O₃, NP, and Q10. Finally, subgroup 6 received NiO NP with Q10. All mice were anesthetized after the treatment at the end of 14 days, and tumors and breast tissues from healthy groups were dissected and subjected to subsequent analyses

MTT assay

Cellular toxicity of Fe₂O₃ and NiO NPs and Q10 antioxidant was determined against 4T1 cell line using MTT test. Briefly, 100 µl of culture medium containing similar numbers of cells (5x10⁴) was cultivated in each well of a 96-well plate. After 24 hours of incubation, the culture medium was replaced with 100 µl of fresh culture medium containing Fe₂O₃ NPs (50, 100, 150, and 200 µg/mL); NiO NPs (10, 20, 30, and 40 µg/mL); and Q10 antioxidant (20, 60, 80, and 100 µg/mL) and was further incubated for 48h at 37°C with 5% CO₂. Then, 20 µl of MTT solution (5mg/ml) was added to each well, and plates were incubated continuously for another 4 hours. Formazan crystals were solved upon adding 100 µl of DMSO. After 15 minutes of incubation at room temperature, the optical densities (ODs) were read using a microplate reader (BioTek-ELx800, USA) at a wavelength of 570 nm. The percentage of cell viability was expressed as $A_{\text{treatment}}/A_{\text{control}} \times 100\%$ (where, A = absorbance). The untreated cells incubated in culture medium alone served as a control. The mean of three absorbance values was calculated for

each concentration. The IC₅₀ values (µg/mL) were determined using Graphpad Prism 6 software (GraphPad Software, La Jolla, CA, USA). All experiments were carried out in triplicates. In this study, mice did not show any mortality after injection with determined IC₅₀ concentrations of all compounds till the end of the observation period of 14 days. Therefore, the lethal dose was not evaluated due to ethical standards (12).

RNA Extraction and cDNA Synthesis

All healthy and tumor-bearing mice were anesthetized intraperitoneally with a ketamine-xylazine (KX) after the treatment at the end of 14 days. Tumors and breast tissues from healthy groups were dissected under sterile conditions, homogenized in ice-cold PBS, and centrifuged at 12,000×g for 15 min at 4°C. Then, the obtained pellet was subjected to RNA extraction using RiboEX kit (GeneAll Biotechnology, Seoul, South Korea) according to the manufacturer's instruction and the amount quantified using a Nanodrop 2000 spectrophotometer (Thermo scientific). Total RNA was reverse transcribed into cDNA using BioFACT cDNA Synthesis kit (Daejeon, South Korea) under the following conditions: 25°C for 10 min, 42°C for 60 min, and 70°C for 10 min.

Analysis of hsa_circ_0001518 expression by Real-Time PCR

The expression level of hsa_circ_0001518 in healthy and breast tumor groups treated with IC₅₀ concentrations of Fe₂O₃ and NiO NPs and Q10 antioxidant and their combination was evaluated using q-RT PCR. qRT-PCR analysis was conducted in triplicate using SYBR Green Master Mix (TAKARA, Japan) on a LightCycler TM 96 (Roche) in 20 µL reactions containing 1×SYBR Green PCR Master Mix, 0.5 µM of each forward and reverse primer and 1.5 µL cDNA template. ACTB (β-actin) was used to normalize the expression of hsa_circ_0001518. The expression levels were measured using the 2^{-ΔΔCt} method (13). All primers were designed using Primer3plus software, and the sequences are listed in Table 1.

Statistical Analysis

Statistical analysis was performed using IBM SPSS statistics (version 21; Chicago, IL) and GraphPad Prism (version 6.0; GraphPad Software, La Jolla, CA, USA). The ex-

Target transcript	Primer type	Sequence (5'→3')
ACTB	Forward	GATCAAGATCATTGCTCCTCTCTG
	Reverse	CTAGAAGCATTTCGGTGGAC
Circ-0001518	Forward	GGCAGAACAGGAAGTTGGTC
	Reverse	GACAGAGAATGGGGCAGAAA

Table 1. Primer sequences used for RT-qPCR

pression data were controlled for normal distribution by the One-Sample Kolmogorov-Smirnov test (K-S test). A one-way ANOVA was used to determine statistical differences in hsa_circ_0001518 expression levels. The significance of all the statistical tests was determined at $p < 0.05$.

RESULTS:

Cellular toxicity via MTT test

The in vitro cytotoxicity effects of Fe₂O₃ and NiO NPs and CoQ10 antioxidant were evaluated against 4T1 breast cancer cell line by MTT assay to adjust the optimal doses (IC50) administered to animals in the next step (Figure 1). As shown in Figure 1 A-C, the IC50 values for Fe₂O₃, NiO NPs, and Q10 were 92.42 µg/mL, 21.49 µg/mL, and 83.47 µg/mL, respectively. The IC50 of NiO NPs was lower than other tested compounds.

hsa_circ_0001518 expression

qRT-PCR analysis was carried out to detect the possible alterations in the expression level of hsa_circ_0001518 in healthy and tumor-bearing mice treated with IC50 concentrations of antioxidant, NPs, and their combination. As shown in Figure 2, the expression level of hsa_circ_0001518 in healthy and tumor-bearing mice after treatment with IC50 concentration of Fe₂O₃ compared to control (healthy and tumor groups without treatment) up-and down-regulated, respectively. In contrast, compared to untreated control, treatment with IC50 concentration of NiO NPs has increased hsa_circ_0001518 expression in both healthy and tumor-bearing mice. According to our results, hsa_circ_0001518 expression was significantly down-regulated with single treatment with Fe₂O₃ and NiO NPs in tumor-bearing mice compared to healthy mice ($P < 0.001$). In addition, hsa_circ_0001518

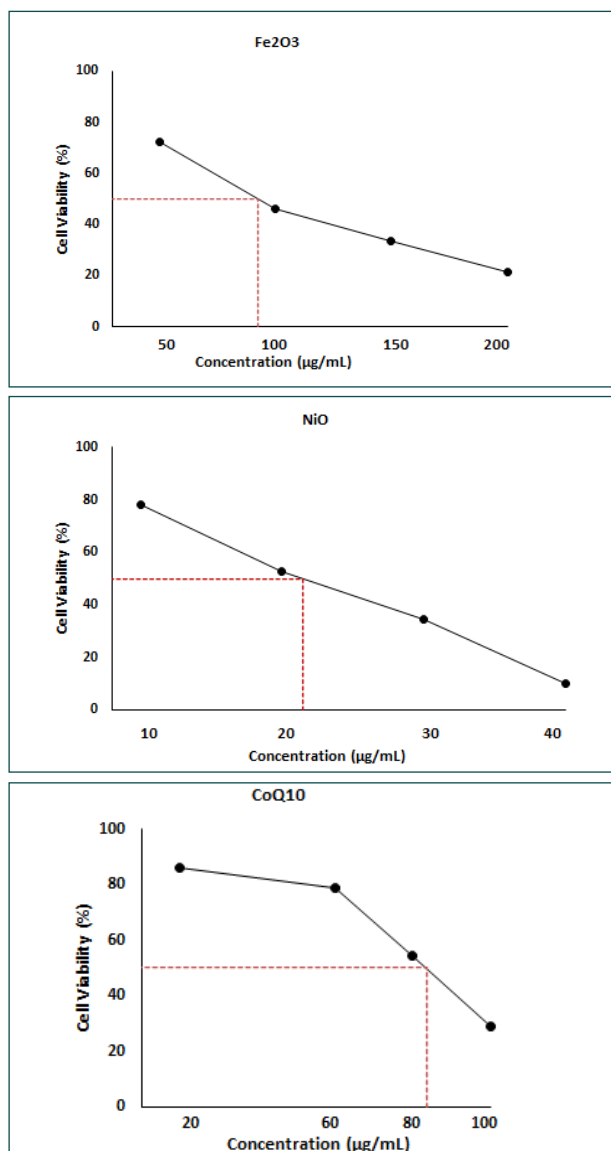


Figure 1. Cytotoxicity of Fe₂O₃ (A), NiO (B), and Q10 (C) after 48h on 4T1 cell line by MTT assay. Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 4T1, mouse mammary carcinoma cell line.

expression was down-regulated following Q10 treatment in healthy mice and was up-regulated in tumor mice. As a result, hsa_circ_0001518 expression levels were up-and down-regulated in healthy and tumor-bearing mice relative to untreated control following combined treatment of Fe₂O₃ NPs and Q10 (P<0.001). In healthy mice, the relative expression levels of hsa_circ_0001518 were elevated to 2.7 (P<0.001) and 18.5 (P<0.001) fold after combination treatment of Fe₂O₃ NPs with Q10, respectively, compared with each agent alone. In contrast, the relative expression levels of hsa_circ_0001518 were downregulated following combined treatment with Fe₂O₃ NPs and Q10 in tumor mice, which was 0.5 and 4.3 folds that of the Fe₂O₃- and Q10-alone groups, respectively. The relative expression levels of hsa_circ_0001518 were down-regulated in both healthy and tumor-bearing mice following combined treatment of NiO NPs and Q10, which further decreased in tumor mice compared to healthy ones (P<0.001). In the present study, the expression level of hsa_circ_0001518 was significantly down-regulated following combined treatment of nickel oxide and Q10, which was 5.2 and 17 fold of the NiO-alone group, and 3.5 and 7.5-fold of the Q10-alone in healthy and tumor mice, respectively (P<0.001). We observed that combined treatment of Fe₂O₃ and Q10 in healthy mice and single treatment of NiO NPs in tumor mice resulted in a further elevated expression level of hsa_circ_0001518 than other treatments.

As shown in Figure 3, hsa_circ_0001518 expression levels significantly increased in tumor-bearing groups treated with Q10, NPs, and their combination compared to untreated control mice. Interestingly among all tested compounds in healthy groups, only combined treatment of NiO and Q10 led to a decrease in the expression level of hsa_circ_0001518 compared to untreated healthy control mice.

DISCUSSION

Today, nanoparticle technology is a promising and widely used strategy for effectively diagnosing and treating many cancers (14). According to reports, metal oxide nanoparticles such as NiO, ZnO, titanium dioxide (TiO₂),

iron oxide nanoparticles, etc., have shown considerable anticancer activity on breast cancer cell lines (15). Rezaei et al. showed that cytotoxic effects of Dextran-coated iron oxide NPs increased against MCF-7 in a time and dose-dependent manner (16). Another study by Perumal Raj et al. synthesized NiO NPs induced significant cytotoxicity against the MCF-7 cell line (17). CoQ10 is used as an endogenous antioxidant, which shows the level of oxidative stress in cells. Due to its antioxidant properties, it can suppress oxidative stress within the cell by accepting electrons and is widely used as a dietary supplement (18). Studies have shown that low plasma Q10 levels may be an independent prognostic factor for breast cancer progression. However, in other conflicting findings, serum Q10 levels were significantly higher in newly-diagnosed breast cancer patients than in the healthy control group, suggesting that Q10 may also be involved in the growth of breast cancer cells. In addition, the antioxidant properties of Q10 protect cancer cells against the toxicity of free radicals produced by radiation therapy resulted in reducing the effect of radiation therapy. Several studies using CoQ10 as adjuvant therapy in human cases have shown that CoQ10 has a desirable impact on achieving regression of tumor masses and remission of distant metastasis of breast cancer (19, 20).

Extensive studies reported the potential of iron oxide nanoparticles (IONs) and their derivatives to induce apoptosis through multiple mechanisms, including a reduction in the expression levels of matrix metalloproteinase-2 (MMP-2) in MCF7 cell line (21) and anti-apoptotic protein Bcl-2 in MDA-MB-231 breast cancer cells (22,23), increased expression of apoptosis-associated proteins such as cytochrome-c, caspase-3, PARP, p53, and Bax in MDA-MB-231 (23). It is broadly recognized that circRNAs play a vital role in inducing apoptosis and preventing metastasis by regulating cancer-related target genes and signaling pathways. Accordingly, multiple studies are carried out on circRNAs to be considered a new diagnostic/prognostic marker and therapeutic target for breast cancer. For example, hsa_circ_006054, hsa_circ_100219, hsa_circ_0001982, hsa_circ_0005230, and hsa_circ_406697 were found highly expressed

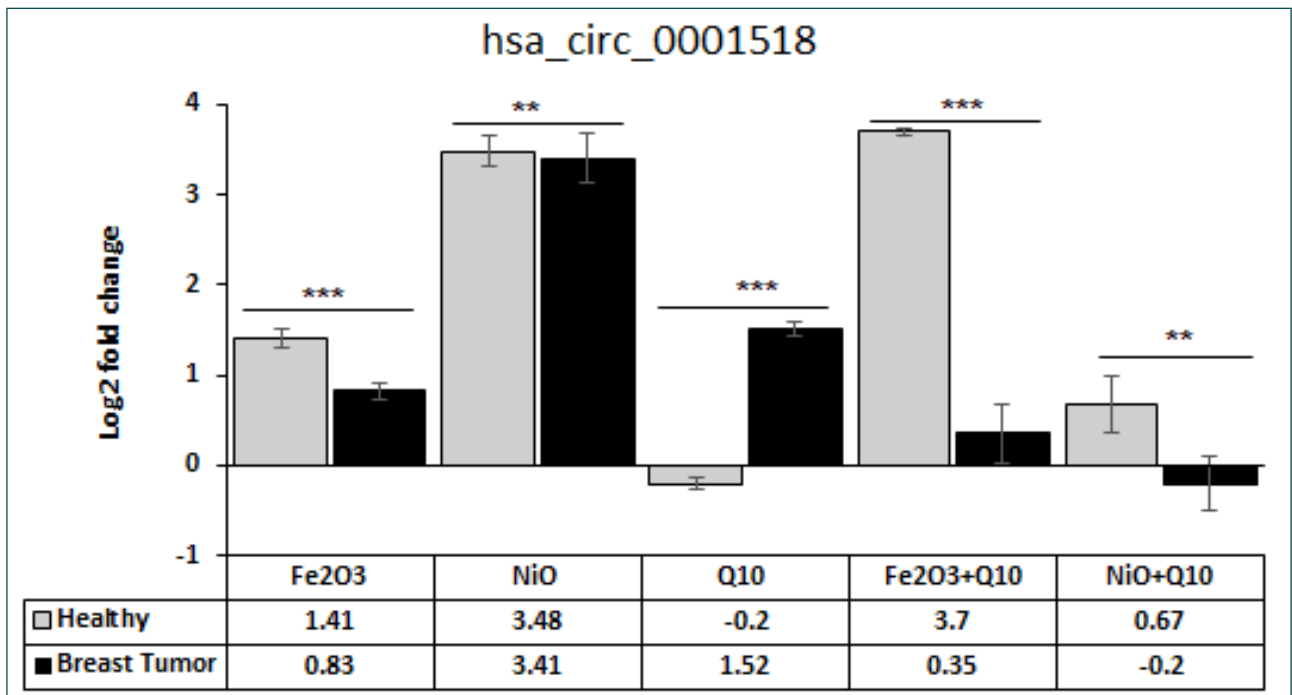


Figure.2. The expression level of hsa_circ_0001518 in healthy and tumor-bearing mice following alone and combined treatment with IC50 concentration of Fe₂O₃, NiO NPs, and CoQ10 antioxidant. Data are presented as means ± SD. (**P>0/001, **P>0/01, and * P>0/05)

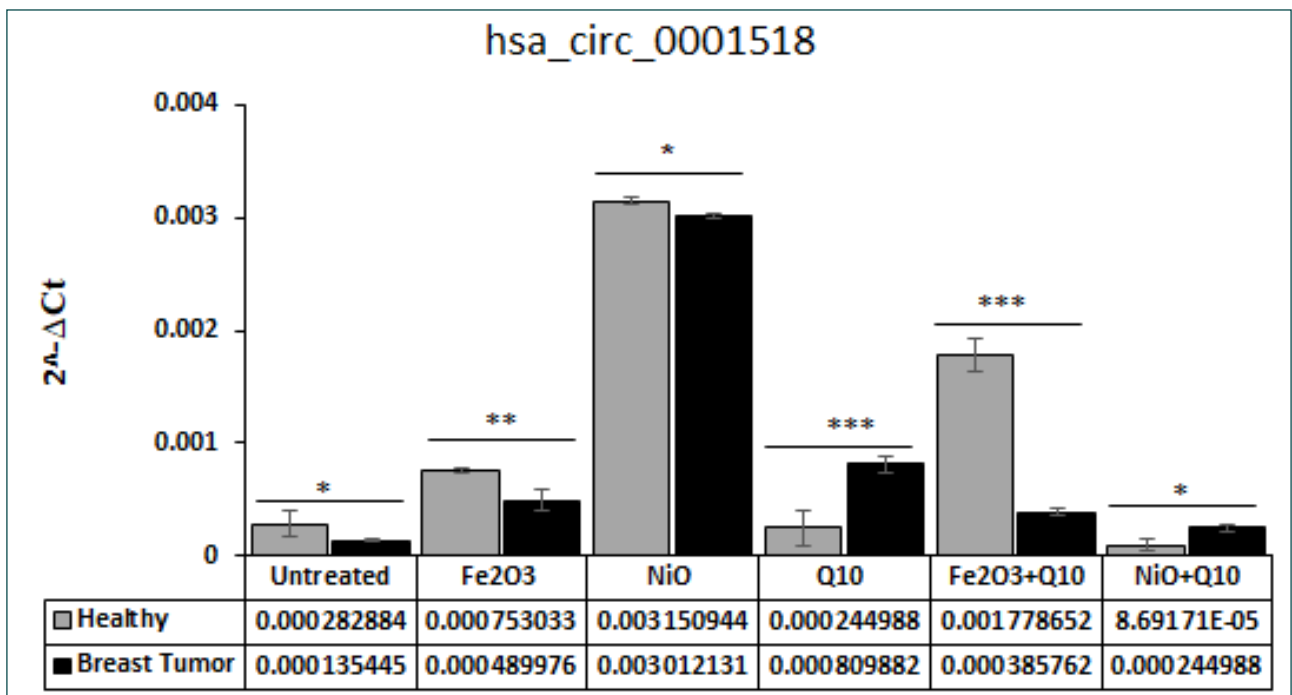


Figure.3. The expression level of hsa_circ_0001518 in healthy and tumor-bearing mice before and after alone and combined treatment with IC50 concentration of Fe₂O₃, NiO NPs, and CoQ10 antioxidant. Data are presented as means ± SD. (**P>0/001, **P>0/01, and * P>0/05)

in breast cancer cells; while, hsa_circ_0011946 was down-regulated (24-26). As revealed by Zhang et al., circRNA_Foxo3 inhibits MDM2 and subsequently induces cellular apoptosis by increasing p53 expression (27). Wang et al. reported that hsa_circ_000911 could sponge miR-449a, which targets the notch1 gene and inhibits cell proliferation, migration, and invasion in breast cancer (28). According to another study, CircRNA_BARD1 (circ_0001098), as a mediator between proapoptotic stress and p53-dependent apoptosis, could increase cell viability by increasing cell apoptosis in breast cancer cells (29).

In the present study, we first quantified IC50 for Fe₂O₃ and NiO NPs, and Q10 antioxidant from the cell viability data obtained using the MTT assay. Our results showed a dose-dependent effect on 4T1 cells where cell viability progressively reduced at higher concentrations of NPs and antioxidants. Our study on the effect of Fe₂O₃ and NiO NPs and Q10 antioxidant on 4T1 cells showed that Fe₂O₃ NPs induced cytotoxicity at a higher concentration (92.42 µg/mL) and Q10 and NiO at lesser concentrations (21.49 µg/mL and 83.47 µg/mL). Consistent with the Pandey et al. study that Fe₂O₃ NPs exhibited lower cytotoxic activity than NiO NPs on lung cancer cell line A549; in our study, the cytotoxic potential of NiO was higher than Fe₂O₃ NPs. To our knowledge, for the first time, we evaluated alterations in the expression level of hsa_circ_0001518 associated with breast cancer predicted through the CircBase algorithm in healthy and tumor-bearing mice following the IC50 treatment of NPs and Q10.

In contrast to NPs, the expression of hsa_circ_0001518 was markedly down-regulated in tumor-bearing mice treated with single-Q10 compared to healthy ones. While combination treatment of Q10 with both NPs resulted in an elevated expression in treated tumor-bearing mice which may be due to the synergic effect of concomitant treatment of NPs and Q10. In this study, combined treatment of NiO NPs and Q10 showed the highest alteration in the expression of hsa_circ_0001518 among all tested compounds, in a way that its expression markedly decreased in tumor-bearing mice.

CONCLUSION

In conclusion, our results suggest that Fe₂O₃, NiO NPs, and CoQ10, especially the combined treatment of NiO NPs and Q10, induce hsa_circ_0001518 expression changes in breast tumor-bearing mice. Thus, they can be considered a promising candidate in breast cancer treatment. However, further studies are needed to elucidate the mechanisms underlying the cytotoxicity of tested compounds.

DISCLOSURE

The authors report no conflicts of interest.

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