Research Article

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The effects of silibinin on oxidative stress and microRNA-10b expression in animal models of breast cancer

[Meme Kanseri Hayvan Modellerinde Silibininin Oksidatif Stres ve MicroRNA-10b Ekspresyonu Üzerine Etkileri]

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Abstract

Background and aims: Among the factors associated with cancer are the oxidative stress and increased expression of some microRNA (miRs). Silibinin has an anti-tumor effect. Therefore, this study evaluates the effects of silibinin on oxidative stress indices and miR-10b expression in the animal models of breast cancer.
Material and methods: In this study, 48 Balb/c mice were divided into six groups (each group contains eight mice): the healthy control, the cancer control, the healthy group receiving 20 mg of silibinin, the cancer group receiving 20 mg of silibinin, the cancer group receiving 40 mg of silibinin and the cancer group receiving 80 mg of silibinin for three weeks. In order to induce cancer, 4T1 cell line was used. After obtaining breast tumor samples, the levels of Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPX) and miR-10b expression in breast tumor biopsy were evaluated. Data were analyzed using one-way ANOVA, Kruskal–Wallis, Mann–Whitney and t-test ($p<0.05$).
Results: The use of silibinin at different doses increased the activity of SOD and GPX (significantly) and the level of TAC (significantly) in the treatment group compared to untreated cancerous mice, but mir-10b and MDA were decreased non-significant and significantly respectively.
Conclusion: Silibinin led to a non-significant reduction of miR-10b in the treatment group compared to untreated cancerous mice. Silibinin has been shown to improve oxidative stress in breast cancer mice.

Keywords: breast cancer; miR-10b; oxidative stress indices; silibinin.

Öz.

Giriş ve Amaç: Oksidatif stres ve bazı artmış microRNA (miR'lerin) ekspresyonu kanserle ilişkili faktörler arasındadır. Silibininin bir anti-tümör etkisi vardır. Bu nedenle bu çalışmanın amacı, meme kanserinin hayvan modellerinde silibininin oksidatif stres indeksleri ve miR-10b ekspresyonu üzerindeki etkilerini değerlendirmektedir.
Gereç ve Yöntemler: Bu çalışmada 48 Balb / c fare 6 gruba ayrıldı (her grup 8 fare içerir): sağlıklı kontrol, kanser kontrolü, 20 mg silibinin alan sağlıklı grup, 20 mg alan kanser grubu 40 mg silibinin alan kanser grubu ve 3 hafta boyunca 80 mg silibinin alan kanser grubu. Kanseri indüklemek için 4T1 hücre hattı kullanıldı. Meme tümör örnekleri elde edildikten sonra, göğüs tümör biyopsisinde Malondialdehid

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(MDA), süperoksit dismutaz (SOD), glutatyon peroksidaz (GPX) ve miR-10b ekspresyon seviyeleri değerlendirildi. Veriler tek yönlü ANOVA, Kruskal–Wallis, Mann–Whitney ve T testi kullanılarak analiz edildi ($p<0.05$).

Bulgular: Farklı dozlarda silibinin kullanımı, tedavi edilmemiş kanserli farelere kıyasla tedavi grubunda SOD ve GPX aktivitesinde artışa neden oldu, ancak TAC'yi önemli ölçüde artırdı ve MDA'yı azalttı.

Sonuç: Silibinin tedavi edilmemiş kanserli farelere kıyasla tedavi grubunda önemli olmayan miR-10b azalmasına yol açmıştır. Silibininin meme kanseri farelerinde oksidatif stresi azalttığı gösterilmiştir.

Anahtar kelimeler: meme kanseri; miR-10b; Oksidatif stres indeksleri; Silibinin.

Introduction

The prevalence of breast cancer in most countries increases one to two percent annually, and an additional one million new cases are added each year [1]. According to studies, the association of oxidative stress with cancer has been confirmed [2]. In other words, oxidative stress is created as a result of disturbance between the production of free radicals (active oxygen and nitrogen species) and the antioxidant defense system [3]. In aerobic biological systems, superoxide dismutase enzymes (SOD), glutathione peroxidase (GPX), catalase and vitamins are important in controlling free radicals [4]. Superoxide dismutase exists in forms of Zn/Cu-SOD and Mn-SOD in cytoplasm, lysozymes and mitochondria, and it catalyzes the dismutation of anion superoxide into H_2O_2 [5]. Moreover, Glutathione peroxidase via glutathione reduces hydrogen peroxide and lipid hydroperoxides into the water and related alcohols. In this process, glutathione is oxidized to glutathione disulfide, which finally turns into a reduced form by glutathione reductase enzyme [6]. The result of lipid peroxidation followed by oxidative stress is metabolites such as malondialdehyde (MDA) which is considered as an index of lipid peroxidation and a major oxidative stress biomarker, and it increases in a variety of cancers, such as breast cancer [7]. In the process of apoptosis and cancer, other molecules are involved among which miRNAs can be mentioned. It seems that up to 50% of all genes are regulated by miRNAs [8]. MicroRNA-10b (miR-10b) extensively increases in metastatic breast cancer cells and stimulates migration and invasion [9]. This molecule has implication in cancer progression, particularly metastatic progression of breast cancer and beside another miRNA such as miR-21, miR-155, and Let-7a is the potential biomarker for the monitoring of breast cancer patients [10].

Lots of studies show that silibinin has an anti-tumor effect on breast cancer, and its toxic effect has been supported in several breast cancer cell lines, and it inhibits their growth [11]. Silibinin antioxidant is a natural polyphenol and a flavonoid which is widely used as a nutritional supplement and forms a major biological component of the active protein derived from *Silibum marianum* (milk thistle) [12]. Therefore, this study investigated the effect of silibinin on oxidative stress indices and mir-10b expression in the animal models of breast cancer.

Material and methods

This study was an experimental one with 48 Balb/c mice (6–7 weeks old) with a weight of 19–24 g. The 4T1 metastatic breast cancer cell line was injected subcutaneously adjacent to the lower left mammary tumor after purchasing and culturing the tissue on 1st day of the experiment, and the treatment was started after two weeks of the tumor appearance [13]. The sample volume was calculated using $n=2+C(s/d)^2$ formula [14] and previous study [15]. Animals were divided into six groups of eight mice: the first group was healthy and intact mice without disease, the second group was mice with breast tumors without treatment that received distilled water, the third group included healthy mice receiving 20 mg/kg of silibinin, the fourth group was cancerous mice receiving 20 mg/kg silibinin, the fifth group was cancerous mice receiving 40 mg/kg silibinin, and the sixth group was cancerous mice which received 80 mg/kg of silibinin. It has been shown that a dose of 120 mg of silymarin whose main component is silibinin is lethal, so the lower doses of silibinin were used [16]. The treatments were administered intraperitoneally and the control group was administered with a normal diet. Recipient groups of silibinin, from the 14th day of cell line injection (the time for tumor appearance) received silibinin for 3 weeks. Intraperitoneal injection is the most common and crucial method for injecting medications in rodents because the peritoneum has plenty of veins in besides having a large size; therefore, solutions that are injected in relatively high volumes enter the general blood circulation shortly after injection [17]. On the other hand, since the solubility of silibinin in the lipids is low, its transport from the membrane of the intestinal tract is limited and, as a result, it has less intestinal absorption; consequently, it was intraperitoneally injected [18]. In the 15th day, 100 mg of tumor tissue was removed to evaluate oxidative stress indices and mir-10b expression.

Cell culture

4T1 cell line was obtained from Pasteur Institute of Iran and cultured in RPMI including 10% fetal bovine serum, 1% penicillin and streptomycin antibiotics at a 4.5 g/L of glucose and oxygen concentration. The CO_2 level of cell culture incubator was set at 5% and its temperature was set at 37 °C. The cell culture flasks were examined microscopically to reach about 80–90% confluency, and no bacterial or fungal infections were observed. The previous cell culture of each flask was discarded and after washing twice with PBS, the trypsin/EDTA solution was added and the flasks were transferred to the incubator at 37 and 25 °C for about 3–5 min. After examining with inverted microscope, we added 2 mL of FBS containing medium to each flask in order to inactivate trypsin. Five-hundred

micro liter of this solution was taken to count the cells and the rest of the solution was transferred to 25 cm square flasks and transferred to the incubator after microscopic examination as before [18]. A cell suspension with a density of 10 million per mL was prepared in PBS buffer in order to be injected. Then, one million cells were injected adjacent to the lower left mammary gland of each Balb/c female mouse. At the end of the 14th day, blood sampling from the heart of the mice were performed, the mice were sacrificed, and the tumor tissue was removed and stored in a nitrogen tanks at a temperature of −70 °C. One-hundred milligram of tumor tissue was placed in a homogenizer containing lysate solution to completely lysis the tissue. Then, the suspension was collected and, after centrifugation, supernatant was used to evaluate the activity of the SOD, GPX, MDA and TAC enzymes.

Tumor volume calculations

The volume of the tumor was calculated using the following equation: where $V=(L \times W \times W)/2$, V=the volume, L=the length and W=the width of the tumor [19].

Assessing the antioxidant indices

Total antioxidant capacity assessment using FRAP method (ferric reducing ability of plasma)

This method relies on the ability of the compound to reduce ferric ions in the presence of TPTZ (Tripyridyl-S-Triazine). The Fe^{2+}-TPTZ complex is a purple colored one whose maximum absorption is at 519 nm. The reduction capacity of the compound was measured by the spectrophotometer at 593 wavelengths by increasing the concentration of the complex, which was accompanied by an increase in the absorbance of the solution [20].

Assessing the superoxide dismutase activity

The activity of the superoxide dismutase enzyme was assessed using the kit (MANUAL/RX MONZA-RANSOD-SD 125, RANDOX Laboratories Ltd. Co. Antrim, UK). In this method, xanthine and xanthine oxidase were used to produce superoxide radicals reacted with phenyltiazolium chloride and formed a red formazan complex, which is measurable by absorbance readings at 505 nm. Superoxide dismutase enzyme inhibits the formation of formazan by converting superoxide radicals into H_2O_2 and molecular oxygen [21].

Assessing the GPX activity

The GPX enzyme activity was measured using GPX MANUAL/Ransel kit, RANDOX Laboratories Ltd. Co. Antrim UK. The GPX catalyzes the reaction of glutathione oxidation (GSH) by cumene hydroperoxide (Cumene Hyroperoxide). In the presence of glutathione reductase NADPH, the oxidized glutathione (GSSG) is converted again to glutathione reduction, which is accompanied by the simultaneous oxidation of the NADPH to $NADP^+$ In this reaction, a decrease in light absorption was measured at 340 nm [22].

Measurement of MDA

The level of lipid peroxidation was assessed with thiobarbituric acid (TBA) method. Oxidative stress increases the lipid peroxidation of unsaturated fatty acids and, by free radicals' attacking to lipids, various aldehydes, such as MDA, are produced which react with aqueous TBA at acidic pH and high temperature. Lipid peroxidation was evaluated by increasing the concentration of MDA with spectrophotometer at 523 nm wavelengths [23].

miR-10b expression assessment

microRNA extraction

Extraction of total RNA containing micro RNA was performed by the miRCURY™ RNA Isolation-Biofluids-Exiqon kit according to manufacturer's instructions. After extracting the RNA, its concentration should be measured, and the required RNA at the cDNA synthesis step was determined for uniformity. To make sure about the quantity of extracted RNA, its concentration was assessed by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The extracted RNA quality was determined using 1.5% agarose gel electrophoresis. The ratio of OD 260–280 nm extracted RNA was considered as the RNA purity index. To monitor the expression of miRNA precursors, the (Exiqon, Denmark) cDNA kit and the Real Time PCR method were employed. This method is based on reverse transcription followed by real-time PCR and LNA™ enhanced primers. Reverse transcription was fulfilled in a 20 µL reaction including 2 µL total RNA, 1 µL of 10 µM RT primer, 8 µL of 100 mM dNTPs, 2 µL 10X PrimeScript buffer and 200 units of PrimeScript RTase. Due to the small size of miRNAs, it is hard to detect these small pieces by common Q-PCR protocols. To prolong the short chain of miRNAs, all miRNAs were initially added a poly-A tail on its 3′ side by *E. coli* poly A polymerase (PAP). The kit provides all the materials needed for the polyadenylation reaction. The sequence of primers is presented in Table 1 which was in

conformity with primers sequences in reference [24] and were made by Sina-Ghan Co., Iran. All experiments were repeated twice, and the temperature condition included: 95 °C for 15 s, 60–55 °C for 60 s, and 72 °C for 30 s. The relative expression of these genes was performed using $2^{-\Delta\Delta CT}$ method. The miR-6U gene was used as an internal control.

Statistical data analysis

The effect of silibinin on MDA and TAC levels, GPX and SOD activity, tumor volume and miR-10b expression in 21st day were analyzed using SPSS software version 18. Dara were compared using one-way ANOVA (to compare the means of the groups), and the t-test method (to compare the mean between the two groups when data has normal distribution). For miR-10b gene expressions, CTs were first calculated for each gene by $2^{-\Delta\Delta CT}$ formula. Because the investigated groups were independent, the normal distribution of the results of the groups was assessed by Kruskal–Wallis test and results between two groups were assessed by Mann–Whitney test to compare median between two groups (because the data were not normally distributed). The significant level was at $p<0.05$.

Results

The results of antioxidants indices in the related groups

As Figure 1 and Table 2 show, there is a significant difference between cancer and healthy groups in regard to MDA levels (p=0.00) and the use of silibinin (40 mg dose) caused a significant reduction of MDA in breast tumor tissue compared with untreated cancerous mice, but this effect was not significant with the other two doses.

As shown in Figure 1 and Table 2, there is a significant difference between cancer and healthy groups in regard to TAC levels (p=0.03) and the use of silibinin increased the TAC in the tumor tissue compared with untreated cancerous mice, and this effect was significant for all doses except for 20 mg. The most significant effect was observed in the concentration of 80 mg silibinin.

Table 1: Primer sequence of used genes.

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
miR-10b	CCCUGUAACCGAAUUUGUGUAA	CCAGTGAGCAGAGTGACG
U6 RNA	CTCGCTTCGGCAGCACA	CCAGTGAGCAGAGTGACG

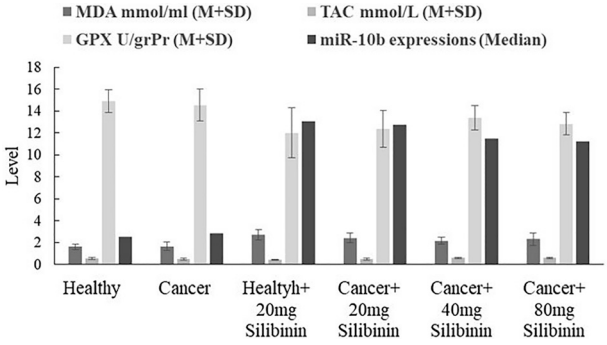

Figure 1: Comparison of TAC, MDA, GPX and miR-10b levels in the groups in 21st day (p between groups were 0.009, 0.001, 0.01 and 0.01 respectively). Use of 40 and 80 mg silibinin increased significantly the TAC in the tumor tissue compared with untreated cancerous mice (both of the p were 0.00). As Figure shows, only the use 40 mg dose of silibinin resulted in a significant reduction of MDA in breast tumor tissue compared with untreated cancerous mice (p=0.04). The use of all dosage of silibinin increased non-significantly the GPX activity and miR-10b expression of the tumor tissue compared with the cancerous untreated group.

Figure 1 and Table 2 show there is a significant difference between cancer and healthy groups in regard to GPX activity (p=0.01) and GPX activity was decreased significantly in the cancer group compared with the healthy control group and the use of silibinin increased the GPX activity of the tumor tissue compared with untreated mice, but effect was not significant in all doses.

Figure 2 and Table 2 show there is a non-significant difference between cancer and healthy groups in regard to SOD activity (p=0.15) and reveal that SOD activity was decreased in the cancer group in compared with the healthy control group and the use of silibinin increased the activity of SOD non-significantly in the breast tumor tissue compared with untreated cancerous mice.

The results for tumor volume measurements

Table 2 and Figure 2 show the results for tumor volume measured in the related groups. As the table and figure show, all dosages of silibinin decreased tumor volume in 21st day that this effect was maximum and significant in cases of 40 and 80 mg silibinin. Eighty milligram dose has a greater inhibitory effect on tumor growth.

Comparison of miR-10b gene expressions in the groups

Figure 1 and Table 2 show there is a significant difference between cancer and healthy groups in regard to miR-10b

Table 2: Results for tumor volume, stress oxidative indice and miR-10b expression measurement in the related groups.

Groups	MDA $_{mmoL/mL}$ (M + SD)	TAC $_{mmoL/L}$ (M + SD)	GPX $_{U/grPr}$ (M + SD)	SOD $_{U/grPr}$ (M + SD)	miR-10b expressions (median)	Tumor volume (mm^3)
Healthy	1.61 ± 0.26	0.54 ± 0.10	14.89 ± 1.05	1208.33 ± 127.65	2.52	
Cancer	1.65 ± 0.38	0.49 ± 0.08	14.56 ± 1.45	1236.67 ± 128.32	2.87	137.50 ± 24.85
Healthy + 20 mg silibinin	2.71 ± 0.49	0.42 ± 0.05	12.02 ± 2.26	1071.67 ± 111.43	13.09	
Cancer + 20 mg silibinin	2.42 ± 0.45	0.49 ± 0.08	12.37 ± 1.71	1140.0 ± 133.56	12.77	108.33 ± 14.37
Cancer + 40 mg silibinin	2.17 ± 0.32	0.59 ± 0.07	13.41 ± 1.11	1211.67 ± 105.53	11.45	92.50 ± 14.05
Cancer + 80 mg silibinin	2.30 ± 0.56	0.59 ± 0.06	12.84 ± 1.01	1140.00 ± 136.23	11.24	83.83 ± 10.77

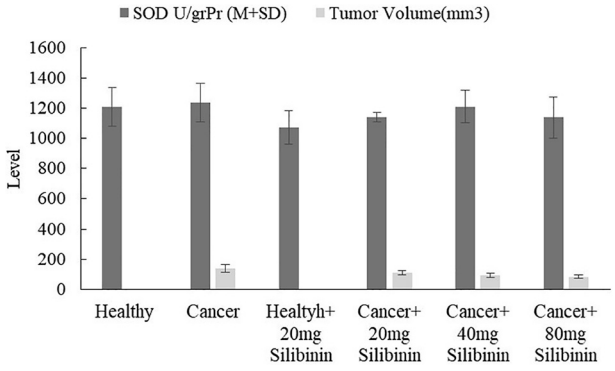

Figure 2: Comparison of SOD activity and tumor volume in the groups 21st day (p between groups was 0.22 and 0.00 respectively). SOD activity was decreased non-significantly in the all treatment groups in compared with the untreated cancerous mice and 40 and 80 mg silibinin dosages decreased tumor volume significiantaly in 21st day (p=0.009 and 0.004 respectively).

expression (p=0.00) and reveal that miR-10b expression was increased in the cancer group compared with the healthy control group and silibinin reduced the miR-10b expression non-significantly in breast tumor tissue compared with untreated cancerous mice.

Discussion and conclusion

Silibinin has a considerable antioxidant, anti-cancer and anti-inflammatory effects, and it is widely used because it is safe and does not have any side effects [25]. By collecting free radicals, particularly through dual bonds in its structure, silibinin increases the activity of antioxidant enzymes such as GPX and SOD, stimulates the expression of antioxidant transcription factors, inhibits free radical releasing enzymes and reduces inflammatory responses with inhibition of NF-κB pathways ,and thereby it increases the antioxidant capacity of the body under oxidative stress conditions [26, 27]. It seems that Silibinin is an important protective factor in repairing damage caused by free radicals in various pathological conditions [27]. Kalemci et al. [28] have reported that silibinin improves the methotrexate-induced lung injury by reducing the oxidative stress. Prabu et al. [29] also have found out that silibinin improves arsenic-induced nephrotoxicity by reducing oxidative stress and by inhibiting inflammation in the kidney. Silibinin protects the heart cells from phenylephrine toxicity through antioxidant mechanisms, which mainly involves inhibition of intracellular signals [30]. Kan et al. [31] have reported that silibinin injections inhibit tumorgenesis in skin and reduces oxidative stress and inflammation by reducing NO and IL-6. In this study, silibinin administration resulted in a significant decrease in the lipid peroxidation index (MDA) and a significant increase in TAC as well. Furthermore, this antioxidant increased the activity of the antioxidant enzymes SOD and GPX. All in all, the activities of this compound reduced the oxidative stress in the breast tumor tissue. Küçükdurmaz et al. [32] have reported that silibinin injection significantly increased SOD and GPX activity and decreased MDA level in ischemic priapism mice. Moreover, Haddad and colleagues have showed that Lipid peroxidation and MDA production were efficiently inhibited by silibinin in a rat model of nonalcoholic steatohepatitis [17]. In addition, their study showed that silibinin was able to significantly reduce O^{2-} in these animals [17]. Vessale and colleagues proved that Milk thistle extract with less extent silymarin can increase GPX activity and decrease lipid peroxidation in renal tissue of diabetic rats [33]. One of the recent discovries about this molecule is its ability to change the expression of different miRNAs of various cells. That's why silibinin has significant beneficial effects, particularly in

the cancer field. It has been shown that silibinin can induce apoptosis and stop the cell division cycle in breast cancer cells by increasing PTEN (an apoptotic and cell cycle stop stimulator) and by decreasing the anti-apoptotic Bcl-2 protein and miR-21 [34]. Ma et al. [9] demonstrated that miR-10b expression was increased in early stage of cancers in metastatic breast tumors, and excessive expression of miR-10b triggers invasion and metastasis in breast cancer models and its expression is asscociated with clinical progression in primary cancer carcinoma. The effect of silibinin on other miRs has been shown that 100 µg/mL of silibinin does reduced significantly miR-21 and miR-155 expression MCM-7 breast cancer cells [10]. Silibinin inhibits growth and cell proliferation through inhibition of oncomers expression. It has been revealed that silibinin in both free and nano forms decreases miR-21 in T47D cell line, which is associated with an increase in apoptosis and a decrease in the cancer cell viability [35].

Conclusion

This study showed that silibinin improves oxidative stress in low doses via increasing antioxidant capacity and decreasing MDA in breast tumors of cancerious mice compared with the control cancerious mice. This effect was not meaningful with regard to miR-10b, SOD and GPX.

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Conflict of interest: Authors state no conflict of interest.

Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: This article does not contain any studies with human participants. All experiments were performed in compliance with the relevant laws and institutional guidelines of Faculty of Veterinary Medicine of Islamic Azad University, Tabriz branch, Tabriz, Iran.

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