INTRODUCTION

The human body regulates hormones for several specific purposes, thus any deficiency or reduced function in these hormones might lead to health problems or diseases. Melatonin is a significant hormone that influences the body's physiological processes. The pineal gland in the brain secretes it in reaction to darkness, and it is associated with relaxation and sleep. Melatonin's biological function includes regulating the sleep-wake cycle and it is utilised for short-term treatment of sleep disorders. Melatonin is produced from tryptophan, an amino acid that is converted into serotonin. Melatonin can serve as an antioxidant in two ways; actively by scavenging free radicals and passively by enhancing the activity of key antioxidant enzymes. Melatonin possesses both direct and indirect anticancer effects.

Melatonin possesses both direct and indirect anticancer effects. Direct anti-cancer efficacy is accomplished by inhibiting tumour cell proliferation and expansion, as well as by preventing healthy cells from developing into neoplastic cells. Many human cancer cell lines are suppressed by melatonin. Melatonin can indirectly reduce chemotherapy toxicity by protecting the lymphoid tissues and bone marrow, therefore potentially impacting cancer treatment. Melatonin is present in various physiological areas such as the digestive tract, kidneys, and retina, and has been detected in

Abstract

Metabolites and antioxidants can be altered in patients with various diseases, particularly in those with cancer. This study aims to measure melatonin and total antioxidant levels in the serum of breast cancer patients and compare them with a healthy control group. Both groups had serum samples collected at 2:00 a.m. and 9:00 a.m. Melatonin levels were determined using High-performance liquid chromatography (HPLC), while total antioxidant levels were assessed by Enzyme-linked immunosorbent assay (ELISA). Levels of melatonin and total antioxidants differed between the groups. In the control group, the levels of melatonin and total antioxidants were considerably greater compared to the diagnostic group, with a p-value of 0.001. Melatonin and total antioxidant levels were consistently greater at 2:00 than at 9:00. The maximum melatonin levels recorded in the diagnostic and control groups were 39.3 pg/mL and 65.9 pg/mL, respectively. Melatonin levels varied based on the age of the participants, with higher amounts observed in younger participants compared to older people in both groups. The BMI affected the levels of melatonin, with a greater BMI leading to elevated melatonin concentrations. The diagnostic group had a total antioxidant level of 39.3 pg/mL, while the control group had a level of 65.9 pg/mL. The elevated levels of melatonin and total antioxidants in the control group indicate a healthy status. The research demonstrated a significant correlation between melatonin and antioxidant levels in breast cancer patients, with reduced melatonin levels and total antioxidant levels in the diagnosed group. Elevated BMI was linked to reduced melatonin levels.

Keywords: Breast cancer, HPLC, ELISA, Melatonin, Total antioxidants, Sleeping disorder.
significant amounts in bone marrow cells. Melatonin's high lipid and water solubility enables it to pass through cell membranes, including the blood-brain barrier. Once it enters the bloodstream, it can quickly spread throughout the body. Melatonin levels fluctuate throughout a 24-hour period, with the highest concentration expected between 2:00 and 4:00 am. Low melatonin levels are associated with the peak hours of endogenous cortisol. The average levels of melatonin in plasma range from 60 to 70 pg/ml, accounting for variations caused by the body's physiology.

Antioxidants are compounds that eliminate free radicals and decrease oxidation in the human body. Oxidation is a chemical process that leads to the creation of free radicals. Free radicals are very unstable molecules due to their unpaired electrons, causing widespread health problems. Cellular damage caused by free radicals is associated with various diseases such as cancer, arthritis, atherosclerosis, and diabetes. Oxidative stress occurs when there is a discrepancy between the production and removal of tissue free radicals due to various internal and external factors. Cells maintain equilibrium between reactive oxygen generation and antioxidant defense in this environment. Free radicals and antioxidants are closely interconnected; elevating free radicals in the body leads to a reduction in antioxidant levels.

Antioxidants can neutralise free radicals, making them ineffective and counteracting radical chain reactions. Research has shown a connection between melatonin and overall antioxidant levels in relation to breast cancer. Epidemiological studies have indicated that those who consume a high-quality diet abundant in antioxidants (such as nuts, fruits, and vegetables) have a reduced risk of cancer. Dietary components may help prevent cancer. The level of metabolites fluctuates during activities or when the body is affected by a disease as part of the body's response to maintain physiological equilibrium. The precise extent of changes in melatonin levels and total antioxidants in breast cancer patients is not fully understood. The study aims to assess the levels of melatonin and total antioxidants in the serum of breast cancer patients and compare them to a healthy group across a 24-hour diurnal cycle. The data was analysed based on the age and Body Mass Index (BMI) of the participants in both groups. This study's data demonstrate the relationship between melatonin and total antioxidants across different groups.

MATERIALS and METHODS

Chemicals
Melatonin, butylated hydroxytoluene (BHT), sodium carbonate (Na\textsubscript{2}CO\textsubscript{3}), methanol (gradient grade, purity: 99.9%), ethanol (96%), ethyl acetate, n-hexane, dimethylformamide (dmf), sodium hydride (nah), benzyl chloride was purchased from Merk (Al Wafi group for Marketing & International Trade, Amman, Jordan).

Sample Collection
Blood samples were collected from 33 patients diagnosed with breast cancer and 22 healthy individuals to analyses the presence of melatonin and total antioxidants in their serum. The patients were admitted to Hiwa Cancer Hospital in Northern Iraq, and the center's ethics committee approved the inquiry. All participants provided written consent and were given the opportunity to withdraw from the study at their discretion. 10 mL of venous blood was collected via venipuncture using a plastic syringe with a 21-gauge stainless needle. 5 mL was used for melatonin analysis and 5 mL for total antioxidant determination. Blood samples were collected at 2:00 a.m. and 9:00 a.m. The serum was obtained from whole blood using centrifugation at 1500 x g for 10 minutes after the blood was separated in a basic polyethylene tube and left to clot at room temperature. The separated serum was kept at -20°C until analysis.

Blood samples were taken from women diagnosed with breast cancer and women without any cancer diagnosis as the control group. The control group participants were non-smoking, non-drinking, and non-medication using relatives of the diagnosed group. The participants varied in age and habitat region. Among the diagnosed group, 23 participants underwent multiple chemotherapy treatments, whereas 10 patients received only one chemotherapy treatment. Furthermore, only 25 patients needed surgical intervention for one breast. The study included participants of diverse ages and disease severities.
Serum Preparation

Serum extraction for melatonin level

One hundred microliters (µL) of BHT and 100 microliters of Na₂CO₃ were combined with 1.3 ml of the serum sample and mixed vigorously for 2 minutes. Subsequently, 2 ml of methanol and 1 ml of ethanol were added to the mixture. The solution was vortexed and centrifuged at 4420 rpm for 1 hour to separate the serum from the solution. Subsequently, 200 µL of ethyl acetate and 200 µL of n-hexane were introduced, mixed vigorously, and then subjected to centrifugation at 1300 rpm for 5 minutes. The resulting supernatant was extracted from the solution and dried using nitrogen gas. The dried solution was dissolved in 300 µL of DMF together with tiny quantities of NaH, and incubated for 30 minutes at 5 °C. After incubation, 50 µL of benzyl chloride was added and incubated for 1 hour at 5 degrees Celsius. The serum solution was filtered using a 0.22 μm PTFE syringe filter into a glass vial, and then stored at -20 °C until being analysed by High-performance liquid chromatography (HPLC).

Serum extraction for total antioxidant level

Eighteen microliters (µL) of serum sample were combined with 300 µL of reagent 1 (Acetate Buffer 0.4 mol/L and PH 5.8) in a cuvette. The absorbance (A1) was then measured after 30 seconds at a wavelength of 660 nanometers. Following that, 45 µL of reagent 2 (ABTS radical cation Prochromogen solution 30 mmol/L) was introduced into the solution, and the absorbance (A2) was recorded after 5 minutes at 660 nm at 37 °C. The Rel Assay Diagnostics ELISA Kit was utilised and the results were juxtaposed with the Trol ox equivalents antioxidant capacity (TEAC). The serum samples were analysed for total antioxidants using the enzyme-linked immunosorbent assay (ELISA) kit from Mega Tıp Sanayi ve Ticaret Limited Şirketi Gaziantep / TURKEY, and the results were compared with the Trolox equivalent antioxidant capacity (TEAC) 22.

Instrumentation

High-performance liquid chromatography (HPLC)

Serum samples were analyzed for melatonin content on LC-2030C NT Shimadzu (Shimadzu, Kyoto, Japan) coupled with 2x LC-20AD pumps, 1x DGU-20A5 degasser, 1x SIL 20A autosampler, 1x CTO-10AS VP column oven, 1x SPD-M20A DAD system, 1x RF-10AXL FLD system and 2x detectors connected in series. These devices were connected via a communication module (Model CBM-20A) and controlled by a Shimadzu LC Solution workstation. The separation was performed using Column, LC, Shim-pack VP-ODS 5-micron, 4.6 x 150mm (Shimadzu, Kyoto, Japan). For injection, 50 µl of melatonin standard solutions and serum samples were injected onto a pre-heated column at 30 °C, with a flow rate of 300 µL/min. The mobile phases of eluent A (acetonitrile + 5 mM potassium dihydrogen phosphate, adjusted with H3PO4 to pH 2.5; 750 + 250, v/v) and eluent B (water + 5 mM potassium dihydrogen phosphate, adjusted with H3PO4 to pH 2.5; 750 + 250, v/v) were used in gradient elution mode. Chromatograms were recorded at 250 nm. For quantification, calibration curves were obtained using at seven standard solutions in the range of 0.1-200 ng/L, by plotting measured analyte peak areas against corresponding analyte concentrations. The data were expressed as mean ± standard deviation, (n=3).

Enzyme-linked immunosorbent assay (ELISA)

Serum total antioxidant level was determined using a commercial ELISA kit (Inova Diagnostics, San Diego, USA) as recommended in the manufacturer’s instructions, which included a microtiter plate with wells coated with antibodies. A limit was defined to the competition between an unknown amount of total antioxidants in the sample and a defined amount of enzyme-labeled antigen for the binding sites of the antibodies coated on the wells. Temperatures, incubation periods, and shaking operations were all set according to the kit’s instructions. The absorbance od the samples was recorded at 660 nm.

Statistical Analysis

The comparison between the diagnosed and control groups was conducted using Student’s t-test in Origin Pro 9.6 software by Origin Lab Inc. based in Massachusetts, USA. The data was displayed as mean ± standard deviation for comparison between the diagnosed and control groups. Statistical
RESULTS and DISCUSSIONS

This study utilised a case-control strategy to investigate the relationship between melatonin and total antioxidant levels in breast cancer patients compared to a healthy control group. Serum samples were taken from the groups at 2:00 am and 9:00 am as part of a diurnal rhythm during a 24-hour period. Figure 1 displays the serum melatonin concentrations of 33 patients with breast cancer and 22 individuals in the control group. The melatonin level was higher in the control group than in the diagnostic group. At 2:00, the melatonin concentration was twice as high in the control group. The control group had the greatest melatonin concentration of 66 pg/mL at 2:00, but the diagnosed group had a peak level of 39 pg/mL at the same time. The peak melatonin levels at 9:00 were 15 pg/mL in the control group and 11 pg/mL in the diagnostic group. At 2:00, the peak melatonin level in the diagnostic group was lower than the lowest melatonin level in the control group. Melatonin levels were considerably lower in the diagnosed group compared to the control group at 2:00 and 9:00 am, with p-values of < 0.001 for both times.

Figure 1. Serum samples from 33 patients with breast cancer and 22 individuals in the control group were analysed for melatonin concentrations (pg/mL). The blue squares, lines within the boxes, and red stars symbolise the medians of melatonin concentrations, medians, and outliers, respectively.

Organising the melatonin concentration data by age revealed that the control group had greater melatonin concentrations, as shown in Figure 2. The results indicates that melatonin levels are typically greater in younger individuals (age group >30 years) compared to older participants (>40 years old and >50 years old). Additionally, the melatonin level in the >40 years old group was higher than in the >50 years old group. Younger participants (age >30 years) had significantly greater melatonin concentrations compared to older participants in both the diagnosed and control groups, with p-values < 0.001. Both the diagnostic and control groups showed greater melatonin concentrations at 2:00 am compared to 9:00 am. For individuals over the age of 50, there was no notable disparity in melatonin levels between the group with the diagnosis and the control group at 9:00.
Figure 2. Serum samples from patients with breast cancer and a control group were analysed for melatonin concentrations (pg/mL) based on age groups: >30 (n= 5 and 5), >40 (n= 17 and 13), and >50 years old (n= 5 and 5). The symbols denote the mean of the data, the whiskers indicate the confidence range of the means, and upward triangles and downward triangles represent the time of the obtained serum samples at 2:00 am and 9:00 am, respectively.

The participants were categorised based on their body mass index (BMI) into three groups: BMI >20, BMI <20, and BMI >30, as shown in Figure 3. The data shows that melatonin concentrations were consistent across the control group, with no significant difference between melatonin concentrations at 2:00 and 9:00. Melatonin concentrations decreased as BMI increased in the diagnosed group at 2:00 and 9:00. Similar results were observed in the control group. The control group consistently exhibited greater melatonin levels compared to the diagnostic group at 2:00 and 9:00 am. This tendency was also observed when analysing the whole data and organising it by the age of the individuals. Higher BMI led to elevated melatonin concentrations in both groups. Increasing the sample size may provide a more comprehensive understanding of the observation. Some subjects were distinguished, particularly in the diagnosed group. For instance, participant 4 had the lowest melatonin concentration while having a BMI of 20. The subject may have experienced these symptoms due to being diagnosed with stage 4 breast cancer at the age of 47, undergoing 4 chemotherapy treatments, and sleeping only a few hours during a 24-hour period.

Antioxidant levels were measured in serum samples from 33 breast cancer patients and 22 individuals in the control group, as shown in Figure 4. The control group consistently exhibited higher total antioxidant levels compared to the diagnosed group at 2:00 and 9:00 am. The control group exhibited the greatest total antioxidant levels, with 2 mmol/L at 2:00 and 1.4 mmol/L at 9:00. The diagnosed group had the highest total antioxidant levels of 1.5 mmol/L at 2:00 and 1.3 mmol/L at 9:00. The total antioxidant levels were considerably lower in the diagnosed group compared to the control group at 2:00 and 9:00 am, with p-values < 0.001 at both times. The control group had a total antioxidant level of 1.3 mmol/L at 2:00, which was similar to the diagnosed group's maximum total antioxidant level of 1.5 mmol/L. The total antioxidant level in the diagnosed group was extremely low, almost reaching the baseline of 0.05 mmol/L.
Melatonin levels (pg/mL) in serum samples of patients with breast cancer and a control group categorised by their body mass index (BMI): >20 BMI (n= 8 and 5), <20 BMI (n= 15 and 12), and >30 BMI (n= 10 and 5). The symbols depict the mean of the data, while the whiskers show the confidence interval of the means. Triangles pointing upward and downward indicate serum samples collected at 2:00 am and 9:00 am, respectively.

Antioxidant levels were measured in the serum samples of 33 breast cancer patients and 22 individuals in the control group. The blue squares, lines within the boxes, and red stars indicate the medians of total antioxidant levels, medians, and outliers, respectively.

Typically, melatonin levels in people fluctuate throughout the day, with secretion of the hormone increasing shortly after darkness sets in, peaking about 2 to 4 a.m., and then progressively decreasing.
thereafter. Previous research has shown that the level of melatonin in human plasma decreases as individuals age. These investigations were cross-sectional, comparing groups of human participants of different ages. In the senior age (60-90 years), the rate of melatonin decline is approximately 40–50% compared to the younger group. Another study did not see any age-related decrease in melatonin levels in older individuals. Although melatonin/total antioxidant levels are complex and their process is not fully understood, a decrease in these levels during abnormal conditions or specific diseases might result in sleep disorders or fatigue. Therefore, it is crucial to treat or adjust the levels of melatonin/total antioxidant and enhance the co-factors to increase melatonin/total antioxidant levels. Melatonin and overall antioxidant levels can be increased in the body by dietary intake of vegetables, caffeine, and certain vitamins, which can have a less intense impact on melatonin generation compared to light exposure. Foods that promote or include melatonin affect the availability of tryptophan, as well as the vitamins and minerals needed as activators in the production of melatonin, and the total antioxidant content. There is a strong desire to incorporate alternative, natural, and safe sources of antioxidants, with a focus on replacing those from plant-based foods. Antioxidants are added to foods to prevent radical chain reactions of oxidation and to postpone the oxidation process. Research involving both animals and humans has confirmed that short-term melatonin use is safe, with no major ill effects identified. Pregnant and breast-feeding women should avoid taking melatonin as a supplement since it has not received clinical approval yet.

CONCLUSION

The study concluded that there is a notable relationship between melatonin and overall antioxidant levels in breast cancer patients as compared to a healthy control group. The diagnosed group regularly had reduced melatonin levels, particularly at 2:00 am, suggesting a disturbance in the day rhythm. Moreover, elevated BMI was linked to reduced melatonin levels in both groups. The diagnosed group showed reduced total antioxidant levels, indicating a possible connection between melatonin, antioxidants, and breast cancer. These findings emphasise the need for more research on the impact of melatonin and antioxidants in preventing and treating breast cancer, as well as the advantages of dietary changes to enhance their levels.

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Data availability

Data available on request from the authors.

Conflict of Interest

The authors declare they have no conflicting interests.

REFERENCES