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Improving anti-tumour activity with melatonin-stimulated mesenchymal stem cell-derived exosomes in metastatic triple-negative breast cancer

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The aim of this study is to increase the bioavailability of melatonin on triple-negative breast cancer (TNBC) cells by loading it into exosomes as well as comparing the therapeutic potentials of melatonin and exosome released from human adipose tissue-derived mesenchymal stem cells. TNBC is one of the most malignant tumours with highly invasive and metastatic features. It is characterized by the absence of estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2. TNBC patients can't benefit from hormonal or trastuzumab-based therapies targeting these receptors. Exosomes are defined as naturally occurring extracellular vesicles. By enabling the transfer of molecules, exosomes play a role in cancer treatment and dynamic intercellular communication between tumour cells and adjacent stromal compartments. The half-maximum inhibitory concentration IC_{50} values were 30.38 μg/mL, 40.49 μg/mL and 1.5 mM at the co-administered melatonin and exosome, AT-Exo and Mel groups, respectively, for 48 h. The percentage of late-stage apoptotic induction was found to be 6.3%, 4.1% and 4.6% for TNBC exposed to co-administered melatonin and exosome (2.5 mM +100 μg/mL Mel/Exo) for 24 h, 48 h, 72 h, respectively. In conclusion, the coexistence of exosomes and melatonin represents a promising therapeutic tool that can interfere with key molecular processes such as cytotoxicity and apoptosis cascade in TNBC.

Keywords: Combination, Exosome, Melatonin, Therapeutic Potential

Breast cancer is the most common tumour model in women, resulting in high malignancy and mortality because of its high rate of metastasis. Clinically, specific subtypes of breast cancer describe the expression of hormone receptors such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2, ERBB2) and the histopathological appearance of these receptors. TNBC lacking these three receptors is an aggressive and highly heterogeneous subtype of breast cancer characterised by a relatively poor prognosis because of the ineffectiveness of hormone receptor-based chemotherapies.Also, TNBC patients do not benefit from hormonal or trastuzumab-based therapies targeting ER, PR, and HER2 cell surface receptors. Therefore, new therapeutic agents are critically needed for this aggressive tumor[1].

It is defined that exosomes are extracellular vesicles (EVs) which are about 20-200 nm in diameter. Exosomes are secreted from endocytic multivesicular bodies (MVBs) into the extracellular space. Exosomes contain biologically active components such as lipids, enzymes, mRNAs, metabolites and various non-coding RNAs represented by miRNAs, long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) includes. It acts as a depot that stores molecules. By enabling the transfer of these molecules, exosomes play a role in cancer treatment progression and dynamic intercellular communication between tumour cells and adjacent stromal compartments[2]. Mesenchymal stem cells (MSCs) have become increasingly popular in regenerative medicine due to their multipotent differentiation potential, and potent immune-modulatory and regenerative functions. MSC-derived exosomes are a novel cell-free alternative to MSCs that have long been of great concern. MSC-derived exosomes are much more helpful in terms of safety, bioactivity, storage and transport compared to MSC transplantation[3].

Melatonin is a hormone secreted from the pineal gland in the centre of the brain. Melatonin has regulatory and protective properties such as improving the ageing process, protecting the body against oxidative stress, and regulating the circadian

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rhythm and immune system[4]. In addition, melatonin cuts the inflammation and redox activity of cancer cells and makes the cells sensitive to chemotherapy[5].

Recent research is on which of the versatile properties of melatonin, which has receptors on many body cells, can be associated with cancer. However, its anti-cancer effect, chronobiological regulator, its antioxidant and immune-supporting properties are also associated with the cancer process[6,7]. Melatonin treatment increases the efficacy of chemotherapy in patients with lung, breast, prostate, gastrointestinal system, head and neck cancers. Besides its mycostatic effect, melatonin reduces anxiety, depression, and toxicity associated with chemotherapy. Melatonin is emerging as a safe and effective treatment for breast and prostate cancer, both alone and in combination with other treatments[8,9]. Melatonin inhibits the growth of positive breast cancer cells (such as MCF-7, T47D, and ZR-75-1) by suppressing the estrogen signalling pathway and vascular endothelial growth factor (VEGF) than more effectively negative breast cancer cells (such as BT-20, MDA-MB- 231, MDA-MB-364, Hs587t). Although the anticancer effects of melatonin in various types of cancer have been widely described, in the current study, besides comparing the therapeutic potentials of melatonin and exosomes released from human adipose tissue-derived mesenchymal stem cells, to increase the bioavailability of melatonin on TNBC cells, were investigated by loading them into exosomes.

Materials and Methods

Cell lines and culture conditions

The cell lines (MDA-MB-231 and MCF10A) of them were gifted by Prof. Servet Özcan. The MDA-MB-231 cells were cultured in the mixture medium DMEM-F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) containing 10% FBS, 1% pen-strep, 0.01 mg/mL insulin andnon-tumorigenic MCF10A cells were cultured in the mixture medium DMEM/Ham's F-12 cell medium supplemented with 100 ng/mL cholera toxin, 20 ng/mL epidermal growth factor (EGF), 0.01 mg/mL insulin, 500 ng/mL hydrocortisone and 20% FBS incubated at 37°C containing 5% CO_2. All media was changed every 3 days. When the flasks reached 80-90%, the cells were removed by incubation with 0.25% trypsin at 37°C for 5 min and prepared for cytotoxicity and apoptosis assay.

Adipose tissue-derived mesenchymal stem cell

The human adipose tissue-derived mesenchymal stem cells, hAT-MSC (PCS-500-011), were gifted by Prof. Servet Özcan. No ethics committee approval is required. The cells were cultured in the mixture medium DMEM-Low Glucose (Dulbecco's Modified Eagle Medium) supplemented with 5% FBS, 1% penicillin-streptomycin, and incubated at 37°C containing 5% CO_2. When the flask reached 80% density, the media was replaced with media-depleted FBS and the cells were incubated for 48 h for exosome isolation.

Exosome isolation and concentraiton

Exosome-derived AT-MSC (AT-Exo) isolation was performed by precipitation method using the System Biosciences EXOTC50A-1 kit. Exosome-derived mesenchymal stem cell was isolated according to the manufacturer's instructions. The antibody-free exosome protein concentration was calculated using System Bioscience's Exocet kit according to the manufacturer's instructions.

Scanning electron microscope (SEM)

Scanning Electron Microscope (SEM) (Zeiss GEMINI 500, Germany) device was used to monitor the membrane integrity of exosomes and measure their average size. To measure their size using the Image J program, they were diluted with phosphate buffer (PBS) at a dilution ratio of 1/1000 and then visualized on gold plating[10]. The diameter of exosomes was measured with the Image J program and the mean exosome size was calculated.

Nanoparticle tracking analysis (NTA)

Isolated exosomes were suspended in 1 mLof 1X PBS and diluted 1:100. Then both size distribution and relative concentration were determined by Nanoparticle Tracking Analysis using a NanoSight NS300 instrument according to the manufacturer's instructions. Size distribution diagrams, mean/mode size values and standard deviations were calculated in nanoparticle tracking analysis (NTA) 3.4 software[11].

Zeta potential

The isolated exosomes were diluted with a 1:1000 dilution ratio with 1XPBS to determine the separation and dispersion stability, and the zeta potential was determined with a zeta sizing analyzer for the potential difference between the electrical and isoelectric point[12].

Cytotoxicity assay

The MDA-MB-231 and MCF10A cells were seeded into96-well plates at 5 × 10^3/100 μL in a mixture medium without FBSincubated at 37°C containing 5% CO_2 for 24 h. The XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity (Biological Industries, Cell Proliferation Kit, XTT based). The percentage of cell viability was analysed according to the manufacturer's instructionsby using an ELISA reader (Bio-Tek Instruments, Inc., USA). The percentage of viable cells treated with melatonin, exosome and co-administered melatonin exosome samples was calculated by counting the number of live cells and comparing them with the untreated control samples. The treatment groups' half-maximal inhibitory concentration (IC_{50}) was calculated for MDA-MB-231 with GraphPad Prism 8 software.

Annexin-FITC/ dead cell double staining

The MDA-MB-231 and MCF10A cells were seeded into6-well plates at 1 × 10^6/mL in a mixture medium without FBS incubated at 37°C containing 5% CO_2 for 24 h. Apoptosis assay was assessed according to the manufacturer's instructionsby using a FACS Aria III flow cytometry to detect apoptotic cells exposed to phosphatidyl serine by staining them with Annexin V-FITC and propodium iodide (PI),dead cell marker 7-Aminoactinomycin D (7-AAD) (Annexin V/ Dead Cells kit, Luminex). Apoptosis graphs were used to show dead cells (Q1-1), late apoptotic cells (Q2-1), live cells (Q3-1), and early apoptotic cells (Q4-1).

Treatment of MCF10A and MDA-MB-231 cells with separate melatonin, AT-Exo, and co-administered melatonin-AT-Exo

After 24 h, melatonin (Mel) (1, 2.5, 5 mM) and AT-Exo (25, 50 and 100 µg/mL) and Mel-AT-Exo (2.5 mM Mel/25 µg/mL AT-Exo , 2.5 mM Mel/ 50 µg/mL AT-Exo and 2.5 mM Mel/100 µg/mL AT-Exo for cytotoxicity and 2.5 mM Mel/50 µg/mL AT-Exo and 2.5 mM Mel/100 µg/mL AT-Exo for apoptosis) were incubated to each tumorigenic and non-tumorigenic cell groups for 24, 48 and 72 h. The evaluation was made with different dose-time dependent for both cytotoxicity and apoptosis assays.

Statistical analysis

The data from independentthree separate experiments for cytotoxicity assay and the data from one experiment for apoptosis assay were evaluated under group analysis using GraphPad Prism 8 program, Two-way ANOVA, Tukey multiple comparisons test- Dunnet's multiple comparisons testbetween and within groups for cytotoxicity, One-way Anova test for apoptosis and the significance value was accepted as $P < 0.05$.

Results

Scanning electron microscope (SEM)

As a result of the precipitation method, it was observed that the exosomes, whose isolation was completed, preserved their structural integrity (Fig. 1). The mean size of the exosomes was measured with the Image J program and calculated to be 98.6 nm±6.9.

Nanoparticle tracking analysis (NTA)

According to the NTA results, the average size of the exosomes was 97.2 nm and 4.59 × 10^6 exosomes per mL were measured and calculated with the NTA device (Fig. 2). In addition, the concentration distribution according to the size of the isolated exosomes was determined (Fig.3). When the NTA results were compared with the SEM image results, it was seen that the results were close to each other and the results supported each other.

Zeta potential

The zeta potential measurement of the isolated exosomes was found to be −39.7 mV and the result was evaluated as good by the device. Depending on the device, the zeta potential being close to −30 indicates that the potential difference between the electrical and isoelectric points of the isolated exosomes is stable (Fig. 4).

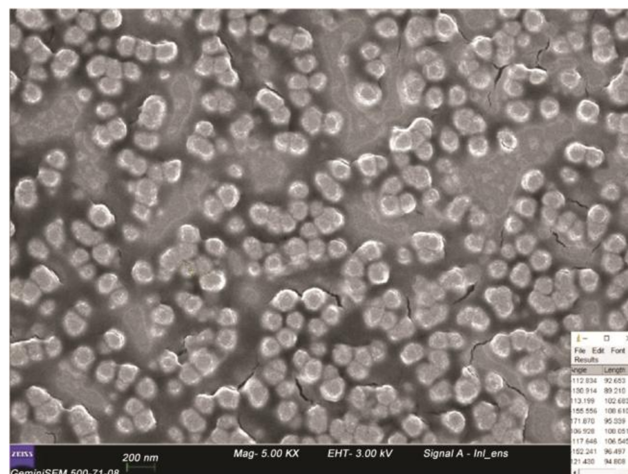

Fig. 1 — SEM image of exosomes and size measurement with Image J, particle size analyzer (98.6 nm±6.9)

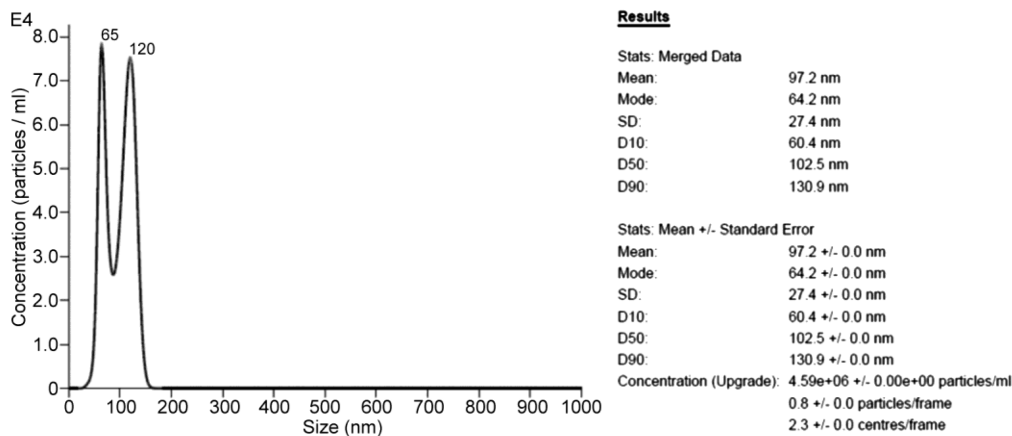

Fig. 2 — Concentration and mean sizes (nm) of exosomes with NTA

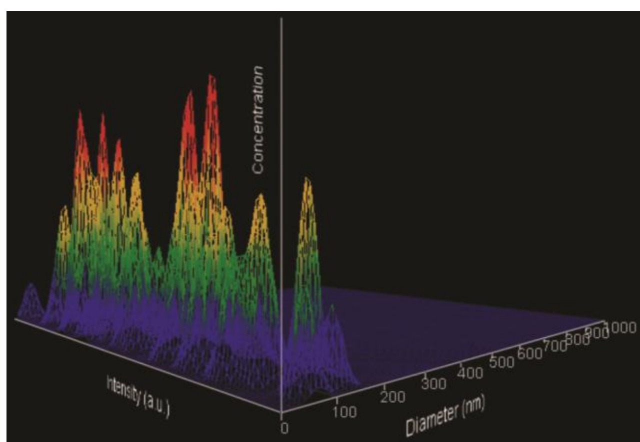

Fig. 3 — 3D size-concentration distribution plot of exosomes with NTA

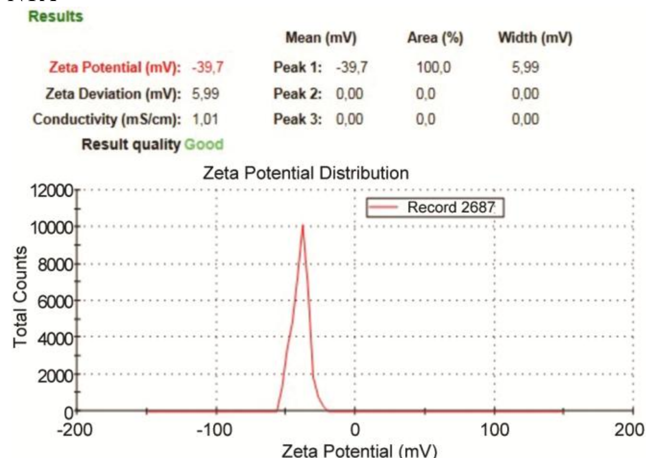

Fig. 4 — Zeta potential measurement of exosomes with a zeta analyzer

Cytotoxicity

The cytotoxic effects of melatonin, AT-Exo and co-administered melatonin-AT-Exo on breast cancer cells *in vitro* were analyzed by XTT test. The IC_{50} dose for each agent was calculated based on time and dose using the statistical program (GraphPad 8) (Fig. 5). In the co-administered melatonin-AT-Exo group, the IC_{50} dose was calculated by keeping melatonin constant, and it was observed that the IC_{50} dose was effective at lower concentrations when compared to the groups in which only exosome and melatonin were administered. While calculating the percentage of cell viability, the control group was accepted as 100% and cytotoxicity results were calculated. Considering the cytotoxicity results, the percentages of cell viability were found as 110.7±3.7 and 92.9 ± 5.4, in the 25 and 50 µg/mL AT-Exo groups at the end of 24 h, respectively. It was found to be 97.9±13 in the 25 µg/mL AT-Exo group for 48 h. When these groups were compared with the control group and statistically evaluated, no significant difference was found between them. *(P >0.05)*. When the control group was compared with each other group depending on time and dose, a statistically significant decrease in the percentage of cell viability was observed *(P <0.001)*. It was observed that the cytotoxic effect was not evenly distributed in all groups. The percentage of cell viability was calculated as 42.7±8.6 and 10.4±4.2, respectively, in the 2.5 mM+100 µg/mL Mel/Exo group administered for 24 and 48 h. The percentage of cell viability was more effective in the melatonin-only groups than in the exosome-only groups. However, cell viability was much lower in the groups that were administered co-administered melatonin-AT-Exo. The most effective cytotoxic effect was observed in the 48 h group that was administered 2.5 mM+100 µg/mL Mel/Exo (Fig.6). There has been an effective decrease in cell viability at each dose applied to the 72 h group, but this is thought to be because the cells were stressed because the cell group was kept in the same culture

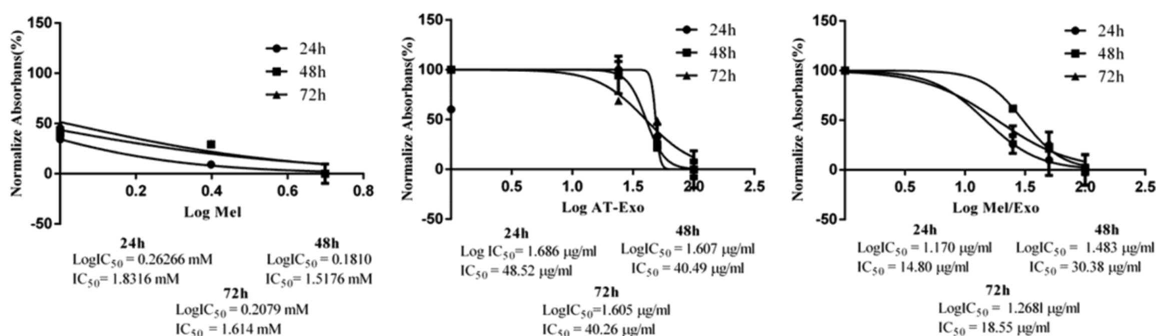

Fig. 5 — LogIC_{50} and IC_{50} values of each dose-time-dependent melatonin, exosome and co-administered melatonin exosomes applied to MDA-MB-231 cells using GraphPad Prism 8 statistical program. Error bars represent the mean standard deviation

conditions without FBS. In addition, a significant increase in the percentage of cell viability was observed in the 72 h groups compared to the 48 h group, and a significant decrease was observed compared to the control group. In the MCF10A groups, the XTT assay showed no cytotoxic response to the MCF10 cells. There was only a minimal decrease in cell viability in the 72 h groups. This situation is thought to be due to the unchanging complex medium.

Apoptosis

The Annexin V/PI method enabled the screening and determination of MDA-MB-231 cells in early and late apoptotic stages. When the results were examined, it was determined that MDA-MB-231 cells, which were applied at different concentrations for both 24 and 48 h, passed into the late apoptotic stage and some cells went directly to the necrotic stage. The late apoptotic graph of the administered groups depending on the dose and time is given in (Fig. 7). Statistically significant changes were observed when the late-term apoptotic percentage results of different applied concentrations were evaluated both among themselves (P <0.01) and depending on time (P <0.05). When the apoptosis results in the control groups are examined, the observed percentage of late apoptotic values shows the expected spontaneous apoptosis during the cell division cycle. It was observed that the percentage of necrotic cells was dose-dependently higher in the 72 h groups compared to the other groups. This situation may be caused by stress because of the absence of FBS and the keeping of the cells in FBS-free medium. When the FACS Aria III results were examined by excluding the 72 h groups, it was observed that the highest percentage of late apoptotic was in the 24 h groups (Figs 8 & 9), while the percentage of necrotic cells was high in the 48 h groups (Figs 10 & 11). As a

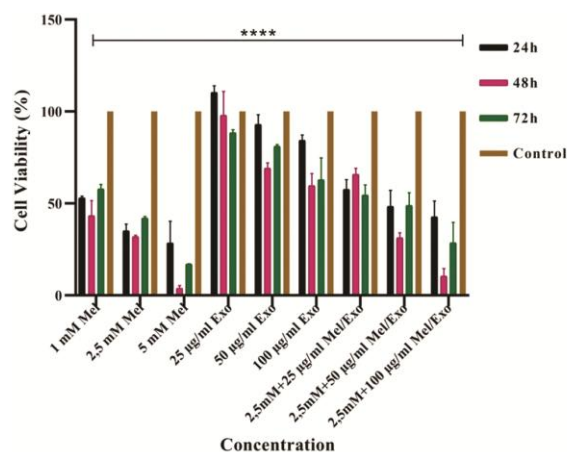

Fig. 6 — The XTT for cytotoxicity response in MDA-MB-231 cells. The effect of melatonin, exosome and co-administered melatonin exosomes on the percentage of cell viability in MDA-MB-231 cells dose-time dependent. Concentrations and time at which cytotoxicity was statistically significant (P <0.0001). The XTT assay showed no cytotoxic response to the MCF10 cells

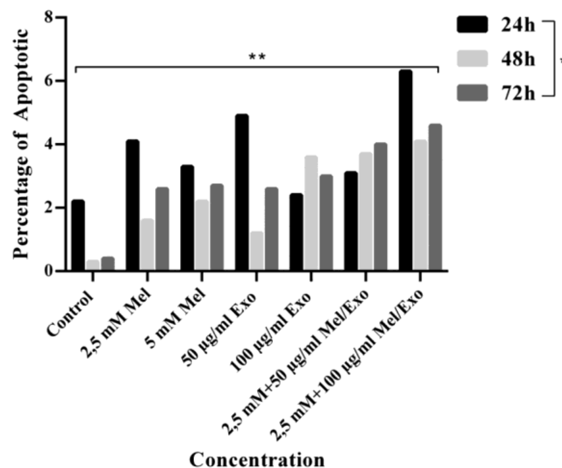

Fig. 7 — The Annexin V/Propidium Iodide for apoptotic effect in MDA-MB-231 cells. The percentage of late apoptotic cells stained with Annexin V (+), 7-AAD (+) by using a FACS Aria III flow cytometry. The percentage of late apoptotic results is statistically significant depending on both concentrations and times (**P <0.01, *P <0.05)

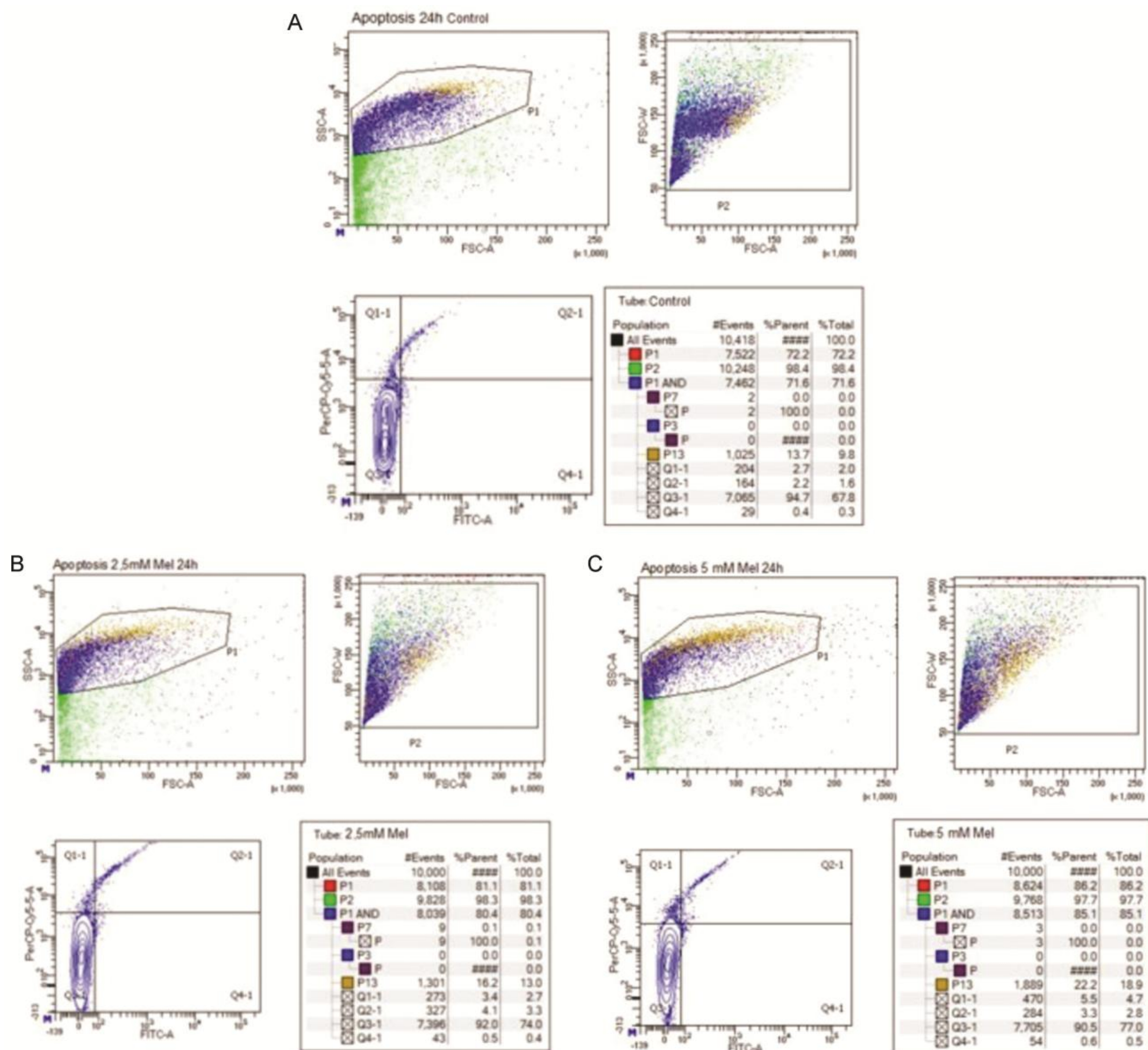

Fig. 8 — The Annexin V/Propidium Iodide for apoptotic effect in MDA-MB-231 cells for 24 h. Apoptosis graphs were used to show dead cells,Q1-1,(Annexin V (–), 7-AAD (+)), late apoptotic cells, Q2-1, (Annexin V (+), 7-AAD (+)), live cells, Q3-1,(Annexin V (–), 7-AAD (–), and early apoptotic cells, Q4-1,(Annexin V (+), 7-AAD (–)).(A) Apoptosis control group; (B) The percentage of dead, late apoptotic, live and early apoptotic cells for the 2,5 mM Mel group; (C) The percentage of dead, late apoptotic, live and early apoptotic cells for the 5 mM Mel group

result of the application of 2.5 mM+100 µg/mL Mel/Exo, the percentage of late apoptotic cells was 6.3% and 4.1% and the percentage of necrotic cells was 7.3% and 28,3% for 24 h and 48 h, respectively.

Discussion

It has been stated that triple-negative breast cancer has the highest incidence in India and constitutes 7-28% of cancer types[13]. TNBC represents an astonishing 30% of all diagnosed cancer cases[14]. TNBC complicates treatment because of its drug resistance, non-responsiveness to treatments targeting surface receptors, and toxicity in current therapies, making it a subtype of cancer with poor prognosis and poor survival[15]. Melatonin's anti-cancer effect is through wide molecular mechanisms and participates in the process with its lncRNAs. Researchers reported that melatonin inhibits TNBC cells *via* lncRNA and is a potential therapeutic for this tumor[16]. It has been reported that melatonin inhibits the proliferation of breast cancer cells at physiological (10^{-9} mol/l) concentrations, and has a cytotoxic effect on breast tumour cells, MCF-7, at pharmacological concentrations (10^{-5} mol/l)[17]. Although melatonin is

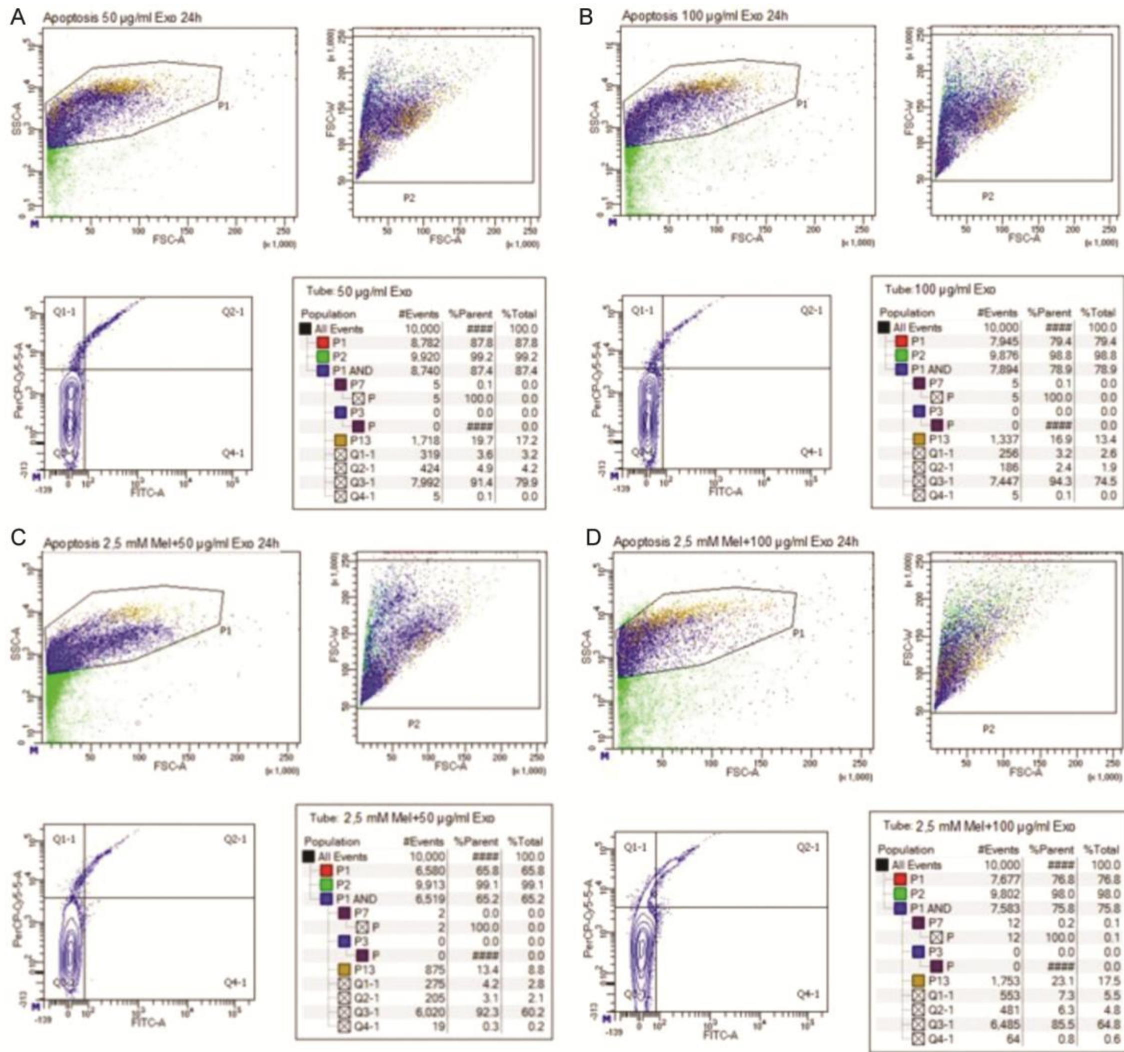

Fig. 9 — The percentage of the dead, late apoptotic, viable and early apoptotic cells in MDA-MB-231 cells for 24 h. (A) 50 µg/mL AT-Exo; (B) 100 µg/mL AT-Exo (C) 2,5 mM /50 µg/mL co-administered melatonin-AT-Exo (D) 2,5 mM /100µg/mL co-administered melatonin-AT-Exo

used positively in the treatment of breast cancer, especially with the MT1receptor, it should be extensively investigated in combination with other treatment methods, considering the increased resistance to treatment and cancer types that do not have the HER2 receptor. Recently, research has increased to understand the biogenesis of exosomes and their functions in cancer therapy[18]. With the understanding of the functions of exosomes, it has been stated that it is appropriate to use exosomes to diagnose and treat diseases [19]. Because TNBC is resistant to chemotherapy and metastatic, research for new treatment methods for drug delivery continues[17]. In recent studies, therapy with mesenchymal stem cells (MSC's) has side effects due to the unlimited proliferation and differentiation of these cells. To avoid this side effect, stem cell-derived biological components were emphasized. Exosomes released from mesenchymal stem cells are extracellular vesicles that have anti-apoptotic, anti-inflammatory and antioxidant properties and show paracrine action[20]. The researchers stated that nano-sized biomolecules function in the specific immune response, and that nanotechnology is used to make immune responses specific[21]. In order to use exosomes, which are of great importance in the definitive diagnosis and treatment of cancer, more effectively in the clinic, there is a need to understand and investigate drug delivery systems. There is also a need to improve the therapeutic potential and delivery

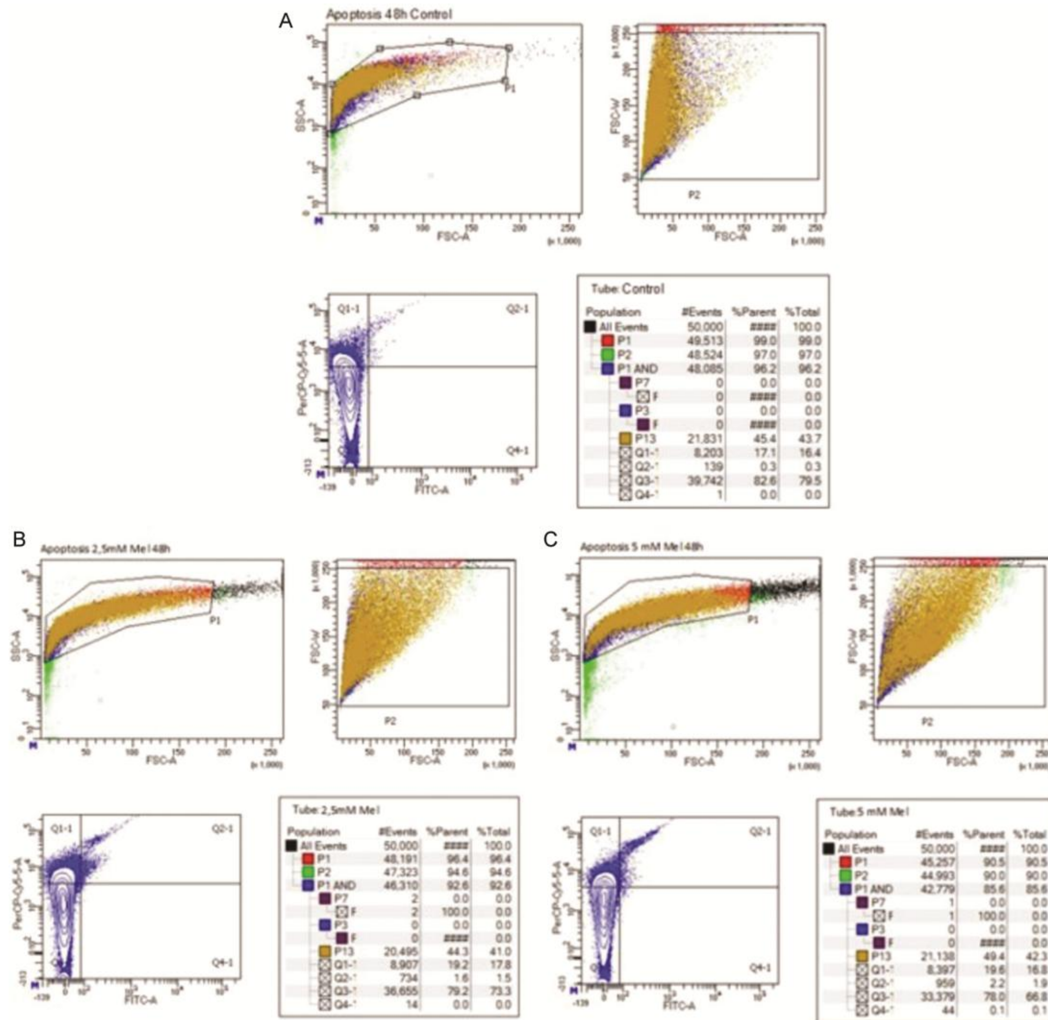

Fig. 10 — The Annexin V/Propidium Iodide for apoptotic effect in MDA-MB-231 cells for 48 h. (A) Apoptosis control group; (B) The percentage of dead, late apoptotic, live and early apoptotic cells for the 2,5 mM Mel group; (C) The percentage of dead, late apoptotic, live and early apoptotic cells for the 5 mM Mel group

efficiency of exosomes. Control of size and zeta potential are therefore important factors for the drug delivery, and efficacy of nanoparticles and allows the identification of cellular targets, for example, liposomes, gold nanoparticles and copolymer micelles[10]. When the results of this study are evaluated, it is seen that the zeta potential is stable. It was observed that the average sizes of the isolated exosomes were appropriate. In addition, the mean sizes of exosomes were also supported by NTA as a result of SEM analysis. Melatonin acts as a glycolytic molecule by providing aerobic glycolysis of damaged cells to maintain normal mitochondrial oxidative phosphorylation within the body[22]. In *in vitro* study, it was stated that inflammation was suppressed by exogenic adipose mesenchymal stem cell (ADMSC)-derived exosome and exosome/melatonin treatment. It has also been shown in an *in vivo* study that it provides oxidative stress and apoptosis. Plasma AST concentration was reported to be the lowest in the exosome melatonin group. It has been stated that combined exosome and melatonin are better at protecting against liver ischemia-reperfusion injury than both melatonin and exosome[23]. The researcher applied melatonin and exosomes released from MSC, Exo, and combined MSCs for renal ischemia-reperfusion injury (I/R). This study also included MSCs previously conditioned with melatonin. He reported that combined therapy provides the best protection for I/R[24].

Another group of researchers reported that the combined treatment of Mel + Exo in their *in vivo*

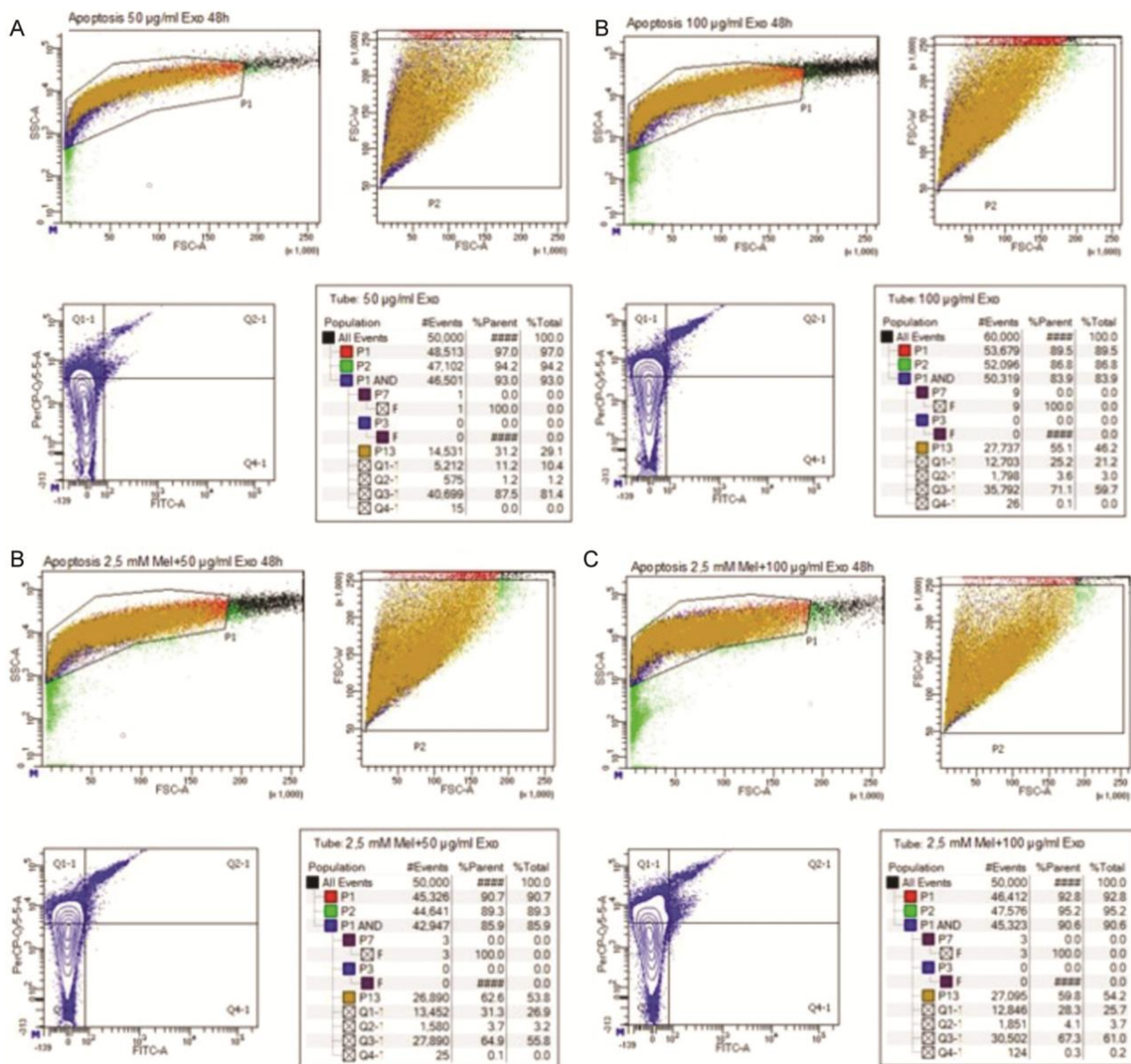

Fig. 11 — The percentage of the dead, late apoptotic, viable and early apoptotic cells in MDA-MB-231 cells for 48 h. (A) 50 µg/mL AT-Exo, (B) 100 µg/mL AT-Exo (C) 2,5 mM /50 µg/mL co-administered melatonin-AT-Exo (D) 2,5 mMMel/100 µg/mL AT-Exo co-administered melatonin-AT-Exo

studies gave much better results for oxidative stress, inflammation and apoptosis parameters in the treatment of kidney damage and has potential use in the treatment[25]. Rong *et al*. investigated the role of melatonin and exosomes originating from adipose tissue in reversing hepatic steatosis. The results of the study stated that melatonin and exosomes extending from adipocytes to hepatocytes *via* exosomes are a new potential target for treating obesity and related hepatorenal syndrome[26]. In a study for diabetic wound healing, it was reported that the combined application of melatonin and exosomes could increase wound healing by activating the PTEN/AKT signalling pathway and suppressing inflammation. In addition, as a result of the findings, it has been reported that combined therapy applied *in vivo* supports angiogenesis and collagen synthesis due to the suppression of inflammation[27]. It has also been reported that combined therapy of exosome and melatonin helps in curing chronic kidney diseases[28], and due to the paracrine effect of the exosome, combined with melatonin helps in curing neurodegenerative diseases[29]. Melatonin acts through MT1 (formerly called Mel1a or ML1A) and MT2 2

(formerly called Mel1b or ML1B) receptors. Human MT1 and MT2 melatonin receptors show different affinity structures and different pharmacological properties[30]. At the cellular level, melatonin and its receptors are poised to influence cancer pathology. MT1 melatonin receptor mediates its oncostatic effect in breast cancer models. The MT1 receptor may be constitutively active in breast cancer cells and inhibit cancer cell proliferation. Therefore, selective MT1 receptor agonists may be effective in the treatment of breast t cancer, either alone or as an adjunct to existing therapeutic approaches[31-34]. Thanks to melatonin's MT1 and MT2 receptors and signalling pathways, melatonin is involved in apoptosis, proliferation, inflammation, angiogenesis, *etc*. It can affect various cellular processes such as cancer and therefore can be used in the treatment of some diseases such as cancer[32]. The current findings of the study showed that combined treatment of Exo and Mel on TNBC cells induced more apoptosis than the other Exo and Mel groups. The combined application of melatonin and exosome at the end of 24 h induces more apoptosis in the cells, while the combined treatment applied for 48 h causes necrosis instead of apoptosis in TNBC cells. Apoptosis plays an important role[35]. Accordingly, it is possible to say that co-administration of melatonin and exosome and repeating these doses every 24 h will further induce apoptosis. It was observed that the percentage of late apoptosis in the 72h group in which 2.5 mM+ 100 µg/mL Mel/Exo was applied was lower than the 24 h result. The percentage of cell viability in the 72 h groups increased compared to the 48 h group and decreased compared to the control group. Based on these results, the effect of melatonin may be lost depending on the culture conditions and the waiting time. As a result, the effect exosomes must have lost intercellular communication due to time constraints as long as they are not renewed *in vitro*. And also the most effective cytotoxic effect was observed in the 48h group that was administered 2.5 mM+100 µg/mL Mel/Exo combined therapy. This exosomal enhancer was mediated by induction of apoptosis, cytotoxic effect and combination with melatonin. To date, no available published data have investigated the effect of gradually increasing melatonin and exosomes and combined exosome-melatonin on TNBC cells. Therefore, this study needs to be done *in vivo* to establish a treatment protocol for TNBC patients.

Conclusion

TNBC is an aggressive and rapidly mutating cancer type. Because of the heterogeneity and biological barriers of tumours, it is often difficult for conventional drugs to combat tumours to strike the balance between optimal drug efficacy and minimal side effects. In clinical practice, most anti-cancer agents with poor bioavailability and poor *in vivo* stability are toxic to cause damage to normal cells. Recently, combined melatonin and mesenchymal stem cell-derived exosomes have provided a promising therapeutic option for the treatment of several diseases, but this treatment approach has not yet been tested against TNBC. This is the first study to report a therapeutic effect by combining MSC-derived exosomes and melatonin on TNBC cells.

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Conflict of interest

The author declares no conflicts of interest.

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