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Melatonin abrogates liver, ovarian, and uterine toxicities induced by tamoxifen in a breast cancer mouse model

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Melatonin is known for its efficacy in breast cancer treatment. However, the safety profile of melatonin, particularly its effect on liver, uterus and ovaries is largely unknown. Here, we explored the safety profile of melatonin using virgin female mice of the Swiss albino strain. Further, we investigated whether melatonin can overcome liver, ovaries and uterine toxicities which are induced by tamoxifen using N,N-dimethylbenzylamine (DMBA) induced breast cancer mouse model? Treatment of tamoxifen after breast cancer induction in mice resulted in reduction of breast masses but severe pathological abnormalities like liver steatosis, hyper ovulation, ovarian cysts, uterine glands dilatations and endometriosis were observed in treated animals. Whereas, melatonin when used in combination with tamoxifen helped to reduce the mouse mammary tumor volume and significantly decreases liver enzymes, steroid hormones and oxidative stress. Melatonin also reverted the liver, ovarian and uterus toxicity induced by tamoxifen. The results have demonstrated that tamoxifen when used as combination therapy with melatonin serve as an effective anti-breast cancer molecule with minimum liver, ovarian and uterus toxicities.

Keywords: Anticancer, Dimethylbenzylamine induced breast cancer, Hyper Ovulation, Endometrium

Breast cancer is the leading cause of mortality in women worldwide, and it is the second most common type of cancer[1]. There were 2,261,419 new cases of breast cancer reported among the women worldwide in 2020 which account 11.7% of all the cancers[2]. The hormone receptors (estrogen, progesterone, human epidermal receptor 2) positive breast cancer accounts almost 70 to 80% of all breast cancers[3]. The effective therapy which could benefit the HR-positive (HR+) breast cancer patients is to inhibit the estrogen synthesis. Selective estrogen receptor modulators such as tamoxifen or aromatase inhibitors are routinely used to treat HR positive breast cancer. Estrogen receptor modulators especially tamoxifen resulted unwanted side effects and toxicities in human breast cancer patients and in breast cancer animal models[4]. Thus, research for safe estrogen modulators with minimum toxicities is an utmost need in modern era to treat breast cancer and to control the breast cancer related mortalities in human.

Melatonin is a hormone produced by the pineal gland during the dark hours at night that regulates sleep and wakefulness. Melatonin acts as a selective estrogen receptor modulator and inhibits transcription of estrogen receptor (ER) alpha. Melatonin protects against variety of diseases such as diabetes, obesity, gastrointestinal disorders, immune disorders, cardiovascular diseases, and neurodegenerative diseases[4] and also implicated in the treatment of various hormone dependent cancers[5]. Melatonin either alone or in combination with other chemotherapies has significantly decreased the tumor frequency, lengthened tumor latency, increased tumor-suppressive effects of used anticancer substance in rat mammary carcinogenesis, and reduced tumor volume in N,N-Dimethylbenzylamine (DMBA) induced breast cancer animal model[6]. Melatonin has been considered as a safe choice of drug to treat breast cancer but the data regarding the experimental evidence about melatonin related toxicities in human patients or animal models is very limited. In this study, we investigated the comparative efficacy of melatonin and tamoxifen against breast cancer and studied the relative level of toxicity induced by these two estrogen receptor modulators on breast, ovaries, uterus and liver using DMBA induced breast cancer animal model.

Material and Methods

Chemicals

N,N-Dimethylbenzylamine DMBA (Product Number 185582) was purchased from Sigma Aldrich (Sigma

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chemicals Co., St. Louis, MO USA. tamoxifen (Nolvadex-D) was purchased from AstraZeneca UK Limited and melatonin was purchased from Puritans Pride, Inc., Holbrook, USA.

Animals

Twelve weeks old virgin female mice of Swiss albino strain were used for this study. The study was approved by the graduate Research Committee of the College of Science, Department of Zoology, King Saud University (Riyadh, Saudi Arabia). The animals were maintained and used following national and international Guide lines for the Care and Use of Laboratory Animals.

Breast cancer induction

Forty-five virgin female mice received single doses (50 mg/kg) of DMBA following the protocol as described previously[7]. The DMBA was injected in the fat pad of the last pair of breasts. Five mice were randomly sacrificed after 10 days post injection and then subjected to histopathological examination to confirm the induction of breast cancer.

Experimental design

The mice were divided into following five experimental groups having 10 mice in each group as indicated here. Control, oral administration of saline; DMBA, 50 mg/kg of DMBA; DMBA + tamoxifen, oral administration of (35 mg/kg) tamoxifen after the induction of breast cancer; DMBA+ melatonin, oral administration of (3 mg/kg) melatonin after the induction of breast cancer; and DMBA+ tamoxifen+ melatonin, oral administration of (3 mg/kg) melatonin and (35 mg/kg) tamoxifen after the induction of breast cancer.

Breast, liver, and ovary indices

At the end of the experimental period, each mouse was weighed and their left breasts, livers, and left ovaries were removed and weighed. Finally, breast, liver, and ovary indices were calculated by dividing the breast, liver, and ovary by the body weight and then multiplying by 100. At the end of experiment mice were subjected to anesthesia then sacrificed. The means and SD were calculated by statistical software SPSS version 16 (SPSS Inc. Released 2007. SPSS for Windows, Version 16.0. Chicago, SPSS Inc)

Biochemical analysis

Blood samples were collected and drawn into centrifuge tubes. The samples were centrifuged at 1008 g-force to separate the sera and stored at −80 ° C until the assay. The serum samples were used for the estimation of liver enzymes alanine amino transferase (ALT) and aspartate amino transferase (AST), sex hormones (estrogen and progesterone). Liver homogenate was used for the estimation of MDA, GSH, catalase, and NO.

Estimation of Aspartate Amino transferase (AST)

Aspartate aminotransferase (AST) activity in treated mice were calculated using aspartate aminotransferase (AST) Activity Assay kit cat# MAK055 from Sigma- Aldrich (St. Louis, MO 63103, USA) following methods essentially as described[8]. Aspartate amino transferase (AST) catalyzes the transfer of the amino group from aspartate to 2-oxoglutarate, forming oxalacetate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm, by means of the malate dehydrogenase (MDH) coupled reaction.

Estimation of Alanine Aminotransferase (ALT) *activity*

Alanine aminotransferase (ALT) activity in treated mice were calculated using alanine aminotransferase (ALT) activity Assay kit cat# MAK052 Sigma (Sigma-Aldrich St. Louis, MO 63103, USA) following instruction of manufacturer. Alanine amino transferase (ALT) catalyzes the transfer of the amino group from alanine to 2-oxoglutarate, forming pyruvate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm, by means of the lactate dehydrogenase (LDH) coupled reaction.

Estrogen and progesterone

To estimate the level of estrogen, the competitive inhibition enzyme immunoassay technique was utilized using 17 beta Estradiol ELISA Kit, cat # ab108667 (Abcam, Cambridge, MA, UK) following protocol as described by manufacturer.

Progesterone ELISA was used for the quantitative measurement of progesterone in mouse serum using Progesterone ELISA kit, Catalog Number SE120102 Sigma-Aldrich St. Louis, MO 63103, USA.

Estimation of levels of antioxidants and oxidative stress markers

The liver homogenate of each animal in all groups was subjected to the determination of oxidative stress level.

Malondialdehyde (MDA)

Lipid peroxidation was estimated according to the method established by and as described previously[9]. About 1.0 mL of trichloroacetic acid 10% and 1.0 mL of thiobarbituric acid 0.67%, was mixed followed by heating in a boiling water bath for 30 min.

The thiobarbituric acid reactive substances were determined by the absorbance at 535 nm and expressed as the MDA equivalents formed.

Glutathione (GSH)

GSH was determined chemically in the liver homogenate using Ellman's reagent and essentially following the same as described previously[10]. The method is based on the reduction of Ellman's reagent 5,5-dithiobis (2-nitrobenzoic acid) with GSH to produce a yellow compound. The chromogen was directly proportional to GSH concentration, and its absorbance was measured at 405 nm.

Nitric oxide (NO)

In an acid medium and in the presence of nitrite, the formed nitrous acid diazotizes sulphanilamide, which is coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish-purple colour, which was measured at 540 nm.

Antioxidant enzyme catalase (CAT)

Catalase reacts with a known quantity of H_2O_2. The reaction was stopped after exactly 1.0 min with a catalase inhibitor. In the presence of horseradish peroxidase, the remaining H_2O_2 reacts with 3,5-dichloro-2-hydroxybenzene sulfonic acid and 4-aminophenazone to form a chromophore with a color intensity inversely proportional to the amount of catalase in the original sample (measured at 240 nm).

Histological examination

The breast pieces of the liver, ovary, and uterus were collected and fixed in 10% neutral buffered formalin. Following fixation, the specimens were dehydrated in an ascending series of alcohols, embedded in wax, and then sectioned to a 5 μm thickness. The sections were stained with hematoxylin and eosin and Masson's trichrome following standard protocol.

Statistical analysis

One-way analysis ANOVA and the statistical comparisons among the groups were completed with Duncan's test using a statistical package program (SPSS version 16.0) to determine the data mean ± Standard deviation (SD). P values ≤0.05 was considered significant.

Results

The organ indexes of treated mice and control group has been shown in Table 1. The mice group injected with DMBA showed significant increase in breast organ index. The organ index of the group injected with DMBA and treated with melatonin showed significant increase in breast index as compared to control group, and also significant decrease in breast index as compared to mice group injected with DMBA alone. The mice group injected with DMBA and received combined doses of tamoxifen and melatonin showed significant decrease in breast index and insignificant decrease in liver and ovary indexes as compared to DMBA alone injected mice group.

Table1 — Organ indexes of control and experimental groups

	Breast Index	Liver Index	Ovary Index
Control	0.29±0.02	6.4±0.3	0.17±0.04
Melatonin	0.31±0.0	6.3±0.3	0.15±0.09
Melatonin+Tamoxifen	0.21±0.04	7.1±0.2	0.17±0.04
DMBA	0.61±0.07[a]	7.0±0.2	0.19±0.03
DMBA+Tamoxifen	0.29±0.05[b]	6.9±0.2	0.20±0.03
DMBA+Melatonin	0.44±0.04[a,b]	6.4±0.2	0.19±0.03
DMBA+Tamoxifen +Melatonin	0.33±0.05[b]	6.7±0.3	0.18±0.01

[Data = mean ± standard error of the mean. [a] significant difference between control and experimental groups; and [b] significant difference between DMBA group and experimental groups]

Table 2 — Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels measured in control and treated mouse groups

	ALTu/l	ASTu/l
Control	37±0.1	110±0.3
Melatonin	40±0.6	112±1.6
Melatonin + Tamoxifen	51±0.6[a]	142±1.2[a]
DMBA	85±0.8[a]	244±6[a]
DMBA + Tamoxifen	76±0.8[a,b]	185±0.9[a,b]
DMBA+Melatonin	69±04[a,b]	170±.1[a,b]
DMBA+Tamoxifen+Melatonin	58±0.5[a,b]	175±.6[a,b]

[Data = mean ± standard error of the mean. [a] significant difference between control and experimental groups; and [b] significant difference between DMBA group and experimental groups]

The effect of melatonin, DMBA, and melatonin combined with tamoxifen on liver enzyme was estimated in treated mice group and compared with control group. As shown in Table 2, Mice group received melatonin displayed insignificant increase of ALT and AST levels as compared to control group. Whereas, mice group received combined dose of melatonin with tamoxifen showed significant increase in ALT and AST levels as compared to control group. Mice group injected with DMBA showed significant increase in ALT and AST enzymes as compared to control group. Mice group received combined dose of melatonin with tamoxifen after breast cancer induction by DMBA injection showed significant increase compared to control group and significant decrease compared to group injected with DMBA. Melatonin exposure did not induce any toxicity in treated mice

group, but rescued the liver damage induced by DMBA by altering ALT and AST levels.

Hormonal analysis

Estrogen

The effect of melatonin treatment on sex hormones level is shown in Table 3. Estrogen level showed significant increase in groups received melatonin as compared to control. The group received combined doses of melatonin with tamoxifen revealed significant decrease compared to control group. Whereas, mice injected with DMBA displayed significant increase in estrogen level as compared to control group. Moreover, mice group received melatonin alone or combined doses of melatonin with tamoxifen after induction of breast cancer by DMBA showed significant decrease in estrogen level as compared with mice group injected with DMBA and control group.

Progesterone

Progesterone level showed insignificant decrease in group received melatonin compared to control group. While mice group received combined doses of melatonin with tamoxifen had no difference compared to control group. The mice group injected with DMBA revealed significant increase in progesterone level as compared to control group. Group received combined doses of melatonin with tamoxifen after cancer induction by DMBA injection showed insignificant increase compared to control group and insignificant decrease compared to DMBA group.

Antioxidant analysis

Malondialdehyde (MDA)

The response of breast cancer inducted animals to oxidative stress upon exposure to melatonin or combined doses of melatonin and tamoxifen was evaluated by measuring the levels of oxidative stress enzymes like malondialdehyde (MDA), Glutathione (GSH), Catalase (CAT), and nitric oxide (NO), in these animals and were compared with control mice groups. The data is presented in Table 4. The breast cancer control animals (which were not injected with DMBA) showed insignificant increase of MDA level upon exposure to melatonin as compared to animals which were not treated with melatonin, while combined treatment of melatonin and tamoxifen raised MDA level significantly in breast cancer control animals' groups. The mice group in which breast cancer was induced by DMBA showed a significant increase in MDA level which was rescued by melatonin alone or combined doses of melatonin with tamoxifen (Table 4).

Glutathione (GSH)

As shown in Table 4, melatonin did not alter the glutathione level in treated mice group when administered alone as compared to control group. The glutathione levels were significantly decrease in mice groups who received either tamoxifen alone or combined doses of melatonin and tamoxifen as compared to control group. The mice group in which breast cancer was induced with DMBA revealed a significant decrease in glutathione levels as compared to mice group which were not injected with DMBA. The glutathione levels elevated significantly in mice group who received melatonin after induction of cancer by DMBA as compared to control group (without DMBA). An increase in glutathione level has also been observed in mice group who received combined doses of melatonin and tamoxifen after induction of breast cancer by DMBA comparing with mice group injected with DMBA but not treated with melatonin and tamoxifen.

Catalase (CAT)

The response of mice group to catalase activity with or without cancer induction and treated with melatonin,

Table 3 — The estimation of estrogen and progesterone among control and experimental groups

	Estrogen pg/mL	Progesterone pg/ mL
Control	63±0.2	0.2±0.07
Melatonin	46±0.05[a]	0.1±0.05
Melatonin + Tamoxifen	45±0.3[a]	0.2±0.05
DMBA	103±0.4[a]	0.8±0.29[a]
DMBA + Tamoxifen	58±0.3[a,b]	0.3±0.24
DMBA+Melatonin	44±0.2[a,b]	0.4±0.19
DMBA+Tamoxifen +Melatonin	50±0.05[a,b]	0.3±0.05

[Data = mean ± standard error of the mean. [a] significant difference between control and experimental groups; and [b] significant difference between DMBA group and experimental groups]

Table 4 — Estimation of MDA, GSH, CAT and NO (mg/g tissue) among control and experimental groups

	MDA	GSH	CAT	NO
Control	31±0.1	52±0.2	6±0.4	70±0.8
Melatonin	40±0.1	50±0.1	4.1±0.02	75±1.8
Melatonin+Tamoxifen	45±0.1[a]	41±0.2[a]	3.7±0.3	94±0.9[a]
DMBA	67±0.2[a]	27±0.1[a]	2±0.2[a]	109±1[a]
DMBA+Tamoxifen	56±0.2[a]	38±0.1[a,b]	3.7±0.3	103±0.3[a]
DMBA+Melatonin	48±0.4[a,b]	47±0.5[a,b]	5±1[b]	73±1.2[b]
DMBA+Tamoxifen +Melatonin	50± 0.3*[a,b]	37± 0.3[a,b]	5± 0.4[b]	88± 0.8[a,b]

[Data = mean ± standard error of the mean. [a] significant difference between control and experimental groups; and [b] significant difference between DMBA group and experimental groups]

tamoxifen and combined doses of melatonin and tamoxifen is presented in Table 4. CAT levels in groups of mice received melatonin, tamoxifen and combined doses of melatonin and tamoxifen showed insignificant decrease as compared to untreated control group. Whereas, group injected with DMBA showed significant decrease compared to control group. The mice groups, received tamoxifen after cancer induction revealed insignificant increase in catalase as compared to group injected with DMBA. Mice treated with melatonin and combined doses of melatonin with tamoxifen after induction of breast cancer by DMBA showed significant increase to catalase as compared to group injected with DMBA alone (Table 4).

Nitric oxide (NO)

NO levels increased insignificantly in group of mice received melatonin as compared to control group (Table 4). Whereas, groups received either tamoxifen or combined doses of melatonin with tamoxifen revealed significant increase in NO levels as compared to control group. In addition, the breast cancer inducted mice group displayed significant increase in NO levels as compared to control group. Moreover, mice group treated with tamoxifen after cancer induction by DMBA showed insignificant decrease in NO levels as compared to group injected with DMBA and not treated with tamoxifen. Mice groups treated with melatonin after cancer induction showed significant decrease in NO levels as compared to group injected with DMBA and not treated with melatonin. The NO levels were significantly decreased in mice group treated with combined doses of melatonin and tamoxifen after breast cancer induction as compared to control group (Table 4). It shows that the NO levels increased significantly in DMBA induced breast cancer mice group.

Histopathology of breast tissue of mice with or without breast cancer induction and treated with melatonin and combined doses of melatonin and tamoxifen

The representative micrograph showing the histopathology of breast tissue of control mice and mice group in which breast cancer were induced by DMBA is shown in Fig. 1. Control breast showed normal breast tissue of virgin mice, revealed few inactive glands with small ducts (Fig. 1 A-C). The control mice group (without breast cancer induction) treated with melatonin alone showed normal histology of breast, having normal adipose connective tissue which contained inactive mammary glands and ducts (Fig. 1 D-F). Breast sections of mice received combined doses of melatonin and tamoxifen had normal breast tissue with inactive mammary glands and the ducts indulged in adipose tissue (Fig. 1 G-I).

The histopathology of breast tissue of mice injected with DMBA to induce the breast cancer revealed multiple foci of tumor nests and ribbons, invasive ductal carcinoma of no specific type (NST) appeared surrounded mammary glands and ducts and infiltrate the adipose tissue (Fig. 1 J-L). High magnification of tumor ribbon revealed atypical cells with variation in shape and size (tripolar, quadripolar and elongated) failed to develop orientation to one another (anaplasia), nuclear irregularity and hyperchromatic resulted in hyperplasia (Fig. 1L). Other sections showed dilated mammary glands and ducts infiltrated by fibers and inflammatory cells, in addition to appearance of small blood vessel nearby tumor referred to angiogenesis process (Fig. 1K).

Breast section of mice group received melatonin after induction of breast cancer have been shown in Fig. 1 M-O. The breast tissue showed marked reduction of tumor masses, dilated ducts infiltrated by some atypical tumor cells with desmoplastic reaction

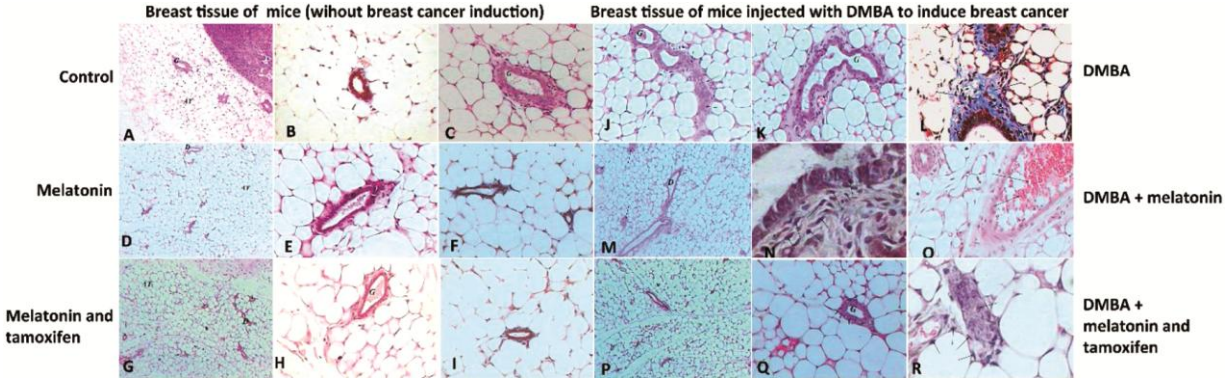

Fig. 1 — Histopathology of breast tissue of mice with or without breast cancer induction and treated with melatonin, and combined doses of melatonin and tamoxifen. H&E: Ematoxylin and Eosin stain, M.Tr: Trichrome staining

as abundant fibrosis was seen (Fig. 1M). High magnification revealed penetration of tumor cells to the basement membrane of the duct (Fig. 1N). The vascular ectasia (appearance of dilatation and congestion of blood vessels) has been observed as well (Fig. 1O).

The breast tissue histology of mice in which breast cancer was induced with DMBA and subsequently treated with combined doses of melatonin and tamoxifen (Fig. 1 P-R) showed healthy breast tissue with inactive mammary glands and ducts glands infiltrated by a few numbers of tumor cells (Fig. 1P), leukocytes and debris of degenerated tumor cells (Fig. 1Q), high magnification revealed a few numbers of atypical and degenerated tumor cells (Fig. 1R).

Histopathology of liver tissue of mice with or without breast cancer induction and treated with melatonin, and combined doses of melatonin and tamoxifen.

Control liver section showed normal structure of liver consists mainly of center vein surrounded by anastomose network of hepatocytes strands, separated from each other by blood sinusoids with abundant kupffer cells (Fig. 2 A & B). A The histology of liver tissue of control mice which were treated with melatonin had normal liver architecture with abundant kupffer cells (Fig. 2C). Section stained with Massons trichrome showed no depositions of collagenous fibers (Fig. 2D). The liver tissue from control mice which were treated with combined doses of melatonin and tamoxifen revealed healthy hepatocytes with active of Kupffer cells , except appearance of steatosis (Fig. 2E). Masson's trichrome stained section showed reduction in collagenous fibers compared to tamoxifen group in addition to a few numbers of leukocytes (Fig. 2F).

Liver of mice injected with DMBA group displayed histopathological alterations manifested by congested central veins infiltrated by leukocytic aggregations, other veins appeared filled with oedema, some hepatocytes looked degenerated (Fig. G).Section stained with Masson's trichrome showed layers of collagenous fibres stained blue with inflammatory cells surrounded congested vein. Moreover, multiple necrotic foci appeared surrounded by collagenous fibers and infiltrative cells mixed with erythrocytes due to hemorrhage (Fig. 2H).

The liver tissue of mice group treated with melatonin after breast cancer induction displayed less pathological signs as compared to untreated group and showed fewer micro vesicles of steatosis and a smaller number of degenerated hepatocytes (Fig. 2I). Liver section stained with Masson's trichrome showed no fibrosis (Fig. 2J). The mice group treated with combined doses of melatonin and tamoxifen had healthy hepatocytes and central vein with a few numbers of inflammatory cells in liver. Moreover, activated Kupffer cells were also observed, in addition to the presence of some macrophages. The collagenous fibers were also fewer as compared to untreated group (Fig. 2 I & J).

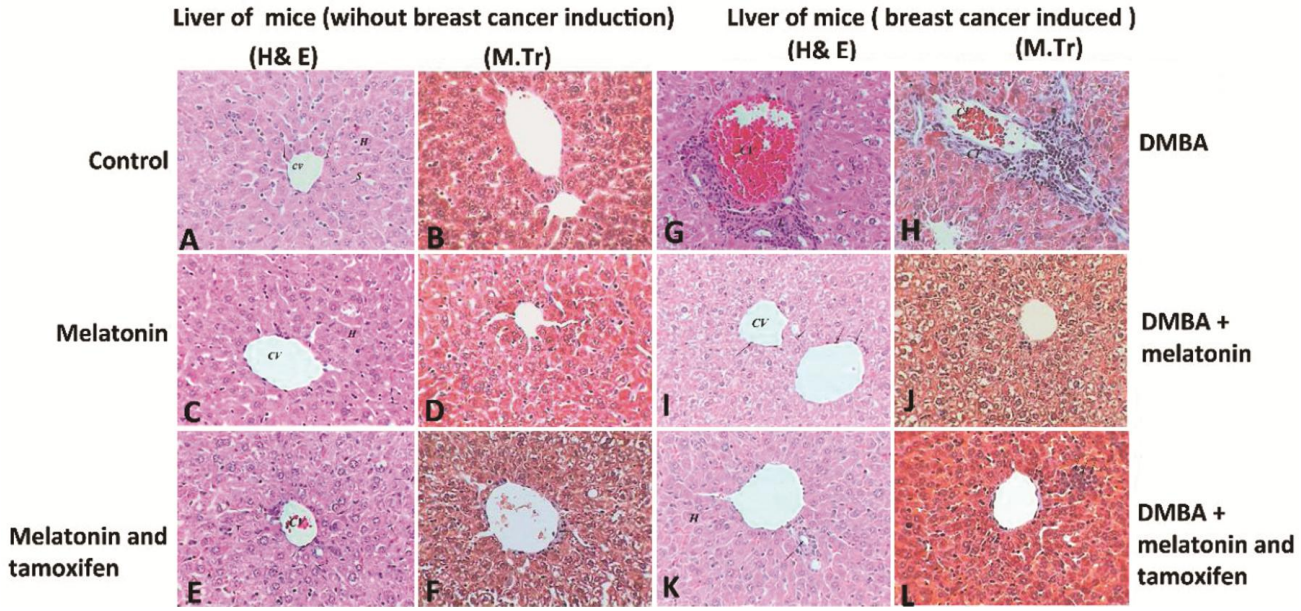

Fig. 2 — Histopathology of liver tissue of mice with or without breast cancer induction and treated with melatonin, and combined doses of melatonin and tamoxifen. H&E: Ematoxylin and Eosin stain, M.Tr: Trichrome staining

Histopathology of ovary of mice treated with melatonin, and combined doses of melatonin and tamoxifen

Control ovary showed small almond shaped structure of normal ovary, that has a cortex which is where ovarian follicles can be formed and a highly vascular medulla with coiled arteries called helicrine arteries (Fig:.3). The ovary cortex contains primordial follicles around the edge (Fig. 3A), there are fewer follicles in different stages of development as primary follicle, antral follicle and corpus luteum (Fig. 3B). Microscopic examination of ovary section of control mice group (without breast cancer induction) which were treated with melatonin revealed healthy ovary having of ovarian follicles at different developmental stages (Fig. 3C), primordial, primary follicles) and antral follicles (Fig. 3D). The control mice group (without breast cancer induction) treated with combined doses of melatonin and tamoxifen had healthy ovaries (Fig. 3 E & G))

The histology of ovaries of mice group in which breast cancer was induced by DMBA had normal and healthy ovary with multiple ovarian follicles (Fig. 3G)), and without fibrosis (Fig. 3 H). The mice group which were treated with melatonin after breast cancer induction had healthy ovaries, having ovarian follicles at different developmental stages, especially primary and antral follicles (Fig. 3 I & J). Moreover, the mice group which were treated with combined doses of melatonin and tamoxifen after breast cancer induction revealed normal number of follicles in different stages, and normal vascularectasia (Fig. 3 K & L).

Histopathology of uterus of mice with or without breast cancer induction and treated with melatonin, and combined doses of melatonin and tamoxifen.

Control uterus section (without cancer induction) showed normal architecture, consisting mainly endometrium and myometrium. Endometrium is lined with columnar ciliated and secretory epithelia. Stroma of the endometrium contains primarily non bundled collagen fibers, uterine glands penetrate the full thickness of endometrium. Myometrium displays the thickest tunic of the uterus, it contains interwoven layers of smooth muscle fibers separated by connective tissue containing venous plexus and lymphatics (Fig. 4 A & B)

Organ histology of uterus sections of un-induced breast cancer mice group which were treated with melatonin showed healthy uterine sections manifested by normal endometrium, uterine glands and myometrium (Fig. 4C). Section stained with Masson's trichrome showed diffuse mucin secretion non-bundled collagenous fibers that stained blue (Fig. 4D). The control mice which were treated with combined doses of melatonin and tamoxifen displayed wide dilatation of uterine glands in endometrium with hypersecretion (Fig. 4 E & F)

The histology of uterus of breast cancer induced mice group revealed healthy uterus with normal endometrium and myometrium (Fig. 4G). Uterus endometrium looked blue due to secretions of mucin (Fig. 4H). Uterus section of the mice group in which breast cancer was induced and then treated with melatonin, showed healthy uterus with limited dilatation of uterine glands in endometrium

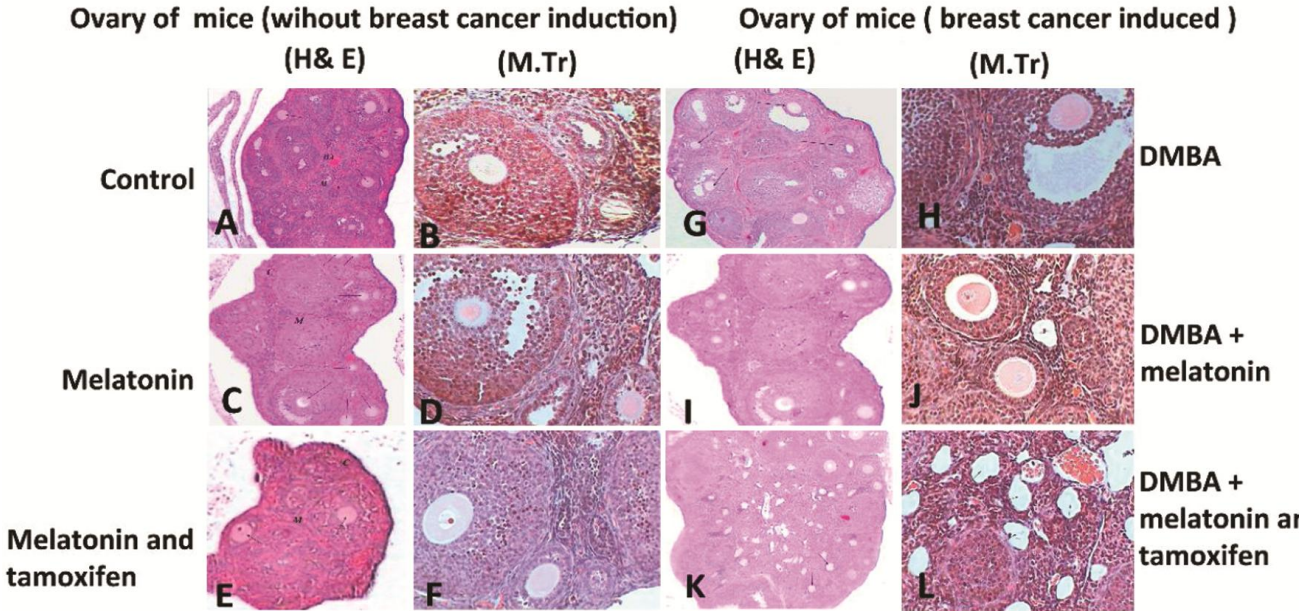

Fig. 3 — Histopathology of ovary of mice with or without breast cancer induction and treated with melatonin, and combined doses of melatonin and tamoxifen. H&E: Ematoxylin and Eosin stain, M.Tr: Trichrome staining

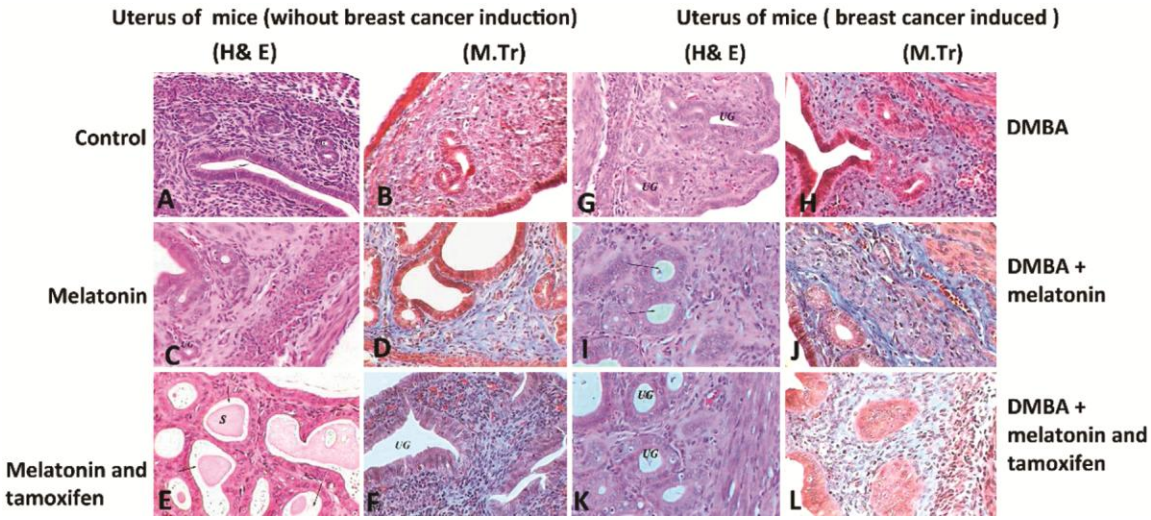

Fig. 4 — Histopathology of uterus of mice with or without breast cancer induction and treated with melatonin, and combined doses of melatonin and tamoxifen. H&E: Ematoxylin and Eosin stain, M.Tr: Trichrome staining

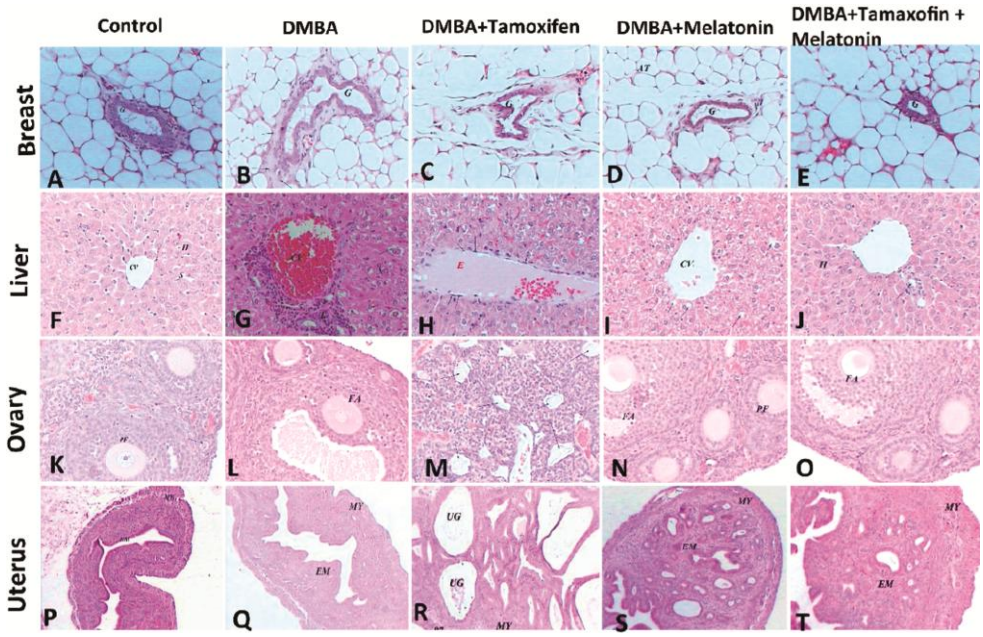

Fig. 5 — Histopathology of liver, ovary, and uterus showing comparative efficacy and toxicities of melatonin and tamoxifen in control and DMBA induced breast cancer in mouse.

and normal myometrium (Fig. 4 I & J). The histology of uterus section in DMBA induced breast cancer mice which were treated with combined doses of melatonin and tamoxifen showed healthy uterus structure with normal endometrium, myometrium and without endometriosis or dilatation of uterine glands (Fig. 4 K & L).

Tamoxifen induced hepatotoxicity in treated mice group

The tamoxifen induced hepatotoxicity in mice group in which breast cancer was induced by DMBA injection and treated with tamoxifen (Fig. 5H). The liver of treated mice had dilated vein congested with edema (E) and kupffer cell (arrows) microvesicles (red arrows). The hepatotoxicity has not been noticed in the mice group in which breast cancer was induced and subsequently treated with melatonin and the liver histology showed that the mice had healthy liver with central vein (CV) and micro vesicles (arrows) (Fig. 5I).

Discussion

Estrogens and estrogen receptors (ERs) play very important role in breast cancer progression [11]. The activation of ERs via estrogen results mammary cell

proliferation in healthy mammary gland. However, altered ER signaling is associated with abnormal cell proliferation as well as the initiation and progression of breast cancer[12,13]. Estrogen activates transcription of estrogen responsive genes by binding to ER-α, and ER-β and thus accelerate tumor cell proliferation[14]. The estrogen or progesterone modulation has been found in majority of breast cancer in women and hence inhibition of estrogen synthesis by using selective estrogen receptor modulators (SERMs) have somehow benefited the patients in past. Tamoxifen is one of the well-known SERM and has shown promising results in clinical trials[15]. However, a number of studies (our unpublished results)[16]; have reported tamoxifen-related toxicities in breast cancer patients and animal models, such as hepatocellular cancer in rats risk of endometrial cancer in experimental models and in human patients[17-19]. The development of resistance against tamoxifen has also been seen in some breast cancer patients[20], which demands switching to other SERMs as safe and an effective alternative to treat breast cancer[21].

Melatonin (N-acetyl-5-methoxy tryptamine) is a naturally occurring derivative of the amino acid tryptophan and was first isolated from bovine pineal gland. Melatonin is well-known regulator for the control of the sleep/wake cycle, as well as for circadian rhythms. Modulation of immune response[22], and antioxidative properties[23-28] has also been attributed to melatonin. Melatonin is also involved in preventing tumor initiation, promotion, and progression [29-30]. Melatonin is a known inhibitor of estrogen and gonadotrophin production and it is postulated that a decrease in melatonin levels might stimulate the growth and development of breast cancer in humans. Moreover, the impaired melatonin secretion has been observed in breast, endometrial, and colorectal cancer patients 30 which support the hypothesis of potential involvement of pineal gland and its secretion "melatonin" in breast cancer progression. Animal studies have shown that melatonin attenuates the incidence of experimentally induced breast cancers[32-35], but there are very few studies which have studied the comparative toxicities between melatonin and tamoxifen in animals models, hence, in the present study we tried to find out the efficacy of melatonin over tamoxifen to curb the experimentally induce breast cancer in animal model and also to find out which one among these two selective estrogen receptor modulators is more safe to use by studying the comparative toxicity profile in experimental animal model system.

The experimental data; the organ indexes (Table 1), blood biochemistry Tables 2, 3 and 4) and organs histology (Fig. 1-4) from this study has indicated that melatonin could be better choice as compared to tamoxifen to treat breast cancer due to the fact that melatonin did not induce toxicities that were typical manifestation of tamoxifen. The data presented in Table 4, clearly showed that the glutathione level in mice treated with melatonin alone after induction of breast cancer were significantly higher as compared with combined doses of melatonin and tamoxifen and also with tamoxifen alone. The present study illustrated that the co-administration of melatonin with tamoxifen after DMBA-induced breast cancer resulted in a reduction in the breast index due to the reduction of tumor masses in the breast tissue, decreased levels of liver enzymes, and the reduction of oxidative stress compared to the breast cancer group and the group treated with tamoxifen. Moreover, a significant reduction in steroidal hormone levels was seen in comparison to the breast cancer group and the group treated with tamoxifen.

The present findings revealed that mice injected with DMBA in fat pad of breast showed severe pathological signs manifested by dilated mammary gland infiltrated by desmoplastic reaction and angiogenesis in the breast and a congested central vein infiltrated by leukocyte aggregations besides degenerated hepatocytes in the liver. Whereas, ovaries and uterus of breast cancer induced mice were normal and had normal follicular antrum, endometrium and myometrium. Tamoxifen helped to reduce the tumour mass in the breast tissue of the mice group treated with tamoxifen after breast cancer induction, but still a dilated mammary gland can easily be visualized in the breast. However, mice breast treated with melatonin after breast cancer induction showed normal inactive mammary glands. Tamoxifen treatment after breast cancer induction by DMBA resulted in hepatotoxicity represented by dilated vein congested with edema and highly activation of kupffer cell. The hepatotoxicity has not been noticed in the mice group in which breast cancer was induced and subsequently

Melatonin has been used for preventing and treating liver injuries and diseases and it also protected the liver damage in experimental animals which were exposed to variety of hepato-toxins[36-37]. The present study illustrated that the administration of

melatonin after breast cancer induction resulted in the reduction of pathological alterations produced in the liver, such as inflammation and fibrosis due to DMBA. Melatonin administration after the induction of breast cancer displayed a decrease in liver enzymes and oxidative stress by reducing the levels of MDA and NO, as well as increased levels of GSH and catalase, compared to the breast cancer group and the group treated with tamoxifen after breast cancer induction by DMBA.

The histology of ovaries of mice group treated with tamoxifen after breast cancer induction showed hyper ovulation as lot of primary follicles (PF) and multiple corpus leutea (arrows) were present (Fig. 5M). The melatonin did not induce ovarian toxicity in mice group treated with melatonin after breast cancer induction. The ovaries showed normal primary follicle (PF) and follicle antral (FA) (Fig. 5N). The tamoxifen also induced uterus toxicity in mice group treated with tamoxifen after breast cancer induction. The organ histology showed wide dilatation of uterine glands (UG) myometrium (MY) (Fig. 5R), while uterus mice treated with melatonin after breast cancer induction showed normal structure to endometrium (EM) and myometrium (MY) (Fig. 5S). The melatonin also ameliorated the tamoxifen associated toxicity. The organ histology of breast cancer induced mice group which were treated with combined doses of tamoxifen and melatonin did not show the toxicities when treated with tamoxifen alone (Fig. 5E, J, O and T). Moreover, the hepatotoxicity, hyper ovulation and endometrium were also observed in tamoxifen treated control mice (mice in which breast cancer was not induced). The tamoxifen associated toxicities in normal mice will be presented somewhere else.

Conclusion

Treatment of tamoxifen after breast cancer induction in mice resulted suppression of tumour, however, severe toxicity hepatotoxicity, hyper ovulation, ovarian cysts, uterine glands dilatations and endometriosis were observed in treated animals. Whereas, melatonin when used in combination with tamoxifen not only helped to reduce the mouse mammary tumour volume but also minimized the toxicities. The data from this study and other publish literature including the clinical trials indicate that melatonin alone or in combination with tamoxifen could be a better choice of treatment for estrogen and progesterone receptor positive breast cancer.

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