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Therapeutic Effect of Microvesicles Derived From BM-MSCS Transplantation And/Or Melatonin In Cuprizone Model of Multiple Sclerosis:A Pharmacodynamic Biochemical Assay

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Abstract

Multiple sclerosis (MS) was induced in mice by cuprizone 0.2% (w/w) for five successive weeks, then mice were divided into five groups, first group was normal mice, second group was MS-induced mice, third group was MS-induced mice treated by a single intraperitoneal dose of microvesicles (0.2 mg/kg), fourth group was MS-induced mice treated by a single intraperitoneal dose of melatonin (10 mg/kg) for three successive weeks, and fifth group was MS-induced mice treated by combination of microvesicles and melatonin. Behavioural functions were evaluated by rotarod test, activity cage and Y-maze. We also measured levels of sirtuin1, sirtuin 3, PGC-1 alpha, complex I, complex II, ATP, MDA, GSH, miRNA 155 and 132. Finally, histological and immunohistochemical examinations of the corpus callosum were performed. Results showed impaired behavioural functions, as well as decreased levels of sirtuin 1, sirtuin 3, PGC-1 alpha, complex I, complex II, ATP and GSH, also increased levels of MDA, miRNA 155 and 132 in MS-induced mice. The behavioural and biochemical parameters mostly returned to normal with treatment using melatonin and microvesicles. Histological examination of the cuprizone treated group revealed loss of normal histological profile. However, a single or combined treatment with microvesicles therapy or melatonin revealed substantial improvement.

Keywords: Multiple Sclerosis; Microvesicles; Melatonin;Situin1&3; Antioxidants; miRNA 155 &132; Behaviour stress tests.

1.Introduction:

Multiple sclerosis (MS), also called disseminated encephalomyelitis, is a demyelinating disease that damages the nerve cells insulating covers in the brain and spinal cord **[1]**. The prevalence varies between 60–200 cases per 100,000 in Northern Europe and North America and 6–20 cases per 100,000 in lower-risk areas. MS is caused by neuronal myelin sheath loss as a result of being attacked by autoantigen-specific immune cells. It typically occurs between the ages of 20 and 40. The incidence of MS showed an obvious increase in the 21st century first decade in the Middle East North Africa region; however, it is less than southern Europe but greatly more than SubSaharan Africa **[2].**

Hypoxia and free radical damage can extremely affect brain tissue due to its increased oxygen usage and elevated levels of both transition metals such as iron and polyunsaturated fatty acids **[3]**. MS is characterized by areas of myelin and oligodendrocyte loss, as well as inflammatory cell infiltrates such as lymphocytes and macrophages[4]. Although the mechanisms of tissue damage mechanisms are unknown, recent evidence shows that mitochondrial injury can have a significant role during axonal loss and demyelination **[5]**.

Numerous studies in the last few years have demonstrated that mitochondria are critical for neuronal cell survival and that mitochondrial dysfunction is a precursor to CNS injury that results in neuronal cell death. In traumatic brain injury, mitochondria play an important role as there will be a decrease in ATP synthesis and increase production of free radicals **[6].**

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Mitochondrial DNA (mtDNA) encodes thirteen hydrophobic polypeptide chains for four enzyme complexes of the inner mitochondrial electron transport chain that are involved in oxidative phosphorylation defects**[7],** aberrant mitochondrial gene expression, impaired mitochondrial enzyme activity, and impaired mtDNA repair activity all seem to be a part of MS lesions development and progression **[8]** due to apoptosis occurring subsequent to high metabolic rate as a result of mitochondrial oxidative stress and impaired phosphorylation **[9].**

The most frequently used treatment for multiple sclerosis is immunomodulation, primarily with interferon or glatiramer acetate **[10]**. While currently available immunomodulatory drugs are effective at alleviating symptoms, they do not slow down ongoing neurodegeneration.

MSCs are a heterogeneous multipotent cell population present in the bone marrow. Also, They are referred to as mesenchymal stromal cells or medicinal signalling cells. They can differentiate into different cell types, including adipocytes, chondrocytes, osteoblasts, and myocytes **[11].**

MSCs have demonstrated promising results in damaged tissue repair in a variety of degenerative diseases, both in human clinical trials and animal models, due to their homing ability, which allows them to migrate into injured sites, their ability to differentiate into local components of injured sites, and their capability to secrete growth factors, cytokines, and chemokines that aid in the regeneration of the tissues **[12].**

Additionally, MSCs secrete extracellular vesicles (MSC-derived microvesicles) (MVs), small spherical membrane fragments included in cell-to-cell communication and acting as a vehicle for delivering proteins and coding and non-coding RNAs to target cells, thereby changing the recipient cells' gene expression, differentiation, and proliferation **[13].** Numerous reports suggest that MVs isolated from the medium in which they were cultured can reproduce the regenerative effect of MSCs **[14]**. The implications MVs and their ability to transport messages between cells indicate that utilizing MVs as a drug delivery system or a therapeutic agent may act as a promising method for identifying and causing modulations in the disease progression. Additionally, MVs are strong candidates for CNS disease therapy due to their capability of BBB crossing**[15]**.

MVs have demonstrated regenerative abilities comparable to those of MSCs in a variety of experimental disease models, including liver cirrhosis, myocardial infarction, endotoxin-induced lung injury, acute kidney injury, and ischemic stroke **[14]**. MVs-based regeneration therapy represents a great chance for the development of novel cell-free therapy approaches which could bypass both risks and limitations linked with using engineered or native stem cells **[16]**.

Melatonin is a hormone whose production is primarily by the pineal gland that plays a role in synchronizing the circadian rhythm, which includes sleep-wake cycles and blood pressure regulation **[17,18].**Numerous clinical observations suggest a connection between melatonin and multiple sclerosis **[19]**. Some of its effects are mediated by activating the melatonin receptors, while others are mediated by its antioxidant properties**[18].**

Recent research indicates that all mitochondria-containing cells, including astrocytes and immune cells, can produce melatonin in some form, offering another important target for treating MS.

Along with its anti-inflammatory and antioxidant properties, melatonin enhances mitochondrial function by increasing oxidative phosphorylation and ATP production. Melatonin also promotes remyelination and inhibits demyelination, indicating that its local regulation in white matter astrocytes by apolipoprotein E4 and serotonin availability is critical in multiple sclerosis aetiology, course, and treatment [**20,21].**

The current study aimed to detect the oxidative stress effect and the effect of treatment using MSC-Mvs, Melatonin, and their combination on the function and distribution of mitochondria in young male mice during experimentally induced demyelination by cuprizone, also it was aimed to study the effect of treatment on behavioural changes of mice including cognitive,locomotive and psychological behavioural changes.

2.Materials and Methods

This research was conducted in Egypt at Cairo University Faculty of Medicine, Unit of Medical Biochemistry and Molecular Biology and at the Pharmacology Department, Medicine and Clinical Studies Research Institute, National Research Centre, Dokki, Giza .

2.1) Preparation of the experimental animal:

This study used 50 male C57BL/6 mice of matched age and weight (eight weeks old) from an inbred strain (20 g). Inbreeding was carried out at Cairo University Experimental Animal Unit. Mice were bred and housed under pathogen-free conditions in an air-conditioned animal house. They were exposed to a 12:12h light/dark cycle and fed a semi-purified diet containing 200 casein, 555 sucrose, 100 fat blends, 100 cellulose, 35 mineral mix, and 35 vitamin mix (gm/kg). Mice were allowed to adapt to these conditions for 2 weeks before beginning the experimental protocol. Experiments were performed according to the National Regulations of Animal Welfare and the Institutional Animal Ethical Committee (IAEC).

Approval of the ethics committee of National organization for Drug Control and Research

(NODCAR) was obtained with registration number NODCAR/111/43/19.

2.2) Experimental design Animals were categorized into five groups (each is ten mice):

Group 1 (Normal control): Ten mice normal healthy mice that had free access to standard diet and water were used.

Group 2 (MS induced by cuprizone): For five weeks, ten mice were ad libitum and fed 0.2% (w/w) cuprizone (Sigma) in ground breeder chow **[22]**. This diet causes selective oligodendrocyte death then axons demyelination in the corpus callosum **[23]**.

The other three groups (each ten mice) received the following treatment for three weeks after cuprizone removal and ensuring development of MS as proven by behavioral stress testing of five mice selected randomly from each group:

Group 3: Ten induced MS mice received MVs (A single i.P injection of MSC-MVs 0.2 mg/kg of body weight) **[24]**.

Group 4: Ten induced MS mice received Melatonin (A single i.P injection of melatonin at dose 10mg/kg for three weeks) **[25].**

Group 5: Ten induced MS mice received both MVs and melatonin in the same regimen as groups 3 and 4.

2.3) Preparing, isolating and identifying Bone Marrow mesenchymal cells (BM-MSCs) in culture:

Ten mice's tibias were flushed with phosphate-buffered saline (PBS) for removing BM cells. Isolated nucleated cells were suspended in a complete culture medium supplemented with 0.5% penicillin and streptomycin using a density gradient Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY). For seven days, cells have been cultured at 5% CO2 and 37°C till an 80–90% confluence have been reached. The morphology and FACS analysis of cultured MSCs were used to determine the negativity of CD45+ and the positivity of CD90+ specific to MSCs.

2.4) Preparing and identifying MVs derived from BM-MSCs:

The supernatants of MSCs cultured overnight in RPMI-Fetal Calf Serum-free medium were used to isolate MVs (FCS).To obtain MVs, cell-free supernatants were centrifuged at 100,000 g at 4°C for one hour (Beckman Coulter Optima L-90K ultracentrifuge), washed in serum-free medium 199 containing 25 mM N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES) (Sigma) then ultracentrifuged for another time **[26].**

Purified MVs Transmission electron microscopy (TEM) analysis confirmed their size and spheroid morphology, as well as integrin gene expression analysis using polymerase chain reaction (PCR).

2.4.1-Detection using transmission electron microscopy: After washing, MVs underwent fixation for two hours with 2.5% glutaraldehyde, ultracentrifugation, then were suspended in 100µ l HSA. Onto a formvar/carbon-coated grid, A volume of 20 µl containing MVs was loaded, and using 3% aqueous phosphotungstic acid, and it was negatively stained for one minute (HITACHI, H7650). TEM of purified MVs confirmed their size and spheroid morphology **[27].**

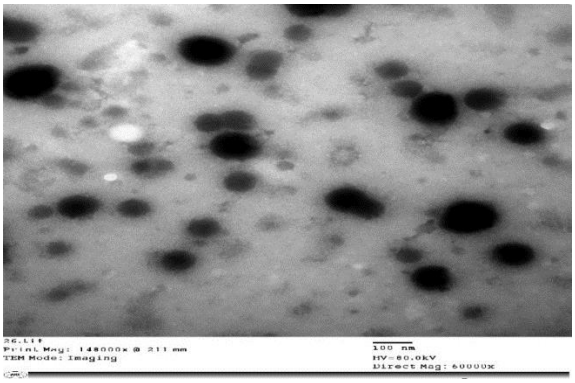

Fig 1: The Electron Microscopic figure of MVs

2.4.2-PCR detection of integrin gene expression: RNeasy purification reagent was used to extract total RNA from isolated MVs (Qiagen). Complementary DNA (cDNA) was synthesized from 5 g total RNA using antisense primer (1 µl (20 pmol)) and 0.8 µl superscript avian myeloblastosis virus (AMV) reverse transcriptase at 37°C for sixty minutes. To perform the PCR, 4 µl cDNA was incubated with 30.5 µl water, 4 µl 25 mM MgCl2, 1 µl dNTPs (10 mM), 5 µl 10 PCR buffer, 0.5 µl (2.5 U) Thermus aquaticus (Taq) polymerase, and 2.5 µl of each primer containing 10 pmol.

The following oligonucleotide primers were used for integrin (gene bank accession number: NM017022.2);Forward, 5′-AATGTTTCAGTGCAGAGC- 3′ and Reverse, 5′-TTGGGATGATGTCGGGAC- 3′. PCR amplification reactions were performed at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s for 30 cycles. The PCR product yielded a 163 bp fragment on 1.5% agarose gel electrophoresis.

2.5) Evaluating behavioural changes of MS mice and the efficacy of treatment was done by Behavioural Stress Tests: motor coordination assessment in the current study was done by the Rotarod (Model No. 7750; Ugo Basile, Varese, Italy), while the Grid floor Activity cage was used in assessing the mice's psychological state (Model No.7430; Ugo Basile, Varese, Italy) **[28],** and the degree of cognition was assessed using a locally made wooden Y-Maze**[29].**

2.5.1) Rotarod Test :for evaluating Motor Co-ordination was done by accelerating the speed .

2.5.1.1.*Training of mice:*Training was done before staring the assessment test of the effect of cuprizone on nervous system and the effect of treatment. Mice were acclimatized for one hour to the test room before doing the test. Mice were placed on the stationary rod.

After being accustomed to the rotarod, mice were trained daily for three training sessions that were separated from each other by ten minutes for three successive days. All mice in the present study were pre-trained on the rotarod apparatus at a fixed speed of four rotations per minute (rpm) in order to reach a stable performance before induction of MS. On the fourth day, the mice were placed on the testing rod and the speed of the rotarod was started at 4 rpm and then increased gradually to reach 40 rpm over 300 s, the average time in seconds spent on the rod for each mouse was detected individually; the time until the mouse fell was a measure of balance of each mouse **[30].**

2.5.1.2. *Behavioural assessment of the effect of cuprizone and treatment*:It was done for all mice in all groups at zero time (baseline record)before adding cuprizone to mice chow, 24 hours following the last dose of cuprizone for ensuring the occurrence of MS in the form of inability of maintanence of mice balance on the rotarod for the same duration as before curizone admistration, which had been given for five successive weeks . Finally the test was done 24 hours after the last dose of treatment with either melatonin alone, BM-MSCs alone or combination of both.

2.5.2) Grid Floor activity cage :for mice psychological state evaluation.

2.5.2.1.*Training of mice:* Mice were acclimatized to the testing room for one hour before starting the test**.**. Each mouse received three five minute training sessions on three consecutive days **[31].**

The training was done before staring the assessment test of the effect of cuprizone on nervous system and the effect of treatment.

2.5.2.2. *Behavioural assessment of the effect of cuprizone and treatment*: It was done for all mice in all groups at zero time (baseline record)before adding cuprizone to mice chow, 24 hours following the last dose of cuprizone for ensuring the occurrence of MS in the form of reduced movement counts across the grid floor cage during the test session,and 24 hours after the last dose of treatment with either melatonin alone, BM-MSCs alone or combination of both. Oscillation information was processed in the activity cage software to provide an index of horizontal movements.The number of mice movements was detected by automatically recording the number of oscillations that take place as a result of horizontal animal movements across the grid floor during the test session which is five minutes for each mouse.

2.5.3) Y-Maze test for evaluating the Cognitive abilities of mice :

2.5.3.1.*Training of mice:* The mice were accustomed to the wooden Y maize by placing each mouse for eight mintes at the centre of the maize and allowing it to move freely and explore the three arms of the maize.Outliers which were mice that didn't move at all or alternated only between two arms or moved longitudinally forth and back across one arm, were excluded from the experiment .

2.5.3.2. *Behavioural assessment of the effect of cuprizone and treatment*: Mice were placed at the centre of the maize and allowed to move freely . The test was done for all mice in all groups at zero time (baseline record)before adding cuprizone to mice chow, 24 hours following the last dose of cuprizone for ensuring the occurrence of MS in the form of disturbed memory characterized by inability to explore all the Y maize arms in the form of either restriction of movements between only two arms without alternation to the third or longitudinal forth and back movements across one arm only during the test session (8 minutes),the final test was done 24 hours after the last dose of treatment with either melatonin alone, BM-MSCs alone or combination of both to evaluate the efficacy of treatment.

Calculations were done by recording the % of spontaneous alternation as a measure of spatial working memory during the 8 minutes test. Percentage of Spontaneous Alternation was calculated %Alternation = [number of alternations/(number of choices-2)]x100 **[29].**

2.6) Statistical Analysis:

ANOVA with two-way analysis of variance was used to compare more than two distinct groups, followed by Tukey Kramer's multiple comparisons test. The groups comparison was made during the same treatment interval. The rotarod and activity cage tests were considered significant at $P < 0.0001$, while the Y-Maze test was considered significant at p 0.1. According to **Jones et al [32]**, for the grid floor activity cage test, a percent (%) of change in movement count was calculated and assumed to be 100% for all mice at baseline, and then a square root transformed per cent was calculated for percent change from the normal base-line value. GraphPad Prism V.6.0 was used for statistical analysis.

2.7) Sample collection and mice scarification:

Animals were sacrificed under anaesthesia by decapitation, and brain tissue was harvested at the planned time (8 weeks) for assessment of the following:

2.7.1- Gene expression of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), Sirtuin-1, Sirtuin-3, miR-132, and miRNA-155 by real-time PCR.

2.7.1.1- Real-time qPCR for miR-132, miR-155, sirtuin-1, sirtuin-3, and PGC-1α gene expression in brain tissue:

Total RNA was extracted from brain tissue homogenates utilizing the SV Total RNA Isolation System (Promega, Madison, WI, USA)

2.7.1.2-Reverse transcription (RT) and real-time quantitative PCR (qPCR):

The reverse transcription of total RNA was carried out in a final volume of 20 µl RT reactions (sixty minutes

at 37 °C followed by five minutes at 95 °C) according to the manufacturer's instructions using the themiScript II RT kit (Qiagen, Valencia, CA, USA). By utilizing a kit named Bioline, a leading life science company headquartered in the United Kingdom (SensiFASTTM SYBRR Hi-ROX One-Step Kit, catalogue no.PI-50217 V), qRT-PCR was done. The primer sequences for the miR-132, miR-155, sirtuin-1, sirtuin-3, PGC-1, and housekeeping gene (18sR) studied in this study are listed in **Table [1]**. The following thermal profile was programmed in the Step One instrument (Applied Biosystems, USA) for reverse transcription-polymerase chain reaction (RT-PCR): 15 minutes at 45 °c for one cycle; 10 minutes at 95 °c for Taq polymerase activation; 40 cycles of 15 seconds at 95 °c, 20 seconds at 55 °c, and 30 seconds at 72 °c for the amplification step. Following reverse transcription-polymerase chain reaction, the data were converted to Cycle threshold (Ct). The Ct values for both the evaluated gene (lncRNA-AF085935) and the housekeeping (reference) gene (18rs) were included in the PCR datasheet. Each target gene's relative quantitation (RQ) is determined by the delta-delta Ct (ΔΔCt) calculation. We determined each gene RQ by taking 2-ΔΔCt.

Table [1]: Primer sequence for the studied target genes.

Symbol of the gene	Primer sequence from 5′- 3′
SIRT1	F:TCAGTGTCATGGTTCCTTTGC R: AATCTGCTCCTTTGCCACTCT
SIRT3	F: GGCCATTTTTGAACTCCCAT R: GGAAATCAACCACATGCAGC
miR-132	F: CGGTGACTCAGCCTAGATGG R: GGACGGGACAGGGAAGGG
miR-155	F: CCGCTTAATGCTAATTGTGATAGG R: GTGCAGGGTCCGAGGT
PGC-1α	F: AAACTTGCTAGCGGTCCTCA R: TGGCTGGTGCCAGTAAGAG
18sRNA	F:CAGCCACCCGAGATTGAGCA R:TAGTAGCGACGGGCGGGTGT

2.7.2-Determination of the mitochondrial complex I (NADH ubiquinone oxidoreductase) and complex II (succinate dehydrogenase (SDH) activity

2.7.2.1.Mitochondrial NADH dehydrogenase activity (complex I) determination:

The specific activity of the enzyme was detected by the usage of the method described by **Birch-Machin et al., 1994[33]**. Complex I activity has been quantified in nanomoles per minute per milligramme of protein. Protein concentrations were determined in accordance with **Lowry et al [34]**.

2.7.2.2.Mitochondrial succinate dehydrogenase (SDH) activity (complex II) determination:

The specific activity of Complex II was determined using the **Birch-Machin et al.,1994 [33],** method following 2,6 dichlorophenol indophenol reduction at 600 nm.

2.7.3. Estimation of MDA, GSH and ATP levels in brain tissue by colorimetry

2.7.3.1. MDA detection:

For determining the MDA concentration, 100 mg of tissue was homogenized with a micro pestle in 1 mL PBS, pH 7.0 **[35]**. To precipitate the protein, adding approximately 20% TCA to the tissue homogenate was done before centrifugation. The supernatants were collected and then treated with a thiobarbituric acid (TBA) solution. The absorbance was measured after boiling for ten minutes in a water bath. MDA concentrations were determined utilizing Elabscience® Catalog No. EBCK025S Colorimetric Assay Kit (TBA method). The standard curve was used to determine MDA concentration (mmol/mg ptn) in homogenate supernatants.

2.7.3.2. GSH detection:

The concentration of GSH was determined in tissue homogenates whose preparation was in phosphate buffer pH 8.0, then precipitated with 5% TCA. After centrifugation, the supernatants of tissue homogenate were added and incubated for one hour with dithiobisnitrobenzoate (DTNB) solution. The absorbance value was determined. The concentration of GSH was determined by the BioVision Colorimetric Assay Kit (Catalog #K261100). The GSH concentration in tissue was determined utilizing a standard curve **[36]-** Sample × 66.66 Mg protein used = mmol/mg protein.

2.7.3.3.ATP content measurement:

The ATP level determination was done using an ATP Assay Kit, Beyotime, Jiangsu, China, as previously described **[37]**, and using a concurrent standard curve, ATP content was detected, then normalized to the protein concentration and expressed as μmol/mg protein.

2.8) Histological examination

2.8.1. Hematoxylin and Eosin staining:

Brain tissues after removal were subjected to washing by saline and fixation in 10 % formalin solution dehydrated in alcohol, clearance in xylene and embedding in wax then sectioning with (5 μm) and staining with hematoxylin and eosin (H&E) stain for histopathological examination **[38].**

2.8.2. Immunohistochemical staining:

Antibodies against caspase-3 were used for detecting apoptotic oligodendrocytes in the corpus callosum **[39]**. On paraffin-embedded sections, immunohistochemical staining was performed. The sections were incubated with 3% hydrogen peroxidase in methanol for seven minutes in a moist container at room temperature before being incubated with caspase-3. Rabbit polyclonal caspase 3 (CPP32) Ab-

4 IgG (RB-1197-R7, Thermo Fisher Scientific; Fremont, USA) antibody was used at +4°C overnight, preserved with secondary antibody using HRP Envision kit for twenty minutes, then incubated with diaminobenzidine for ten minutes, and counterstaining was done by Mayer's hematoxylin, followed by dehydration and clearing in xylene.

2.8.3. Morphometric study:

Calculating the mean area percent and mean optical density of caspase-3 immunoexpression (IE) in ten high power fields (HPF) in immunostained sections using an interactive measurements menu and computer-assisted image analysis with Leica Qwin 500 LTD (Cambridge, UK).

3.Results:

3.1.) Behavioural Stress Tests

3.1.1.Results of the assessment of the locomotor system of mice by the rotarod in this study revealed that induction of MS by cuprizone (0.2%w/w) for five successive weeks for all mice in all groups except the normal group led to a significant reduction in the duration in seconds of sustained balance of mice on the accelerating speed rotarod by 63.33% compared to the normal group, which denotes severe affection of the locomotive system of mice. The duration of balance of mice that were left without treatment for three successive weeks (positive control group) decreased more significantly compared to the normal group. Treatment of mice with a single intraperitoneal (IP) injection of MSC-MVs (0.2 mg/kg), Melatonin (10mg/kg) single intraperitoneal dose for three successive weeks, and the combination of a single dose of MSC-MVs together with Melatonin given for three successive weeks increased the duration of balance of mice significantly in comparison to the cuprizone administered mice, as the duration was restored as normal group. The efficacy of all treatment regimens was 63.33% and 82.23%when compared to the same group before starting treatment and to the untreated cuprizone group, respectively **(table2, fig2 A).**

3.1.2.Regarding assessing the mice psychological state, through the usage of the grid floor activity cage that recorded the ambulation of mice across its bars for five minutes, also the administration of cuprizone to all mice for five successive weeks except the normal group caused significant deterioration of the psychological state of mice compared to the normal group as what was manifested by a significant reduction in the number of movements of all mice that received cuprizone across the grid floor. The number of movements of the cuprizone positive control group that was left without treatment for the following three weeks continued to be significantly less than the normal group. However, treatment with a single dose of MSC-MVs, melatonin for three successive weeks and the combination of a single dose of MSC-MVs together with Melatonin given for three successive weeks significantly improved the psychological state of mice compared to both normal and positive control groups as manifested by an increase in the number of movements across the activity cage. The efficacies of MSC-MVs injected in a single IP dose was 39.2%, Melatonin single IP dose was 45.32%, and the combination of both single IP dose of MSC-MVs and Melatonin single IP dose for three successive weeks was 51.59% **(table3, fig 2 B)**.

3.1.3. As for the cognitive abilities of mice that were evaluated by calculating their percent (%) of alternation within the Y-Maze, admission of cuprizone for five successive weeks to all groups except the normal group was not significantly different in comparison to the normal group. However, the positive control group, which was left without treatment for the following three weeks, had a significant cognition deterioration in comparison to the normal group. Only treatment with a single IP dose of MSC-MVs, significantly improved cognition compared to the positive control. Its efficacy was 22.23%. Treatment with Melatonin single IP dose only for three successive weeks didn't improve cognition as the % of alternations was significantly less than MSC-MVs and normal groups and almost the same as the positive control group. Treatment with a combination of a single IP dose of MSC-MVs together with melatonin given as a single IP dose for three successive weeks improved cognition by 14.73% compared to the positive control group **(table 4, fig 2 C)**.

Table 2: **Evaluation of Loco-motor activity of MS-induced mice treated with MSC-MVs, Melatonin and combination of both by using Accelerating Speed Rotarod**

	Groups				
	Normal	**Positive control (Cuprizone 0.2%W/W)**	**MSC-MVs (0.2mg/kg)**	**Melatonin (10mg/kg)**	**MSC-MVs+ Melatonin**
Baseline	300	300	300	300	300
5 weeks induction of MS	300	110^{a} ± 5.7	110^{a} ± 5.7	110^{a} ± 5.7	110^{a} ± 5.7
3weeks Treatment	300	53.3^{a} ± 3.33	300^{b}	300^{b}	300^{b}

Results of Rotarod. N=8, results were expressed as means of duration of balance of mice on accelerating speed rotarod calculated in seconds ±S.E. Statistical analysis was done by two-way analysis of variance followed by Tukey Kramer test for multiple comparisons. $p< 0.0001$.(a)significantly different from the normal group; (b) significantly different from the positive control group.

Table 3: Evaluation of the Psycho-motor state of MS-induced mice treated with MSC-MVs, Melatonin and combination of both through Grid floor Activity cage usage.

	Groups				
	Normal	**Positive control (Cuprizone 0.2%W/W)**	**MSC-MVs (0.2mg/kg)**	**Melatonin (10mg/kg)**	**MSC-MVs+ Melatonin**
Baseline	1	1	1	1	1
5 weeks (induction of MS)	0.99 0.004	0.77^{a}±0.07	0.77^{a}±0.07	0.77^{a}±0.7	0.77^{a} ±0.07
3weeks(Treatment)	1±0.07	0.76^{a}±0.01	1.25^{ab}±0.01	1.39^{abc}±0.13	1.57^{abcd} ±0.008

Results of grid floor activity cage: N=8, results were expressed of square root transformed % of the number of movements / 5 minutes ±S.E.N=8. A two-way analysis of variance was used, followed by the Tukey Kramer test for multiple comparisons. p< 0.0001.(a)significantly different from the normal group; (b) significantly different from the positive control group;(c) significantly different from the MSC-MVs group;(d) significantly different from the melatonin group.

Table 4: Evaluation of Cognitive abilities of MS-induced mice treated with MSC-MVs, Melatonin and a combination of both by using the Y-Maze test

	Groups				
	Normal	**Positive control (Cuprizone 0.2%W/W)**	**MSC-MVs (0.2mg/kg)**	**Melatonin (10mg/kg)**	**MSC-MVs+ Melatonin**
Baseline	69.76±3.38	69.76±3.38	69.76±3.38	69.76±3.38	69.76±3.38
5 weeks(induction of MS)	71.12±2.9	63.09±2.91	63.09±2.91	63.09±2.91	63.09±2.91
3weeks (Treatment)	71.58±3.68	56.66^{a}±2.35	72.86^{b}±3.14	56.69^{ac}±2.05	66.45±2.49

Results of Y maze test. N=8, results were expressed as means of % of alternations of mice across the maze arms±S.E. Two way analysis of variance was used, followed by Tukey Kramer test for multiple comparisons. P< 0.1. (a) significantly different from the normal group; (b) significantly different from the positive control group;(c) significantly different from the MSC-MVs group.

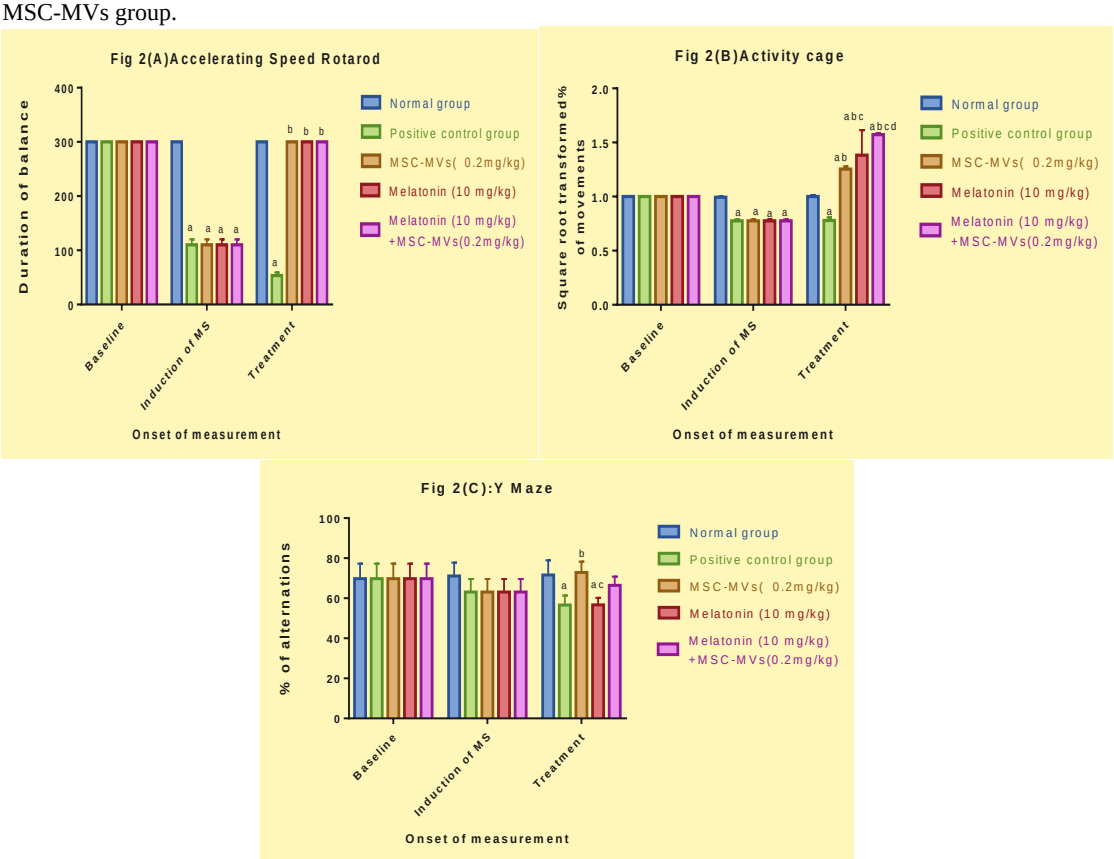

Fig 2:(A) Results of Accelerating Speed Rotarod were expressed as means of duration of balance of mice on the rotarod calculated in seconds ±S.E. p< 0.0001 (B): Results of Grid Floor Activity Cage were expressed as means of square root transformed % of the number of movements / 5 minutes ±S.E p< 0.0001. (C): Results of the Y maze test were expressed as means of % of alternations of mice across the maze arms±S.E. p< 0.1. N=8, Two-way analysis of variance was used followed by Tukey Kramer test for multiple comparisons. (a) significantly different from the normal group; (b) significantly different from the positive control group; (c) significantly different from the MSC-MVs group;(d) significantly different from the melatonin group.

3.2.) Biochemical results:

3.2.1. Decreased level of Diagnostic biomarkers of MS, miR-132 and miRNA-155, in response to melatonin and MVs treatment:

As shown in table (5), MS-induced mice showed a significant increase in MS markers (miR-132 and miRNA-155) if compared to normal mice. Fortunately, treatment with Microvesicles and melatonin significantly decrease the elevated MS markers when compared to MS-induced mice. But this correction is partial as there is still a significant difference between all mentioned parameters versus normal mice. The combination of both regimens, microvesicles and melatonin, showed a better correction over the two single regimens in miR-132, miRNA-155 levels.

3.21.2.Combined MVs and melatonin intake improves mitochondrial biogenesis markers expression (SIRT1, SIRT3, PGC-1α):

As shown in table (6), MS-induced mice showed that SIRT 1, SIRT 3 and PGC-1α significantly decreased in comparison to normal. Fortunately, treatment with microvesicles and melatonin significantly elevate the depressed SIRT 1, SIRT 3 and PGC-1α, when compared to MS-induced mice. But this correction is partial as there is still a significant difference between all mentioned parameters versus normal mice .The combination of both regimens, microvesicles and melatonin, showed a better correction over the two single regimens in SIRT 1, SIRT 3 and PGC-1α levels.

3.2.3.Cuprizone oxidative damage showed a marked decrease secondary to combined MVs and melatonin treatment:

As shown in table (7), MS-induced mice revealed disturbed oxidative stress state in the form of significant elevation of MDA associated with significant depression of GSH in comparison to normal mice. Treatment with either microvesicles or melatonin partially alleviated this disturbance by the observed significant decrease in MDA and a significant increase in GSH when compared to MS-induced mice, but all are still significant in relation to normal levels .The combination regimen showed better results than both single types of treatment shown by more significant depression in MDA and more significant elevation in GSH. Besides, the combined regimen was able to regain the normal level of GSH.

3.2.4.Improvement of mitochondrial function in MS-induced mice secondary to combined MVs and melatonin treatment (Complex1, Complex2, ATP)

As shown in table (8), MS-induced mice revealed significant depression complex 1, complex 2 and ATP in comparison to normal mice. Treatment with either microvesicles or melatonin partially alleviated this disturbance by the observed significant increase in complex 1, complex 2 and ATP when compared to MS-induced mice, but all are still significant in relation to the normal level. The combination regimen showed better results than both single types of treatment shown by a more significant elevation in complex 2 and ATP. Complex 1 was the only one that showed an insignificant difference between the combined regimen and microvesicles treated mice.

	Control (I)	MS-induced Group (II)	MS Group treated by Microvesicles (III)	MS Group treated by Melatonin (IV)	MS Group treated by a combination (IV)	F	P-value
Tissue SIRT1 (nmol/g)	**1.03 ± 0.03**	**0.25[a] ± 0.03**	**0.73[a,b] ± 0.02**	**0.71[a,b] ± 0.04**	**0.84 [a, b,c,d]± 0.01**	69.867	**< 0.001***
Tissue SIRT3 (mmol/g)	**1.03 ± 0.01**	**0.15 [a] ± 0.01**	**0.54 [a,b] ± 0.01**	**0.56 [a,b] ± 0.02**	**0.79 [a,b,c,d] ± 0.009**	290.140	**< 0.001***
Tissue PGC-1alpha (nmol/ml)	**1.01 ± 0.006**	**0.15 [a] ± 0.01**	**0.65 [a,b] ± 0.009**	**0.69 [a, b] ± 0.02**	**0.89 [a,b,c,d]± 0.01**	356.048	**< 0.001***

Table 5: Decreased level of Diagnostic biomarkers of MS, miR-132 and miRNA-155, in response to melatonin and MVs treatment.

Parameters	Control (I)	MS-induced Group (II)	MS Group treated by Microvesicles (III)	MS Group treated by Melatonin (IV)	MS Group treated by combination (IV)	F	P- value
Tissue MIR 132 (nmol/ml)	**1.01± 0.005**	**4.6 [a] ± 0.1**	**2.51 [a, b] ± 0.2**	**2.38 [a,b] ± 0.1**	**1.80 [a,b,c,d] ± 0.07**	75.828	**< 0.001***
Tissue MIR 155 (nmol/ml)	**1.01± 0.007**	**6.05 [a] ± 0.2**	**3.12 [a, b] ± 0.03**	**3.19 [a,b] ± 0.2**	**2.22 [a,b,c,d] ± 0.07**	78.469	**< 0.001***

Data was presented as mean ±SEM .a, b, c, d: significant values in comparison to control, multiple sclerosis (MS) induced group, Microvesicles treated group and melatonin treated group respectively. Significant values when P<0.05, using one-way ANOVA followed by Tukey multiple comparison test.

Table 6:Combined MVs and melatonin intake improves mitochondrial biogenesis markers expression (SIRT1,SIRT3, PGC-1α)

Data was presented as mean ±SEM.a, b, c, d: significant .values in comparison to control, multiple sclerosis (MS) induced group, Microvesicles treated group and melatonin treated group respectively.

Table 7:Cuprizone oxidative damage showed a marked decrease secondary to combined MVs and melatonin treatment.

	Control (I)	MS-induced Group (II)	MS Group treated by Microvesicles (III)	MS Group treated by Melatonin (IV)	MS Group treated by a combination (IV)	F	P-value
Tissue MDA(nmol/g)	**30.00± 1.77**	**114.48 [a] ± 1.75**	**53.88 [a,b] ± 3.86**	**49.48 [a, b]± 2.05**	**41.83 [a,b,c,d]± 1.37**	164.882	**< 0.001***
Tissue GSH (mmol/g)	**67.45 ± 3.72**	**26.63 [a] ± 1.47**	**50.45 [a,b] ± 2.22**	**50.13 [a,b] ± 2.53**	**59.33 [b,c,d] ± 1.64**	32.357	**< 0.001***

Data was presented as mean ±SEM a, b, c, d: significant values compared to control, multiple sclerosis (MS) induced group, Microvesicles treated group and melatonin treated group respectively.

Table 8: Improvement of mitochondrial function in MS-induced mice secondary to combined MVs and melatonin treatment (Complex1, Complex2, ATP).

Parameters	Control (I)	MS-induced Group (II)	MS Group treated by Microvesicles (III)	MS Group treated by Melatonin (IV)	MS Group treated by a combination (IV)	F	P-value
Tissue Complex 1 (nmol/ml)	**43.30 ± 1.77**	**14.33 [a] ± 0.89**	**27.85 [a,b] ± 1.26**	**24.81 [a,b]± 1.36**	**32.30 [a,b,d]± 1.59**	47.008	**< 0.001***
Tissue Complex 2 (nmol/ml)	**80.63 ± 1.37**	**35.55 [a] ± 1.36**	**50.50 [a,b]± 1.97**	**52.68[a,b] ± 2.33**	**69.71 [a,b,c,d] ± 2.60**	64.886	**< 0.001***
Tissue ATP (nmol/ml)	**117.7 ± 1.41**	**52.75 [a] ± 3.28**	**90.73 [a,b] ± 2.48**	**88.41 [a,b] ± 1.29**	**103.75 [a,b,c,d] ± 1.43**	107.578	**< 0.001***

Data was presented as mean ±SEM a, b, c, d: significant values compared to control, multiple sclerosis (MS) induced group, Microvesicles treated group and melatonin treated group, respectively.

3.3.)Histological result:

Microscopic examination of corpus callosum from the control group showed normal white matter nerve fibre and oligodendrocytes **(Fig 3a, 3b)**. In contrast, the cuprizone treated group showed corpus callosum with loss of normal histological profile represented in severe disruption and vacuolation of nerve fibre, an area with loss of nerve fibre and apoptosis of oligodendrocytes were seen **(Fig 3c)**. Notable improvement could be observed after treatment with microvesicles where nerve fibre appeared normal with a mild vacuolated area **(Fig 3d)**. In the melatonin treated group, the corpus callosum tissues revealed moderate improvement, where most of the nerve fibre and oligodendrocytes appear intact, with mild apoptotic oligodendrocytes occasionally seen **(Fig 3e)**. Microvesicles+ melatonin treated group showed marked improvement and corpus callosum tissues apparently comparable to control group **(Fig 3f).**

3.4.) Immunohistochemical results:

Brain section of control mice demonstrated –ve immunoexpression (IE) of oligodendrocytes in the corpus callosum **(fig 4a)**. In the cuprizone treated group, multiple +ve IE was found **(fig 4b)**. at the same time, few +ve IE in melatonin treated group **(fig 4c)**. In microvesicles and combined groups, +ve IE in occasional oligodendrocytes was observed **(fig 4d&4e)**.

3.5.) Morphometric results:

Compared to the control group, the mean area percent of caspase 3 immunoexpression significantly increased in the cuprizone-treated group. In the melatonin treated group, a slight increase in mean area% in comparison with the control was detected. But, microvesicles, combined groups and control groups were not significantly different **(Table 9) & Fig (5)**.

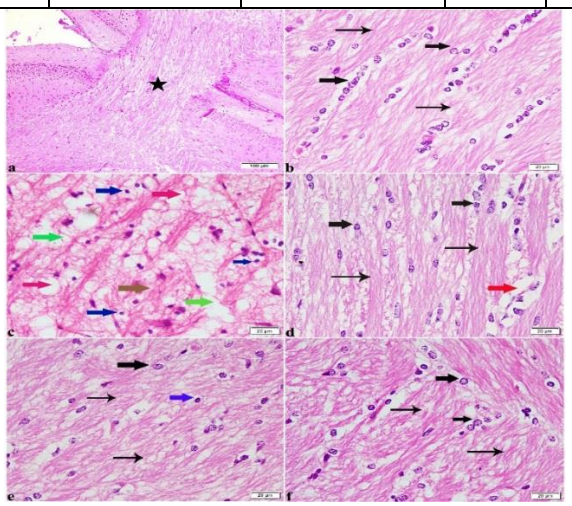

Fig. (3): Photomicrograph of brain section. Showing corpus callosum (a.b): control group. (c): group treated by cuprizone (d): group treated by microvesicle. (e): group treated by melatonin. (f): microvesicle+ melatonin group. corpus callosum (Asterix) nerve fiber (thick arrow), oligodendrocytes (thin arrow), vacuolated area (red arrow), apoptotic oligodendrocytes (blue arrow), loss of nerve fiber (green arrow), disrupted nerve fiber (brown arrows) (a, x:10, scale bar 100 µm. (b-f) x:400, scale bar:20 µm)

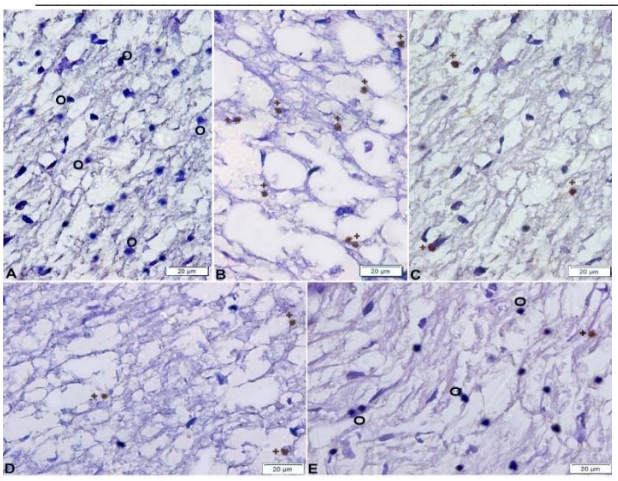

Fig. (4): Caspase- 3 immunostaining (x:400) brain section showing corpus callosum (a): control group. (b): group treated by cuprizone. (c): group treated by microvesicle. (d): group treated by melatonin. (e): microvesicle+ melatonin group. (O): intact oligodendrocytes. (+): +veimmunoexpression.

Table 9: Morphometric results:

Group	Mean area % of caspase -3
control	0
cuprizone	23.4± 5.2***
microvesicles	10.1± 1.4*
melatonin	4.1± 1.0
microvesicles+ melatonin	0.5± 0.1

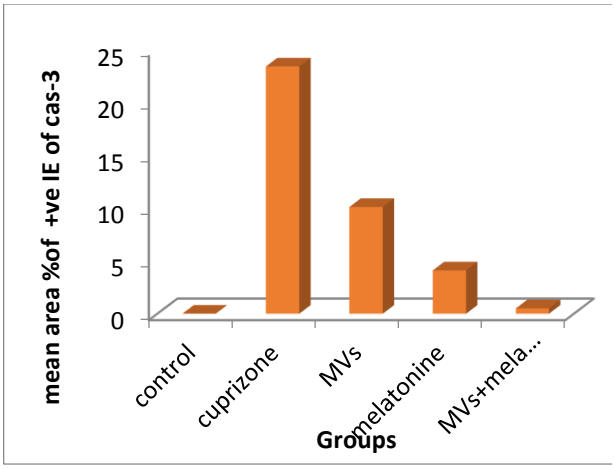

Fig (5): Histogram showing means area % of +ve caspase -3 immunoexpression in different experimental groups.

4.Discussion:

Multiple sclerosis (MS) is a central nervous system (CNS) disease caused by an autoimmune response. It is characterized by inflammation and neurodegeneration. **[5].** Current therapies are very effective in reducing neuroinflammation in some patients, but many other patients are less responsive to these treatments and continue to decline with progressive neurodegeneration. Thus, additional treatment targeted at neuroprotection might provide a new direction for curing MS**.** Promoting remyelination and protecting myelin from demyelination are critical goals in multiple sclerosis research **[40].**

In the current study, cuprizone ingestion, a copper chelator, for five successive weeks without giving the subsequent antidote, resulted in significant impairment of mice ambulation and psychological state as well as cognitive deficits as manifested by the reduced duration of balance on accelerated speed rota-rod, reduced number of movements across the grid floor activity cage and decreased percentage of alternations across the Y maze arms. These manifestations can be explained by what was mentioned in the study of **Zhan et al [41]**, who revealed the occurrence of cuprizone induced CNS deficits in experimental animals after being administered for five to six weeks, to the immediate induction of apoptosis of oligodendrocytes that is followed by astrocyte and microglial cells activation within the brain and consequently the development of white and grey matter demyelination; a picture similar to pattern III MS lesions, which were also proven by the histological and immunopathological examination in our study. Also, the current study revealed that cuprizone caused marked deterioration in the biochemical parameters measured to verify the therapeutic effects of MVs and/or Melatonin. Therefore, the Cuprizone model was proven to be useful in studying the pathogenesis of primary demyelination resulting from mitochondrial dysfunction, as also a former study by **Ruhling et al [42]**, had demonstrated spheroidal mitochondrial deposits at the site of axon lesions due to intoxication by cuprizone. The current research aims to determine the effect of MSC-Mvs, Melatonin, and their combination as a novel strategy for treating multiple sclerosis, as well as their effect on the distribution and function of mitochondria in young male mice following experimentally induced demyelination with cuprizone.

MVs are membranous vesicles that are submicron-sized. They are shed from cell surface membranes or actively released from cells via secretory compartments. They are synthesized by a diverse range of cell types and serve as carriers of biological information (e.g., miRNA, mRNA, and protein) to distant cells, exerting an effect on their gene expression, function, differentiation, and proliferation **[13].**

MVs may either transport transcripts from stem cells to injured cells, minimizing tissue injury and inducing resident cell cycle re-entry. They may also transport transcripts from injured cells to stem cells, reprogramming their phenotype to take on the characteristics of a particular tissue, resulting in tissue

self-repair. In numerous experimental models, MVs mimic the effect of mesenchymal stem cells by apoptosis inhibition and cell proliferation promotion **[43]**.

Administration of MSC-derived-MVs supported renal function recovery in a unilateral acute kidney injury (AKI) rat model. Through RNA transfer, MVs cause RNA delivery into injured tubular cells and induce the production of HGF, facilitating tubular cell differentiation and regeneration **[44]**.

In another study by **Lee et al [24],** they found that MVs extracted from brain MSCs have been shown to improve behavioural recovery and reduce infarct size in a rat model of ischemia by promoting angiogenesis, neurogenesis, and anti-inflammation.

Melatonin which is an indolamine synthesized endogenously is used for combating neurodegenerative diseases because it is a potent natural antioxidant that is capable of crossing the blood-brain barrier, and it is directed towards the mitochondria to prevent reactive oxygen species production at their site of generation through cutting mitochondrial electron transport chain **[21].**

In our study, treatment of MS mice models with Melatonin alone for three successive weeks resulted in significant improvement of locomotion and psychological state of mice. However, melatonin alone didn't improve cognition which was the case with **Roostaei et al [45],** who stated that in a clinical study, patients suffering relapsing/remitting MS didn't show cognitive improvement. On the other hand, when combining melatonin with a single MVs injection in our study, cognition was markedly improved in addition to the other stress test parameters for locomotion and psychological state. These manifestations were confirmed with the histopathological results as well as the biochemical parameters. Our results agreed with **Vakilzadeh et al [46]**; who demonstrated that intraperitoneal administration of melatonin to cuprizone induced MS rats improved their clinical assessment and increased their movements. This is because it is capable of detoxifying metal intoxication **[47].**

The therapeutic effects of melatonin towards MS in our study are due to several mechanisms, including receptor-dependent and independent pathways.

Receptor dependent pathway is through binding with specific targets intracellularly as ion binding proteins, mitochondria, and transporters **[48].**

The receptor-independent pathway of Melatonin incorporates reaction with hydroxyl radical, resulting in the formation of less toxic and less reactive indolyl radical cation [**21**], as well as metal chelation **[47].**Thus Melatonin is considered as a potent antioxidant, anti-inflammatory, natural remedy for protection against neuronal degenerative and autoimmune diseases, which enforced the results of our study in evaluating the effects and underlying mechanisms of melatonin in MS mice.

Numerous studies on mitochondrial dysfunction have been conducted and indicated that mitochondrial dysfunction and accumulation of reactive oxygen species plays a cardinal role in neurodegeneration in MS **[49].**

To study the role of mitochondrial biogenesis and its dysfunction in the pathogenesis of MS in mice model, we measured Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), Sirtuin1 (SIRT1), in addition to Sirtuin3 (SIRT3) gene expression. Our results show that MS-induced mice showed a significant decrease observed in SIRT1, SIRT3 and PGC-1alpha when compared to normal. Fortunately, treatment with Microvesicles and melatonin significantly elevated the depressed SIRT1, SIRT3 and PGC-1alpha, when compared to MS-induced mice. But this correction is partial as there is still a significant difference between all mentioned parameters versus normal mice. The combination of both regimens (microvesicles and melatonin) showed a better correction over the two single regimens in SIRT1, SIRT3 and PGC- 1alpha.

Sirtuins are nicotinamide adenine dinucleotide (NAD)-dependent deacetylases which participate in a wide variety of molecular pathways, including neurodegeneration, inflammation, ageing, metabolism and cancer **[50-54].**

In relation to multiple sclerosis (MS), Silent information regulator 1 (SIRT1) is the family member that has been the most extensively studied in a variety of animal models. Previous studies have demonstrated that activating SIRT1 has a significant neuroprotective role in neurodegenerative diseases **[55]**, as well as significantly reducing the levels of reactive oxygen species (ROS) and increasing mitochondrial metabolism **[56,57]**. SIRT1 was proposed as a final mediator of the antioxidant activity changes observed in PMBCs treated using melatonin in another research that involved peripheral blood mononuclear cells (PMBCs) from patients with multiple sclerosis **[58]**. This establishes a direct link between SIRT1 and the well-established melatonin action on antioxidant enzymes **[59].**

SIRT3, a major mitochondrial deacetylase, acts as a metabolic sensor that can respond to cellular changes in the energy status, and it is involved in the epigenetic regulation of complex I proteins in the respiratory chain **[60,61]**, as well as mitochondrial translation regulation **[62]**. Increased respiration and decreased production of reactive oxygen species are observed when SIRT3 is overexpressed in cultured cells **[63].**

Rice et al [64], examined the function of the mitochondrial sirtuin. They identified these proteins as a novel target for repairing and protecting against multiple sclerosis (MS), and examined post-mortem human brain tissues using immunohistochemistry and discovered that MS-affected brains had lower levels of SIRT3 expression than control samples.

Huifeng et al [65], demonstrated that melatonin's hepatoprotective effect in cadmium-induced toxicity is mediated by the mitochondrial sirtuin. Cadmium induces autophagic cell death via the mitochondria, accompanied by a decrease in SIRT3 expression. This demonstrates that SIRT3 has a critical role in melatonin antioxidant activity and reveals that a large number of antioxidant enzymes' indirect actions can be explained by direct stimulation of the SIRT3 gene and/or activity.The actions so far noted of SIRT1 and, to a lesser extent, SIRT3 suggest that these deacetylases may be critical in explaining a number of melatonin functions.

PGC-1α is a subcellular, transcriptional coregulator. It is referred to as the master regulator of mitochondrial biogenesis and the liver gluconeogenesis primary regulator, increasing the expression of genes involved in gluconeogenesis **[66].**

Dang et al [67], proved that PGC-1α up-regulation reduces susceptibility to ROS, attenuates ROS production, and protect neurons from undergoing apoptosis. These findings show that PGC-1 α is a promising therapeutic target for further investigations seeking to identify ways to provide neuroprotection in MS.

Additionally, **Yu et al [68],** demonstrated that melatonin caused a significant increase in PGC-1 and SIRT3 genes expression in diabetic rats, indicating that melatonin promotes mitochondrial biogenesis and mitochondrial anti-oxidative enzymes deacetylation.

Micro RNAs (mi RNAs) are short single-stranded oligonucleotides capable of regulating gene expression post-transcriptionally, thus affecting a variety of biological processes. They are responsible for the emergence of many pathological conditions due to their presence in exosomes and ectosomes **[69].** Numerous research has revealed abnormal miRNA profiles in CNS glial cells and peripheral blood immune cells in patients with multiple sclerosis compared to controls **[70,71],** suggesting the possibility that such altered miRNA expression is able to account for immunological characteristics associated with MS pathogenesis.

In our study, we measured gene expression of miRNA-132 and -155, aiming to find a correlation with the pathogenesis of MS to use them later as markers for diagnosis and possibly for prognosis of the disease. Our results showed that MS-induced mice showed a significant increase in miRNA-132 and 155 if compared to normal mice. Fortunately, treatment with Microvesicles and melatonin significantly decrease the elevated MS markers (MIR-132 and MIR-155) when compared to MS-induced mice. But this correction is partial as there is still a significant difference between all mentioned parameters versus normal mice .The combination of both regimens (microvesicles and melatonin) showed a better correction over the two single regimens.

miR-132 was first identified as a critical regulator of neuronal survival, maturation, and differentiation and has since been linked to a variety of immune responses **[72].** Additionally, miR-132 is thought to have an essential role in neurodegenerative diseases such as caspase-dependent apoptosis, synaptic signalling blockade, and excessive axonal damage, as well as in inhibiting or activating downstream cascades, resulting in irreversible devastation in humans. Its increased or decreased expression within certain nerve tissues has a significant effect on normal neuron functions and activity **[73].**

Miyazaki et al [74], discovered that abnormally increased secretion of lymphotoxin and tumour necrosis factor by MS B cells is associated with abnormally increased expression of miR-132. In normal B cells, overexpression of miR-132 significantly increased lymphotoxin and tumour necrosis factor production. Additionally, overexpression of miR-132 suppressed sirtuin-1, a miR-132 target.miR-155 has been described as a master regulator of the immune response, specifically driving myeloid cell polarisation toward a pro-inflammatory state, and it was revealed that miR-155 expression in multiple sclerosis patients is significantly increased **[71].**Increased miR-155 expression across the profiled neurodegenerative diseases, in combination with its well-known role in neuroinflammation, shows that higher miR-155 expression may aggravate the pathology of neurodegenerative diseases through an inflammatory mechanism**[75].**In a study performed by **Thamilarasan and colleagues [76]**, they found that in both patients with MS and Mice with Experimental Autoimmune Encephalomyelitis (EAE), miR-155 expression was significantly increased**,** suggesting that miR-155 may be used as a diagnostic marker for patients with MS.

To figure out the effect of MVs and melatonin treatment on mitochondrial function in MS-induced mice, we measured the activity of mitochondrial complex I, complex II and ATP content. Our results revealed significant depression in their levels in **MS-induced mice** compared to normal mice. Treatment with either microvesicles or melatonin partially alleviated this disturbance by the observed significant increase in complex I, complex II and ATP when compared to MS-induced mice, but all are still significant in relation to normal levels .The combination regimen showed better results than both single types of treatment shown by a more significant elevation in complex II and ATP. Complex I was the only one that showed an insignificant difference between the combined regimen and microvesicles treated mice. **Kashani et al [77]** findings, support our findings on the regulation of respiratory chain enzyme subunits. After cuprizone exposure, they observed a significant down-regulation of all four complex

subunits, which was reversed by melatonin. Notably, melatonin restored basal levels while also enhanced complexes I–IV expression. This would allow for the cells to be compensated for any oxidative stress, and the energy balance would be improved.

In plants, animals, fungi, and some bacteria, glutathione (GSH) acts as an antioxidant, protecting against damage to critical cellular components due to reactive oxygen species such as peroxides, free radicals, and heavy metals **[78].**Glutathione exists in two states: oxidized (GSSG) and reduced (GSH). The ratio of reduced to oxidized glutathione in cells serves as a proxy for cellular oxidative stress, with a higher GSSG-to-GSH ratio indicating increased oxidative stress **[79].**MS changes are believed to be caused by free radicals generated by cuprizone exposure **[80].**GSH is the main intracellular and thus mitochondrial antioxidant in the brain **[81].**

Reactive oxygen species are capable of degrading polyunsaturated fatty acids, resulting in the formation of malondialdehyde (MDA). This compound is a reactive aldehyde that induces toxic stress in cells; its production is used as a biomarker to determine an organism's level of oxidative stress **[82].**

To show the effect **of MVs and melatonin treatment on** oxidative stress in MS-induced mice, we measured GSH and MDA levels. Our result revealed a disturbed oxidative stress state in the form of a significant elevation of MDA associated with significant depression of GSH in comparison to normal mice. Treatment with either microvesicles or melatonin partially alleviated this disturbance by the observed significant decrease in MDA and the opposite significant increase in GSH when compared to MS-induced mice, but all are still significant in relation to normal levels. The combination regimen showed better results than both single types of treatment shown by more significant depression in MDA and more significant elevation in GSH. Besides, the combined regimen was able to regain the normal level of GSH.

As confirmed in our study, Kashani et al. (2014) demonstrated that cuprizone-induced oxidative stress is manifested by decreased reduced glutathione (GSH) levels in the brain.

Melatonin treatment prevented the decline in the GSH/GSSG ratio during ageing in both male and female mice, indicating that these mitochondria were capable of maintaining a low oxidant status during ageing. Melatonin's ability to prevent GSH depletion with age is most likely due to its effect on the activities of the GSH redox cycle enzymes **[83].**These findings are consistent with observations made by other authors **[84,85]**.

Additionally, in another study, the MDA levels normalization following melatonin treatment could have been caused by melatonin inhibitory effect on the lactoperoxidase enzyme, which catalyzes the oxidation of a variety of inorganic and organic substrates by hydrogen peroxide **[86]**.

According to the histological results of this study, our work revealed pathological alterations in corpus callosum tissues of mice treated with cuprizone in the form of severe disruption and vacuolation of nerve fibre, loss of nerve fibre with notable apoptotic oligodendrocytes. This observation was confirmed by quantitative morphometric data, which revealed a significant increase in the mean area% of +ve cells caspase- 3 immunoexpression. These results agree with **Zhan et al [41],** who observed that the apoptosis of oligodendrocytes appears days after initiation of the cuprizone intoxication protocol. They used gene array analysis to compare the expression levels of messenger RNA in the white matter tract of the corpus callosum from control mice and animals that had been intoxicated with cuprizone for two days in a newly published study, and found that the expression levels of many mRNAs were raised which are mainly expressed by oligodendrocytes, it might be suggested that cuprizone mostly damages homeostasis of mature oligodendrocytes **[15]**. It has been reported that the cuprizone diet stimulates caspase-3 expression on oligodendrocytes which play a curial role in cell death of (experimental autoimmune encephalomyelitis) EAE model of demyelination. Also, the cuprizone diet activates astrocytes which motivate (nuclear factor-κ B) NF-κ B upregulation led to oligodendrocytes death **[46].**

In the melatonin treated group, examination of corpus callosum tissues showed marked improvement accompanied by a slight increase in mean area% of caspase-3 immunoreactivity in comparison with control. Several studies revealed that melatonin has the ability to prevent apoptosis by regulating the function of mitochondrial complex, which leads to reduction of electron leakage and free radicals generation, reduce Bax pro-apoptotic protein and rise Bcl-2 anti-apoptotic protein level **[87].**

Also, our results revealed that the treatment with MVs showed marked restoration of corpus callosum tissues. Similar results were documented by **Xiao et al [88],** who observed that BMSCs- derived exosomes prevent apoptosis of oligodendrocytes via exosomal miR- 134 by negatively regulating the caspase- 8-dependent apoptosis pathway and maybe a novel possible therapeutic target for ischemic stroke therapy.

5.Conclusions

Our results support the therapeutic role of melatonin and microvesicles in MS-induced mice and correct the oxidative damage that happens during the course of the disease through the antioxidant activity of melatonin and alteration in levels of miRNAs. Melatonin reduced oxidative stress in the CNS and subsequently suppressed inflammatory reaction, neuronal apoptosis, and demyelination. Moreover, melatonin improved mitochondrial function maintenance of ATP level.All

these promising biochemical effects of treatment resulted in improved locomotion, psychological condition and impaired cognition that were deteriorated as a result of oxidative stress , inflammatory reaction, neuronal apoptosis, and demyelination induced by cuprizone that led to development of MS signs in mice.

Also, our results suggest that miRNA -155 and 132 could be used as diagnostic and possibly prognostic factors for MS and may be used as therapeutic targets.

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7.Conflict of interest: Authors declare that there is no conflict of interest regarding the present work

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