Heliyon 9 (2023) e20385

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Maternal stress-induced changes in adolescent and adult offspring: Neurobehavioural improvement and telomere maintenance

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ARTICLE INFO

Keywords:
Maternal stress
Telomere
Shelterin complex
Anxiety-like behaviour
Inter-limb coordination
Bacopa monnieri
L- carnosine

ABSTRACT

Maternal stress (MS) during gestation is known to increase the risk for the development of behavioural and physiological disorders and advances cellular aging. In this study, we investigated whether the supplementation of standardized *Bacopa monnieri* extract (CDRI-08/BME) or L-Carnosine (L-C) to the mother exposed to social stress during gestation modify the effect on their offspring's neurobehaviour, antioxidant defence gene expression, telomere length, and telomere biology. To test this, timed pregnant rats were subjected to social stress during the gestational day (GD) 16–18. A subset of stressed pregnant rats received either BME [80 mg/kg in 0.5% gum acacia (per-orally; p.o)] or L-C [1 mg/kg (p.o)] every day from GD-10 to until their pup's attained postnatal day (PND)-23. We observed that MS induced anxiety-like behaviour, altered inter-limb coordination, antioxidant defence genes [Superoxide dismutase (SOD1,2), Catalase (CAT), Glutathione peroxidase-3 (GPX3)], telomerase reverse transcriptase (TERT), shelterin complex subunits (TRF1, RAP1B, POT1) protein level and shorten telomere length. Notably, supplementation of BME/L-C dampens the MS, thus the effect on neurobehaviour, antioxidant defence gene expression, and telomere biology is minimized in their offspring. Together, our results suggest that supplementation of BME/L-C during gestation dampens the MS and reduced oxidative stress-mediated changes in telomere shortening/biology and associated neurobehaviour in offspring born following MS.

1. Introduction

Social stress experienced by the mother during gestation may be transmitted to the developing embryo through the stress-related maternal-placental-fetal (MPF) pathway [1,2]. The adverse stress stimuli primarily activate the hypothalamic-pituitary-adrenal (HPA) axis, which leads to the release of the hypothalamic corticotrophin-releasing hormone (CRH) into the bloodstream along with the placental CRH [3,4]. The presence of elevated levels of free corticosteroids passes to the fetus, and the presence of excess corticosteroids has been known to exert an effect on brain development. At the cellular level, the hormonal cascade synergistically alters the

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https://doi.org/10.1016/j.heliyon.2023.e20385
Received 24 December 2022; Received in revised form 22 July 2023; Accepted 20 September 2023
Available online 21 September 2023

cellular physiology, as a result, increases the production of free radicals and oxidative stress [5–7]. Oxidative stress has been known to alter the balancing mechanism of antioxidant defence enzymes and cause damage to the biological molecules, including DNA-protein cross-link and single-stand DNA at the telomeric region [8]. To neutralize the effect, antioxidant enzymes i.e. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx) are naturally activated in the system and protect the cellular physiology [9,10]. The stress defence mechanism is inherent in the developing brain to protect against cellular damage [11], changes in defence mechanism/elevation of reactive oxygen species (ROS) can contribute to neuronal damage, alter neuro-behavioural changes, and induces genomic instability associated with telomere length [12–14]. Telomere length and associated regulatory molecules have been recognized as biological markers for cellular aging and psychological stress. Earlier studies in the animal model and humans established that psychological stress shortens the telomere length [15–18]. Telomere length (TL) and its regulation are governed by three key components, telomerase enzyme, shelterin complex [a six-subunit protein complex, telomere repeat factor 1 & 2 (TRF1 & 2); interacting nuclear factor 2 (TIN 2); repressor/activator protein 1 (RAP 1); protection of telomeres (POT1) [19]. Telomerase reverse transcriptase (TERT) which is maintaining the telomere length by the addition of the telomere repeat (TTAGGG) [20]. Further, the shelterin complex binds with the telomere to prevent activation of DNA damage response (DDR) and degradation [21]. The interaction of TRF1 & 2 with telomeric DNA has been reported to be stabilized by TIN2 and RAP1 [22,23], which is essential to maintain the telomere length and structural integrity. In addition, POT1 protects or initiates the extension of telomere by recruiting telomerase [24]. Stress-mediated reduction in telomerase activity and telomere shortening in the brain led to suppression of neurogenesis and induces depression/anxiety-like behaviour [25], however, the effect of stress molecules that maintain the telomere length is not reported.

Bacopa monnieri (Linn.) has been used as a nootropic agent in ayurvedic herbal formulations (medhyarasayana). *B. monniera* extract contains flavonoids like apigenin, luteolin, quercetin dammarane-type triterpene, and aminoglycosides, triterpene saponins have been named bacosides and bacopasaponins [26,27]. The characterization and structural elucidation of *B. monnieri* extract (CDRI-08) reveals the presence of bacosides [bacoside A (bacogenins A1, A2, A3, and A4), bacoside B, bacopacoside II, bacopasaponin X, and bacopasaponin C] [28], which contain the neuropharmacological property. The bacoside-enriched standardized extract of *B. monniera* (CDRI-08, which contains 55 ± 5% bacosides) is reported as BME in this article. Pardridge [29] demonstrated that through lipid-mediated passive diffusion, non-polar glycosides (Bacosides) possibly cross the blood-brain barrier (BBB). Subsequently, radiopharmaceutical analysis confirmed the biodistribution of bacosides in the brain [30]. Earlier, we have shown that supplementation of BME improved memory by acting through the serotonergic system [31], regulates signaling molecules through microRNA (miR)- 124 [32], epigenetic modifications [33,34], and regulates the synaptic plasticity [35]. Earlier studies reported that *B. monnieri* supplementation enhances cognitive performance in patients with neurodegenerative disorders [36–39]. Studies in animal models and in elderly people demonstrated that supplementation of *B. monnieri* extract (CDRI-08) reduces oxidative stress, depression, and anxiety-like behaviors [35,40–42]. Carnosine (β-alanyl- L-histidine) is synthesized from β-alanine and L-histidine by the enzyme carnosine synthase [43] and is an effective scavenger of reactive oxygen species and aldehydes. It prevents lipid peroxidation and protein oxidation and acts as an antioxidant activity [44,45], antidepressant activity [46], and protects the DNA damage in fibroblast cells [47].

In this study, first, we designed the experiment to test whether maternal stress (MS) induced oxidative stress accelerates neuronal aging/genomic instability, and anxiety/depressive-like bahviour in their male offspring. Second, whether the supplementation of standardized *B. monnieri* extract (CDRI-08) or L- Carnosine to the pregnant mother dampen the maternal stress (MS) induced changes in their offsprings. To test this, the expression of antioxidant defense genes [Superoxide dismutase (SOD1,2), Catalase (CAT), Glutathione peroxidase-3 (GPx3)], telomerase reverse transcriptase (Tert), shelterin complex subunits (Terf1, Rap1B, Pot1), telomere length, anxiety-like behaviour, and inter-limb coordination was examined.

2. Materials and methods

2.1. Study animal

Healthy adult female rats (*Rattus norvegicus*) were selected and allowed to acclimatize in the laboratory for a week. After the careful monitoring of the estrous cycle, female rats were housed individually with a sexually active male rat. On the day when sperm present in the vaginal smear consider gestational day (GD)-0. Pregnant rats were housed individually at standard laboratory conditions (24 ± 2 °C; light/dark cycle for 12 h (7:00–19.00), 50 ± 5% of Rh with *ad libitum* of food and water. All the experimental group rats were handled minimally to avoid handling effects.

2.2. Social stress-induced maternal stress

The pregnant rats were subjected to social defeat (SD) to induce social stress during GD-16 to GD-18 in the specially designed SD cage to induce maternal stress (MS) [35,48]. SD cage constructed with two chambers [intruder chamber (IC), resident chamber (RC)] with equal size (30 × 30 cm). The standard laboratory cage was connected with the RC of the SD cage through the transparent plastic pipe (60 cm in length and 10.5 cm in diameter) to easily access food and water. RC and IC were partitioned with sliding doors made up of wire mesh to initiate the aggressive interaction between the resident aggressor and the pregnant intruder. The senescence male rat was habituated at RC for 10 days as a resident male (Aggressor) for the social defeat. The pregnant rat was introduced to the IC and allowed to explore for 5 min, and the sliding door was opened to facilitate the interaction with the aggressor for 10 min/day and then the pregnant rat was carefully transferred to their home cage. The control group-pregnant rats were allowed to explore the IC in the

absence of the aggressor.

2.3. Drugs

Freshly prepared aqueous suspension of standardized extract of *Bacopa monnieri* (CDRI-08; referred to as BME in this study) or L-Carnosine (Cat #C9625, Sigma-Aldrich, India) or vehicle (gum acacia aqueous suspension and saline) were supplemented orally to the dams each day (10.00–11.00 h) from GD-10 to their pup's postnatal day (PND)-23 except on the day of parturition (≈8 h after parturition). The bacoside (bacopaside A1, A2, A3, A4, bacopacoside II, bacopasaponin X, and bacopasaponin C) enriched extract was obtained from Dr. Hemant Singh as a generous gift, Lumen marketing company, Chennai, India (Batch# C15030294).

2.4. Experimental groups

The pregnant rats were assigned into five groups i) Control (CON) ii) Maternal Stress (MS), iii) MS received BME [(80 mg/kg) + 0.5% gum acacia (per-orally, p.o) (MS + BME)]; iv) MS + saline [(MS + PBS) (0.5% gum acacia+ 0.9% Saline/p.o)] and v) MS + received L- Carnosine [(1 mg/kg) (p.o) (MS + L- C)]. The dosage of BME [31] and L-Carnosine [49] was decided based on the earlier report. The day of parturition was noted as PND-0 and the pups were weaned on PND-24. Five dams that produced an equal number of pups were randomly assigned to each experimental group, from each dam four male pups were selected. From the assigned group, six active animals were tested during adolescence (PND -31-33) and the remaining were allowed to grow and tested during adult age (PND- 84–86) (Supplementary Fig. 1). Therefore, the animals are not tested repeatedly and inactive male animals and females are not included for the analysis.

2.5. Behavioural test

Light/dark-box test: The apparatus consists of two chambers of equal size (44 X 21 × 21 cm) separated by a partition with a rectangular opening (12 × 5 cm) at the floor level. One chamber is illuminated with bright light and the other one is covered with black stickers. Animals were transported to the experimental room 1 h before the experiment and left undisturbed, and the experiment was conducted under bright illumination (900 lux). The test animals were placed in the light chamber and allowed to explore for 5 min and video recorded. Individual animals spent time in light and dark chambers and time taken to assess the risk to enter the dark chamber (light-dark transition) was calculated.

Ladder rung walking Test: The rung walking apparatus was designed based on the previous report [50]. Rung walking was carried out for all the experimental groups (Control; MS; MS + BME; MS + PBS and MS + L-C) during adolescence (PND -31, 32 &33) and adult (PND-84, 85 & 86). The entire apparatus was elevated 30 cm from the ground level. Male rats were individually trained (on PND-31 &32) to walk on ladder rungs placed in regular patterns (1–40 continuous rung patterns with a 2 cm distance) starting from the neutral platform to the end. On PND-33, experimental group individuals were tested on irregular rung patterns (15,17,19,21,23,25, 27,30,31, 34,35). Limb placement on the rung was recorded and analyzed [50].

2.6. Sample preparation

Immediately after the behavioural test (PND- 33 &86; n = 6 from each group) animals were sacrificed and samples were collected. The whole brain was carefully cut off and placed on the ice-cold Petri dish and then the prefrontal cortex (PFC) was dissected out. One portion of the tissue was used to isolate genomic DNA and RNA and the other for total protein.

2.7. RNA isolation

RNA was isolated from one portion of the prefrontal cortex (PFC). Total RNA was extracted using TRI Reagent (Cat. # FATRR 001; Favogen Biotech Corp, Taiwan) and stored at −80 °C. Total RNA (2 μg) was incubated with DNase I (1Unit, Cat#: 2270A, Takara) for 30 min at 37 °C followed by heat-denaturation of the enzyme for 5 min at 80 °C, then the sample was used for the synthesis of cDNA (Cat.# 170–8891; Iscript cDNA synthesis kit, Bio-Rad laboratories Inc., USA)

2.8. DNA isolation

Genomic DNA was isolated from one portion of the prefrontal cortex (PFC) following the manufacturer's instruction (Cat #K0512: Thermo Scientific, USA). The concentration of DNA samples was estimated using Biophotometer plus (Eppendrof Inc., Germany) and stored at −80 °C.

2.9. Total protein isolation

Tissue was homogenized with ice-cold lysis buffer [50 mM Tris- Hydrochloric acid (Tris-HCL)-pH 7.5, 150 mM Sodium chloride (NaCl), 5 mM Ethylenediaminetetraacetic acid (EDTA), 1.0 mM Dithiothreotol (DTT), 0.1% Tergitol (NP-40), 0.2 mM Sodium orthovanadate (Na_3VO_4), 0.23 mM Phenylmethylsulfonyl fluoride (PMSF)] and protease inhibitor cocktail (4μL/mL) (Sigma- Aldrich, Saint Louis, MO, USA). The collected homogenate was centrifuged at 4 °C (10,000×*g*) after the incubation of 30 min in ice. Then

supernatants were collected in a fresh tube and subjected to centrifugation at 4 °C (12,000×*g*) for 20 min. The final extraction of samples was aliquot and stored at −80 °C. Extracted protein was quantified by the Bradford method (cat. #5000006; Bio-Rad Protein Assay kit, Bio-Rad Laboratories Inc., Hercules, CA, USA) using Biophotometer Plus (Eppendorf Inc, Hamburg, Germany).

2.10. Quantitative real-time PCR

The reaction mixture (SYBR green super mix, Bio-Rad laboratories Inc.) contains cDNA (0.2 μg) and specific primers (100 pmoles) were prepared and used. Specific primers (Supplementary Table 1) were used to estimate the expression of superoxide dismutase −1 & 2 (SOD1 &2); glutathione peroxidase −3(GPX3), catalase (CAT) and normalized with internal glyceroldehydes-3-phosphate dehydrogenase. Specific PCR product amplification has been confirmed by observing the dissociation curve followed by melting curve analysis (CFX-96 Touch Real-Time PCR; CFX manager ver. 2; Bio-Rad Laboratories Inc., USA). The triplicate data were normalized with internal control and presented in the form of a mean fold change relative to the control group.

The telomere length (TL) was measured by quantitative real-time PCR (qRT-PCR) using Acyl- CoA Oxidase (ACX) and Telomere (Tel) [51,52]. PCRs were performed in 20 μl reaction containing SYBR green master mix (BioRad Universal SYBR® Supermix, USA), ACX/Tel primers (100 pmols), and different concentrations of genomic DNA (0.5 μg; 1.0 μg, 1.5 μg; 2.0 μg; 2.5 μg) [53]. The PCR reaction started with initial denaturation (95 °C for 3 min), and then 40 cycles of denaturation (95 °C for 15secs), annealing [Tel- 60 °C for 60 s; ACX-50 °C for 30 s], extension 72 °C for 10 s. Further, the relative amount of telomeric DNA to the single copy gene (T/S) ratio was calculated using the C_t value of T relative to S (Average C_t of telomere) – (Average C_t of ACX). The relative telomere length was calculated according to the $2^{-\Delta\Delta Ct}$ method, Where $\Delta\Delta C_{\ t} = \Delta C_{t\ sample} - \Delta C_{t\ calibrator\ sample}$ and $\Delta C_{\ t\ sample} = C_{\ t\ Tel} - C_{\ t\ ACX}$ [52].

2.11. Western blot analysis

Equal concentration of proteins (60 μg) was resolved on 10% Sodium dodecyl sulphate Polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, IPVH00010, Burlington, MA, USA) using Trans-Blot® Tribo™ blotting system (Bio-Rad Laboratories Inc, USA). After the transfer of protein, membranes were then placed in the blocking solution (5% non-fat dry milk in tris-buffered saline (TBS) containing 0.1% Tween-20; TBS-T) for 3 h at room temperature (RT). The blocking solution was discarded, and the membranes were incubated at 4 °C overnight with any one of the primary antibodies (Supplementary Table 2) then washed with TBS-T followed by incubated in alkaline phosphatase (ALP) conjugated secondary antibody. Then, 5 bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) and nitro blue tetrazolium chloride (NBT) (Merk Life science, ES006) substrate was used to detect the ALP activity by following the manufacturer's instruction. Image acquisition was done by Image Lab 2 software (Chemi Doc XR + System; Bio-Rad Laboratories, Inc., USA). The optical density of each band was measured and the data were represented as fold relative to internal control.

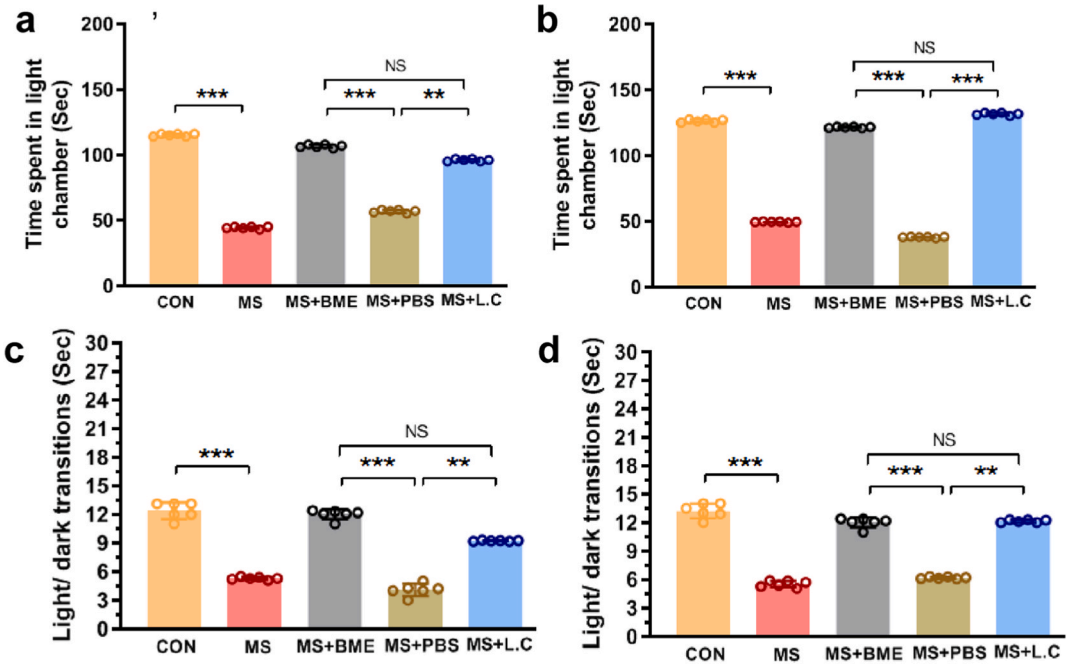

Fig. 1. Supplementation of *B. monnieri* extract or L- Carnosine reduces the maternal stress-induced anxiety-like behaviour in their adolescent and adult offspring. The MS/MS + PBS group adolescent (a) and adult (b) less time in the light chamber compared to BME/L-C supplemented siblings. In addition, the adolescent (c) and adult (d) took more time in the light-dark box transition compared to BME/L-C supplemented siblings.

2.12. Statistical analysis

Graphical representation was produced by plotting the Data [mean, standard error of the mean (SEM)] in Kyplot (Ver 5.0). One way- ANOVA in Sigma Stat (Ver 11.0) followed by pairwise *t*-test with Bonferroni correction post-hoc test, in order to identify differences between the groups [i.e., Control, maternal stress (MS), MS with vehicle supplemented (MS + PBS), MS supplemented with B. monniera extract (MS + BME), MS supplemented with L-Carnosine (MS + L-C)] considering their interaction. Differences were considered as significant if ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$ and NS denotes difference was not-significant between groups.

3. Results

3.1. Supplementation of BME/L-C dampens the maternal stress-induced anxiety-like behaviour

At first, our behavioural analysis using a light/dark box showed a significant difference in their behavioural profile among adolescents ($F_{(4,39)} = 20.015$, $p < 0.001$) and adults ($F_{(4,39)} = 100.221$, $p < 0.001$) experimental group animals. *Post hoc* analysis showed that the MS group spent significantly less time in the light chamber than the Con group ($p < 0.001$). Similarly, MS + PBS animals stayed significantly less time in the light chamber compared to MS + BME/MS + L-C group adolescents ($p < 0.001$) (Fig. 1a) and adult rats ($p < 0.001$) (Fig. 1b). In addition, we have observed significant differences between groups in light-dark transition in adolescents ($F_{(4,39)} = 10.980$, $p < 0.001$) and adult rats ($F_{(4,39)} = 16.750$, $p < 0.001$). Estimated *post hoc* analysis revealed that the MS group light-dark transition is significantly less compared to Con. Likewise, the MS + PBS group rat's light-dark transition was significantly less than MS + BME/MS + L-C group ($p < 0.001$) (Fig. 1c and d). Observed behavioural profile i.e. time spent at light chamber/light-dark transition of MS/MS + PBS group rat suggest that they have less preference for the light chamber, which could be linked with anxiety-like behaviour induced by MS. Interestingly, supplementation of BME or L-C dampen the effect, hence, they made more number of light-dark transition and spent more time in light chamber.

3.2. Supplementation of BME/L-C dampens the maternal stress-induced effect on limb coordination

A ladder rung-walking test was conducted to examine the forelimb and hindlimb coordination and accuracy of limb placement. We found that limb placement was significantly different between experimental adolescent groups training ($F_{(4,119)} = 215.06$; $p < 0.001$) and testing ($F_{(4,119)} = 169.43$; $p < 0.001$). *Post hoc* analysis revealed that MS and MS + PBS group rats made significantly more misplacement than the control ($p < 0.001$) and MS + BME/MS + L-C ($p < 0.001$) in respective comparisons (Fig. 2a,c). Likewise, limb placement of adults was significantly different during training ($F_{(4,119)} = 134.87$; $p < 0.001$) and testing ($F_{(4,119)} = 118.43$; $p < 0.001$). *Post hoc* analysis demonstrates that MS and MS + PBS rat misplacement was significantly more compared to control and MS + BME/

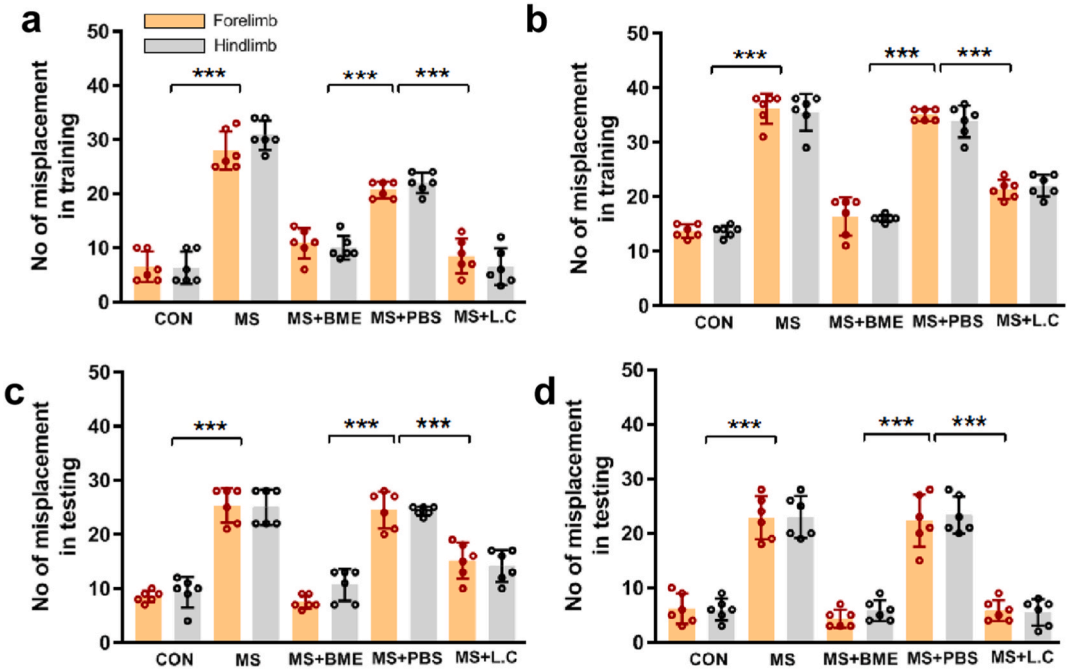

Fig. 2. Ladder rung walking task analysis shows the experimental group animal's skilled limb coordination. MS/MS + PBS group adolescent rats made more misplacement during training (a) and testing (b). MS-induced effect possibly long-lasting, thus, adult MS/MS + PBS rats made more number misplacement during training (c) and testing (d), but the effect was modified by supplementation of *Bacopa monnieri* extract or L- Carnosine.

MS + L-C respectively (Fig. 2b,d). However, we did not detect a significant difference between FL and HL during training ($F_{(1,119)} = 0.00520$; $p = 0.943$) and testing ($F_{(1,119)} = 0.524$; $p = 0.471$). Observed limb misplacement data suggest that MS affects fine motor control during development, which extended up to adulthood. Notably, supplementation of BME/L-C significantly dampens the MS-induced effect on motor control.

3.3. Supplementation of BME/L-C dampens the maternal stress-associated changes in antioxidant defence gene expression

To examine the maternal stress-induced changes in cellular defence mechanism, we measured the expression level of major antioxidant defence genes. In adolescent rats, we found that the level of SOD1 expression was significantly different between groups ($F_{(4,14)} = 9.683$, $p < 0.002$), *post hoc* analysis indicates that the level SOD 1 was significantly low in MS and MS + PBS groups compared to control ($p < 0.001$) and MS + BME/MS + L-C respectively ($p < 0.001$) (Fig. 3a). Similarly, the level of SOD2 was significantly different between groups ($F_{(4,14)} = 12.804$, $p < 0.001$). As shown in Fig. 3b, MS significantly reduced the SOD2 level compared to, whereas supplementation of BME/L-C suppressed the MS effect, and all other comparisons depicted. Therefore, we found significantly lower levels of SOD2 in MS and MS + PBS groups compared to the control and MS + BME/MS + L-C respectively. The estimated level of CAT was significantly different between groups ($F_{(4,14)} = 177.14$, $p < 0.001$) (Fig. 3c). Subsequent *post hoc* analysis demonstrated that the estimated level of CAT was significantly low in the MS and MS + PBS with the respective comparison of control and MS + BME/MS + L-C groups. Supplementation of BME/L-C dampens the MS-induced effect (Fig. 3c). Further, the estimated level of GPX3 was significantly different between groups ($F_{(4,14)} = 150.70$, $p < 0.001$)(Fig. 3d). (Supplementary Table 3) Post-hoc analysis revealed that the estimated level was significantly low in MS and MS + PBS groups with respective comparisons of control and MS + BME/MS + L-C. In adolescent rats, estimated antioxidant enzymes suggest that social stress-induced maternal stress significantly influences the antioxidant defence mechanism, whereas, the supplementation of BME or L-C dampens stress.

In adults, we found that the level of SOD1 ($F_{(4,14)} = 69.149$, $p < 0.001$) and SOD2 ($F_{(4,14)} = 30.273$, $p < 0.001$) expression was significantly different between groups. Further, *post-hoc* analysis indicates that the estimated level of SOD1/SOD2 in the MS and MS + PBS group was significantly low compared to the respective control ($p < 0.01$) and MS + BME/MS + L-C group ($p < 0.01$) (Fig. 4a and b). The estimated level of CAT was significantly different between groups ($F_{(4,14)} = 98.360$, $p < 0.001$) (Fig. 4). The subsequent *post-hoc* analysis demonstrated that the estimated level of CAT was significantly low in MS and MS + PBS groups when compared with the control ($p < 0.001$) and MS + BME and MS + L-C ($p < 0.001$) supplemented group respectively. Further, the estimated level of GPX3 was significantly different between groups ($F_{(4,14)} = 41.433$, $p < 0.001$) (Fig. 4d). *Post-hoc* analysis revealed that the level of GPX3 was significantly low in the MS group than control ($p < 0.001$). Likewise, compared to the MS + BME and MS + L-C groups estimated level of GPX3 was significantly low in the MS + PBS group ($p < 0.001$). Overall analysis suggests that antioxidant defence gene expression was suppressed by MS even in adults, but the supplementation of BME/L- C resilient the MS effect on the offspring.

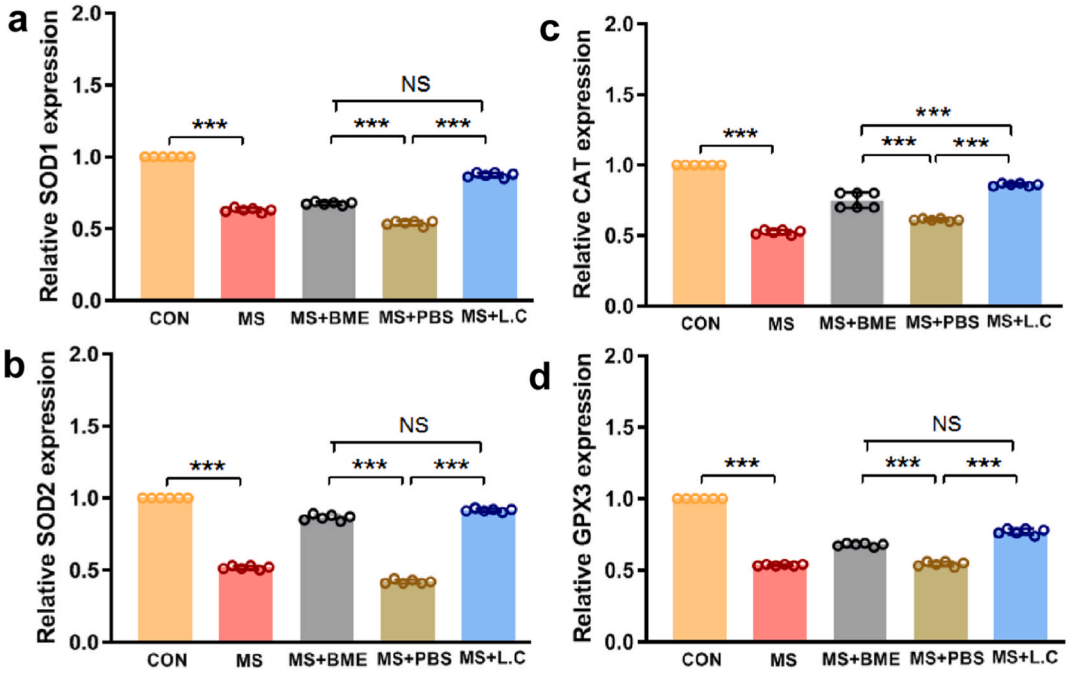

Fig. 3. Quantitative real-time PCR analysis showing that maternal stress differentially alters the expression of antioxidant genes (SOD1-2, CAT, and GPX3) in adolescents. The altered expression was restored by supplementation of *B. monnieri* extract or L- Carnosine. Experimental group's individual calculated relative expression level of (a) Superoxide dismutase-1, (b) Superoxide dismutase-2, (c) Catalase, and (d) Glutathione peroxidase-3.

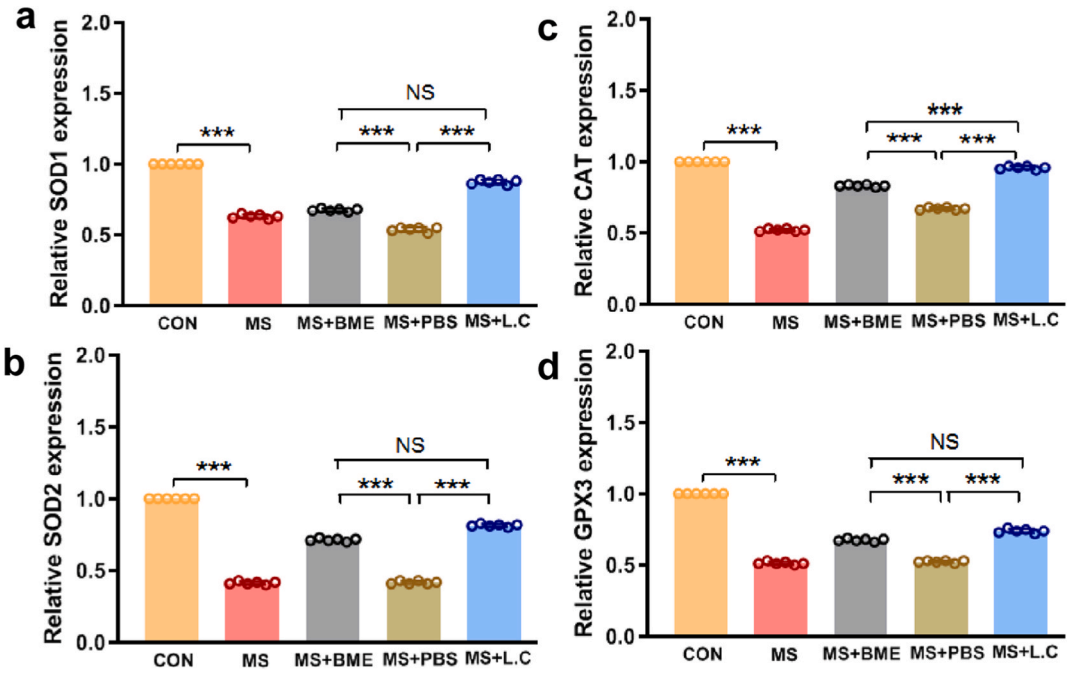

Fig. 4. Quantitative real-time PCR analysis showing that maternal stress alters the expression of antioxidant defence genes (SOD1-2, CAT, and GPX3) in adults. The altered expression was restored by supplementation of *B. monnieri* extract or L- Carnosine. Experimental group's individual calculated relative expression level of (a) Superoxide dismutase-1, (b) Superoxide dismutase-2, (c) Catalase, and (d) Glutathione peroxidase-3.

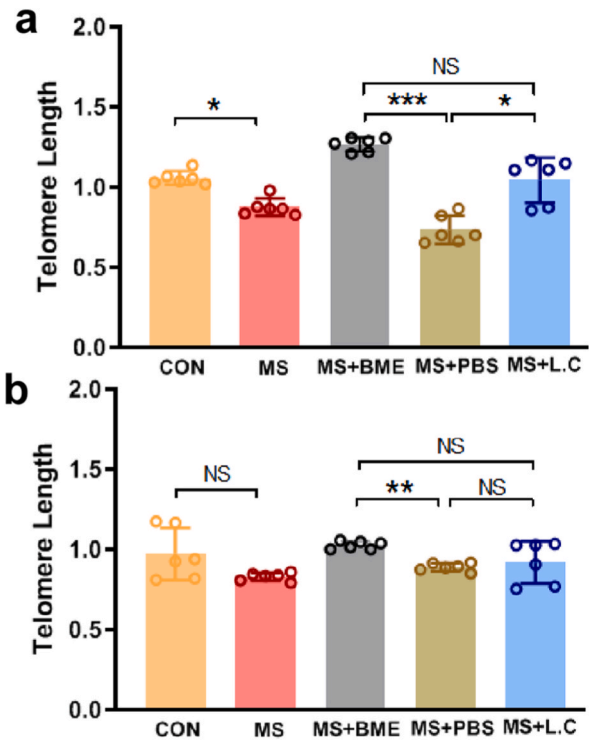

Fig. 5. Quantitative real-time PCR analyses showing that maternal stress alters the telomere length, and the effect was modified by supplementation of *B. monnieri* extract or L- Carnosine. The change was measured as relative amount of telomeric DNA to the single copy gene (T/S) ratio in adolescents (a) and adults (b).

3.4. Supplementation of BME/L-C dampens the maternal stress-induced effect on telomere length

As predicted, the telomere length was significantly altered by MS in adolescents ($F_{(4,14)} = 14.668$, $p < 0.001$) and adults ($F_{(4,14)} = 14.761$, $p < 0.001$). In comparison, the post-hoc analysis depicts that telomere length was significantly shorter in the MS group compared to the control. Similarly, the estimated telomere length was significantly shorter in the MS + PBS group compared to MS + BME and MS + L-C groups ($p < 0.001$) (Fig. 5a and b). To confirm the obtained telomere length, we performed an independent standard curve for telomere repeat and ACX gene. Obtained semi-log plot of DNA concentration versus cycle threshold, both sets of curves were linear. [(Tel: CON $r^2 = 0.99$; MS: $r^2 = 0.86$; BME: $r^2 = 0.99$; PBS: $r^2 = 0.98$; LC: $r^2 = 0.99$/ACX Con: $r^2 = 0.99$; MS: $r^2 = 0.99$; BME: $r^2 = 0.98$; PBS: $r^2 = 0.98$; LC: $r^2 = 0.99$) and adults (TEL: CON $r^2 = 0.94$; MS: $r^2 = 0.98$; BME: $r^2 = 0.95$; PBS: $r^2 = 0.96$; LC: $r^2 = 0.97$/ACX CON: $r^2 = 0.99$; PBS: $r^2 = 0.88$; BME: $r^2 = 0.99$; PBS: $r^2 = 0.97$; L-C: $r^2 = 0.98$)] for the four DNA concentrations in the experimental groups (Fig. 6a and b). At each DNA concentration, the C_t of ACX occurred later than (approximately for adolescents: 6–8 cycles; for adults: 8–10 cycles) C_t of TL. This analysis revealed that along with aging, MS plays a role in telomere shortening, whereas supplementation of BME or L-C minimizes the effect.

3.5. Supplementation of BME/L-C dampens the maternal stress-associated changes in telomerase reverse transcriptase (Tert)

MS-associated changes in TERT level were examined during adolescence and adulthood (Figs. 7a and 8a; uncropped images in supplementary fig. 2,3). We found a significant difference in the expression of TERT among the experimental groups in adolescents ($F_{(4,14)} = 571.42$, $p < 0.001$) and adults ($F_{(4,14)}$ 1242.310, $p < 0.001$) (Figs. 7b and 8b). The *post-hoc* analysis suggests that MS-induced expression of Tert, therefore the level of TERT was significantly higher in the MS group compared with control ($p < 0.001$) groups. Similarly, the estimated level was higher in MS + PBS than in MS + BME and MS + L-C groups ($p < 0.001$). Observed results suggest that MS induces the expression of TERT, but the supplementation of BME/L- C suppresses the MS-induced effect.

3.6. Supplementation of BME/L-C dampens the maternal stress-associated changes in telomeric repeat binding factor 1(Terf1)

Elevation of TERT has been known to influence the shelterin protein complex to participate in DNA repair and telomere function. Western blot analysis (Figs. 7a and 8a; uncropped images in supplementary fig. 2,3) showed that the significant differences in TERF1 expression between the experimental groups during adolescent ($F_{(4,14)} = 705$, $p < 0.001$) (Fig. 7c) and adult ($F_{(4,14)} = 1780$, $p < 0.001$) (Fig. 8c). The TERF1 expression pattern was similar in both adolescents and adults, *post-hoc* analysis suggests MS induces expression of Terf1, hence, the level of TERF1 protein was significantly higher in MS groups compared to the control ($p < 0.001$). In comparison, the estimated level TERF1 was significantly low in MS + BME and MS + L-C groups than in MS + PBS ($p < 0.001$). Observed results suggest that MS alters the expression of Terf1, which is extended up to adults but the supplementation of BME/L-C suppresses the MS-associated changes in TERF1.

3.7. Supplementation of BME/L-C dampens the maternal stress-associated changes in shelterin complex subunit Rap1 expression

Estimated extra telomeric RAP1 binding protein was significantly different among the experimental groups in adolescents ($F_{(4,14)} = 212.627$, $p < 0.001$) (Fig. 7a,d; uncropped images in supplementary fig. 2), and adults ($F_{(4,14)} = 395.534$, $p < 0.001$) (Fig. 8a,d; uncropped images in supplementary fig. 3). Further, the post-hoc analysis showed that the expression of Rap1 was suppressed by MS,

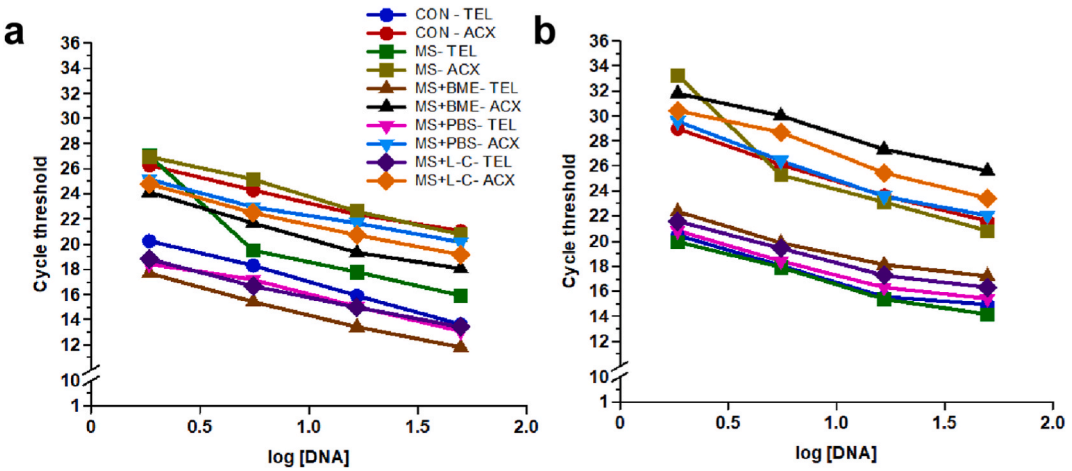

Fig. 6. Maternal stress alter the telomeric DNA length, which was measured in absolute telomere length (C_T, cycle threshold for TL, and single copy gene ACX) for each experimental group with different concentration for adolescent (a) and adult (b) was detected. The correlation coefficient within the linear range was 0.98–0.99 and 0.94–0.99 in adolescents and adults, respectively.

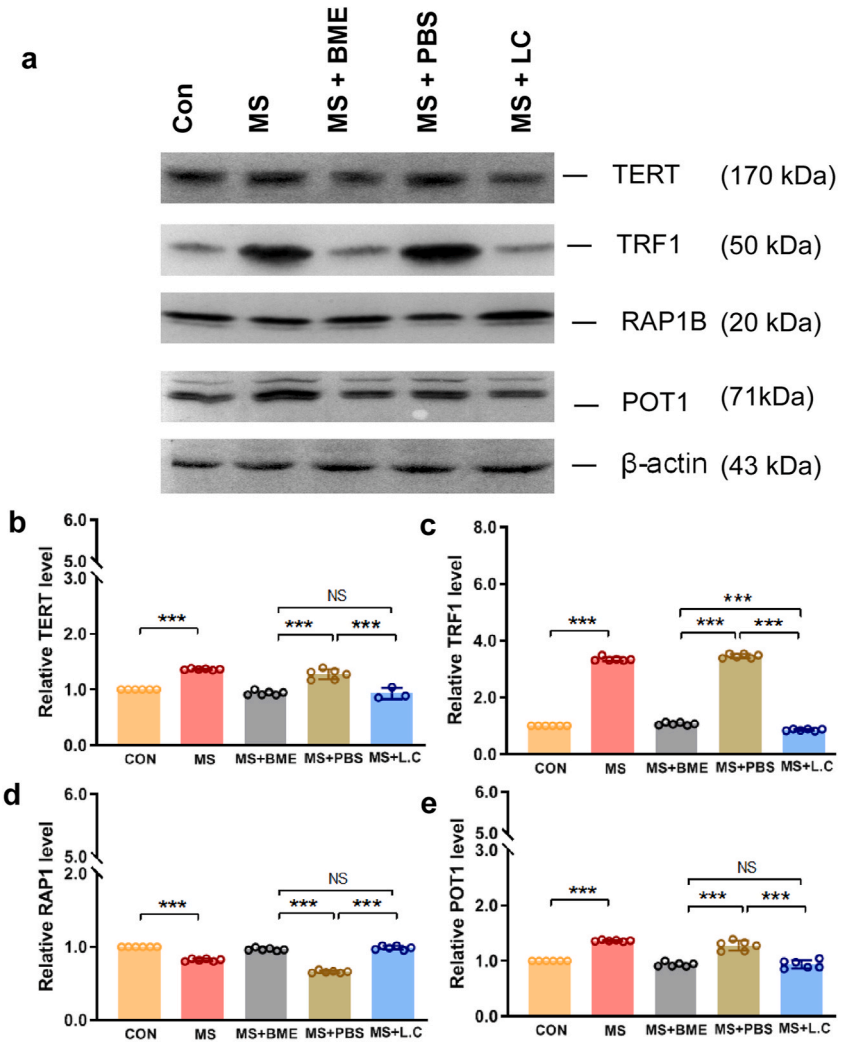

Fig. 7. Maternal stress alters the expression of TERT and shelterin protein complex subunit gene but the effect was modified by supplementation of *B. monnieri* extract or L- Carnosine. (a) Representative western blots showing the experimental group's individual expression during their adolescent age (uncropped westernblot images in supplementary information as supplementary fig. 2); the estimated levels of (b) Telomerase reverse transcriptase (c) Telomere repeat factor 1 (d) Repressor/activator protein 1B and (e) Protection of telomeres 1.

the expression pattern was similar in both adolescents and adults. The estimated level of RAP1 in the MS group was significantly lower than the control ($p < 0.001$). Whereas the level of RAP1 was significantly lower in MS + PBS than in MS + BME and MS + L-C groups ($p < 0.001$). These results suggest that MS-induced suppression of shelterin complex subunit Rap1 expression was effective up to adult age, but the supplementation of BME/L-C dampen the effect.

3.8. Supplementation of BME/L-C dampens the maternal stress-associated changes in shelterin complex subunit Pot1 expression

The component of shelterin complex Pot 1 expression has significantly differed among the adolescent experimental groups ($F_{(4,14)} = 147.666$, $p < 0.001$) (Fig. 7a,e; uncropped images in supplementary fig. 2) and adults ($F_{(4,14)} = 259.328$, $p < 0.001$) (Fig. 8a,e; uncropped images in supplementary fig. 3). The expression pattern of Pot1 was the same in both adolescents and adults, and *post-hoc* analysis reported that MS significantly increased the level of POT1 protein, which was significantly higher in the MS group than control ($p < 0.001$). Whereas, the estimated POT1 level was significantly higher in MS + PBS than in BME (MS + BME) or L-C (MS + L-C) supplemented groups ($p < 0.01$). The observed changes in POT1 showed that BME and L-C supplementation dampens the effect of MS-induced expression of Pot1.

4. Discussion

Alterations in neurobehaviour, physiology, and genomic instability have been associated with prenatal stress in human and animal

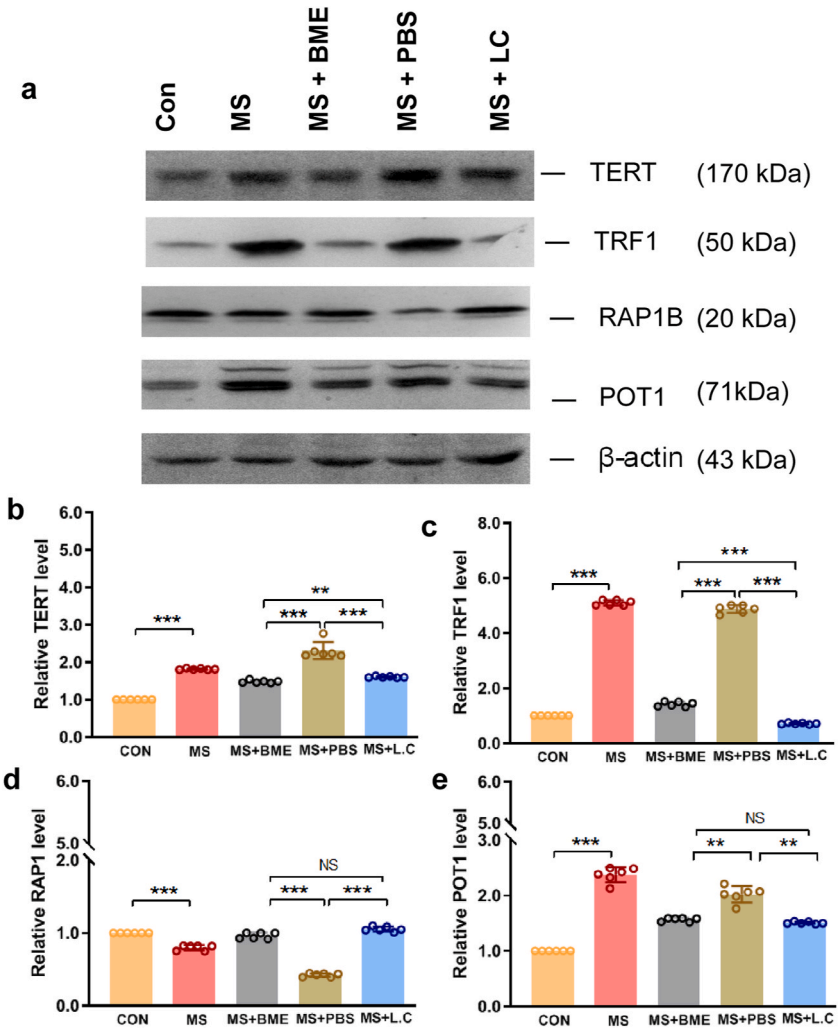

Fig. 8. Maternal stress alters the expression of TERT and shelterin protein complex subunit gene but the effect was modified by supplementation of *B. monnieri* extract or L- Carnosine. (a) Representative western blots showing the experimental group's individual expression during their adult age (uncropped westernblot images in supplementary information as supplementary fig. 3); the estimated levels of (b) telomerase reverse transcriptase (c) Telomere repeat factor 1 (d) Repressor/activator protein 1B and (e) Protection of telomeres 1.

models, and are observed in offspring born following maternal stress [14]. In this study, we investigated whether the supplementation of standardized *Bacopa monnieri* extract (CDRI-08/BME) or L-Carnosine (L-C) to the mother experienced social stress during gestation modified the effect on their offspring's neurobehaviour, antioxidant defence gene expression, telomere length, and telomere biology. We observed that MS/MS + PBS group's rat pups displayed anxiety-like behaviours such as less preference for light chamber and less light-dark transition. Observed behavioural phenotype is similar to other reported stress models [54,55]. In line with earlier reports [56–58], supplementation of BME/L-C dampen the MS-induced stress and reduces the anxiety-like behaviour. Subsequently, we examined the sensorimotor mediated inter-limb coordination in their offspring at adolescent and adult age. We found that individuals representing MS/MS + PBS group made more number of limb misplacements in the ladder rung walking test than control, BME/L-Carnosine supplemented individuals. Alterations of the cellular oxidative status might be an important factor underlying challenging early life conditions, which possibly advance aging, damage motor cortical neurons, reduced cortical excitability [59], which may impair compensatory adjustment of fore-and hindlimb placement, stepping and inter-limb coordination [60,61]. Therefore, we estimated the level of antioxidant defence gene expression during their adolescent and adult age. We found that the level of antioxidant defence genes (SOD1,2, CAT, and GPX3) expression has reduced in adolescent and adult individuals who experienced MS, but the expression level was elevated in supplemented with BME/L- C group. Our results may be linked with the earlier reports, which showed that BME and L-C possess antioxidant properties, protect the neuron, and reduced neuronal degeneration [35,41,42,62,63].

Note to mention that telomeric DNA is highly susceptible to oxidative damage and maternal stress altered the telomere dynamic through oxidative stress [15]. In addition, earlier studies reported that stress shortens the telomere length [15,16,64]. Therefore, we examined the telomere length and the regulatory molecules that maintain the telomere length. We found that shortening of telomere

length in individuals who experienced MS. Supporting our observation, independent studies linking the psychological stress during gestation with telomere length in the brain of the newborn [65] young adulthood, and adult offspring [14]. Interestingly, BME/L-C supplementation dampens the MS-induced effect, which possibly suppresses oxidative stress, and neuronal aging [62,66], and thus, telomere length is maintained [41,47]. Even though, earlier studies demonstrated that telomere length is shortened by stress, the underlying regulatory molecules and their mechanism are not reported. Therefore, subsequently, we have analyzed the associated molecules. Telomeric DNA replication is primarily regulated by TERT, therefore, at first we have examined the level of TERT in experimental group animals. We found that the level of telomerase was significantly higher in the MS group compared to the control, whereas BME or L-C supplementation dampen the effect compared to the MS + PBS group. Observed upregulated level of Up- TERT in MS/MS + PBS animals possibly induced against oxidative stress as a non-canonical function to reprogram the pluripotent state of cells or suppress the DNA damage response (DDR) [67].

Interestingly, BME/L-C supplemented individuals' level of TERT was basal level, which suggests that antioxidant properties of BME/L-C possibly dampen the MS-induced effect [42,62]. Further, the length of the telomere was regulated by telomere binding proteins TRF 1, we examined the level of TRF 1 and found that the level was significantly high in MS and MS + PBS animals. Observed higher level of TRF 1 are associated with prolonged stress [68] and acts as a negative regulator by inhibiting the progression of telomeric repeats and shortening the telomere length [69,70]. Notably, supplementation of BME/L-C dampen the effect of MS and normalized the expression of TRF1. The TRF 1 complex has been known to recruit POT1 to the telomeric DNA, we found that the level of POT1 was significantly higher in MS and MS + PBS. POT1 is known to bind with single-strand telomeric DNA and act as a natural inhibitor of telomerase [71]. Thereby, disrupting the binding of telomerase and elongation of the telomere, which could be linked with observed shortened telomere length in MS and MS + PBS groups. On the other hand, supplementation of BME/LC dampen the MS-induced effect and diminished the POT1 to the basal level. RAP1 is the sixth subunit of the shelterin complex binds to telomeric repeats through its interaction with TRF2 and regulates telomere length and DDR [72]. Observed short telomere length in MS and MS + PBS animals possibly by the reduction in RAP1 level was suppressed by MS, which may lead to shortening of telomere, enhanced DDR [19], and DNA double-strand breakage [72]. Interestingly, BME/L-C supplementation dampens the effect of MS, thus we observed the level of RAP1 at a basal level.

5. Conclusion

Taken together, our analysis demonstrates that maternal stress (MS) during gestation can afflict the developing offspring. Maternal stress (MS) altered limb coordination, influences antioxidant defence gene expression (SOD1,2; CAT, GPx3), and shortens the telomere length. Notably, TERT and shelterin complex subunits (TRF1, POT1 RAP1) that regulate the telomerase were altered by MS. Supplementation of BME/L-Carnosine dampen the effect, which regulates balanced expression of cellular defence gene, telomerase, and shelterin complex subunits, to protect telomere length. The antioxidant property of BME/L-Carnosine could be one of the possible mechanisms that prevent the MS effect.

6. Limitation of this study

In this study, we have not tested the behaviour of pregnant rats to confirm whether social defeat induces stress in pregnant mother rats. However, we have observed that few social defeat-experienced mother rats ate their pups on the same day of parturition.

Funding statement

K.E.R. thank Rashtriya Uchchatar Shiksha Abhiyan (RUSA) 2.0 – Biological Sciences and Tamil Nadu State Council of Higher Education (TANSCHE) RGP/2019-20/BDU/HECP-0072. The department of animal science is supported by the Department of Science and Technology (DST)- Fund for Improvement of S&T Infrastructure (FIST) and DST- Promotion of University Research and Scientific Excellence (PURSE). The research was partially supported by Chiang Mai University, Chiang Mai, Thailand.

Institutional review board statement

All the experimental procedure was recommended by the Institutional Animal Ethics Committee (IAEC), Bharathidasan University, Tiruchirappalli, India, and approved (Ref. No. BDU/IAEC/Re02/2021 dt September 4, 2021) by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Informed consent statement

Not applicable.

Author contribution statement

Karunanithi Sivasangari: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Bhagavathi Sundaram Sivamaruthi: Analyzed and interpreted the data.
Chaiyavat Chaiyasut: Analyzed and interpreted the data; Wrote the paper.

Koilmani Emmanuvel Rajan: Conceived and designed the experiment; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

K.S. is the recipient of the ICMR- SRF research fellowship (2021-14874-F1/Dt 1st April). We thank Dr. Hemant Singh (Lumen Foundation, Chennai, India) for his generous gift of *Bacopa monnieri* extract (CDRI-08). B.S.S. and C.C. thank Chiang Mai University, Chiang Mai, Thailand, for the support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20385.

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